PhD Thesis

The Endocrine Role of the Skeleton – Osteocalcin, Glucose Metabolism and Fertility

Submitted by

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Affidavit

I hereby declare that this dissertation is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this dissertation. Due acknowledgement has been made in the text to all other material used. Throughout this dissertation and in all related publications I followed the guidelines of “Good Scientific Practice”.

Graz, October 2013

Acknowledgement

I would like to thank my supervisors and my family for their support.

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Abbreviations

%ucOC=% of undercarboxylated osteocalcin of total osteocalcin

AUC=area under the curve

BMD=bone mineral density

BMI=body mass index

cOC=carboxylated osteocalcin

CTX=CrossLaps

DHEAS=dehydroepiandrosterone sulfate

EIA=enzyme immunoassay

EIA=enzyme immunoassay

ELISA=enzyme-linked immunosorbent assay

ER=estrogen receptor

GIP=glucose-dependent insulinotropic polypeptide

GLP-2=glucagon-like peptide 2

GnRH=gonadotropin-releasing hormone

HOMA-IR=homeostatic model assessment of insulin resistance

HTR=serotonin receptor

OC=osteocalcin

OGTT=oral glucose tolerance test

OPG=osteoprotegerin

OR=odds ratio

P1NP=procollagen -1 N-terminal telopeptide

PBS=phosphate buffered saline

PCOS=polycystic ovary syndrome

RANKL=receptor activator of nuclear factor κ B ligand

SNS=sympathetic nervous system

T2DM=type 2 diabetes mellitus

TRACP5b=tartrate-resistant acid phosphatase

ucOC=undercarboxylated osteocalcin

uNTX=urinary N-terminal telopeptide of type I collagen

WHR=waist-hip ratio

ΔOC/ΔucOC/Δ%ucOC/ΔcOC=the decline from baseline to 120 minutes of the respective parameter

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Abstract

Osteocalcin (OC), released by osteoblasts and known as a marker of bone turnover, influences both energy metabolism as well as male fertility in murine models. Results from clinical studies are controversial. Single nucleotide polymorphisms in the OC gene have been investigated and a haplotype of three SNPs associated with body mass index (BMI).

In the first part, we investigated the effect of a 75g oral glucose tolerance test (OGTT) on total OC, undercarboxylated (ucOC) and carboxylated OC (cOC) in insulin-resistant (IR) and non-insulin-resistant (nIR) premenopausal women. Further, the relationship of changes in OC, ucOC, and cOC with AUCinsulin and Matsuda index were examined. In this cross-sectional study, OGTT were performed in 105 premenopausal women, 18 of which were insulin-resistant (HOMA-IR > 2.6) and 87 of which were not. Changes in total OC, ucOC, and cOC were evaluated 60 and 120 minutes after glucose load.

At baseline, total OC, cOC, and ucOC were significantly lower in insulin-resistant women. In non-insulin-resistant women, total OC, ucOC and cOC decreased significantly while in insulin-resistant women, none of the parameters of OC did. In stepwise linear regression analyses adjusted for age and BMI, the declines in OC and cOC significantly predicted AUCinsulin and Matsuda.

Glucose intake in non-insulin-resistant women is accompanied by decreases in the levels of OC, ucOC, and cOC, the extent of which predicts insulin resistance and sensitivity in premenopausal women. In insulin-resistant women these parameters appeared suppressed. Osteoblast responses to oral glucose in insulin-resistant and non-insulin-resistant women might differ – OC reflects this finding.

The aim of the second part was to assess the association of OC, ucOC, cOC with testosterone and sperm count in 159 men from infertile couples. For this purpose, testosterone, LH, OC, ucOC were measured and semen analysis performed.

cOC and OC correlated weakly but significantly with testosterone, but not after adjustment for BMI and age. No significant correlations were observed between parameters of OC and sperm count, semen volume and number of vital sperms. In binary logistic regression analyses, none of the parameters of OC were predictors of oligozoospermia after adjustment for age and BMI or WHR. The current data show that OC is only modestly associated with male fertility in young men from infertile couples.

The third aim was to test the association of three OC polymorphisms with BMI and with parameters of glucose and lipid metabolism in women with polycystic ovary syndrome

(PCOS). Metabolic and anthropometric measurements and oral glucose tolerance tests were performed in 680 PCOS women and genotyping of three OC SNPs (rs2758605, rs1543294, rs2241106) was carried out.

As for rs2241106, 340 women were carriers of the CC genotype, 278 of the CG genotype, and 62 of the GG genotype. CC genotype carriers had a significantly higher BMI compared to CG genotype carriers, but not compared to women carrying the GG genotype. No difference between the groups could be identified concerning the parameters of glucose and lipid metabolism analyzed. As for rs1543294 and rs2758605, there was no difference between the genotypes concerning the parameters investigated. These data confirm the association of the SNP rs2241106 with BMI in a cohort of women with PCOS. The three OC SNPs did not show an association with parameters of glucose and lipid metabolism in this cohort.

To conclude, bone and glucose metabolism seem to be related via OC. In humans, a stronger role of the carboxylated fraction of OC is suggested. OC polymorphisms are associated with BMI in healthy individuals and women with polycystic ovary syndrome, while data on other patient cohorts are still lacking. The association of OC with testosterone is weak. OC and parameters of semen analysis do not seem to be associated so far – however, more data are needed to support these findings.

Zusammenfassung

Osteocalcin (OC), ein von Osteoblasten freigesetzter Knochenumbauparameter, beeinflusst im Tiermodell sowohl den Glukosestoffwechsel als auch die männliche Fertilität. Die Ergebnisse klinischer Studien sind jedoch umstritten. Polymorphismen im OC-Gen wurden untersucht, wobei ein Haplotyp aus drei Polymorphismen mit dem BMI assoziiert war.

Das Ziel des ersten Teils war es, den Effekt eines 75g-Glukosetoleranztests (oGTT) auf OC gesamt, untercarboxyliertes OC (ucOC) und carboxyliertes OC (cOC) bei insulin-resistenten und nicht-insulinresistenten prämenopausalen Frauen zu untersuchen. Weiters wurde eine mögliche Assoziation zwischen dem Ausmaß der Veränderung von OC, ucOC und cOC mit AUCInsulin und Matsuda getestet. In dieser Querschnittsstudie wurden bei 105 prämenopausalen Frauen (18 waren insulin-resistent mit einem HOMA-IR >2.6; 87 nicht-insulin-resistente) oGTTs durchgeführt. Die Veränderungen in OC gesamt, ucOC, cOC wurden 60 und 120 Minuten nach Glukoseeinnahme untersucht.

Zum Zeitpunkt 0 waren OC gesamt, ucOC und cOC signifikant niedriger bei insulin-resistenten Frauen. Nicht-insulin-resistente Frauen zeigten einen signifikanten Abfall der genannten Parameter, insulin-resistente nicht. In einer schrittweisen linearen Regressionsanalyse (adjustiert für Alter und BMI), waren die Veränderungen von OC und cOC signifikante Prädiktoren von AUCInsulin und Matsuda.

Glukoseeinnahme bei nicht-insulin-resistenten Frauen wird also begleitet von Abfällen der OC-, ucOC- und cOC-Level. Das Ausmaß dieses Abfalls ist ein signifikanter Prädiktor von Insulinresistenz und –sensitiviät bei prämenopausalen Frauen. Bei insulin-resistenten erscheinen diese Parameter supprimiert. Die Antwort der Osteoblasten auf Glukose könnte in Abhängigkeit vom Vorliegen einer Insulinresistenz variieren. OC scheint dieses Phänomen widerzuspiegeln.

Das Ziel des zweiten Teils dieser Dissertation war es, eine mögliche Assoziation von OC gesamt, ucOC und cOC mit Testosteron und Spermienzahl bei 159 Männern von infertilen Paaren zu untersuchen. Zu diesem Zweck wurden Testosteron, LH, OC, ucOC gemessen und Spermiogramme durchgeführt.

cOC und OC korrelierten schwach aber signifikant mit Testosteron, eine Assoziation, die jedoch nach Adjustierung für Alter und BMI verschwand. Keine signifikanten Korrelationen konnten gezeigt werden für OC-Parameter und Spermienzahl, Ejakulationsvolumen und Anzahl der vitalen Spermien. In einer binären logistischen Regressionsanalyse war keiner der OC-Parameter ein signifikanter Prädiktor für

Oligozoospermie nach Adjustierung für Alter und BMI. Die vorliegenden Daten zeigen, dass OC nur schwach mit der Fertilität von jungen Männern von infertilen Paaren assoziiert ist.

Das dritte Ziel war es, die Assoziation von 3 Polymorphismen des OC-Gens (rs2758605, rs1543294, rs2241106) mit BMI und Parametern des Glukose- und Lipidstoffwechsels bei Frauen mit polyzystischem Ovarsyndrom (PCOS) zu testen. Metabolische und anthropometrische Messungen und oGTTs wurden in 680 Frauen durchgeführt.

340 Frauen hatten den CC Genotyp des Polymorphismus rs2241106, 278 CG, 62 GG. CC Genotyp-Trägerinnen hatten einen signifikant höheren BMI verglichen mit CG Trägerinnen. Keine Unterschiede in den Gruppen konnten für die Parameter des Glukose- und Lipidstoffwechsels gezeigt werden. Für rs2758605 und rs1543294 gab es keine Unterschiede zwischen den Genotypen. Diese Daten unterstützen die Assoziation des Polymorphismus rs2241106 mit dem BMI bei prämenopausalen Frauen mit PCOS.

Zusammenfassend scheint eine Verbindung des Knochen- und Glukosestoffwechsels sehr wahrscheinlich. Klinische Studien zeigten bis dato eine größere Rolle von cOC. OC Polymorphismen waren mit dem BMI assoziiert, sowohl bei weitgehend Gesunden als auch bei Frauen mit PCOS. Die Assoziation von OC mit Testosteron ist schwach. OC und Spermiogramm-Parameter scheinen nicht miteinander in Verbindung zu stehen, wobei erst wenig Daten vorliegen.

1 INTRODUCTION

1.1 Endocrine mechanisms

Typically, cells being affected by a hormone return signals such that the hormone-producing cell is influenced in a feedback loop. Various organ systems function in the described way.

For the skeleton, we know that it is influenced by various pathologic conditions, these being for instance diabetes mellitus type 1 and 2 or hypogonadism, or by physiologic situations such as the decrease in estrogen during menopause. This may suggest, as proposed by Karsenty and colleagues, that not only glucose metabolism and reproductive functions could have an effect on the skeleton, but that bone might interact with the respective organs to return a signal, thereby keeping up a cross-talk between skeleton, pancreas, muscle, liver, and gonads. The introduction of this thesis will cover the well known side of this feedback loop and will then focus on the role of bone in glucose metabolism and reproduction.

1.2 Diabetes and bone

Diabetes mellitus is a disease with an increasing prevalence worldwide and can be accompanied by numerous complications involving different organ systems including the skeleton. However, type 1 and type 2 diabetes mellitus affect bone differently.

Patients with type 1 diabetes mellitus, which mostly manifests in young adults or adolescents, bone formation seems to be impaired and accrual of peak bone mass is inadequate (1,2). In contrast, patients with type 2 diabetes mellitus have higher bone mineral density compared to healthy individuals but an increased risk of fractures due to impaired bone quality (2–5).

1.2.1 Bone mineral density (BMD)

In type 1 diabetes, BMD at the spine equals BMD in individuals without diabetes in two studies (6,7), while BMD at the femoral neck is lower (6,7). Most studies investigating BMD in type 2 diabetes have shown higher BMD values at all measurement sites (1,2).

1.2.2 Fractures

A systematic review and a meta-analysis have shown a stronger association of hip fracture incidence with type 1 diabetes. The odds ratio (OR) for hip fractures in type 1 diabetes was 6.3-6.9, for type 2 diabetes 1.4-1.7 (8,9).

The risk of fracture is also elevated in diabetes mellitus. In type 1 diabetes OR for any fracture was 1.3, in type 2 diabetes it was 1.2 in a case-control study including 124 655 patients (9). Additionally, type 2 diabetes patients show an increased risk of forearm fractures with an OR of 1.2. Type 1 diabetes is associated with an elevated risk of spine fractures (OR 2.5). Spine fractures were not elevated in type 2 diabetes (10).

An analysis of data from three prospective observational studies has shown an association of femoral neck BMD T-score and FRAX score with hip and nonspine fracture risk in patients with type 2 diabetes (11). Glycemic control, duration of diabetes, and the presence of vascular complications influence the skeletal status in patients with diabetes (1,2).

The reasons for the occurrence of fractures in diabetes are presumably a combination of clinical reasons and skeletal properties. The first include conditions enhancing the risk of falls such as polyneuropathy, impaired vision due to retinopathy and hypoglycemia (12). Further, diabetic cardiovascular consequences such as arrhythmias and heart failure may enhance the risk of falling (2) as does vitamin D deficiency (12). In diabetes, the skeleton itself also undergoes alterations that may contribute to the increased fracture risk (13). Firstly, the accumulation of advanced glycosylation endproducts (AGEs) in the organic bone matrix has been identified to render bone more brittle (13). Thus, less deformation can occur before fracturing (13). Secondly, bone formation may be weakened by AGEs due to direct interference with osteoblast development. Thirdly, cortical bone, comprising more than 80% of the whole skeleton, seems to be more affected in diabetes than trabecular bone (13). This notion is supported by the finding that cortical bone is mostly found at nonvertebral sites, which are the typical locations for fractures in diabetics (13). Cortical porosity goes undetected by osteodensitometry, which emphasizes the fact that fracture risk is increased in spite of increased BMD in T2D (13). Thiazolidinediones and insulin probably do not account for an increased risk of fracture per se, even though the use of insulin has been associated with increased fracture risk. These findings, however, most likely reflect severity of diabetes rather than the effect of insulin by itself, as summarized by Leslie and colleagues (13). In the ACCORD study no difference in fracture rate was observed between patients on the intensive or standard glycemic control, despite the fact that thiazolidinedione use was higher in the intensive group (14).

Reduction of peak bone mass in type 1 diabetes might be due to the lack of the osteoanabolic effects of insulin (2). The lack of the osteoanabolic factors amylin and preptin, also produced by ß-cells in the pancreas, might also account for a decrease in bone formation in type 1 diabetes (2).

1.3 Male reproduction and bone

It has been well investigated that androgens play a major role in young adults in the building of the skeleton and in the prevention of bone loss in the elderly man. Men with prostate cancer receiving therapy with gonadotropin-releasing hormone (GnRH) agonists leading to hypogonadism were found to have significantly lower BMD at the lateral spine and forearm in comparison to eugonadal men with prostate cancer (15). Further, a case-control study has shown that testosterone levels in men with hip fractures were significantly lower than in controls. 20, i.e. 71%, of the men with hip fractures and only 9, i.e. 32% of the controls had testosterone deficiency (16). 49 of 112 patients with 47,XXY Klinefelter syndrome, characterized by primary hypogonadism due to progressive testicular failure, showed low bone mass, either osteopenia or osteoporosis (17).

Androgens exert their actions on bone directly via the androgen receptor found on osteoblasts and, to a greater part, indirectly after aromatization to estrogens and by binding to estrogen receptor (ER) α and ERβ (18). As more women suffer from osteoporosis than men and since testosterone is metabolized into 17-beta-estradiol, estrogens seem to be more important for bone than androgens.

The effects of diabetes and androgens on bone have been extensively studied. In recent years, experimental data have revealed that the skeleton not only receives impact from glucose metabolism and sex hormones but also is able to influence glucose metabolism and reproduction in return, suggesting an endocrine role of the skeleton and a feedback loop between the respective organs. In an intervention study, eliminating both testosterone and estradiol reduced serum OC, while both sex hormones increased OC levels (19).

1.4 Bone as an endocrine organ

Stabilization, locomotion and protection of our body were the classical functions attributed to the skeleton for a long time. Further, bone was regarded a site for hematopoiesis and an important organ for calcium and phosphorus homeostasis until recently another role of the

skeleton was revealed: an endocrine role (20). Parts of this introduction were published in an altered form in Schwetz et al. (20).

It is known that obese individuals develop osteoporosis less frequently and that hypogonadal patients are at increased risk of developing osteoporosis (21). Based on these observations, Karsenty and colleagues developed a hypothesis in which they postulated a common regulation of bone, energy metabolism, and reproduction (22,23). By suggesting that adipose tissue could influence bone, they discovered that leptin, an adipocyte-derived hormone, is an important mediator of bone remodeling, exerting its functions on osteoblasts via two neural pathways (23). Bone by itself was suggested to be involved in the regulation of glucose metabolism and reproduction (21) by one of the few osteoblast-produced proteins (24).

The introduction describes how adipose tissue acts on bone mass, how the skeleton seems to influence the organs in our body, the regulatory feedback between pancreas and osteoblasts. Data from animal studies and from studies in humans are set into context.

1.4.1 Adipose tissue acts on bone mass

In their hypothesis, Karsenty and his colleagues suggested a common regulation between bone, glucose metabolism and reproductive functions. As both appetite and reproduction are controlled by the hypothalamus, it was assumed that bone metabolism also underlies the same regulatory center.

The hormone leptin is produced in the adipose tissue and inhibits appetite and favors reproductive functions in the brain (22). Leptin-deficient *ob/ob* and leptin receptor-deficient *db/db* mice (23) are obese and sterile. But in spite of being hypogonadic and having elevated glucocorticoid levels they have increased bone formation. After infusion of leptin – but not phosphate buffered saline (PBS) – into the third ventricle of *ob/ob* mice, they fully recovered their high bone phenotype, suggesting a central mechanism. When leptin was infused into wild-type mice it was also shown to lower bone mass (22), suggesting a central catabolic function of leptin on bone metabolism (22,23).

In a study with *Cre/LoxP* transgenic mice, the leptin receptor on neurons and osteoblasts was deleted (25). Neuronal deletion caused an increase in bone mass, a deletion of the receptor on osteoblasts did not affect bone remodeling (25). l/l mice with a partial gain of leptin signaling function had low bone mass at the trabecular bone (25,26), but normal appetite, energy expenditure and reproductive function (25). These findings imply that

leptin signaling causes a decrease of bone mass accrual via the sympathetic nervous system (SNS) (25). As bone mass accrual is decreased, but appetite and reproduction are unaffected, the threshold of leptin signaling needed to affect bone metabolism is assumed to be lower (27).

Studies using higher doses of leptin, however, have shown a peripheral anabolic action of leptin on bone mass. In vitro, leptin enhances proliferation of isolated fetal rat osteoblasts expressing the signaling form of the leptin receptor (28). In vivo, injection of leptin diminished bone fragility in adult male mice (28). In addition to that, cell culture experiments have shown that leptin is an inhibitor of osteoclast formation (29). In *ob/ob* mice, both i.c.v. and s.c. administration of leptin enhanced bone mineral density, bone mineral content, bone area and mineral apposition rate (30). In microcomputed tomographic analyses, *db/db* mice had significantly lower cortical and trabecular bone volume and thickness and a lower trabecular number in the tibias and the vertebrae (31). Summarizing the above mentioned studies, they clearly demonstrate a bone anabolic function of leptin via a central and peripheral mechanism.

Regarding the mechanisms of the suggested central regulation of bone metabolism by leptin it is important to note that independent of these considerations, selective serotonin reuptake inhibitors (SSRI) have been associated with increased appetite, risk of falling, diminished bone mineral density (BMD) at the hip and by trend at the spine (32). Also, an association with an increased rate of fractures (33) was observed, even after adjusting for BMD and depression (33). These findings suggest a connection between the mechanisms of action of serotonin and leptin.

To exert its functions, i.e. to inhibit appetite and bone mass accrual, leptin relies on the integrity of hypothalamic neurons, not, however, on the presence of the receptor ObRb on these neurons (34). This finding suggests the existence of an intermediary neurotransmitter.

As a matter of fact, leptin inhibits the synthesis and the release of serotonin from the raphe nuclei, thus avoiding the hypothalamic receptors (upper part of Figure 1) (27). When brain-derived serotonin is released, it binds to the serotonin receptor (HTR) 2C of ventromedial hypothalamic neurons located in the hypothalamus. Thus, signaling of the sympathetic nervous system (SNS) is decreased and bone mass accrual enhanced (34,35). Appetite is increased by the binding of leptin to two different receptors in the arcuate nuclei of the hypothalamus, i.e. HTR1A and HTR2B (34).

Serotonin thus functions as a mediator in the regulation of bone mass and appetite. Leptin decreases serotonin synthesis, which causes an increase in the signaling of the SNS. The SNS activates two different cascades via β2 adrenergic receptors on osteoblasts (36) – one is inhibition of osteoblast proliferation, the other promotion of receptor activator of nuclear factor κ B ligand (RANKL) exression (TNFSF11) (23). In total, bone resorption is increased (23).

When binding to receptors of the neurons of the arcuate nuclei, leptin triggers the expression of CART (cocaine- and amphetamine-regulated transcript) (37,38). *RANKL* expression by osteoblasts is decreased by CART and thus bone resorption hampered (39) (upper part of Figure 1). Interestingly, the functions of CART are rather those of a circulating factor than a neuropeptide as an increase in the circulating levels rescues the low bone mass phenotype of *Cart* -/- mice (40). Whether the mechanism by which CART affects gene expression in osteoblasts is direct or indirect is unknown until now (40).

In summary, via two different pathways – SNS and CART – leptin decreases bone mass accrual (23). However, leptin plays an even further-reaching role as depicted in the lower part of figure 1. On the one hand, via the SNS and β2 adrenergic receptors leptin increases the expression of *Esp* in osteoblasts, which encodes an intracellular protein tyrosine phosphatase responsible for the regulation of OC bioactivity (24,41,42), as described in further detail below. Mice in which the respective receptor was deleted showed increased serum insulin and low postprandial serum glucose levels and increased expressions in insulin, glucokinase and cyclin-dependent kinase 4 (CDK4) (42). On the other hand, leptin directly hampers insulin secretion by the β-cells (43,44).

Leptin → serotonin ↓ → SNS ↑ → osteoblast proliferation ↓ and RANKL ↑ → bone resorption ↑ (45)
Leptin → CART ↑ → RANKL ↓ → bone resorption ↓ (39)
Leptin → serotonin ↓ → SNS ↑ → ESP ↑ → decarboxylation of OC ↓ → OC bioactivity ↓ (29)
Leptin → direct influence on β-cells → insulin secretion ↓ (35,43)

Table 1: Mechanisms of action of leptin.

The common regulation of bone metabolism, appetite and reproduction might have evolutionary reasons. Constant bone remodeling and repair, i.e. after fractures, was and is

essential for mobility but costly in terms of the energy needed and thus relies on a constant supply (34).

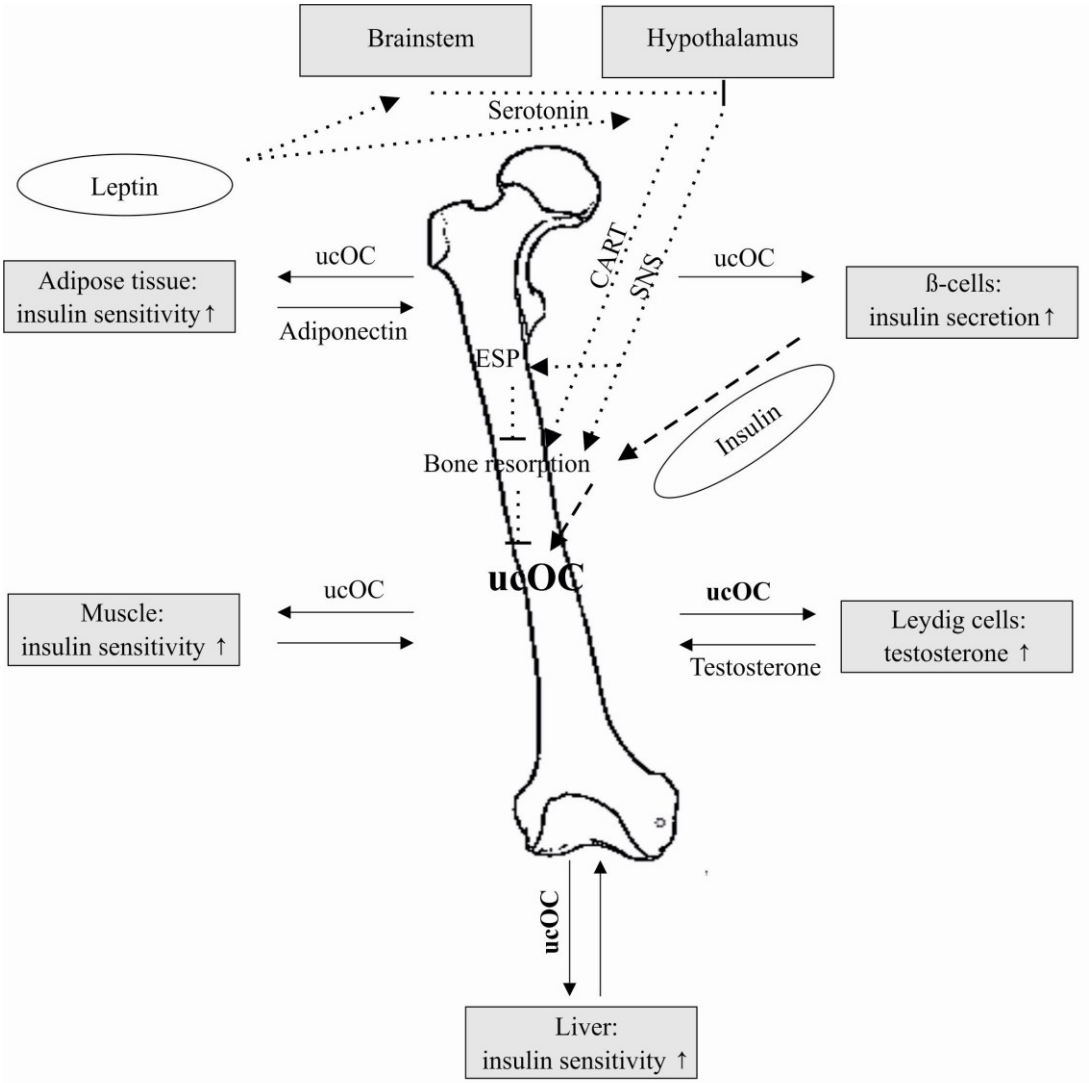


Figure 1: Bone as an endocrine organ (slightly adapted from (20)).
OC=osteocalcin, ucOC=undercarboxylated osteocalcin, SNS=sympathetic nervous system.
Upper part: Adipocyte-produced leptin inhibits serotonin synthesis in the brain. Thus, the sympathetic tone and bone resorption are enhanced. If not inhibited, serotonin would diminish sympathetic nervous system activity.
In the arcuate nuclei, leptin induces the expression of cocaine- and amphetamin-regulated transcript (CART) which decreases bone resorption. Via CART and SNS, leptin causes a decrease in bone mass accrual.
Lower part: OC bioactivity is regulated by the two hormones leptin and OC, which have opposing mechanisms. The actions of leptin are even further-reaching than depicted above.

Leptin enhances the expression of *Esp* which encodes the intracellular protein tyrosine phosphatase OST-PTP. Thereby it favors the carboxylation of OC. A decrease in *Esp* expression implies a reduction in OC bioactivity. *Esp-/-* mice have higher levels of ucOC and thus show a gain of OC function.

When insulin binds to its receptor on osteoblasts, osteoprotegerin (OPG) expression is decreased as is OPG/RANKL ratio. Bone resorption is thus enhanced and OC decarboxylated. The effects of ucOC include increase of insulin secretion in β-cells, enhancement of insulin sensitivity in the organs muscle, liver and adipose tissue. In Leydig cells, ucOC is said to increase testosterone production.

1.4.2 Bone acts as an endocrine organ via osteocalcin

The protein osteocalcin (OC), or bone GLA protein, is produced by osteoblasts and odontoblasts and has been known and used in clinical routine as a marker of bone turnover (46,47). Posttranslationally, OC is carboxylated by the enzyme γ-glutamyl carboxylase on three glutamic acid residues. The resulting carboxylated amino-acid γ-carboxyglutamic acid (Gla) can bind to free calcium and calcium-containing surfaces (48,49) and thus to bone matrix. OC has three Gla residues, thus a high affinity to bone matrix and is the predominant bone Gla protein (49), i.e. the most abundant noncollagen protein in bone (50). On the contrary, in the decarboxylated state, the affinity to hydroxyapatite decreases. As the process of carboxylation is vitamin K-dependent, vitamin K depletion increases levels of undercarboxylated OC (ucOC) and supplementation of vitamin K decreases levels of ucOC (51).

OC is released into the systemic circulation both in its carboxylated (cOC) as well as in its undercarboxylated form (24). cOC is considered the biologically inactive form and ucOC the active form with hormonal characteristics. These are cell specificity of its gene, release in a circadian pattern and synthesis as a pre-pro-molecule (49). Animal models have shown that ucOC might play an important role in energy metabolism as it enhances β-cell proliferation, insulin secretion and sensitivity and energy consumption (24). Thereby, the skeleton seems to have an effect on glucose metabolism.

1.4.3 Osteocalcin and glucose metabolism

The effects of the osteoblast-produced protein OC on energy metabolism were shown in *OC-/-* mice. These mice have lower insulin secretion and sensitivity, less ß-cell

proliferation, decreased energy expenditure and a higher fat mass than the wild-type (24). They develop insulin resistance and glucose intolerance (24), but have a normal bone phenotype (21). By contrast, mice lacking *Esp* – *Esp* being a gene expressed in osteoblasts and Sertoli cells only, encoding a receptor-like protein tyrosine phosphatase called OST-PTP (or protein tyrosine phosphatase 1B (PTP 1B as the counterpart in humans) – have increased ß-cell proliferation, insulin secretion and sensitivity (24). They show the opposite metabolic phenotype compared to *OC-/-* mice (24). At birth, *Esp-/-* mice have severe hypoglycemia and also as adults, their glucose levels are low. C-peptide and insulin are higher, as is glucose tolerance and insulin sensitivity in spite of hyperinsulinemia, compared to wild-type mice (24). The reasons for these differences is that the number of islets, islet size, and ß-cell mass are increased in *Esp-/-* mice (24). In addition, these mice possess lower liver fat content, body weight and triglyceride levels, while energy expenditure and serum adiponectin levels are higher (24). Due to a decrease in osteoprotegerin (OPG), bone resorption is increased in these mice (52).

Esp-/- mice missing one allele of OC or *Esp-/-* mice treated with an antiresorptive drug, show a reversal of their metabolic phenotype (52). As *OC-/-* and *Esp-/-* mice demonstrate opposing metabolic phenotypes, it can be concluded that in the latter there is a gain of OC activity. OC expression in these mice is normal, as OST-PTP does not regulate OC expression, but rather regulates the degree of OC gamma-carboxylation and thus OC activity (24) as described below.

1.4.4 Mechanisms of action of osteocalcin

For this to happen, insulin must first bind to its receptor – a tyrosine kinase – on osteoblasts (Figure 2). The insulin signaling cascade can be interrupted by the tyrosine phosphatase OST-PTP encoded by *Esp*. If not, the binding of insulin lowers OPG expression and the OPG/RANKL ratio and thus bone resorption is increased. Bone resorption causes acidification of the bone extracellular matrix (52). The acidic milieu in the resorption lacunae enables the release of OC from the bone matrix and induces its decarboxylation. The undercarboxylated form of OC (ucOC) is released into the systemic circulation where it exerts its effects on insulin-sensitive tissues (52).

Insulin, as a regulator of OC bioactivity, is thus a major counterplayer of leptin and takes on a pivotal role in linking bone remodeling and energy metabolism.

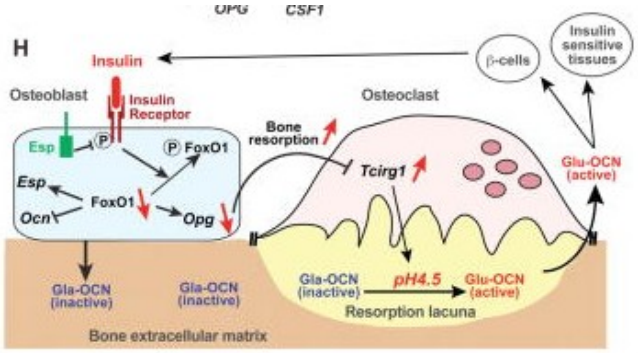


Figure 2: Bone resorption-dependent activation of OC (52).
Insulin signaling in osteoblasts decreases in a FoxO1-dependent manner *Opg* expression (52). Insulin signaling can be inhibited by *Esp* (52). If not, bone resorption is promoted, *Tcigr1* expressed and bone extracellular matrix acidified (52). Thus OC is decarboxylated, released into the systemic circulation and influences β-cell proliferation, insulin secretion and insulin sensitivity (52).

1.4.5 Bone and adipose tissue hormones

OC increases the expression of the gene Adipoq encoding adiponectin in adipose tissue, thereby enhancing insulin sensitivity (24,41). By binding to its receptors on osteoblasts adiponectin induces their proliferation and differentiation (53).
As suggested by Foresta and colleagues (54), adipocytes might also secrete OC, which expands the model by Karsenty. Foresta and others could show that visceral and subcutaneous adipose tissue expresses OC mRNA and genes involved in carboxylation (γ-glutamyl carboxylase, GGCX, and both isoforms of vitamin K-epoxide reductase complex, subunit 1, VKORC1 isoforms 1 and 2) (54) in gene expression and immunohistochemistry analyses. In vitro experiments showed that adipose tissue releases OC in its undercarboxylated and carboxylated form. It was also shown that androgens increase the expression of both forms of OC in adipose tissue.
As androgens are lower in obese men (55–58), this relationship might explain low OC levels in the same patients. More studies are necessary to investigate the contribution of osteoblast- and adipocyte-derived OC to glucose metabolism.

1.4.6 Osteocalcin and reproduction

OC seems not only to influence muscle, adipose tissue, pancreas and the liver, but, as shown in animal models, is suggested to influence male reproduction (22,23). Again, a

feedback loop between the skeleton and the testis seems likely, while female reproductive functions are not influenced by OC (59). OC is, however, solely produced by osteoblasts, but not by the testis itself (59).

When OC-/- mice are crossed with wild-type mice, litter size is significantly smaller than when crossing wild-type with wild-type (59). In OC-/- mice, the frequency of litters, testis size and weight, the weights of epididymides and seminal vesicles, sperm count and levels of testosterone were significantly decreased as compared to gain-of-function mice. Levels of luteinizing hormone were higher (59). No difference between OC-/- and wild-type mice was identified in the percentage of motile sperm, in the percentage of abnormally shaped or dead sperm (59).

By contrast, female OC-/- mice had unaltered fertility, ovary weight, uterus morphology, number of follicles and levels of sex steroids hormones (59). Thus, OC influences male fertility but has no effect on female reproductive functions. The receptor for OC is the G-protein coupled receptor GPRC6A which is, in accordance with these findings, expressed in Leydig cells but not in the ovaries (60).

The fact that OC-/- mice showed increases in LH raised the question whether OC acts downstream of LH or whether two regulatory pathways exist that determine male fertility (61). Oury and colleagues speculated that if LH were the regulator of OC reproductive functions, LH-deficient mice would show low levels of ucOC. Further, the injection of ucOC should improve the low levels of testosterone in these mice. However, neither of the two assumptions were confirmed. Additionally, ucOC injection did not improve spermatogenesis or reverse Leydig cell hypoplasia in LH-deficient mice (61). From these and further experiments the authors concluded that LH functions in an OC-independent manner (61). Also, as the OC receptor GPRC6A is not expressed in the hypothalamus and pituitary, but in Leydig cells only, OC is suggested to exert its effect on testosterone synthesis independently of the hypothalamus-pituitary-axis (61). Thus, male fertility is regulated by the hypothalamus-pituitary-axis and by a pancreas-bone-testis axis.

1.5 Clinical studies on osteocalcin and reproduction

Out of 59 men with primary testicular failure and a normal karyotype, Oury and colleagues could identify 2 men with a heterozygous amino acid substitution in a transmembrane domain of GPRC6A, a point mutation that is not observed in healthy individuals (61). This mutation prevents the receptor from localizing in the cell membrane and thus causes a loss

of function. In support of their findings from mouse models, these men were infertile, and interestingly, presented with glucose intolerance (61). Other clinical studies found ambiguous results. In the study by Kirmani et al. (62), correlations of total OC with testosterone in boys aged 11-14 years could be identified, an association that could not be shown in boys aged between 4 and 10 years or between 15 and 20 years. Between 11 and 14 years testosterone levels may be rising due to the beginning of puberty, OC levels might increase due to the phase of skeletal growth (62). Total OC and cOC correlated weakly with total testosterone in a cohort of 204 Finnish men aged between 18 and 20 years (63). In 69 men with type 2 diabetes mellitus who were about 60 years of age, a positive association was shown between ucOC and ucOC/total OC ratio with free testosterone and a negative association with LH. This association was independent of age, duration of diabetes, BMI and HbA1c (64). The authors' conclusion was that a reduction in ucOC would lead to a decline in testosterone levels and an increase in LH (64). In a cohort including 1338 men between 25 and 86 years of age from the Study of Health in Pomerania and 110 outpatients with bone disorders between 18 and 85 years of age, a positive association between total OC and total testosterone was revealed (65). In 2400 Chinese men aged 20 to 69 years, OC was positively associated with total testosterone after adjusting for age (66). After adjustment for SHBG and age, OC was associated positively with total testosterone, free testosterone and bioavailable testosterone in men with central obesity or with any components of the metabolic syndrome (66).

Other studies, however, show opposing results. No association between OC and testosterone was found in a study by Legrand which included 40 healthy male adults and 80 men with osteoporosis (67). Bolland and colleagues figured that if the connection OC-testosterone has clinical relevance in humans, antiresorptive treatment in 43 HIV-infected men with zoledronate, which lowers OC levels, should induce a decline in testosterone levels as well (68). However, no iatrogenic hypogonadism could be observed in the respective study population after a 2-year antiresorptive therapy (68). Data on semen quality was not available in any of the studies mentioned.

1.6 Clinical studies on osteocalcin and glucose metabolism

1.6.1 Evidence for total osteocalcin

One of the earliest reports on the association of OC and glucose metabolism in a clinical setting dates from 1998 (69). In this study (69), patients with type 2 diabetes mellitus were compared to healthy subjects and were found to have significantly lower baseline levels of serum OC, as was also shown in the MrOS Sweden study (70). After an improvement in glycemic control, serum levels of insulin-like growth factor 1 (IGF1) and OC increased significantly (69). Further, significant inverse correlations were shown between total OC and BMI, fat mass, plasma glucose (70,71), fasting insulin (71), insulin resistance (71–73), systemic inflammation (IL6, CRP) (71), HbA1c (53,74), adiposity measures (75) and a positive correlation with adiponectin (53,76). Negative associations of OC were also observed with ALT, AST, HOMA-IR and hepatocyte ballooning in patients with nonalcoholic fatty liver disease, who have lower levels of total OC compared to healthy individuals (77).

Weight loss in obese children caused an increase in OC levels and a decrease in leptin and insulin-resistance (78). Similarly, in adults weight loss with reduction in visceral fat mass and increase in exercise was paralleled by an increase of OC (72). In line with these results, weight loss associated with bariatric surgery was followed by an increase in total OC levels during the first 12-24 months, an effect that was independent of the procedure applied (79). After this time period, levels started decreasing (79). After glucose load, total OC was observed to decrease from baseline to 120 minutes in healthy young adults (80). In contrast to these findings, in a 7-h stepped insulin infusion accompanied by glucose clamp and somatostatin infusion, bone turnover markers, OPG and ucOC levels did not differ after intermediate or high-dose insulin infusions compared to low-dose infusions (81). Changes in insulin after a meal thus do not cause changes of the respective parameters (81).

Aerobic training in obese men and diabetics induced increases in OC and ucOC together with decreases in glucose levels (82). After power training men showed similar decreases in glucose, but only slight increases in OC and ucOC (82), while other studies investigating the effect of training on OC could not detect any change (83–86).

Pregnant women with gestational diabetes had higher OC levels than pregnant women with normal glucose tolerance. 3 months after birth, no difference in OC between the groups

could be observed (87). These findings might suggest a compensatory increase in OC during pregnancy in the face of gestational diabetes (87).

In a study of 3542 older men, the first and the fifth quintile of total OC was associated with higher all-cause and cardiovascular mortality (88). Similarly, in 2271 men at high cardiovascular risk referred to coronary angiography, a U-shaped association of OC was observed with fatal events (89). The association of low OC with all-cause, cardiovascular and non-cardiovascular mortality remained significant after adjusting for BMI, insulin resistance, prevalent diabetes and adiponectin, but was attenuated for high levels of OC (89). These findings may be based on the relationship of low OC with obesity and disturbances in glucose metabolism (89).

1.6.2 Osteocalcin, leptin, adiponectin

Clinical studies investigating the role of leptin have shown that it is strongly associated with insulin resistance and secretion in a cohort of nearly 600 men and women. Leptin and OC were inversely, adiponectin and OC were positively associated (90). Another study investigating whether the change in ucOC in response to osteoporosis treatment is associated with metabolic parameters could not show that ucOC is associated with leptin (91), but indeed positively with adiponectin (91). This is in line with cell culture experiments which have shown that ucOC enhances adiponectin expression from adipocytes (24,42).

1.6.3 Evidence for undercarboxylated and carboxylated osteocalcin

Most studies have investigated the association of total OC with parameters of glucose metabolism. Data from animal experiments have shown that it is the undercarboxylated fraction of OC hormonal characteristics are attributed to. The first study (92) investigating the role of ucOC in humans, included 199 men aged 25 to 60 years. Men in the upper tertiles of ucOC and cOC had lower fasting and stimulated glucose levels. Additionally, individuals in the highest ucOC tertile had higher HOMA-β levels (representative of beta-cell function), the highest tertile of cOC was associated with lower HOMA-IR values (92). In men, a negative association was found between ucOC and fasting plasma glucose, HbA1c, percent trunk fat and visceral/subcutaneous fat ratio, even after adjustment for possible confounders (93).

Studies in children found that higher cOC was associated with lower levels of adiponectin and leaner children with a higher ucOC/cOC ratio had higher levels of HOMA-ß (94). Heavier children showed relations of higher ucOC/cOC ratio with higher adiponectin (94). On the contrary, children with prediabetes had lower levels of ucOC compared to overweight children with normal glucose tolerance (95). In both groups cOC was associated with insulin sensitivity. Analysis of a cohort of school children aged 11 to 14 showed an inverse association of adiposity measures with total OC (75). The acute insulin response to glucose load correlated negatively with ucOC in all children investigated, the lipid risk score with ucOC only in whites (75).

Postmenopausal women with rheumatoid arthritis on antiresorptive bone-active medication have lower levels of ucOC, mirroring suppression of bone turnover (96,97). Patients with glucocorticoid therapy likewise had lower levels of ucOC (97).

The logical consequence of the findings from studies like the ones by Aonuma and Mokuda and colleagues would be that antiresorptive drugs cause a decrease in ucOC and thus disturbances in glucose metabolism. This, however, could not be shown so far and remains to be clarified (98).

Recent studies have shown results that question the extent of the influence of OC on glucose metabolism. 1,229 nondiabetic men were observed for 8.4 years, 90 (7.3%) of which developed type 2 diabetes mellitus. Differences between OC tertiles were observed in baseline BMI, percentage of body fat, triglycerides, HOMA-IR and HDL, but not glucose levels, HbA1c and, most interestingly, development of type 2 diabetes mellitus after 8.4 years (99). Another study investigating 64 daughter-premenopausal mother-maternal grandmother trios showed a noncommensurate change in parameters of OC, glucose and adipokines with age (100). The authors concluded that the association between OC and glucose metabolism is minor and age-specific in non-diabetic women (100).

All of these studies, however, have only shown associations but not causality regarding OC and glucose metabolism. Only one intervention-like study exists in humans so far. In this study, two patients with osteoid osteoma, benign tumors of the skeleton assumed to secrete OC, underwent surgical resection (101). OC levels decreased significantly after surgery and glucose levels increased, whereas other bone turnover markers did not change one day after the procedure (101). This study shows that OC has an influence on glucose metabolism in humans in the short time frame reported and that osteoid osteomas may be considered osteocalcinomas (101).

This case study provides another hint that the connection of OC with energy metabolism might also exist in humans. However, how do we reconcile the divergent results from the many differing clinical studies – association with ucOC, association with cOC, no association – and the inability of the studies in humans to reproduce the findings in murine models? Reasons may be methodical difficulties such as inhomogeneity and lack of specificity of the respective commercially available assays or species differences as to the extent of the role of OC in glucose metabolism (98). Species differences might also account for the findings that in humans parameters of glucose and lipid metabolism are associated with cOC rather than ucOC (102). Some authors suggest that cOC may affect insulin sensitivity in humans and ucOC ß-cell function, especially in prediabetic and diabetic patients (95).

1.6.4 Osteocalcin and arteriosclerosis

As a logical consequence of the association of OC with glucose and lipid metabolism, associations have also been observed between OC and parameters indicating arteriosclerosis. In the study by Kanazawa, the brachial-ankle pulse wave velocity and intima-media thickness were associated with OC in 179 men with type 2 diabetes after adjustment for multiple confounders (74). Evaluation of the abdominal aortic calcification score (ranging from 0 to 24) on a lateral lumbar radiograph showed that men, but not women, with a score of 3 or greater had significantly lower levels of OC and ucOC than men with a score of 0-2 (103). In the MINOS cohort including 774 men aged 51–85 years, higher baseline OC levels were associated with a lower abdominal aortic calcification progression rate, evaluated at 3.5 or 7 years, with an odds ratio of 0.74. Higher OC levels at baseline implied lower 10-year all-cause mortality in this cohort with a hazard ratio of 0.62 (104). In 817 men and women, OC was independently associated with carotid intima-media thickness and low OC inferred a high risk of carotid atherosclerotic plaque with an OR of 1.77 after adjustment (105).

1.6.5 Osteocalcin and bariatric surgery

Studies evaluating the effect of Roux-en-Y gastric bypass on bone turnover markers could observe an increase of OC (106) and ucOC after 6 months (107). The first study included 10 men and 10 women (106), the latter 32 individuals with a BMI of around 50 kg/m^2 (107). Thus surgically induced weight loss induced a significant increase of both OC and

ucOC after six months. 1 year after Roux-en-Y gastric bypass in 30 subjects and 1 year after gastric banding in 10 patients, OC had also significantly increased in both groups (108). A study with 23 patients undergoing Roux-en-Y gastric bypass also showed a significant increase in OC already 3, 6 and also 12 months after surgery (109).

1.6.6 Genetic variants and osteocalcin

Six genomic region variants of OC were tested in a cohort of 230 families consisting of 1112 healthy men and women (110). The association between these polymorphisms and body composition phenotypes was investigated (110). A haplotype of three single nucleotide polymorphisms (SNPs) (rs2758605-rs1543294-rs2241106) was associated significantly with BMI and circumference measurements (110). The association with BMI was subsequently confirmed in a cohort of 2244 unrelated adults (110).

1.7 Osteocalcin as a treatment in preclinical trials

To demonstrate a causal relationship between OC and glucose metabolism intervention trials are necessary. Up until now, these have only been possible in murine models, as OC cannot be administered to humans. When injecting recombinant ucOC into mice fed a normal diet, serum glucose levels decrease, insulin sensitivity increases (42,111), partly due to an increase in β-cell mass and insulin secretion (111). Moreover, when mice on a high-fat diet were treated with recombinant ucOC, insulin senstivity and glucose tolerance improved. Also, these mice were protected from diet-induced obesity due to enhanced energy expenditure. Additionally, hepatic steatosis caused by high-fat diet completely regressed upon ucOC administration (111).

As mentioned, no similar studies exist in humans up until now. An intervention-like study was achieved by administering parathyroid hormone and alendronate; the first causes a rise in ucOC, the latter a decrease (91). Three-month changes in ucOC were inversely associated with a change in body weight and fat mass over 12 months and positively with a change in adiponectin over the same time period (91).

All of the above mentioned studies present evidence that the role of OC in humans is likely to exceed that of a marker of bone turnover. Variations in the levels of OC may result from a change in bone turnover but may also represent a transient metabolic adaptation (98).

1.8 A further-reaching endocrine role

Yoshikawa and colleagues (112) have shown that partial ablation of osteoblasts in mice results in hypoinsulinemia, hyperglycemia, glucose intolerance, decreased insulin sensitivity as well as decreased gonadal fat and increased energy expenditure. Administration of ucOC reversed glucose intolerance, blood glucose and insulin levels returned to normal. However, insulin sensitivity was only partially restored, while improved gonadal fat weight and energy expenditure were not influenced at all (112).
This suggests that osteoblasts influence energy metabolism only partly via OC. The rest may be regulated in an OC-independent manner, perhaps via yet unidentified other osteoblast-produced hormones (112).

1.9 Conclusion of introduction

Both studies in mice and humans have supported the hypothesis of a link between bone, energy metabolism and reproduction via OC in recent years. Not only glucose metabolism and sex hormones influence the skeleton, but in a feedback mechanism, bone seems to have an effect on adipose tissue, muscle, liver, testis and pancreas. Which fraction of OC is responsible for the proposed effects remains to be clarified – in animal models it clearly seems to be the undercarboxylated fraction of OC. In humans, data are less clear.
It remains to be elucidated whether OC is a long-term protector against metabolic disturbances and whether it reacts to rises in glucose or insulin levels. The latter question is examined in the first part of this thesis. We further aimed to elucidate the role of OC in male fertility in humans, reflecting the second part of the thesis. To understand which fraction of OC has a more pronounced role in humans, we performed analyses with both the carboxylated as well as the undercarboxylated fraction of OC. The third aim of the thesis involves the role of genetic variants of OC in bone and glucose metabolism.

2 AIMS

To elucidate the clinical value and the importance of OC in humans regarding glucose metabolism and reproduction, this thesis included three aims.

2.1 Aim 1 – Osteocalcin and glucose metabolism

The first aim was to explore the proposed regulatory loop between insulin and OC, i.e. between the pancreas and osteoblasts. We aimed to elucidate whether patients with insulin resistance react differently to glucose load in comparison to healthy individuals. The hypothesis was that insulin resistance and its associated metabolic and hormonal conditions might hamper OC release from the osteoblasts upon oral glucose load. Oral glucose tolerance tests were performed in women investigated for polycystic ovary syndrome (PCOS) as these women are at increased risk of an adverse metabolic profile.

2.2 Aim 2 – Osteocalcin and male fertility

The aim of the second part of the thesis was to elucidate whether the assumed association of OC with testosterone exists in young adult men from infertile couples. This association has not been investigated in young otherwise healthy young men so far. Further, we aimed to clarify whether OC, ucOC, or cOC serve as predictors of sperm count.

2.3 Aim 3 – Osteocalcin and genetics

The third aim was to test the association of three OC polymorphisms with BMI in a cohort of women with polycystic ovary syndrome (PCOS). Moreover, as these women often have adverse metabolic profiles, we aimed to evaluate the possible association of OC polymorphisms with parameters of glucose and lipid metabolism. These included AUCinsulin, AUCglucose, Matsuda, QUICKI (indices for insulin sensitivity), HOMA-IR (index for insulin resistance, levels of triglycerides, total cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL).

3 METHODS

3.1 Measurement of total osteocalcin

Many different methods for the determination of total OC, cOC and ucOC exist and aggravate the comparison between studies. To give an idea of the reference ranges, the different levels of OC in men and women and of the conditions altering total OC, the publication by Hannemann and colleagues (113) is helpful. The authors determined total OC by means of IDS-iSYS N-Mid Osteocalcin assay in a healthy reference population including 1107 men, 545 premenopausal women, 498 postmenopausal women (113). The central 95% range (2.5th-97.5th percentile) was used as a reference interval. In men, median serum OC concentration was 15.4 ng/mL, in premenopausal women 14.4 ng/mL and in postmenopausal women 18.6 ng/mL (113). The highest OC concentrations were found in men and in premenopausal women between 25 and 29 years of age (113). During midlife, values were stable but started rising when women started entering menopause and exceeded the age of 65 (113). Conditions lowering OC concentrations were intake of oral contraceptives or hormone replacement therapy after menopause, diabetes mellitus and BMI <18 or >30 kg/m2 (113).

Measurement of the undercarboxylated fraction of OC has posed even more problems in terms of reliability and comparability of results. Several methods have been developed based on the assumption that affinity of OC for hydroxyapatite or barium sulfate depends on its number of carboxylated residues (114). The proportion of OC that does not bind to hydroxyapatite or barium sulfate is considered undercarboxylated OC (114). Important to note is that firstly, the amount of hydroxyapatite that is needed to bind OC depends on the source of hydroxyapatite. Secondly, the absolute amount of OC that is bound varies with the concentration of the protein in the serum. This implies that the percent unbound increases with the proportion of undercarboxylated OC. It is thus better to express the undercarboxylated fraction of OC as percent of total OC. Even when doing that, there were differences in the unbound fraction due to the total amount of OC present in the sample. The error based on the total amount of OC in the sample can range between 5 and 15% (114).

Caution is warranted using hydroxyapatite. High concentrations will absorb a significant portion of the undercarboxylated fraction. The unbound OC could thus likely be at or

below the detection limit of the assay used. If concentrations of hydroxyapatite used are too low, other serum proteins can bind and thus displace OC from hydroxyapatite (114).
Another factor that might influence determination of ucOC is the carboxylation status in the serum, which has not been tested. It may be assumed that it is similar to OC that is purified from bone which is incompletely carboxylated at the first gla residue (residue 17), ranging between 55 and 89%. Residues 21 and 24 are carboxylated in more than 90% (114).
Lastly, it should be known that various fragments of OC circulate, a major fragment being that spanning residues 1-43. These fragments might be created by proteolysis in the circulation or during sample processing. If only intact OC is measured by an assay, the unbound fraction may be lower than expected. The 1-43 fragment will bind to hydroxyapatite as does intact OC (114).

3.2 Biochemical analysis of total osteocalcin

Total OC was measured by electrochemiluminescence immunoassay (Cobas, Roche, Mannheim, Germany) (115) in serum detecting all forms of OC with a similar affinity.
Principle:
OC can be found in the serum in its unstable intact form (amino acids 1-49) and as its stable fragment (amino acids 1-43). This electrochemiluminescence immunoassay uses two monoclonal antibodies, directed specifically against epitopes on the 1-43 fragment of OC and the N-terminal fragment of OC. It thus detects the stable fragment 1-43 as well as the yet intact OC and is independent of the unstable C-terminal fragment (amino acids 43-49).

Reagents provided with the kit (115):
1. Streptavidin-covered microparticles
2. Anti-OC-Biotin monoclonal antibody
3. Anti-OC monoclonal antibody marked with ruthenium

Procedure (115):
1. Incubation of 20 µl of sample with one Anti-OC-Biotin monoclonal antibody and one ruthenium-complex marked monoclonal OC specific antibody. A sandwich complex is formed.

2. Addition of streptavidin-covered microparticles. A complex is formed via Biotin-Streptavidin interaction.
3. The microparticles bind to the surface of the electrode via a magnetic mechanism. The unbound substances are removed. Electric tension induces chemiluminescence emission which is measured by means of a photo multiplier.
4. Results are determined by use of the calibration curve.

Reference range (115):

	OC ng/ml 50^{th} percentile	OC ng/ml 5^{th} to 95^{th} percentile
Premenopausal healthy women >20 years of age	23	11-43
Postmenopausal healthy women (no hormone replacement therapy)	27	15-46
Patients with osteoporosis	27	13-48
Healthy men 18-29 years of age	40	24-70
Healthy men 30-50 years of age	25	14-42
Healthy men >50-70 years of age	24	14-46

Table 1: Reference values for total OC determined by electrochemiluminescence immunoassay (115).

3.3 Determination of undercarboxylated osteocalcin

Two methods are most commonly used for the determination of the undercarboxylated fraction of osteocalcin – the hydroxyapatite-binding assay and direct determination by ELISA specific for ucOC (Takara Bio Inc, Japan). The hydroxyapatite-binding method is based on the higher affinity of cOC towards hydroxyapatite compared to the undercarboxylated fraction. Before and after incubation with hydroxyapatite, the serum sample is measured by EIA detecting all forms of OC with a similar affinity (Cobas, Roche Diagnostics).

3.3.1 Measurement of undercarboxylated osteocalcin by means of the hydroxyapatite-binding method

Total OC is determined in serum. Afterwards, the carboxylated fraction of OC is separated from the undercarboxylated fraction by adsorption on hydroxyapatite adapted from Pietschmann and Price (116–118). After incubation with hydroxyapatite, the serum sample is again measured by EIA (Cobas, Roche Diagnostics). ucOC levels are expressed as percentage of total OC (%ucOC) measured before incubation with hydroxyapatite (114,119) as the ability of the assay to detect ucOC may depend on the concentration of total OC in the serum (114). Values for cOC were obtained by subtracting ucOC from total OC.

Protocol:

1. Add 20mg of hydroxyapatite to 200µl of serum sample.
2. Vortex.
3. Turn end-over-end for 30 minutes at room temperature.
4. Centrifuge with 1000 rounds per minute (rpm) for 5 minutes.
5. Retrieve supernatant, containing unbound (i.e. undercarboxylated) OC.
6. Throw away pellet containing hydroxyapatite and carboxylated OC.

To validate the hydroxyapatite-binding method, we performed measurements of the same 35 serum samples by means of three different methods. Firstly, ucOC was determined by means of the hydroxyapatite-binding method in Graz which showed a coefficient of variation of 7.4. Secondly, hydroxyapatite-binding method was performed in Lyon according to the protocol of Prof. Chapurlat. Thirdly, a commercially available enzyme immunoassay (EIA) (Takara Bio Inc., Japan) was used for determination of ucOC. We observed a Spearman correlation coefficient of 0.95 between our hydroxyapatite-binding method and the EIA ($p<0.001$).

ucOC determined by means of the hydroxyapatite-binding method in Graz (mean uOC 3,15 ± 1,16) and EIA (mean uOC 3,57 ± 1,76) showed a correlation according to Spearman of r=0,95 ($p<0,001$). ucOC levels measured by hydroxyapatite-binding method in Graz and Lyon (mean ucOC 4,94 ± 1,87), showed a correlation of r=0,95 (Pearson correlation coefficient, $p<0,001$). The Passing-Bablok regression line equation was y = 0,58 + 0,84 x (y = HA method Graz, x = EIA) (Figure 1). The 95% CI for the intercept ranged from 0,22

to 0,68 and for the slope from 0,70 to 1,02. The methods differed systematically by a constant amount. Yet there was no proportional difference between the methods. There was no significant deviation from linearity (p = 0,71) (Figure 3).

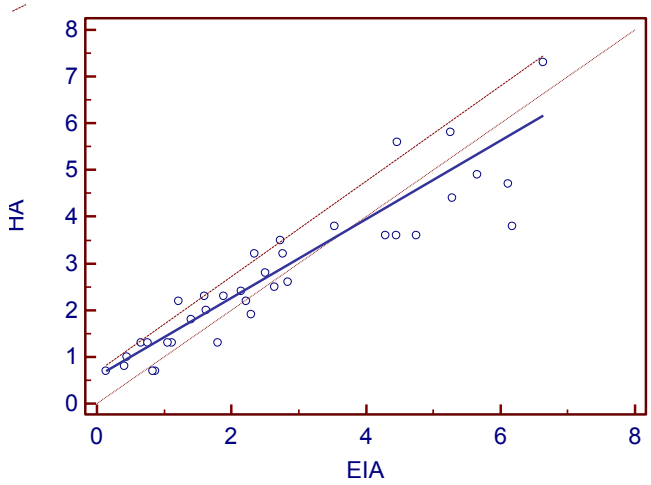


Figure 3: Passing-Bablok regression analysis between OC analysis techniques. HA=hydroxyapatite method, EIA=enzyme immunoassay.
This is a scatter diagram with the regression line (solid line), the confidence interval for the regression line (dashed lines), and the identity line (x=y, dotted line) based on both techniques.

Additionally, 10 serum samples measured for the comparison of methods were stored at -80°C and re-evaluated two months later to test for reproducibility of our results.
OC values revealed a correlation coefficient according to Pearson of r=0,988 (p<0,001) when taking the same amount of serum at both time points (500µl and 500µl) and of r=0,980 (p<0,001) when reducing the amount of serum at time point 2 (200µl) (Figure 4). The coefficient of variation (CV) to determine the reproducibility of measurements was 5,02% when taking 500µl of serum for both determinations, and 9,76% when reducing the amount of serum to 200µl at time point 2.

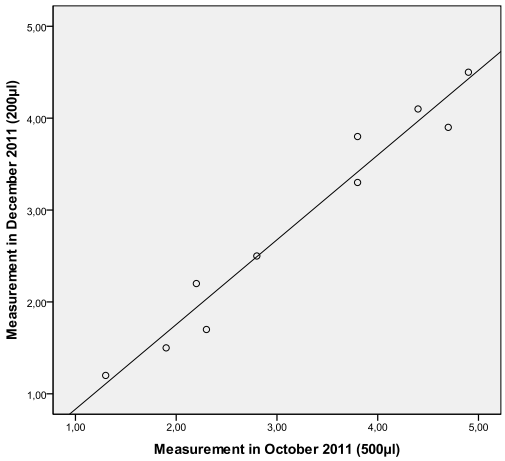


Figure 4: Correlation (r=0,980, p<0,001) between OC measurements before and after 2 months of freezing at -80°C.
For the first measurement 500µl of the samples, for the second measurement 200µl of sample were used. Results were well comparable even after 1 freeze-and-thaw cycle.

The hydroxyapatite-binding method and EIA provided comparable results. ucOC levels determined by hydroxyapatite-binding method were very well reproducible in the same samples after storage at -80°C. However, while the direct EIA for ucOC is highly specific for the undercarboxylated fraction of OC, it may not well be used in conjunction with total OC assays to determine the percentage ucOC of total OC. The reason is a difference between the assays in sensitivity to circulating OC fragments (114). Together with the reliable determination of ucOC by hydroxyapatite-binding, these were the reasons to choose this method for analysis of our samples.

3.3.2 Undercarboxylated Osteocalcin (Glu-OC) enzyme immunoassay (Takara Bio Inc., Japan) (120)

Principle

The enzyme immunoassay (EIA) for the detection of ucOC is based on a sandwich method and works in a two-step procedure. The microtiter plate is covered with immobilized mouse monoclonal anti-ucOC, which is blocked against non-specific binding. Samples and standards are pipetted into the wells of the microtiter plate. After incubation, the plate is washed and afterwards the second anti-ucOC antibody is added. The second antibody is labelled with peroxidase (POD). ucOC is thus bound to both antibodies. The reaction between POD and substrate (H2O2 and 3,3',5,5' tetramethylbenzidine) causes colour development in the wells proportional to the amount of ucOC present in the samples and

standards. Quantification of ucOC is achieved by measuring the absorbance using an EIA plate reader. By comparing the absorbances of the samples with the standard curve, the accurate sample concentrations can be determined.

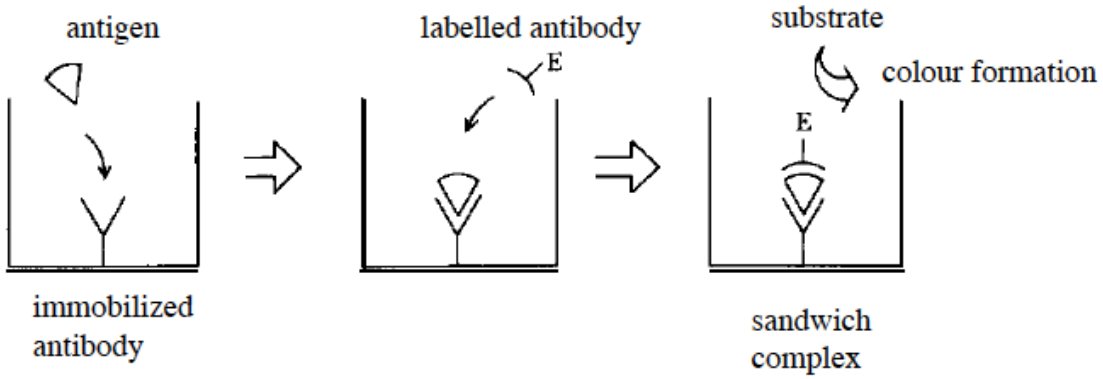


Figure 5: The principle of the EIA for the detection of ucOC based on a sandwich method (120).
The microtiter plate is covered with immobilized mouse monoclonal anti-ucOC. After addition of the serum sample ucOC binds to the antibody. After washing, the second anti-ucOC antibody labelled with peroxidase is added. The labelled antibody binds to the antigen. The reaction between peroxidase and substrate causes colour development.

Reagents and materials

Provided by the manufacturer:

- Antibody coated microtiterplate (coated with murine monoclonal antibody to ucOC)
- Antibody-POD conjugate (lyophilized horseradish peroxidase (POD) conjugated murine monoclonal antibody to OC)
- Standard (lyophilized synthetic ucOC)
- Sample diluent (protein in a buffered solution for zero standard, for dilution of the standard and samples which are above the calibration curve).
- Substrate solution (hydrogen peroxide and tetramethylbenzidine in a buffered solution)

Required:

- Washing Buffer: Phosphate-buffered Saline (PBS)

- Stop solution : 1 N H2SO4

Sample handling:
Serum samples can be stored for 24 hours at 4°C, for longer storage samples should be frozen at -20°C.

Preparation:

- The antibody-POD conjugate solution is prepared by dissolving the contents in 11 ml distilled water.
- The standard solution, containing 8 ng of ucOC/ml is mixed with 1 ml of distilled water.
- All of the kit's contents should be brought to room temperature.

Procedure:

- Pipette 100 µl sample and standard into one well within 5 minutes, mix, seal the microtiter plate with a foil and incubate for 2 hours at room temperature.
- Remove sample solution and wash the wells 3 times with ca. 400 µl of PBS.
- Antibody-POD conjugate incubation: Pipette 100 µl of antibody-POD conjugate solution, mix, seal the microtiter plate with a foil and incubate for 1 hour at room temperature.
- Remove the sample solution and wash the wells 4 times.
- Substrate incubation: Add 100 µl of substrate solution into each well and incubate at room temperature for 15 minutes.
- Add 100 µl of stop solution (1N H2SO4) into each well in the same order as for substrate and tap plate gently to mix.
- Measure the absorbance at 450 nm with a plate reader as soon as possible after the completion of the assay.

3.4 Statistics

Data are mostly presented as median with interquartile range. Kolmogorov-Smirnov test and descriptive statistics were applied for the evaluation of the distribution of data.
Parametric tests were performed for normally distributed data. For non-normally

distributed data nonparametric tests were applied or parameters were logarithmically transformed when parametric tests were performed. A p-value of 0.05 was considered statistically significant. Analyses were performed using SPSS version 18.0 (SPSS Inc., Chicago, IL).
More detailed descriptions of the analyses used are given in the according chapters.

3.5 Genetics

3.5.1 Common genetic variants

90% of the genetic differences between individuals are considered to be based on single nucleotide polymorphisms (SNPs) (121).These are single base pair positions in genomic DNA at which different sequence alternatives (alleles) can exist. The least frequent allele has to have an abundance of 1% or greater to be speaking of polymorphisms, in contrast to the more seldom point mutations (121,122). Theoretically, all four different bases (the purin bases cytosine and thymidine and the pyrimidine bases adenine and guanine) of the DNA could occur in any of these positions. As a matter of fact, however, most SNPs are bi-allelic (121). The most frequent allele in a population is referred to as the wild-type allele, the less frequent is referred to as the mutant allele, or the variant. Thus, the genotype can be homozygous for the wild-type as well as the mutant allele or heterozygous, implying that each allele carries a different nucleotide. Homozygosity implies that each allele carries the same nucleotide. Being heterozygous for a certain polymorphism may imply a reduced amount of gene product compared to the wild-type. Homozygosity may infer that no or only impaired protein is produced. If homozygous individuals have a more pronounced phenotype than heterozygous individuals, and these in turn have a more pronounced phenotype than wild-types, this is termed gene-dose effect.
One single SNP may cause a disease in either a recessive (both alleles need to be mutated in order for a disease to occur) or a dominant manner (one mutated allele is sufficient to cause the disease). Many characteristics, however, do not follow simple (Mendelian) patterns of inheritance, but have a genetically inherited component and are therefore termed complex traits (123) (page 563). These traits are often polygenic, i.e. influenced by many genes (123). The DNA of any two individuals differs in millions of polymorphisms, accounting for phenotypical properties such as height but also for the risk of diseases.

The human genome consists of about 3 billion base pairs – a SNP is found approximately every 1000 base pairs, while in some areas of the genome the occurrence is 100-fold higher (122).

SNPs can be used as markers if they are closely linked to a disease gene, meaning if the disease-causing gene and the marker SNP are close together on the chromosome and thus inherited together (123) (page 560f).

3.5.2 Preparation of DNA

With the NucleoSpin® Blood method genomic DNA can be isolated from whole blood, cultured cells, serum, plasma or other body fluids. For lysis, whole blood is incubated in a solution containing chaotropic ions and proteinase K. By adding ethanol to the lysate, DNA can bind to the silica membrane of the column. Washing steps are necessary to remove contaminations. In a slightly alkaline elution buffer, genomic DNA is finally eluted.

Isolation of DNA (124)

Chemicals and reagents:

- Buffer B3*
- Wash buffer BW*
- Wash buffer B5 (Concentrate)*
- Elution buffer BE*
- Proteinase K (lyophilized)*
- Proteinase buffer PB*
- 96–100 % ethanol
- Aqua destillata

*Contents of the NucleoSpin Blood Kit ® from Macherey-Nagel

Equipment:

- Automated pipetting system (ep-Motion 5070, Eppendorf Austria)
- Manual dispenser (Multipette ® plus, Eppendorf Austria)
- Pipettes (Eppendorf reference ® 100-1000µl, 10-100µl, 1-10µl, Eppendorf Austria)
- Centrifuge with plate adapter (Eppendorf 5810R with swing bucket and plate rotor, Eppendorf Austria)

- Shaking incubator (Microplate Shaker TiMix5 Control, Edmund Bühler, Germany)
- Incubator (BD53, Germany)
- Personal protection equipment (lab coat, disposable gloves)

Consumables:

- 96 well spin column plates (AcroPrep ™ 96 Filter Plate, Pall Life Sciences, Austria)
- 96 well deep well plate (DWP), 2 ml for sample lysis, DNA elution and waste (Nerbe plus 04-072-0020)
- Adhesive PCR film (Thermo Scientific, UK)
- Disposable filter pipette tips (Eppendorf dual filter tips 0.1-10µl, 2-100 µl, 50-100 µl, Eppendorf Austria)
- Disposable dispenser tips (Combitips plus: 0.5ml, 2.5ml, Eppendorf Austria)
- Tips for the automated pipetting system (epT.I.P.S.® Motion pipette tips with filters 20-300 µl, Eppendorf Austria)

Preparation of working solutions:
Wash buffer B5 (NucleoSpin® Blood): Add 80 mL of ethanol (96–100 %) to wash buffer B5 concentrate. Mark the label of the bottle to indicate that ethanol was added. Store wash buffer B5 at room temperature (18–25 °C) for up to one year.
Proteinase K: Add 3.35 mL proteinase buffer PB to dissolve lyophilized proteinase K. Proteinase K solution is stable at -20 °C for up to 6 months.

Procedure of DNA purification:
The standard procedures were adapted for old and/or clotted blood samples.

Before starting the preparation:

- Define the pattern of DNA on the 96 deep well plate.
- Check if buffer B5 and proteinase K were prepared accordingly
- Set an incubator or water bath to 70 °C.
- Preheat elution buffer BE to 70 °C.

1. Lysis of blood sample:

- Prepare a deep well plate with 100 µl blood and 100 µl aqua destillata, following the

predefined pattern.

- Add 15 µl proteinase K.
- Add 200 µl buffer B3.
- Centrifuge with 2000 rounds per minute (rpm) for 1 minute (min).
- Put samples in the shaking incubator with 1400 rpm for 10 min.
- Incubate samples at 55°C for 20min.

2. Adjustment of DNA binding conditions:

Add 200 µl ethanol (96-100%).

Put samples in the shaking incubator with 1400 rpm for 1 min.

3. Binding of DNA to the silica membrane:

Transfer the lysate from the lysis deep well plates into the 96 well spin column plates on top of an empty deep well plate for the collection of waste.

Centrifuge with 4400 rpm for 1 min.

4. Washing of silica membrane:

Prewash: Add 200 µl of buffer BW and centrifuge with 4400 rpm for 1 min.

1st wash: Add 200 µl of buffer B5 and centrifuge with 4400 rpm for 1 min.

2nd wash and drying of silica membrane: Add 200 µl of buffer B5 and centrifuge with 4400 rpm for 3 min. During this longer centrifugation step, residual ethanol is removed.

5. Elution of DNA:

Place the 96 well spin column plates on top of a deep well plate according to the predefined pattern of samples.

Add 50 µl of preheated buffer BE.

Incubate at room temperature for 3 min.

Centrifuge with 4400 rpm for 1 min.

The DNA has now been eluted into the deep well plate, which should be closed accurately with a PCR film. The filterplate can be discarded. The isolated DNA can be stored at 4°C for a short period of time, at -20°C for medium-term storage and at -80°C for long-term storage.

3.5.3 SNP genotyping: 5'3' Exonuclease assay (Taqman ®)

Principle:

Taqman ® 5'3' exonuclease assay is a diagnostic assay for the determination of the mutational status of a DNA sample in one step by the combination of PCR amplification and detection (125). In this assay, an oligonucleotide probe is needed in addition to the PCR amplification reaction with the primers. This probe hybridizes to the target sequence during the annealing/extension step of the PCR (125) (denaturation = disruption of hydrogen bonds between complementary bases, yielding single-stranded DNA molecules; annealing = primers bind to the single-stranded DNA template; extension/elongation = synthesis of a new DNA strand complementary to the template by the DNA polymerase). When the Taq polymerase encounters the probe, which needs to be specifically hybridized to the target sequence, the doubly-labelled probe is cleaved by the 5'3' exonuclease activity of the Taq polymerase (125). The probe is an oligonucleotide labelled with a fluorescent reporter dye and a quencher dye. As long as the probe is intact, the quencher and the reporter are in close proximity. Thus, the fluorescence energy is absorbed by the quencher by fluorescent resonance energy transfer (FRET). Upon cleavage, however, the reporter dye is released and fluorescence intensity enhanced (125). Meanwhile, polymerization of the strand continues. In summary, the detection method is based on the increase in fluorescence intensity when a reporter fluorophore is released from its quencher as the doubly-labelled probe is cleaved during PCR (126). An increase in reporter fluorescence implies that the probe-specific target has been amplified (125).

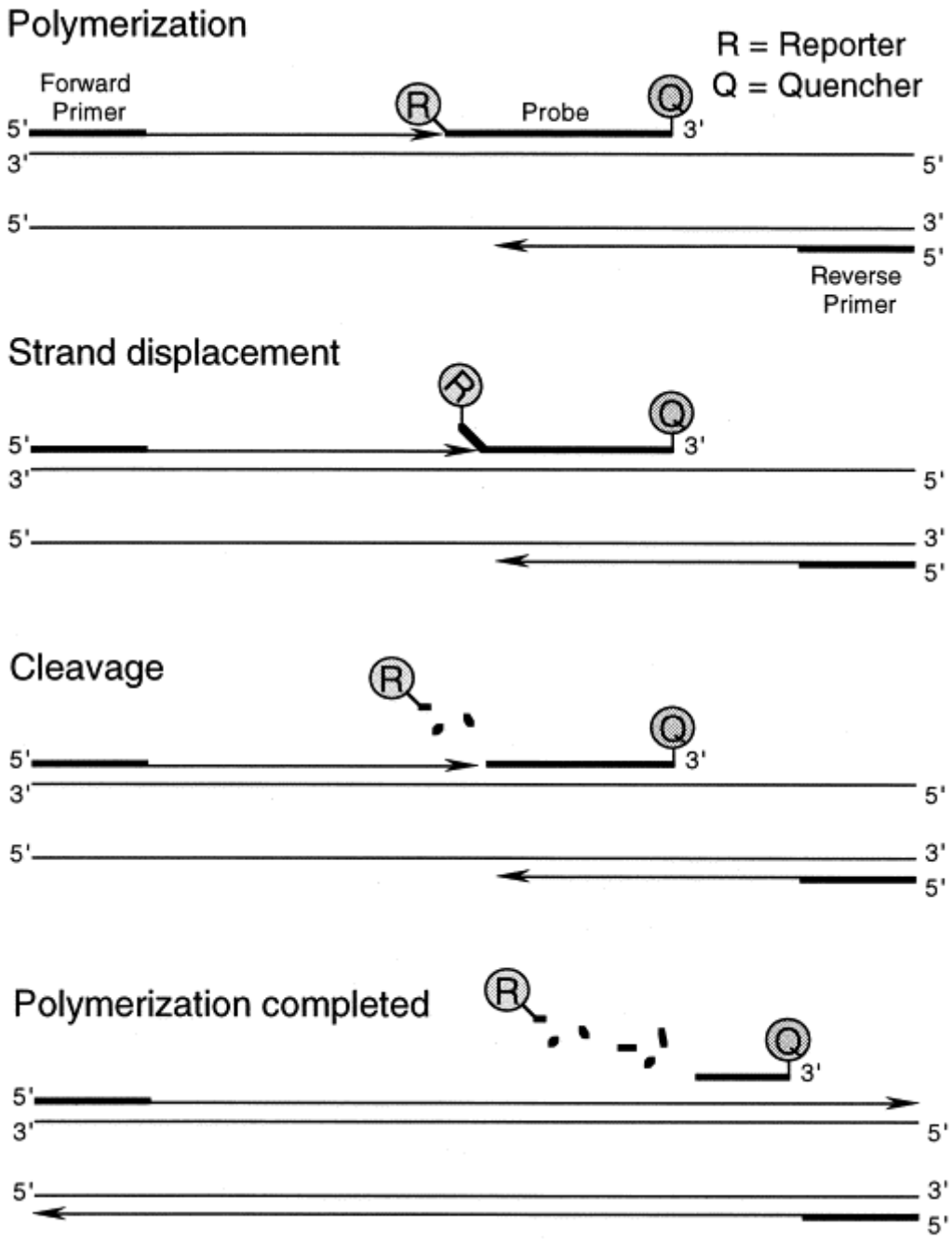


Figure 6 (125): 5′ to 3' nuclease activity of Taq polymerase acting on a fluorogenic probe during one extension phase of PCR.

5′ to 3' nuclease activity of Taq DNA polymerase acting on a fluorogenic probe is shown. Attached to the fluorogenic probe are two dyes, a fluorescent reporter R and a quencher Q. As long as the dyes are attached to the probe, emission is quenched. During the extension cycle, the reporter dye is cleaved from the probe. Thus, the reporter dye can emit its fluorescence (125).

For bi-allelic polymorphisms, two allele-specific probes (125,126), in our case labelled with either the reporter dye VIC (red) for the mutant sequences or the reporter dye 6-carboxyfluorescein (FAM, green) for the wild-type sequences, are included in the PCR assay. All probes contain the non-fluorescent quencher and a minor groove binder (MGB). The purpose of the MGB is to minimize the probe length and to enhance the specificity of the probe (126). Substantial increase in VIC or FAM fluorescent signal implies homozygosity for the VIC- or FAM-specific allele, while an increase in both signals shows homozygosity (125). Fluorescence is detected in real-time PCR thermal cycler or as endpoint measurement.

According to the protocol by Applied Biosystems (127), the Taqman ® gene expression master mix contains AmpliTaq Gold ® DNA Polymerase, UP (Ultra Pure), Uracil-DNA Glycosylase (UDG), deoxyribonucleotide triphophates (dNTPs, i.e. dATP, dCTP, dGTP, dTTP; they are added to the template DNA strand in a complementary manner by the DNA polymerase during PCR) with deoxyuridine triphosphate (dUTP), ROX™ Passive Reference, and buffer compounds.
The AmpliTaq Gold ® DNA Polymerase, UP, requires a thermal incubation step for its activation. This provides that the enzyme is only activated when the DNA is fully denatured. At room temperature, the enzyme is inactive, not capable of primer extension and thus mispriming is prevented (127).
ROX™ Passive Reference is an internal reference for normalization of the reporter-dye signal during data analysis (127).

Chemicals and Reagents:

- Taqman® SNP genotyping assay for rs2241106 (Applied Biosystems by Life Technologies, Invitrogen, Life Tech Austria, Austria), long time storage -20°C.
- Taqman® SNP genotyping assay for rs2758605 (Applied Biosystems by Life Technologies, Invitrogen, Life Tech Austria, Austria), long time storage -20°C.
- Taqman® SNP genotyping assay for rs1543294 (Applied Biosystems by Life Technologies, Invitrogen, Life Tech Austria, Austria), long time storage -20°C.
- Aqua destillata (DEPC treated, USB Corporation, UK)
- Mineral oil (Sigma-Aldrich Chemie GmbH, Germany)

Equipment:

- 12-channel pipette (Research plus pipette, 12-channel, 0.5-10µl, Eppendorf Austria)
- Manual dispenser (Multipette ® plus ,Eppendorf Austria)
- Pipettes (Eppendorf reference ® 100-1000µl, 10-100µl, 0.5-10µl, Eppendorf Austria)
- Microcentrifuge: (Hermle Z233M, Hermle Labortechnik, Germany)
- Centrifuge with plate adapter: (Heraeus Megafuge 1.OR, Heraeus Instruments, Germany)
- Vortexer (lab dancer vario, IMLAB, France)
- Personal protection equipment (lab coat, disposable gloves)
- Cycler (Mastercycler gradient, Eppendorf Austria and Duocycler, VWR International Vienna)

Consumables:

- 96 well plates (96 well thin wall PCR plates, Simport, Austria)
- Adhesive PCR films (Eppendorf, Austria)
- Disposable filter pipette tips (Eppendorf Dualfilter T.I.P.S. 0.1-10µl, 2-100µl, 50-100µl, Eppendorf Austria)
- Disposable dispenser tips (Combitips plus: 0.1ml, 0.5ml, Eppendorf Austria)
- 1.5ml reaction tubes (Safe-Lock Tubes 1.5ml, Eppendorf Austria)

Procedures:

The standard protocol has been modified and adapted for rs2758605, rs1543294 and rs2241106. Primer and probe sets were designed and manufactured by Applied Biosystems by Life Technologies (Life Tech Austria, Vienna, Austria).

Preparation:

Defrost the 96 well plates with the DNA as well as the assay mixes.

Reaction mix:

Set up a 5 µl reaction per well in a 96 well plate and overlay with 5µl of mineral oil:

- Transfer 2 µl of mixed isolated DNA from the 96 deep well plate into a 96 well plate by using a 12-channel pipette.

- Prepare the Mastermix for 96 reactions and add 11 reactions as a pipetting reserve, as shown in the table below.
- Pipette 3µl of Mastermix into each well of the 96 well plate by using a Multipette.
- Mix DNA and Mastermix.
- Overlay the reaction mix (a total of 5µl) with 5 µl of mineral oil.
- Close the 96 well plate with a PCR film.
- Spin down the reaction volume to the bottom of the well by centrifuging up to 1000 rpm.

Mastermix in µl	**x1**	**x105**
UMM (2x)	2.5	262.5
A.d.	0.42	44.1
AM (40x)	0.08	8.4
Total	3.0	

Table 2: Components of the Mastermix.

PCR reaction:

Perform 40 cycles of PCR according to the following temperature profile:

95°C 10 min	1x
92°C 15 sec 60°C 1 min	40x
4°C ∞	1x

Table 3: Cycler conditions for the TaqMan reaction.

rs-number	Gene	SNP type	Poly-morphism	Context Sequence [VIC/FAM]
rs2758605	PMF1	intron, transversion substitution, intragenic	C/G	AAAAGAGTTCATTTAACTTGAGTTT**[C/G]**TCTGTCCAAGAGGAATGAGCCATTG
rs1543294	BGLAP	intron, transition substitution, UTR 3, intragenic	C/T	AATCTGTGAGCATGCTTCCTCTGCC**[C/T]**TGGCCTCTCTTGGGCCAAGACCTGC
rs2241106	BGLAP	transversion substitution, intron, intragenic	C/G	CAAATTGCCTGCTTCTTGTTCTGTT**[C/G]**CATCCACTTCAAAGAAGCCTTTCCA

Table 4: Characteristics of investigated SNPs. All probe sets were designed and manufactured by Applied Biosystems by Life Technologies, Invitrogen (Life Tech Austria, Austria).
UTR=untranslated region.

Detection of fluorescence:

Endpoint fluorescence of the 96 well plates is measured in the Fluoroskan Ascent Microplate Fluorescence Reader (Thermo-Labsystems, Helsinki, Finland). FAM dyes are excited with a wavelength of 485 nm and emit light at 520 nm, VIC dyes with a wavelength of 520 nm and 520 nm, respectively.

After measurement, fluorescence data are exported into Excel format and displayed as a scatter plot. Relative fluorescence units of VIC dyes are shown on the y-axis, relative fluorescence units of FAM dyes on the x-axis. Each point represents a different sample. Four distinct clusters of samples are depicted.

The first cluster, located next to the y-axis, represents homozygotes for VIC, i.e. wild-types. The cluster next to the x-axis accordingly shows samples homozygous for FAM, i.e. homozygous mutated samples. The cluster in the middle depicts samples which show both alleles and are thus heterozygous. The researcher alters the borders between the clusters manually. Samples, which cannot be unequivocally assigned a genotype, are manually excluded from analysis. Samples located below the area of the three clusters do not show any amplification and thus no genotype can be assigned. These samples are coded as 91.

All results demonstrated in the scatter plot are automatically listed in a table and titled by their position on the 96 well plate.

An example of the raw data of VIC and FAM fluorescence measurements (Table 5) and of the scatter plot with the genotypes (Table 6) is given below.

Sample	VIC	FAM
Blank	0,38	0,77
Blank	0,27	0,59
WT	0,26	0,56
HE	0,25	0,55
HO	0,21	0,33
A06	1,16	4,68
A07	1,21	4,64
A08	0,40	5,90
A09	1,50	0,79
A10	1,26	0,69
A11	0,54	8,47
A12	1,36	5,52

Table 5: Raw data of VIC and FAM fluorescence measurement.

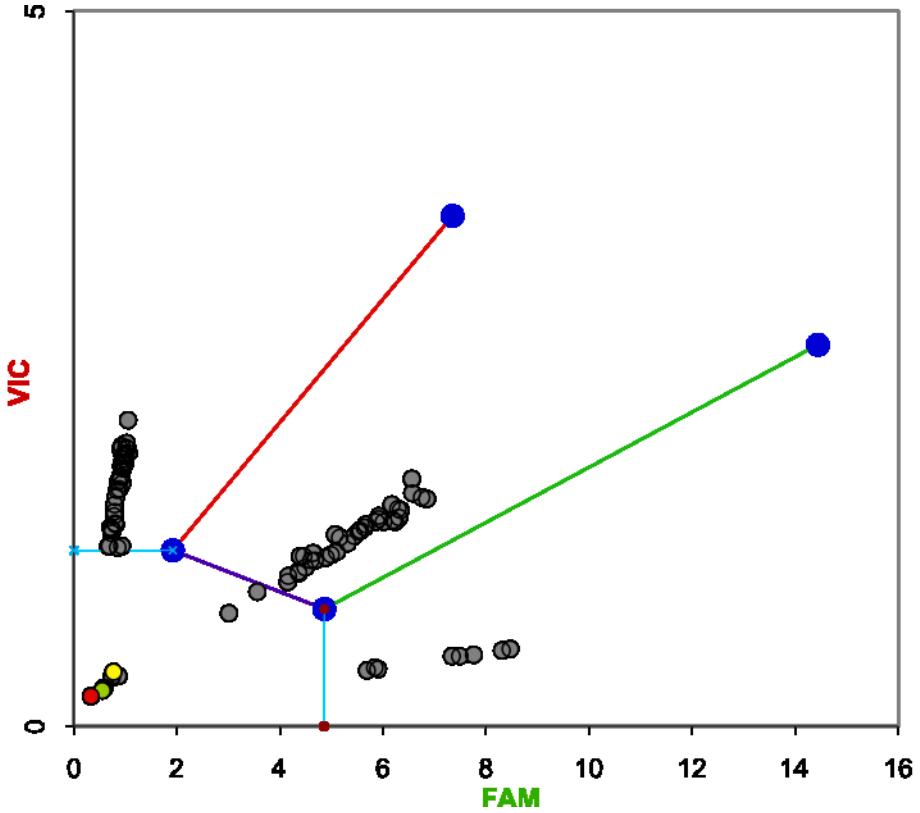


Figure 7: Scatter plot of VIC and FAM fluorescence data of rs2241106.

Quality controls (as performed in the Research Laboratory of the Division of Endocrinology and Metabolism) (128):

No-target controls:

Each 96 well plate that is analysed has a specific number and pattern of empty well positions, which are filled with aqua destillata, referred to as no-target controls. These no-target controls help to identify contaminations, sample confusions, plate confusions and distortion of plates during pipetting or measuring.

Repetition of genotyping:

For each cohort, a certain number of samples are genotyped twice to assess genotyping quality by determining the number of discrepancies between the repeatedly analysed samples.

Plausibility check:

The genotype frequencies of each plate are compared with the mean genotype frequencies of all plates taken together. A strong deviation from the mean suggests that an error may have occurred, such as the use of a wrong Mastermix for the plate in question.

Hardy-Weinberg equilibrium:

The Hardy-Weinberg equilibrium is a principle that proposes that the genetic variation in a population will not change from one generation to the next provided there are no disturbing factors (129). The theorem states that: 1. Genotype and allele frequencies will remain constant in a large population from generation to generation (130). 2. If the allele frequencies with two alleles at a certain locus are p and q, the expected genotype frequencies are p^2, 2pq, and q^2 (130).

These two assumptions only apply if the population in question fulfils certain premises:

1. Natural selection is not acting on the respective locus (130).
2. Neither mutation nor migration causes the appearance of new alleles (130).
3. There is no genetic drift to cause random changes in allele frequencies (130). Genetic drift means that allele frequencies increase or decrease by chance, typically in small populations (129).
4. Individuals in the population mate randomly (130).

These premises imply that a number of forces can disturb the Hardy-Weinberg equilibrium, which describes an idealized state and rarely applies in reality. The forces could be mutations, natural selection, non-random mating, genetic drift and gene flow (129). Gene flow is the introduction of new alleles into a population due to breeding between two different populations (130).

The Hardy-Weinberg equilibrium was checked by a Chi-square goodness-of-fit test.

4 AIM 1 – OC AND GLUCOSE METABOLISM: OSTEOCALCIN LEVELS ON ORAL GLUCOSE LOAD IN WOMEN BEING INVESTIGATED FOR POLYCYSTIC OVARY SYNDROME

4.1 Introduction to thesis part I

OC, produced by osteoblasts and odontoblasts, has been known as a marker of bone turnover (46,47) until recent studies have suggested a hormonal role of OC in regulating glucose and energy homeostasis (24). Possibly, even a regulatory circuit exists between the pancreas and osteoblasts in the bone (52).

Especially murine models have shown positive effects of the undercarboxylated form of OC on insulin-sensitive tissues, even though the carboxylated form is also found in the systemic circulation. Decarboxylation of OC takes place after the binding of insulin to its osteoblastic insulin receptor, which triggers a signalling cascade (52). After release into the blood stream, ucOC increases insulin sensitivity in liver, muscle and adipose tissue and insulin secretion and β-cell proliferation in the pancreas (24,42,111).

Accordingly, knock-out mice lacking the gene of OC are obese, insulin resistant and glucose intolerant (24). Upon injection of uncarboxylated OC in mice fed a normal diet serum glucose levels decreased and insulin secretion improved (42,111).

These data from animal models have shown clear insulin-sensitizing effects of OC. Data from studies in humans are more controversial. No intervention studies have been carried out, as OC cannot be administered to humans, which is why we rely on association studies only. A causal relationship between OC and insulin sensitivity could thus not be demonstrated in humans so far. Inverse correlations between OC and many parameters related to glucose and lipid metabolism have been shown. Amongst others, OC is inversely associated with insulin (71), insulin resistance (71–73), glucose, fat mass, BMI (70,71). Positive associations between ucOC and adiponectin levels (91,93), negative associations with glucose levels in men with T2DM were demonstrated (93). Data by Shea and colleagues revealed associations of insulin resistance with total OC and cOC, but not ucOC (131).

An intervention-like study was performed by Confavreux et al. (101). They observed that in young men with osteoid osteoma, or osteocalcinomas, the resection of the tumour went along with a decrease in OC concentration in the serum and an increased blood glucose

level (101). Inverse associations were observed between three-month changes in ucOC levels and changes in body weigth and fat mass (91).

In spite of the number of cross-sectional studies depicting associations of OC with parameters of glucose and energy homeostasis, clinical data on the regulatory circuit between the skeleton and glucose metabolism are rare. It has been shown that bone resorption markers respond to feeding with a decrease (132,133). The reaction of OC and other bone formation markers to feeding is still vastly unexplored. In individuals with normal glucose tolerance and normal insulin response during an oral glucose tolerance test, OC and cOC decrease upon glucose load (80).

The aim of the first part of the thesis (134) was to explore the proposed regulatory loop between insulin and OC, i.e. the pancreas and osteoblasts, and to examine whether OC in patients with insulin resistance reacts differently to glucose load than in healthy individuals. The hypothesis was that insulin resistance and its associated metabolic and hormonal conditions might hamper OC release from the bone in response to oral glucose load. We performed oral glucose tolerance tests to answer this question as it is feasible, easy to perform and cost-effective. The population we investigated are women investigated for polycystic ovary syndrome (PCOS), as women with this condition are at increased risk of an adverse metabolic profile. Many of these women have elevated insulin levels with mostly normal glucose values.

4.2 Subjects and Methods of thesis part I

4.2.1 Subjects

Premenopausal women who came to our outpatient clinic between 2009 and 2012 for evaluation of PCOS were recruited for the study after obtaining written informed consent. Diagnosis of PCOS was established in the presence of at least two of the following three criteria according to the Rotterdam criteria (135): clinical and/or biochemical signs of hyperandrogenism, oligo- and/or anovulation, polycystic ovaries. None of the women had a history of recent atraumatic or traumatic vertebral or nonvertebral fracture, none of the women took medications affecting glucose metabolism, mineral metabolism or endocrine parameters. Five of the women included in the study showed impaired glucose tolerance, two had type 2 diabetes according to the results obtained by the oral glucose tolerance test.

The ethics committee of the Medical University of Graz approved of the study protocol. Written informed consent was obtained from each patient.

4.2.2 Procedures

Standard anthropometric data such as weight, height, waist and hip circumference and blood pressure were collected from each patient. In the morning between 8 and 9 a.m. after an overnight fast, blood samples were obtained and a 75g-oral glucose tolerance test (OGTT) performed. After glucose load, blood samples were collected at 30, 60 and 120 minutes for the determination of glucose and insulin the same day. Afterwards, samples were stored at -70°C. Samples drawn at baseline, after 30, 60 and 120 minutes were batch analyzed for OC and ucOC.

Impaired glucose tolerance was diagnosed in case of 2h-glucose levels between 140 and 199mg/dl in the OGTT. Diabetes was defined as 2h-glucose-levels levels >= 200mg/dl. Insulin resistance was approximated using the homeostatic model assessment of insulin resistance index (HOMA-IR) which is determined as follows: fasting insulin [μU/ml] x fasting glucose [mg/dl]/405 (136). We used a cut-off of 2.6 to define insulin resistance in accordance with Ascaso and colleagues (137). They included 65 individuals in their study and chose the 75^{th} percentile to define the cut-off for insulin resistance which corresponded to a HOMA-IR index of 2.6 (137). Tripathy et al. analysed 5396 persons (138). Participants with normal glucose tolerance had a HOMA-IR index of 1.73 +- 0.03, while subjects with impaired fasting glucose had HOMA-IR values of 2.64 +- 0.08 (138).

We performed the same analyses after dividing the women into two groups according to Matsuda index to differentiate between insulin-sensitive and non-insulin-sensitive women. For this purpose, we applied a cut-off of 5 (139).

The area under the curve (AUC) of insulin or glucose during OGTT was determined by means of the standard trapezoid method. Matsuda index was applied to estimate whole-body insulin sensitivity and was calculated as 10,000/square root of the following: [(fasting glucose x fasting insulin) x (mean glucose x mean insulin during OGTT)] (140).

4.2.3 Biochemical analysis

Insulin and C-peptide were determined by enzyme-linked immunosorbent assay (ELISA) (Siemens, Erlangen, Germany), CTX and P1NP were measured by electrochemiluminescence immunoassay (Cobas, Roche, Mannheim, Germany). DHEAS

(LDN Labor Diagnostika Nord GmbH) and androstenedione (DiaMetra; BioVendor) were measured by ELISA, total testosterone (Siemens) by luminescence immunoassay and free testosterone by radioimmunoassay (Dia-Chrom).

4.2.4 Statistical analysis

Data are presented as median with interquartile range which is presented in square brackets if mentioned in the text. For evaluation of the distribution of the data, Kolmogorov-Smirnov test and descriptive statistics were applied.

Parametric tests were performed for normally distributed data, for non-normally distributed data nonparametric tests were applied. That means, to compare between groups, the Student's t-test was used for parametric variables, the Mann-Whitney-U-test for non-parametric variables. For nominal variables we used Fisher's exact test or Chi-square test.

For evaluation of the changes in OC, ucOC, and cOC upon oral glucose load, the paired Student's t-test was applied for normally distributed data; for non-parametric variables we used the Wilcoxon signed-rank test. Univariate general linear model analyses were performed when adjustment for BMI and age was needed.

The Pearson correlation coefficient was used for normally distributed parameters, for non-normally distributed data, Spearman correlation was applied.

We performed multivariable stepwise linear regression analyses using AUCinsulin and Matsuda as dependent variables and age, BMI, and OC, ΔOC (calculated as OC at 120 minutes minus OC at baseline), cOC, and ΔcOC (calculated as cOC at 120 minutes minus cOC at baseline) as independent variables. All non-normally distributed continuous parameters were logarithmically transformed when regression analysis was performed.

A p-value of 0.05 was considered statistically significant. Analyses were performed using SPSS version 18.0 (SPSS Inc., Chicago, IL).

4.3 Results of thesis part I

4.3.1 Comparison between the groups: baseline characteristics, OC, P1NP, and CTX

The women participating in the study were divided into non-insulin resistant and insulin-resistant according to HOMA-IR index calculated from fasting insulin and fasting glucose. A cut-off of 2.6 was used to discriminate between insulin resistant and non-insulin-resistant state. The baseline characteristics of the two groups of insulin-resistant women (n=18) and non-insulin-resistant women (n=87) are shown in Table 6: Characteristics of study participants. In 12 of the 18 insulin-resistant women a diagnosis of PCOS could established, which accounts for 66.7% of the group. Of the 87 non-insulin-resistant women examined, 40 women had PCOS, which is 46.0% of the group. This difference between the group was not statistically significant (p=0.090). No other endocrine abnormalities could be established in these patients.

	Insulin-resistant women n=18 **NGT=14** **IGT=2** **DM=2**		**Non-insulin-resistant women n=87** **NGT=84** **IGT=3** **DM=0**		
PCOS patients	**12/18(66.7%)**		**40/87(46.0%)**		**p=0.090**
Variable	Median	Interquartile range	Median	Interquartile range	p-value
Age (years)	26	20-40	28	23-35	0.462 (U)
Weight (kg)	99.0	90.0-103.3	63.3	56.3-73.5	<0.001 (U)
Height (cm)	168	163-171	168	161-171	0.905 (U)
BMI (kg/m2)	35.6	32.1-39.3	22.3	20.9-25.3	<0.001 (U)
Waist	105	99-109	74	71-85	<0.001

circumference (cm)					(U)
Hip circumference (cm)	118	111-122	99	94-108	<0.001 (U)
WHR	0.91	0.87-0.95	0.76	0.73-0.82	<0.001 (T)
Systolic BP (mmHg)	139	126-146	118	110-126	<0.001 (U)
Diastolic BP (mmHg)	93	82-101	80	76-89	<0.001 (U)
Free testosterone (pg/ml)	2.11	1.68-3.26	2.06	1.65-2.63	0.100 (U)
Testosterone (ng/ml)	0.54	0.39-0.66	0.41	0.30-0.64	0.328 (U)
Androstenedione (ng/ml)	2.97	1.79-6.57	3.41	1.73-6.04	0.955 (U)
DHEAS (μg/ml)	1.55	0.79-2.23	1.97	1.33-2.67	0.090 (T)
Energy metabolism					
Fasting glucose (mg/dl)	89	81-100	79	76-84	<0.001 (U)
Glucose (mg/dl) 1h	136	124-189	108	85-133	<0.001 (U)
Glucose (mg/dl) 2h	106	93-135	85	72-99	0.001 (U)
Fasting insulin (μU/ml)	16.0	14.7-19.9	3.6	2.5-5.4	<0.001 (U)
Insulin (μU/ml) 1h	130.5	74.1-201.8	39.5	19.8-60.2	<0.001 (U)
Insulin (μU/ml) 2h	83.6	37.9-145.4	21.9	13.8-39.2	<0.001 (U)
HOMA-IR	3.7	3.0-6.0	0.7	0.5-1.1	<0.001 (U)
AUCinsulin	237.5	128.4-287.8	64.7	41.4-83.2	<0.001 (U)

AUCglucose	255.4	228.6-304.3	203.8	178.1-232.6	<0.001 (U)
Matsuda	2.32	1.78-3.27	10.82	8.28-17.41	<0.001 (U)
CRP (mg/dl)	3.3	2.2-6.5	0.7	0.6-1.2	<0.001 (U)

Table 6: Characteristics of study participants.
The p-values for the unadjusted intergroup comparisons of the variables are shown on the right. Values are presented as median with interquartile range. Student's t-test (T) or Mann-Whitney U-test (U) were used to compare between groups. Fisher's exact test was applied to compare the number of women with PCOS in the two groups.
NGT=normal glucose tolerance, IGT=impaired glucose tolerance, T2DM=type 2 diabetes mellitus, BMI=body mass index, WHR=waist-to-hip ratio, HOMA-IR=homeostatic model assessment of insulin resistance, DHEAS=dehydroepiandrosterone sulfate, AUC=area under the curve, CRP=C-reactive protein.

In comparison to non-insulin-resistant women, insulin-resistant women had significantly lower levels of total OC and cOC, as shown in Table 7, but not after adjustment for BMI and age. Levels of %ucOC and ucOC, P1NP (procollagen type 1 amino-terminal propeptide) and CTX (carboxy-terminal collagen crosslinks) at baseline and 120 minutes after glucose load did not differ at all between the groups.
To exclude that our findings originate from PCOS itself, even though the number of women affected in the group of insulin-resistant and non-insulin-resistant did not differ statistically, we performed additional analyses. However, the results remained materially unchanged when analyzing PCOS and non-PCOS women separately. That means we compared parameters of OC between PCOS patients with and without insulin resistance and we compared parameters of OC between non-PCOS women with and without insulin resistance and obtained comparable results (data not shown).

	Insulin-resistant women **n=18**	**Non-insulin-resistant women** **n=87**	

Variable	Median	Interquartile range	Median	Interquartile range	p-value
Osteocalcin					
Total (ng/ml) baseline	14.3	11.6-15.3	18.0	14.5-24.7	0.001 (U)
Total (ng/ml) 60 min	12.7 (p=0.472)	11.1-16.7	14.5 (p<0.001)	11.1-17.9	0.296 (T)
Total (ng/ml) 120 min	13.1 (p=0.744)	11.3-15.7	14.6 (p<0.001)	10.9-17.8	0.521 (U)
ucOC (ng/ml) baseline	2.4	1.8-3.5	3.2	2.1-4.5	0.079 (U)
ucOC (ng/ml) 60 min	2.4 (p= 0.047)	2.0-3.3	2.6 (p<0.001)	1.8-3.4	0.140 (U)
ucOC (ng/ml) 120 min	2.3 (p=0.006)	1.7-3.1	2.5 (p<0.001)	1.7-3.5	0.358 (U)
ucOC (%) baseline	18.3	15.8-22.4	16.8	14.7-19.5	0.176 (U)
ucOC (%) 60 min	18.3 (p=0.278)	16.0-20.0	17.8 (p<0.001)	15.1-21.2	0.622 (U)
ucOC (%) 120 min	17.4 (p=0.006)	15.0-19.7	17.2 (p=0.061)	14.5-20.5	0.376 (U)
cOC (ng/ml) baseline	11.4	9.5-12.5	14.9	12.1-20.4	<0.001 (U)
cOC (ng/ml) 60 min	10.6 (p=0.379)	9.1-14.0	11.7 (p<0.001)	9.1-14.4	0.063 (U)
cOC (ng/ml) 120 min	11.0 (p=0.845)	9.5-13.2	11.1 (p<0.001)	9.0-14.5	0.655 (U)
Bone metabolism					
P1NP (ng/ml) baseline	42.8	19.9-56.1	46.4	33.8-62.9	0.133 (U)
P1NP (ng/ml) 120 min	45.0 (p=0.098)	30.1-57.1	41.6 (p<0.001)	27.2-55.7	0.829 (U)
CTX (ng/ml)	0.26	0.18-0.46	0.35	0.24-0.49	0.183 (U)

baseline					
CTX (ng/ml) 120 min	0.13 (p=0.001)	0.11-0.20	0.15 (p<0.001)	0.11-0.20	0.992 (U)

Table 7: Changes in parameters of OC, P1NP, and CTX after glucose load in insulin-resistant and non-insulin-resistant women.
The p-values for the intragroup comparisons refer to the change compared to baseline and are listed below the value at 60 or 120 minutes. The p-values for the intergroup comparison of the variables are listed in the column on the right. Values are presented as median with interquartile range. Student's t-test (T) or Mann-Whitney U-test (U) were used to compare between groups.
OC=osteocalcin, ucOC=undercarboxylated osteocalcin, cOC=carboxylated osteocalcin, P1NP=procollagen type 1, CTX=carboxy-terminal collagen crosslinks.

4.3.2 Comparison between the groups: OC, P1NP, and CTX in response to oral glucose

After a 75g oral glucose load, non-insulin resistant women showed significant decreases in total OC, ucOC, and cOC but not in %ucOC between baseline and 60 and 120 minutes, as shown in Table 7. More precisely, a decrease of total OC between baseline and 120 minutes of 19% (p<0.001) was observed, of ucOC a significant decrease of 26% (p<0.001) was observed. Details are displayed in Table 7 and Figure 8.
In insulin-resistant women, no decrease in any parameters of OC could be observed (see Figure 8 and Table 7). The baseline levels of total OC, ucOC, and cOC in insulin-resistant women are similar to the levels in non-insulin-resistant women after 120 minutes.

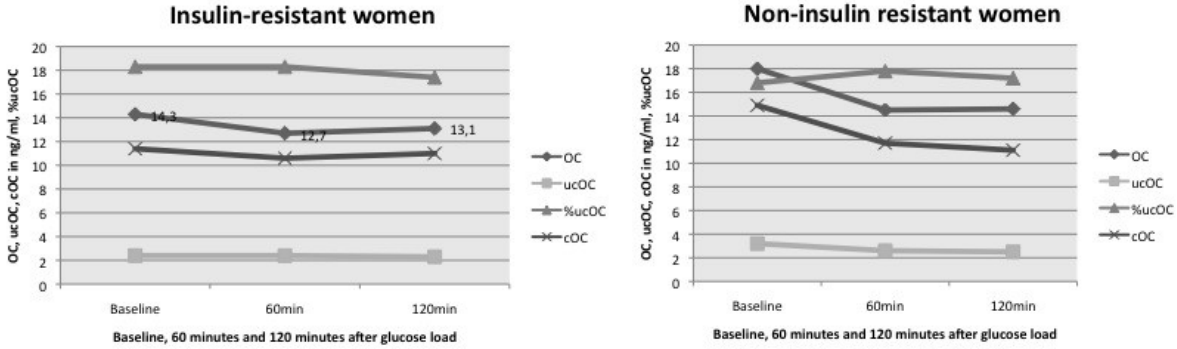


Figure 8: Parameters of OC at baseline, 60 minutes and 120 minutes after glucose load in insulin-resistant and non-insulin-resistant women.
OC=osteocalcin, cOC=carboxylated osteocalcin, ucOC=undercarboxylated osteocalcin, %ucOC=percentage of ucoC of total OC.
In non-insulin-resistant women, there was a significant decrease in levels of P1NP by 10% and in levels of CTX by 57% (p<0.001, respectively) between baseline and 120 minutes (Table 2, Figure 2). In insulin-resistant women, CTX also decreased significantly by 50% (p=0.001), while P1NP did not show a significant change (p=0.098) (
Table 7 and Figure 9).

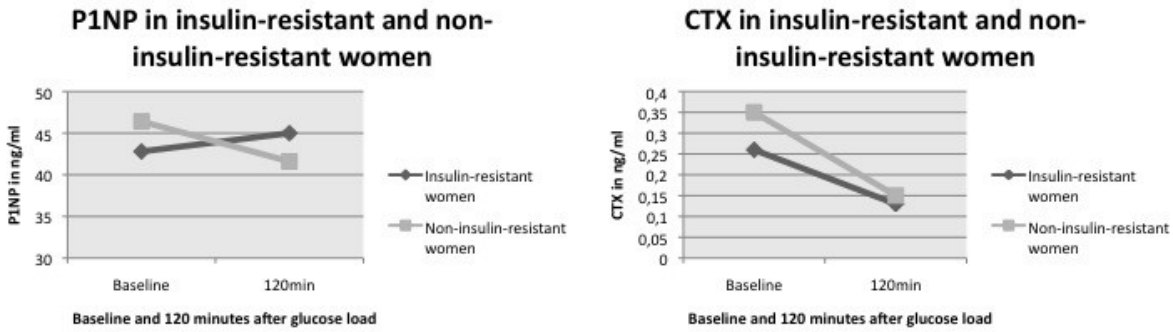


Figure 9: P1NP (ng/ml) and CTX (ng/ml) at baseline and 120 minutes after glucose load in insulin-resistant and non-insulin-resistant women.
P1NP=procollagen -1 N-terminal telopeptide, CTX=CrossLaps.

In addition to using HOMA-IR index to discriminate between insulin-resistant and non-insulin resistant women, we applied a second index to define insulin sensitivity in order to check the reliability of our results. We used Matsuda index to differentiate between insulin-sensitive and non-insulin-sensitive women, applying a cut-off of 5 (139).
22 women had a Matsuda index of equal to or below 5, 78 women of greater than 5. The women with Matsuda <=5 had a median total OC of 13.8 (IQR 11.2-15.7), the ones with Matsuda >5 of 18.4 (IQR 14.8-24.9). This difference was statistically significant (p=0.002). cOC was also significantly higher in insulin-sensitive women. The other parameters of OC were not different between the groups, similar to the results obtained when using HOMA index.

Women with a Matsuda of <=5 did not show a decline of P1NP, OC and cOC after glucose load, while women with Matsuda >5 did show this decline, comparable to our results in Table 8. The results thus remained materially unchanged and are all depicted in Table **9**.

	Matsuda <=5 n=22		**Matsuda >5 n=78**	
	Median	IQR	Median	IQR
Total OC (ng/ml)	14.4	12.2-16.4	18.2	14.9-24.7
Total OC 60 min (ng/ml)	13.8 (p=0.153)	11.4-16.8	14.5 (p<0.001)	11.3-18.0
Total OC 120 min (ng/ml)	13.6 (p=0.211)	11.3-16.5	15.1 (p<0.001)	11.0-17.8
ucOC (ng/ml)	2.7	1.8-3.7	3.1	2.1-4.6
ucOC 60 min (ng/ml)	2.7 (p=0.016)	2.0-3.4	2.7 (p<0.001)	1.9-3.4
ucOC 120 min (ng/ml)	2.5 (p=0.001)	1.7-3.1	2.5 (p<0.001)	1.8-3.5
%ucOC	17.0	15.8-21.9	16.9	14.7-19.4
% ucOC 60 min	18.3 (p=0.526)	16.0-20.4	17.9 (p<0.001)	16.2-21.4
%ucOC 120 min	17.2 (p=0.007)	15.0-20.0	17.1 (p=0.042)	14.5-20.5
cOC (ng/ml)	11.6	9.9-12.9	15.1	12.4-19.9
cOC 60 min (ng/ml)	11.3 (p=0.135)	9.7-14.0	11.9 (p<0.001)	9.0-14.5
cOC 120 min (ng/ml)	11.1 (p=0.465)	9.5-13.8	11.7 (p<0.001)	9.1-14.8

P1NP (ng/ml)	46.5	25.7-58.2	45.7	33.5-62.9
P1NP 120 min (ng/ml)	46.4 (p=0.391)	33.7-64.2	42.9 (p<0.001)	28.0-55.7
CTX (ng/ml)	0.29	0.20-0.56	0.35	0.24-0.48
CTX 120 min (ng/ml)	0.14 (p<0.001)	0.11-0.22	0.14 (p<0.001)	0.10-0.20

Table 9: Changes in parameters of OC, P1NP, and CTX after glucose load in insulin-sensitive and non-insulin-sensitive women.
The p-values for the intragroup comparisons refer to the change compared to baseline and are listed below the value at 60 or 120 minutes. The p-values for the intergroup comparison of the variables are listed in the column on the right. Values are presented as median with interquartile range. Student's t-test (T) or Mann-Whitney U-test (U) was used to compare between groups.
OC=osteocalcin, ucOC=undercarboxylated osteocalcin, cOC=carboxylated osteocalcin, P1NP=procollagen type 1, CTX=carboxy-terminal collagen crosslinks.

4.3.3 Pooled data from insulin-resistant and non-insulin resistant women – associations between OC, anthropometric parameters, serum androgens, AUCinsulin, and Matsuda

Data from insulin-resistant and non-insulin-resistant women were pooled to analyze possible associations of OC with insulin resistance, anthropometric and metabolic parameters. As shown in Table 10, significant negative correlations were found between total OC and BMI, waist and hip circumference, and WHR. The same associations were observed with the carboxylated fraction of OC, as well as with the decline in OC (ΔOC) and cOC (ΔcOC) between baseline and 120 minutes in the OGTT. Weaker, but also significant negative correlations were found between BMI, waist and hip circumference and ucOC, but not for %ucOC.
OC, cOC, ΔOC and ΔcOC correlated positively with Matsuda index. cOC correlated negatively with AUCglucose and HOMA-IR. ΔOC showed a weak negative association with AUCinsulin, ΔOC showed weak negative associations with HOMA-IR and

AUCinsulin. No significant associations were found between serum androgens or pituitary hormones and any parameters of OC (data not shown).

	Total OC		**ucOC**		**%ucOC**	
Variable	r	p-value	r	p-value	r	p-value
BMI (kg/m2)	-0.393	<0.001	-0.209	0.036	0.159	0.112
Waist circumference (cm)	-0.439	<0.001	-0.234	0.035	0.169	0.131
Hip circumference (cm)	-0.421	<0.001	-0.242	0.029	0.113	0.311
WHR	-0.360	0.001	-0.154	0.169	0.231	0.038
HOMA-IR	-0.179	0.070	-0.015	0.883	0.188	0.057
AUCinsulin	-0.088	0.383	0.084	0.401	0.201	0.043
AUCglucose	-0.178	0.070	-0.068	0.493	0.122	0.215
Matsuda	0.216	0.031	0.031	0.759	-0.194	0.053

	cOC		**ΔOC**		**ΔcOC**	
Variable	r	p-value	r	p-value	r	p-value
BMI (kg/m2)	-0.405	<0.001	0.247	0.013	0.237	0.018
Waist circumference (cm)	-0.457	<0.001	0.405	<0.001	0.392	<0.001
Hip circumference (cm)	-0.441	<0.001	0.313	0.004	0.285	0.010
WHR	-0.384	<0.001	0.425	<0.001	0.435	<0.001
HOMA-IR	-0.205	0.036	0.178	0.071	0.201	0.042
AUCinsulin	-0.137	0.171	0.237	0.017	0.269	0.007
AUCglucose	-0.197	0.044	0.147	0.135	0.154	0.119

Matsuda	0.254	0.011	-0.254	0.011	-0.287	0.004

Table 10: Unadjusted correlations between parameters of OC and BMI, waist circumference, hip circumference, WHR, HOMA-IR, AUCinsulin, and AUCglucose and Matsuda index.
As the parameters were not normally distributed, correlations were calculated according to Spearman. OC=osteocalcin, ucOC=undercarboxylated osteocalcin, cOC=carboxylated osteocalcin, ΔOC=OC at 120 minutes minus OC at baseline, ΔcOC=cOC at 120 minutes minus cOC at baseline, BMI=body mass index, WHR=waist-to-hip ratio, HOMA-IR=homeostatic model assessment of insulin resistance, AUC=area under the curve.

P1NP showed negative correlations with BMI, CTX correlated negatively with BMI, waist and hip circumference and WHR (Table 11).

	P1NP		**CTX**	
Variable	r	p-value	r	p-value
BMI (kg/m2)	-0.225	0.026	-0.256	0.011
Waist circumference (cm)	-0.185	0.103	-0.310	0.006
Hip circumference (cm)	-0.214	0.056	-0.253	0.024
WHR	-0.102	0.371	-0.255	0.024
HOMA-IR	0.118	0.242	0.061	0.545
AUCinsulin	0.177	0.081	0.164	0.106
AUCglucose	-0.069	0.494	-0.050	0.620
Matsuda	-0.113	0.271	-0.075	0.465

Table 11: Unadjusted correlations between P1NP and CTX and BMI, waist circumference, hip circumference, WHR, HOMA-IR, AUCinsulin, and AUCglucose and Matsuda index.

As the parameters were not normally distributed, correlations were calculated according to Spearman. P1NP=procollagen type 1, CTX=carboxy-terminal collagen crosslinks, BMI=body mass index, WHR=waist-to-hip ratio, HOMA-IR=homeostatic model assessment of insulin resistance, AUC=area under the curve.

4.3.4 Pooled data – decline in OC as a predictor of AUCinsulin and Matsuda index

Based on the significant correlations we found between parameters of OC and parameters of insulin resistance and sensitivity, stepwise linear regression analysis was performed. Data were again pooled to investigate whether parameters of OC can be identified as predictors of insulin sensitivity and resistance.

In the first analysis, AUCinsulin was used as dependent variable. Age (beta=-0.371, $p<0.001$), BMI (beta=0.364, $p<0.001$) as well as ΔOC (beta=0.301, p=0.001) could be identified as independent predictors of AUCinsulin (R2=0.318), while OC did not predict AUCinsulin. Similarly, age (beta=-0.355, $p<0.001$), BMI (beta=0.356, $p<0.001$) as well as ΔcOC (beta=0.315, $p<0.001$) were independent predictors of AUCinsulin (R2=0.327), while cOC was not.

In the second analysis, we used Matsuda index as dependent variable. Age (beta=0.240, p=0.002), BMI (beta=-0.600, $p<0.001$), as well as ΔOC (beta=-0.235, p=0.003) were independent predictors of Matsuda index (R2=0.462). Likewise, age (beta=0.228, p=0.003), BMI (beta=-0.594, $p<0.001$), and ΔcOC (beta=-0.245, p=0.002) independently predicted Matsuda index (R2=0.467), while OC or cOC did not.

Both ucOC and %ucOC did not reach statistical significance as independent predictors of AUCinsulin or Matsuda in age- and BMI-adjusted analyses. BMI (beta=0.371, $p<0.001$), age (beta=-0.341, $p<0.001$) as well as Δ%ucOC (beta=-0.226, p=0.014) significantly predicted AUCinsulin (R2=0.297), whereas ΔucOC did not. BMI (beta=-0.600, $p<0.001$), age (beta=0.221, p=0.006) and Δ%ucOC (beta=0.181, p=0.027) predicted Matsuda index (R2=0.450), while ΔucOC did not.

Results did not change materially when we adjusted our analyses for WHR instead of BMI. Patients with diabetes or impaired glucose tolerance were included in the analysis, as their exclusion did also not alter the results.

As 52 of the 105 patients (49.5%) had a diagnosis of PCOS, we repeated linear regression analyses with adjustment for free testosterone, testosterone, androstenedione, and DHEAS

to account for a possible influence of androgens on insulin sensitivity and resistance. When using Matsuda as dependent variable, none of androgens showed statistical significance in predicting Matsuda. Results thus remained materially unchanged.

As for AUCinsulin, free testosterone independently predicted AUCinsulin, did not alter the results of the other predictors though. Beta-coefficients and p-values after the inclusion of free testosterone were as follows: AUCinsulin: R2=0.345, ΔOC: beta=0.247, p=0.006; age: beta=-0.261, p=0.008; BMI: beta=0.322, p=0.001; free testosterone: beta=0.232, p=0.017; AUCinsulin: R2=0.357, ΔcOC: beta=0.272, p=0.002; age: beta=-0.244, p=0.011; BMI: beta=0.306, p=0.001; free testosterone: beta=0.233, p=0.015.

4.4 Discussion of thesis part I

Our data show an acute decrease of total OC, ucOC, and especially cOC upon oral glucose load in non-insulin-resistant women, but not in insulin-resistant women who have suppressed levels of all parameters of OC. The extent of the decrease of OC and cOC upon glucose load might serve as a predictor of insulin resistance and sensitivity in premenopausal women.

As glucose intolerant, insulin resistant mice can be effectively treated with recombinant uncarboxylated OC (42,111), it seems clinically important to elucidate the role of undercarboxylated and carboxylated OC in glucose metabolism in humans.

Murine models have suggested the undercarboxylated fraction of OC as the metabolically actice one, studies in humans have shown controversial results. Our results show the strongest associations between BMI, WHR, and Matsuda and parameters of OC for cOC. This finding might suggest a more important role of the carboxylated fraction of OC in glucose metabolism, particularly concerning insulin sensitivity and resistance. Other studies have also pointed towards a possibly more pronounced role of cOC in humans, such as a longitudinal study by Shea and colleagues (131). HOMA-IR was not associated with ucOC in their study, but cOC was negatively associated with 3-year changes in HOMA-IR (131). Others have shown lower levels of total OC in diabetic patients (69,70) and lower levels of total OC and ucOC in prediabetic children (95). Many publications have demonstrated correlations of total OC and metabolic parameters and parameters of energy metabolism (e.g. BMI, fat mass, glucose levels, etc.) (54,70–73). As total OC is mostly made up of the carboxylated fraction of OC, these findings might indeed suggest an important role of cOC in the clinical setting.

The reasons for these differences in the findings between murine models and studies in humans are subject to debate. One possible explanation might be species-specific differences, as suggested by Gundberg et al. (102). Other authors have proposed that in humans the carboxylated fraction of OC might be crucial for insulin sensitivity, the undercarboxylated fraction might affect ß-cell function (95). The latter association, however, has only been observed in prediabetic and diabetic patients and might not play any role in healthy individuals (95). From these assumptions we can derive for our cohort, comprised of non-insulin-resistant and insulin-resistant women with mostly normal glucose levels, that indeed the carboxylated fraction of OC may be of greater relevance.

Upon oral glucose load, decreases of CTX, P1NP, OC, cOC, and ucOC, but not of %ucOC were observed in non-insulin resistant women. Insulin-resistant women show a decline in CTX but not in P1NP, OC, ucOC or cOC was found. The changes in these parameters in non-insulin-resistant women are supported by two previous studies which measured bone turnover markers in healthy participants (80,141). In response to oral glucose, decreases of CTX, P1NP, TRACP5b (tartrate-resistant acid phosphatase), total OC, and cOC were found in 23 healthy young adults (80); in 15 healthy subjects decreases of CTX, P1NP, uNTX (urinary N-terminal telopeptide of type I collagen), OC, and PTH from baseline to 4 hours were observed. These decreases were inhibitable by octreotide (141). In addition, Christgau (142) observed an average variation in CTX of ±13.6% in fasting individuals and of ±34% under non-fasting conditions in 11 premenopausal women. In summary, all of these studies show that bone turnover markers decrease after feeding in healthy individuals. To the best of our knowledge, this effect has not been studied in insulin-resistant patients before.

Upon feeding, bone resorption markers tend to decrease to a greater extent than bone formation markers (143). From our findings we deduct that in the condition of insulin resistance, bone resorption markers behave as they do in healthy individuals, while parameters of OC are suppressed. The change in P1NP in insulin-resistant women is not significant, perhaps due to the small sample size. P1NP would be suspected to act as in non-insulin-resistant women. These findings suggest that the osteoblast response to oral glucose differs in insulin-resistant and non-insulin-resistant women, a phenomenon that might be reflected by cOC and OC.

Calciotropic hormones and cortisol have been excluded as mediators of the decrease in bone turnover markers in healthy individuals. Gut and pancreatic peptides (143) and several hormones, among them insulin, have also been suggested as regulators of this

effect. However, even though insulin is likely to have long-term effects on bone formation and bone mass (as in type 2 diabetic patients), a role in mediating the acute effect of feeding on bone resorption is unlikely (143) as also shown by a study by Basu and colleagues (81). They evaluated the effects of a 7-h stepped insulin infusion accompanied by a glucose clamp (81). P1NP, CTX, OPG, OC, and ucOC were not altered by changes in insulin concentrations during this time period. Possibly limiting the value of the study is the small number of participants (7 diabetic, 7 healthy) (81).

In contrast to these findings, Bjarnason et al. (144) do propose that part of the response in bone resorption markers to feeding might be caused by variations in insulin. GLP-2 could be a second key mediator of bone turnover processes (145,146). OC levels, however, remain unaffected by GLP-2 administration (146).

In contrast to the study by Basu and others (81), our data did reveal differences in the response of bone turnover markers to glucose load between insulin-resistant and non-insulin resistant women. The most overt differences between the groups are baseline as well as stimulated insulin levels.

Interestingly, levels of OC and ucOC in insulin-resistant women at baseline are similar to the stimulated levels at 120 minutes in non-insulin-resistant women. This may be clinically relevant in indicating a possibly preserved potential to react to rising glucose and insulin levels in non-insulin-resistant women. Insulin-resistant women seem to have suppressed levels of OC, ucOC and cOC that cannot react to glucose challenge, perhaps mediated by the constantly elevated insulin levels. In summary, osteoblast response to oral glucose in insulin-resistant and non-insulin-resistant women might differ - OC is not the mediator of this difference but could reflect this finding.

Furthermore, in our cohort of women investigated, the declines in OC, cOC and %ucOC were independent predictors of AUCinsulin and Matsuda after adjustment for BMI and age. Greater changes in OC and cOC (and smaller changes in %ucOC) between baseline and 120 minutes after oral glucose load infer a smaller AUCinsulin and higher Matsuda index. The results for Δ%ucOC oppose the ones obtained for ΔcOC which is in support of the role of the carboxylated fraction of OC.

Limitations of our study include the relatively small sample size, which might be the reason why some associations failed to reach statistical significance. Further, the mechanisms behind our observations could not be elucidated, partly because leptin, adiponectin, GIP and GLP were not determined. It was thus not possible to clarify whether acute changes in glucose or insulin or gut-derived hormones such as GIP or GLP account

for the changes in OC observed. Further experiments such as euglycemic hyperinsulinemic clamps are needed to unravel the mechanisms behind our observations. Another limitation is that we did not measure vitamin K, which influences the carboxylation of OC.

In addition, our results apply to premenopausal women only. Moreover, half of the women we included in the study had a diagnosis of PCOS, which we tried to take into account by adjusting for serum androgens in regression analyses. To check for bias, we analyzed PCOS and non-PCOS women separately – results remained materially unchanged, however.

In conclusion, these data show a decrease in OC and cOC upon glucose load in non-insulin-resistant women. The extent of the decrease is associated with insulin resistance and sensitivity in premenopausal women. In insulin-resistant women, OC and cOC are suppressed. These findings suggest an association of glucose homeostasis and the carboxylated fraction of OC in humans.

5 AIM 2 – OC AND FERTILITY: OSTEOCALCIN IS NOT A STRONG DETERMINANT OF SERUM TESTOSTERONE AND SPERM COUNT IN MEN FROM INFERTILE COUPLES

5.1 Introduction to thesis part II

4-17% of couples seek medical advice due to infertility (147,148). About 7% of all men are affected by fertility problems (149), while exclusive male causes of infertility are found in approximately 20% of involuntarily childless couples (150). In 26% problems can be found both in the male as well as in the female partner (150). 15-20% of infertile men are azoospermic, 10% have a sperm density below 1 million/ml (150). In 40-60% of all infertile men, a cause cannot be identified; most infertile men have idiopathic oligozoospermia (150). Rare, but treatable causes of infertility are gonadotropin deficiency, obstruction and coital disorders (150). The most common genetic causes are Klinefelter syndrome or Y chromosome microdeletions (150). Fertility might also be affected by certain environmental factors (149).

Recently, an influence of OC on male reproductive functions has been shown in murine experiments. After its release into the systemic circulation (52), ucOC is said to influence insulin sensitivity in muscle, liver and adipose tissue (24) and insulin secretion in the beta cells (52). In humans, associations of OC, ucOC, but also cOC with parameters of glucose metabolism (summarized in (20)) and mortality (89) have been reported.

The endocrine role of the skeleton seems to be even further reaching. Bone remodelling by the gonads has proposed a feedback loop between the gonads and the skeleton. One part of the suggested feedback loop is better known – the fact that androgens have beneficial effects on bone (18). Eliminating both testosterone and estradiol in 59 elderly men diminished serum OC, while both sex hormones enhanced OC levels (19). The other part of the feedback loop assumes that OC in turn has an influence on testosterone production, i.e. that bone influences reproductive functions at least in males (22,23). As such, the testes have been indentified as a target organ of OC (59).

In Leydig cells, OC stimulated testosterone production and as a consequence germ cell apoptosis was reduced (59). OC did not, however, have any influence on oestrogen or testosterone production in the ovaries (59), possibly because the relevant G protein-coupled receptor (GPRC6A) is not expressed (60).

Oury et al. (59) found that testosterone production in mouse Leydig cells was higher when the cells were cultured in the presence of supernatants of wild-type (WT) rather than OC -/- osteoblasts. Treatment of Leydig cells with uncarboxylated OC resulted in a dose-dependent increase of testosterone production – as did the injection of uncarboxylated OC into WT mice.

In OC knock-out mice the proposed importance of OC in male fertility became evident. These mice had significantly smaller litter sizes. Testes size and weight were significantly decreased. Furthermore, the weights of epididymides and seminal vesicles as well as sperm count were decreased as compared to Esp-/- mice, who show high levels of ucOC and the opposite metabolic phenotype compared to OC-/- mice. OC-/- mice had decreased levels of testosterone, but increased levels of luteinizing hormone (LH).

In constrast, the percentages of motile, abnormally shaped, and dead sperm did not show any differences between OC-/- and wild-type mice. OC production was only found in osteoblasts, not however, in the testis itself (59). Importantly, OC production can only be found in osteoblasts, not however, in the testis itself (59).

In a clinical study with boys aged 11-14, total OC correlated significantly with testosterone (62). Weak correlations of total OC and cOC with total testosterone could also be shown in 204 18-20-year old Finnish men (63). A study by Kanazawa and colleagues (64) also showed a positive association with serum free testosterone levels and ucOC and ucOC/total OC ratio in men with type 2 diabetes. ucOC and ucOC/total OC ratio were also negatively associated with LH, suggesting that a reduction of ucOC would decrease testosterone levels, in turn raising LH via the feedback loop (64). Furthermore, in 1338 men (25-86 years) from the population-based epidemiological Study of Health in Pomerania and in 110 male outpatients with bone disorders (18-85 years) positive associations between OC and total testosterone were also observed (65).

Of course, contradictory studies exist concerning the association of testosterone with bone turnover markers. For instance, in one study no effect of testosterone stimulation on OC production or even a decrease was observed (18). Legrand and others also could not find any association of OC with testosterone in 40 healthy men and 80 men with osteoporosis.

None of the studies mentioned, showing associations of OC and testosterone or not, has examined parameters of semen analysis. For this reason, the aim of this part of the thesis was to examine whether the assumed association of OC with testosterone exists in young adult men from infertile couples – an age group that has not been investigated yet – and

whether OC, ucOC, or cOC serve as predictors of sperm count. The results of the second part of this thesis were published in (151).

5.2 Subjects and methods of thesis part II

5.2.1 Subjects

Male adults who were referred to semen analysis were recruited from the outpatient clinic of the Department of Gynecology and Obstetrics at the Medical University of Graz between 2010 and 2012. These men were either part of an infertile couple (i.e. unable to achieve pregnancy despite unprotected intercourse for a period of greater than 12 months (152)) or desiring medical check-up in advance as they were wishing to conceive and therefore came for semen analysis. Patients with diabetes or a history of bone disease or fractures were not included in the study. None of the men we included took medications known to affect bone, mineral, energy metabolism or spermatogenesis. Also, men on vitamin D supplementation were excluded. As such, 11 patients on bone-active medication or on medication known to effect fertility could not be considered for analysis. The ethics committee of the Medical University of Graz approved the study protocol and written informed consent was obtained from each patient.

5.2.2 Procedures

Standard anthropometric data, including weight, height, waist circumference, hip circumference, and blood pressure, were obtained from each study participant. Blood samples were drawn in the morning between 8 and 9 a.m. after an overnight fast. Testosterone, free testosterone, and LH were measured as samples were collected, while total OC and ucOC were batch analyzed later. Until analysis, serum samples were stored at -70°C. The free androgen index (FAI) was calculated as (total testosterone : SHBG) x 100. Semen analysis was carried out as recommended by the WHO (153). Participants were told to collect a complete sample in a private room near the laboratory by masturbation into a container after a minimum of 2 days and a maximum of 7 days of abstinence and avoidance of large changes in temperature (153). No more than 3 hours passed between the collection time of the semen sample and microbiological investigation. As suggested by the WHO in 2010, oligozoospermia was defined as a sperm count of less than 15mio/ml

(153). In men with oligozoospermia, semen analysis was repeated – the mean values of semen volume, sperm count and vital sperms were used for analysis.

5.2.3 Biochemical analysis

Total OC and ucOC were measured as described in the general Methods section. SHBG (Roche, Basel, Switzerland) and total testosterone (Siemens, Erlangen, Germany) were measured by luminescence immunoassay. LH was determined by enzyme immunoassay (Radim diagnostics, Italy).

5.2.4 Statistical analysis

Data are presented as median with interquartile ranges. Kolmogorov-Smirnov test and descriptive statistics were applied to evaluate the distribution of data. Parametric tests were performed for normally distributed data, for non-normally distributed data nonparametric tests were applied. Alternatively, continuous parameters were logarithmically transformed for parametric tests.

Accordingly, Spearman correlation analyses were performed for non-normally distributed data, Pearson correlation analyses for normally distributed data. Stepwise linear regression was used to analyze whether parameters of OC predict testosterone or LH. Binary logistic regression analyses were used to determine whether oligozoospermia (yes/no) is predicted by parameters of OC. Analyses were adjusted for age and BMI/WHR. A p-value of <0.05 was considered statistically. All analyses were performed using SPSS version 18.0 (SPSS Inc., Chicago, IL).

5.3 Results of thesis part II

The baseline characteristics of our study cohort are shown in Table 12. 48 of 159 (30.2%) patients had oligozoospermia, 111 of 159 (69.8%) had normozoospermia. cOC and OC but not ucOC or %ucOC showed negative correlations with body mass index (BMI) (OC: r=-0.209, p=0.008, cOC: r=-0.206, p=0.010) and waist-hip ratio (WHR) (OC: r=-0.218, p=0.006, cOC: r=-0.221, p=005). No associations were observed between parameters of OC and blood pressure, HbA1c, and fasting glucose.

Testosterone showed weak but significant correlations with cOC and OC but not ucOC (OC: r=0.165, p=0.040, cOC: r=0.193, p=0.017), while %ucOC correlated inversely with

testosterone (r=-0.172, p=0.034). This emphasizes the positive correlation of cOC with testosterone.

In the associations concerning free testosterone, LH, and FAI with parameters of OC, only %ucOC, but not OC, cOC or ucOC, correlated inversely with LH (r=-0.184, p=0.023). No correlation of free testosterone with OC (r=-0.016, p=0.846), cOC (r=-0.011, p=0.893), %ucOC (r=-0.109, p=0.178) or ucOC (r=-0.054, p=505) was found. In stepwise linear regression analyses with adjustment for age and BMI or WHR, however, using testosterone as dependent variables, only age, but none of the parameters of OC were independent predictors of testosterone. OC, ucOC, and cOC did not predict LH after adjusting for age and BMI/WHR. Only %ucOC remained statistically significant as a weak predictor of LH (beta=-0.186, p=0.022, WHR/BMI and age were not significant). BMI/WHR and age are relevant confounders due to their correlation with testosterone and free testosterone.

	Median	**IQR**
Anthropometric data		
Age (years)	35	31-41
BMI (kg/m2)	25.3	23.5-28.1
WHR	0.88	0.84-0.95
Systolic blood pressure (mmHg)	135	122-145
Diastolic blood pressure (mmHg)	88	80-94
Parameters of semen analysis		
Sperm count (mio/ml)	34.4	12.1-74.9
Semen volume (ml)	3.0	2.0-4.2
Vital sperms (%)	33	22-48
Laboratory parameters		
Testosterone (ng/ml)	3.85	2.72-5.36
Free testosterone (pg/ml)	10.11	7.77-12.38
FAI	11.6	8.9-16.8
LH (mIE/ml)	3.15	2.12-4.14
FSH (mIE/ml)	3.79	2.72-5.87

Total OC (ng/ml)	21.1	17.1-27.1
ucOC (ng/ml)	3.2	2.5-3.9
%ucOC (%)	14.8	12.9-17.2
cOC (ng/ml)	17.9	14.6-22.7
25-OH-vitamin D (ng/ml)	26.6	17.1-39.6
Fasting glucose (mg/dl)	88	80-96
HbA1c (%)	5.3	5.1-5.4

Table 12: Characteristics of study participants (n=159).
BMI=body mass index, WHR=waist-to-hip ratio, FAI=free androgen index, LH=luteinizing hormone, FSH=follicle-stimulating hormone, OC=osteocalcin, ucOC=undercarboxylated osteocalcin, %ucOC=percentage of ucOC from total OC, cOC=carboxylated osteocalcin. Total OC was available in 158 subjects, as was ucOC (missing in one other patient). The other parameters were available in all 159 patients.

Age showed negative correlations with semen volume (r=-0.254, p=0.001), vital sperms (r=-0.213, p=0.007), testosterone (r=-0.201, p=0.012), free testosterone (r=-0.307, p<0.001), FAI (r=-0.307, p<0.001), FSH (r=0.206, p=0.011), BMI (r=0.168, p=0.035), and WHR (r=0.345, p<0.001). No correlation with age was, however, observed for sperm count (r=-0.018, p=0.826) or LH. Further, no correlations were found between any of the parameters of OC and sperm count, semen volume, and number of vital sperms.
We performed binary logistic regression analyses (crude model in brackets, for corrected model see Table 13) using oligozoospermia/normozoospermia as dependent variable and OC (β=-1.001, p=0.455), ucOC (β=-0.623, p=0.510), %ucOC (β=-0.607, p=0.703), and cOC (β=-0.627, p=0.649) as independent variables. None of the parameters of OC were predictors of sufficient or insufficient sperm count after adjustment for age and BMI or WHR.

	Beta	**p-value**
Corrected model		
OC	-1.196	0.391
Age	-0.202	0.920
BMI	-0.780	0.783

Corrected model		
cOC	-0.880	0.537
Age	-0.587	0.773
BMI	-0.980	0.730
Corrected model		
ucOC	-0.737	0.445
Age	-0.597	0.770
BMI	-0.939	0.739
Corrected model		
%ucOC	-0.596	0.708
Age	-0.428	0.834
BMI	-0.688	0.806

Table 13: Results of binary logistic regression analyses with oligozoospermia (yes/no) as dependent variable and parameters of OC (i.e. OC, cOC, ucOC, %ucOC) as independent variables. Correction for possible confounders (age and BMI) was performed. After adjusting for WHR results remained materially unchanged.

5.4 Discussion of thesis part II

Our data show significant correlations of cOC and OC with testosterone and of %ucOC with LH. After adjustment, only the weak association between %ucOC and LH remained significant. As no associations with testosterone and sperm count could be identified, the clinical significance of the association between %ucOC and LH seems minor. None of the parameters of OC significantly prediced oligozoospermia. Our data can currently not strengthen the assumed association of OC, testosterone, and human male fertility as observed in murine models (59).

Our results do not seem in line with the studies by Kirmani et al. (62), Kanazawa et al. (64) and Hannemann et al. (65). The first study (62) showed a significant association of total OC with testosterone in boys aged 11-14. The study by Kanazawa (64) found a significant

correlation between ucOC and ucOC/total OC ratio with serum free testosterone levels in men with type 2 diabetes. Interestingly, in the first study no correlation between testosterone and OC was found for boys younger than 11 or older than 14. Therefore, these findings might be derived from the fact that boys aged 11 to 14 experience a phase of rapid skeletal growth and are in puberty. These data must not necessarily support the postulated influence of OC on testosterone and fertility in human adults. Differences of our results to the study by Kanazawa et al. (64) might originate from an age difference of about 25 years between the study groups and the presence of diabetes in the cohort of men examined by Kanazawa. Regarding the association of total testosterone and OC in the study by Hannemann, a possible stimulatory effect of total testosterone on OC secretion may possibly be the underlying mechanism of the observed associations, as also postulated by the authors (18,65). In our cohort of patients, we find a weak association of testosterone and total OC as well as cOC before adjustment. This result can be interpreted in a two-fold manner – either the only weak association is due to the small sample or that OC is only a weak determinant of testosterone levels in humans.

In previous publications on the relationship between OC and testosterone, no data were available on semen analysis to the best of our knowledge. Previous studies (70,71) have only shown associations of OC with glucose metabolism. Our findings regarding the associations of cOC and OC with BMI and WHR are totally in line with these data.

The origin of the discrepancy regarding the data on male fertility between our results and the study by Kanazawa (64) is subject to speculation. Some of the difference may be explained by the different age groups and the diverging pre-existing conditions (non-diabetic vs. diabetic) in the study participants. The differing results reflect the controversial data on the role of OC in glucose metabolism. A recent study, for instance, could not demonstrate an association of OC with the development of type 2 diabetes in middle-aged males (99). Other authors suggest only a minor and age-specific association of OC and glucose metabolism in non-diabetic women (100).

In contrast to what has been shown in animal models, OC might have only a slight effect on testosterone levels in humans. It is likely that both the sample sizes of the study by Kanazawa as well as of our own cohort (69 and 159) are too small to satisfyingly answer this question. However, data exists supporting our assumptions. Antiresorptive bone-active medication inhibits osteoclasts and suppresses bone turnover. As such, antiresorptives can lower serum OC levels. Osteoporotic men (often hypogonadic with secondary osteoporosis) on antiresorptive treatment should therefore – if there is a strong causal

connection between OC and testosterone – have lower levels of testosterone than before treatment and the consecutive clinical signs. However, no such observations were made in any of the clinical trials (154,155). Admittedly, decreases in sperm count would presumably go undetected as semen analyses were not performed in any of the studies on antiresorptive treatment.

The choice of patients of two of the studies (62,64) that have shown human correlations between OC and testosterone is debatable. The first (62) included adolescents, the second had enrolled 60 year-old diabetics (64). Our study is the first in this setting on young adult men from infertile couples who supposedly have normal bone turnover. A normal bone turnover is necessary to minimize the effect of confounders.

Limitations of our study include the cross-sectional character of our analysis. In addition, no other bone-turnover markers other than OC were measured. This might impact the quality of our study. Furthermore, we did not evaluate alcohol consumption possibly affecting male fertility (149,156). Even though we were able to recruit a number of men, the sample size might still be too small to infer clinical consequences. However, our sample size exceeds the sample sizes of many previous studies on the topic.

Another limitation is that the high prevalence of oligozoospermia in our cohort of men might have confounded a possible association with OC. The large biological variability in semen quality might also impact our results (157). We tried to overcome this problem by repeating semen analysis in men with oligozoospermia. The mean of semen volumen, sperm count and vital sperms were then used for further analysis.

Despite these limitations, our study is the first to examine the association of OC with parameters of semen analysis in young adult men from infertile couples or from couples wishing to conceive. We find that in our cohort of men OC may not be considered a strong determinant of testosterone and semen quality. To take into account possible carboxylation-specific effects of OC we determined the carboxylated as well as the undercarboxylated fraction of OC. Further studies with larger sample sizes are warranted to elucidate the discussed findings on OC and male fertility.

6 AIM 3 – OC AND GENETICS: OSTEOCALCIN SINGLE NUCLEOTIDE POLYMORPHISM RS2241106 IS ASSOCIATED WITH BMI IN WOMEN WITH POLYCYSTIC OVARY SYNDROME

6.1 Introduction of thesis part III

After the endocrine role of OC was suggested by the results from murine experiments, many clinical studies were published showing associations of OC with parameters of glucose and lipid metabolism. Associations of OC with characteristics of body composition such as BMI and fat mass and of obesity-related phenotypes such as insulin resistance and glucose levels (70–73) were demonstrated.

OC knock-out mice were shown to be insulin resistant and glucose intolerant (24). Genetic studies in humans regarding the OC gene bone gamma-carboxyglutamate protein (BGLAP) located on chromosome 1q22 are rare. In a study by Das and Elbein, this gene was linked to type 2 diabetes (158). When sequencing the gene, 17 SNPs in African Americans and two known SNPs in Caucasians were identified, none of which were associated with T2DM (159). The authors could thus exclude BGLAP variants as major risk factors for T2DM in Caucasians (159). In African Americans, coding variants in exon 4 of this gene might play a role in glucose homeostasis and diabetes risk (159). Another study investigated the OC HindIII genetic polymorphism located at the promoter region upstream from exon 1 in 328 premenopausal Chinese women (160). Homozygote and heterozygote genotype carriers had a 2.73% and a 1.27% higher BMI than the wild-type genotype, respectively (160).

Korostishevsky and colleagues have tested whether BGLAP genomic region variants are associated with a variation of body composition phenotypes (110). They performed an association analysis between six SNPs and five obesity-related phenotypes in an ethnically homogeneous discovery sample of 230 European families composed of 1112 apparently healthy individuals (561 males and 551 females) (110). The most significant results were observed between the haplotype composed of three SNPs (rs2758605-rs1543294-rs2241106) and BMI as well as nine body circumference measurements (110). The association with BMI could be replicated in 2244 unrelated adult US Caucasians (110). Non-additive interactions between the three SNPs concerning their association with BMI are likely (110). The best association results could be shown for the G-C-G haplotype,

composed of the most frequent alleles in the SNPs rs2758605, rs1543294, rs2241106 with BMI (110).

The aim was to test the association of three OC polymorphisms with BMI in a cohort of women with PCOS. Moreover, as these women show an adverse metabolic profile, we aimed to evaluate the possible association of OC polymorphisms with parameters of glucose and lipid metabolism: AUCinsulin, AUCglucose, Matsuda, QUICKI (quantitative insulin sensitivity check index) (indices for insulin sensitivity), HOMA-IR (index for insulin resistance), levels of triglycerides, total cholesterol, HDL and LDL.

6.2 Subjects and methods of thesis part III

6.2.1 Subjects

The study cohort consisted of 680 women with PCOS and of 145 BMI-matched control women. Women with PCOS were routinely referred to our outpatient clinic for PCOS evaluation between 2009 and 2012. Control women – i.e. women with normal thyroid function, regular menstrual cycle, normal serum androgens, and without clinical signs of hyperandrogenism – were referred to our outpatient clinic for thyroid evaluation between 2009 and 2010. None of the women took any medication affecting carbohydrate metabolism, serum lipid profile or endocrine parameters for at least three months before being included in the study.

PCOS was diagnosed according to the Rotterdam criteria (135). Accordingly, women were diagnosed with PCOS if at least two of the following criteria were fulfilled: (1) polycystic ovaries by ultrasound, (2) oligo- and/or anovulation, (3) clinical and/or biochemical signs of hyperandrogenism.

Written informed consent was obtained from each patient after the local ethics committee had approved the study protocol.

6.2.2 Procedures

Standard anthropometric data, i.e. weight, height, waist circumference, hip circumference, and blood pressure were obtained from each participant. Basal blood samples were drawn in the morning between 8 and 9 a.m. after an overnight fast. Metabolic parameters –

glucose, insulin, total cholesterol, HDL, LDL and triglycerides – were determined. A 75g OGTT was performed and blood samples collected after 30, 60, and 120 minutes for glucose and insulin determination. Glucose and insulin were measured as samples were collected, and then stored at -70°C.

Insulin resistance was estimated using HOMA-IR calculated as fasting insulin [μU/ml] x fasting glucose [mg/dl]/405 (136). Integration of the insulin glucose tolerance test curve [i.e. area under the curve (AUC)] was calculated by the standard trapezoid method. Whole-body insulin sensitivity was estimated using the Matsuda index, calculated as 10,000/square root of the following: [(fasting glucose x fasting insulin) x (mean glucose x mean insulin during OGTT)] (140) and QUICKI. QUICKI is calculated as 1/log fasting insulin (mcU/ml) + log fasting glucose (mg/dl) (161).

In 295 patients, measurements of subcutaneous adipose tissue (SAT) thickness were performed on 15 body sites using a patented optical device (EU Patent No. 0516251) (162,163). Thus, total body fat, fat mass, lean mass, SAT mass and visceral adipose tissue (VAT) mass were calculated. Further details are given in the general Methods section.

6.2.3 Biochemical analysis

Insulin and C-peptide were measured by enzyme-linked immunosorbent assay (ELISA) (Siemens, Erlangen, Germany). DHEAS (LDN Labor Diagnostika Nord GmbH) and androstenedione (DiaMetra; BioVendor) were measured by ELISA, total testosterone (Siemens) by luminescence immunoassay and free testosterone by radioimmunoassay (Dia-Chrom).

6.2.4 Genotyping

Blood samples were drawn and collected in tubes containing EDTA as anticoagulant. Genomic DNA was extracted using silica membrane technology (NucleoSpin Blood; Machery-Nagel GmbH & Co., Dueren, Germany). OC polymorphisms rs2758605, rs1543294, rs2241106 were analysed using a TaqMan fluorogenic 5'-exonuclease assay. Primer and probe sets were designed by Applied Biosystems by Life Technologies (Life Tech Austria, Vienna, Austria). Fluoroskan Ascent Microplate Fluorescence Reader (Thermo-Labsystems, Helsinki, Finland) was used to measure endpoint fluorescence. Fluorescence data were analysed as scatter plot in Excel format (for an example see Figure

12). To provide a quality control, genotyping was repeated in 94 samples by two different operators. No genotype discrepancies were observed.

6.2.5 Statistical analysis

Data are presented as median with interquartile range. Kolmogorov-Smirnov test and descriptive statistics were used to evaluate the distribution of data. Parametric tests were performed for normally distributed data. For non-normally distributed data nonparametric tests were applied or continuous parameters were logarithmically transformed for parametric tests. Within the groups, ANOVA with post hoc analysis (Bonferroni) was performed for normally distributed variables, Kruskal-Wallis test with post hoc analysis (Mann-Whitney U test) was applied for non-normally distributed variables. Nominal variables were analysed using the Chi-square test. A p-value of <0.05 was considered statistically significant. All analyses were performed using SPSS version 18.0 (SPSS Inc., Chicago, IL).
Hardy-Weinberg equilibrium was tested by a Chi-square goodness-of-fit test.

6.3 Results of thesis part III

The baseline characteristics of the study participants are shown in Table 14.

Variable	Median	Interquartile range
Age (years)	24	27-32
Weight (kg)	59.8	69.5-82.6
Height (cm)	162	168-170
BMI (kg/m2)	21.4	24.2-27.6
Waist circumference (cm)	82	72-97
Hip circumference (cm)	85	76-96
WHR	0.74	0.79-0.85
Systolic BP (mmHg)	112	121-133
Diastolic BP (mmHg)	76	83-90
Free testosterone (pg/ml)	1.86	2.49-3.26
Testosterone (ng/ml)	0.44	0.57-0.76

Androstenedione (ng/ml)	1.83	2.69-4.67
DHEAS (µg/ml)	2.23	1.48-2.89
Cholesterol (mg/dl)	168.0	182.5-201.3
HDL (mg/dl)	55.8	65.5-78.0
LDL (mg/dl)	81.5	98.0-117.3
Triglycerides (mg/dl)	59	79-103
AUCinsulin	38.1	59.2-84.9
AUCglucose	150.6	174.9-199.4
QUICKI	0.35	0.37-0.42
HOMA-IR	0.6	1.1-1.9

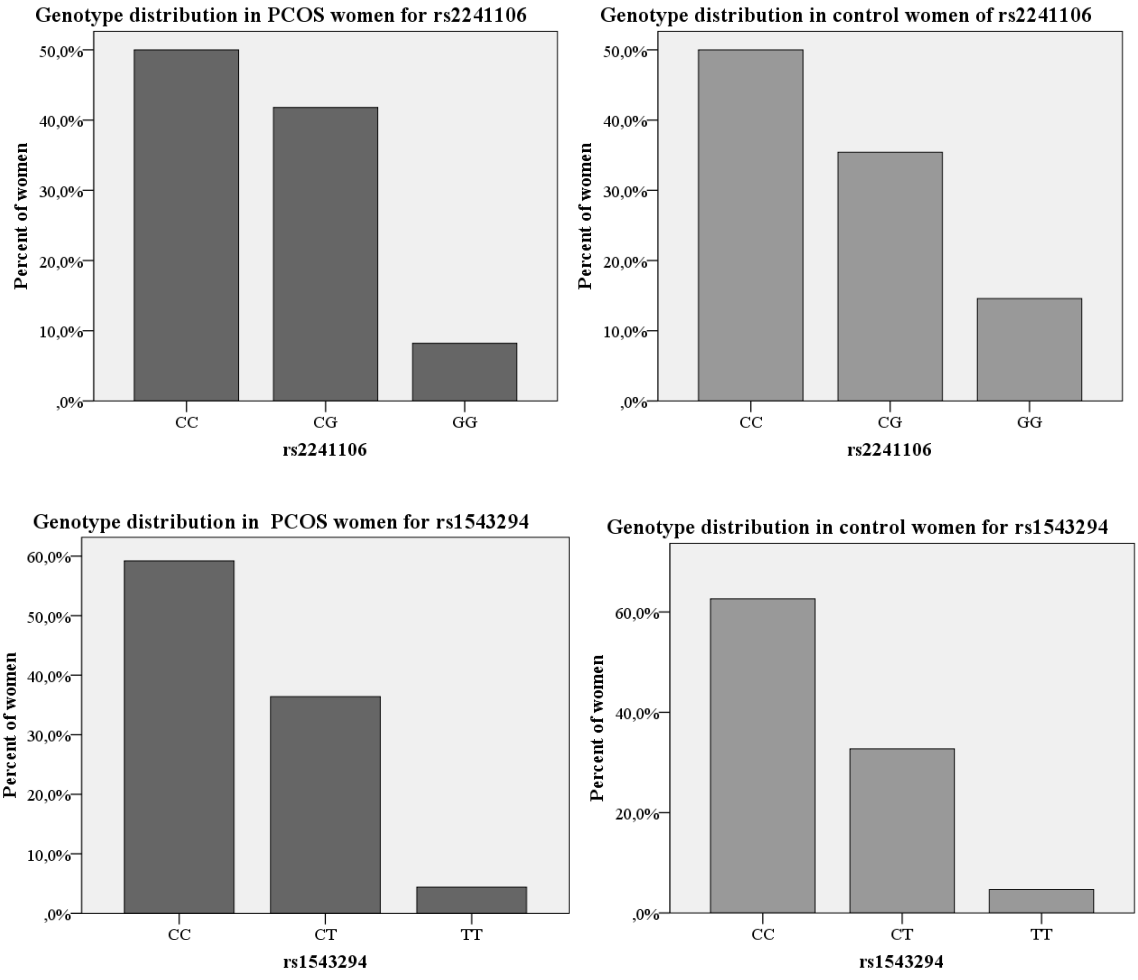
Table 14: Baseline characteristics of women with PCOS.
BMI=body mass index, WHR=waist-to-hip ratio, BP=blood pressure, DHEAS= dehydroepiandrosterone sulfate, HDL=high-density lipoprotein, LDL=low-density lipoprotein, AUCinsulin=area under the curve insulin, AUCglucose=area under the curve glucose, QUICKI=quantitative insulin sensitivity check index, HOMA-IR=homeostatic model assessment of insulin resistance.

The genotype frequencies of all three SNPs did not show a significant difference between PCOS and control women. Genotype distributions for all three SNPs are displayed in Figure 10. As for rs2241106, 50% (n=292) of PCOS women had the genotype CC, 41.8% (n=244) CG, 8.2% (n=48) GG. Of the control women, 50% (n=48) had the genotype CC, 35.4% (n=34) CG, 14.6% (n=14) GG (p=0.106). Concerning rs1543294, 59.2% (n=322) of PCOS women had the genotype CC, 36.4% (n=198) CT, 4.4% (n=24) TT. 62.6% (n=67) of the control women had the genotype CC, 32.7% (n=35) had the genotype CT, 4.7% (n=5) had the genotype TT (p=0.768). As for the polymorphism rs2758605, 11.2% (n=54) of the PCOS women had the genotype CC, 46.9% (n=227) CG, 41.9% (n=203) GG. Of the control women, 10.1% (n=10) had the genotype CC, 36.4% (n=36) CG, 53.5% (n=53) GG (p=0.099).

In the group of PCOS women and in an overall analysis, rs2241106 CC genotype carriers had a significantly higher BMI (25.0, IQR 21.9-30.9) compared to CG genotype carriers (23.8, IQR 20.8-28.9, p=0.041; p-value for PCOS only p=0.014) (Figure 11), but not compared to women carrying the GG genotype (23.7, IQR 21.0-28.0). However, in the

group of control women, this difference did not remain significant (Figure 11), presumably due to the low amount of subjects in this group. In an overall analysis rs2758605 CG genotype carriers had significantly higher cholesterol levels (182, IQR 162-203) compared to the other genotype carriers (CC: 173, IQR 156-194, GG 174, IQR 155-199, p=0.038, p-value for PCOS women only p=0,024) a difference that might be attributable to chance, as this is a single result and does not reflect a gene-dose-effect. No difference between the genotype carriers could be observed concerning any of the other parameters of glucose and lipid metabolism analysed. As for rs1543294 and rs2758605, no difference between genotypes could be observed concerning the glucose and lipid parameters investigated.

In the 295 patients for whom lipometry was performed, no differences in total body fat, fat mass, lean mass, SAT mass and VAT mass could be observed between genotypes of all three polymorphisms.



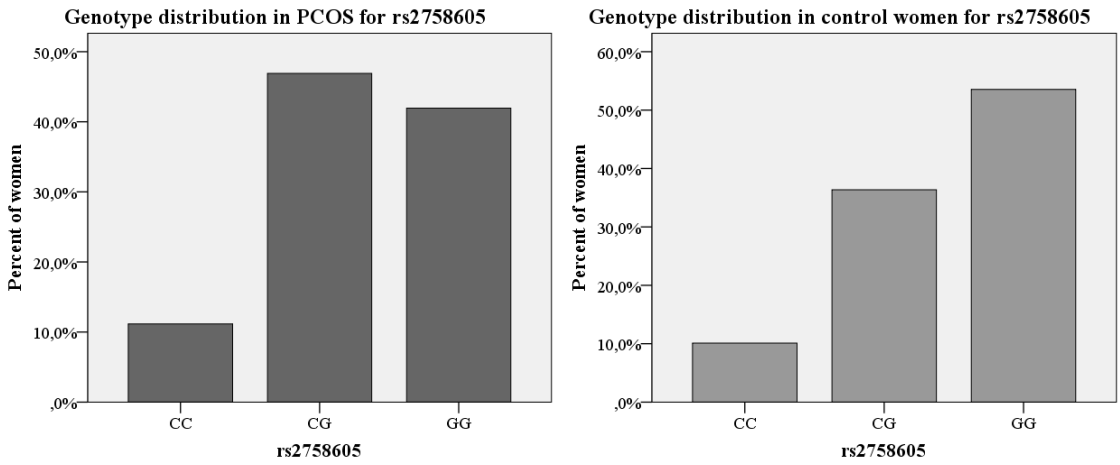
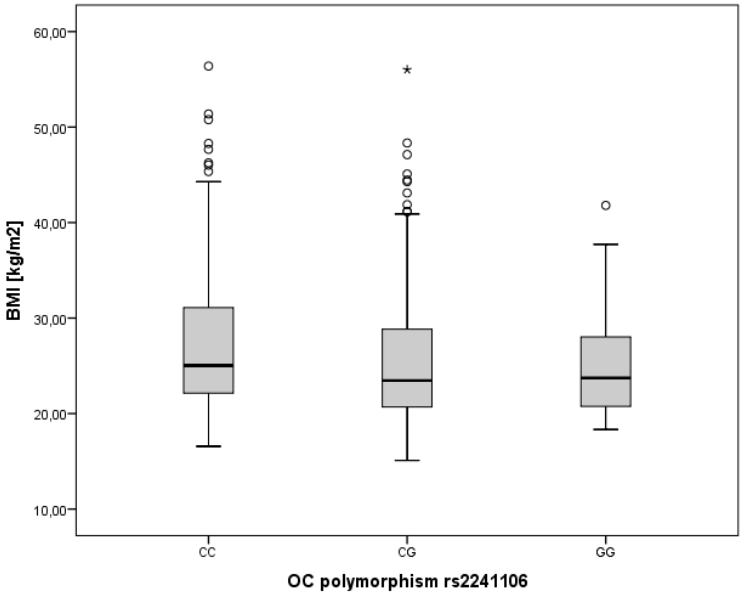
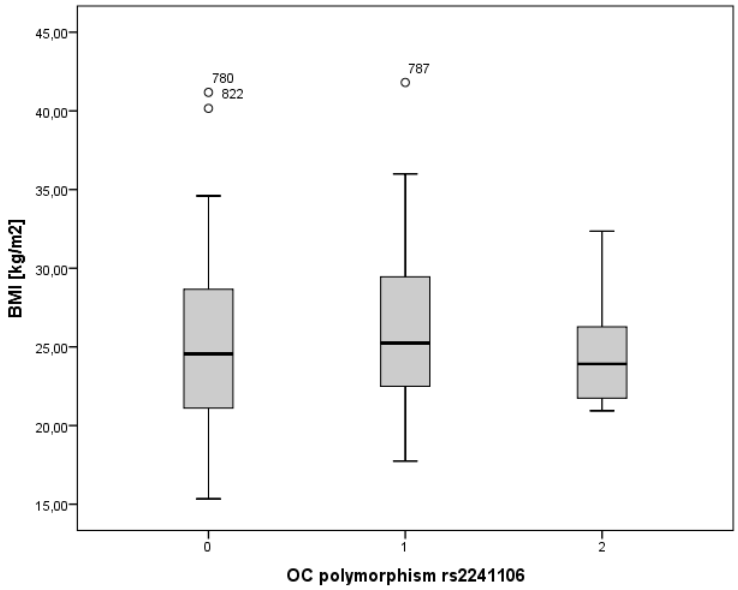


Figure 10: Genotype distribution in PCOS (dark gray) and control women (light gray) for the three SNPs rs2241106, rs1543294, rs2758605.



PCOS women



Control women

Figure 11: In an overall analysis (p=0.041) and in PCOS women only (p=0.014), CC genotype carriers had significantly higher BMI compared to CG genotype carriers, but not compared to women carrying the GG genotype. In control women, no significant difference was observed.
OC=osteocalcin, BMI=body mass index.

6.4 Discussion of thesis part III

Our data show an association of OC polymorphism rs2241106 with BMI in a cohort of PCOS women. None of the polymorphisms were associated with parameters of glucose and lipid metabolism or total body fat, fat mass, lean mass, SAT mass and VAT mass. Our data thus support the notion proposed by Korostishevsky et al. (110) that OC genomic variants are associated with BMI but less likely with adipose tissue itself.

Associations of OC SNPs and BMI have been observed in the large cohort of healthy Caucasians by Korostishevsky (110) and in a smaller cohort of premenopausal healthy Chinese women (160). To date, to the best of our knowledge, OC SNPs have not been analyzed in patients who are at an adverse metabolic profile due to an underlying disease or syndrome. As such, our study provides hints that OC polymorphisms might also play an influencing role in the BMI of women with PCOS who are at increased cardiovascular risk.

Of course, BMI is a complex trait determined by many individual genes and their interactions, as has been shown in many genome-wide association studies (164,165).

Our results substantiate the results obtained in previous studies showing a role of OC polymorphisms in determining BMI. The causative variation in the BGLAP locus, however, still remains to be determined. In the Human Gene Mutation Database (www.hgmd.cf.ac.uk/), there are no reports on disease-causing mutations or phenotype-associated/functional polymorphisms in BGLAP (110). Korostishevsky and colleagues propose that not only BGLAP gene polymorphism cause the associations observed (110). Several other SNPs that might play a role are located next to the area of the polymorphisms investigated, which include complex locus PAQR6, encoding progestin and the adipoQ receptor family member VI as well as PMF1, encoding the human polyamine-modulated factor 1 (110). Definite conclusions as to the causative variation can definitely not yet be drawn.

There are limitations to our study. Firstly, even though the number of PCOS women is high, the sample size for a genetic association study, especially concerning the control group, is limited. Secondly, we did not measure OC, cOC or ucOC in this study. It would have been highly interesting to determine whether OC SNPs have an influence on serum levels of total OC, cOC or ucOC. Thirdly, potential confounding factors for BMI such as caloric intake or socioeconomic status were not considered in our study.

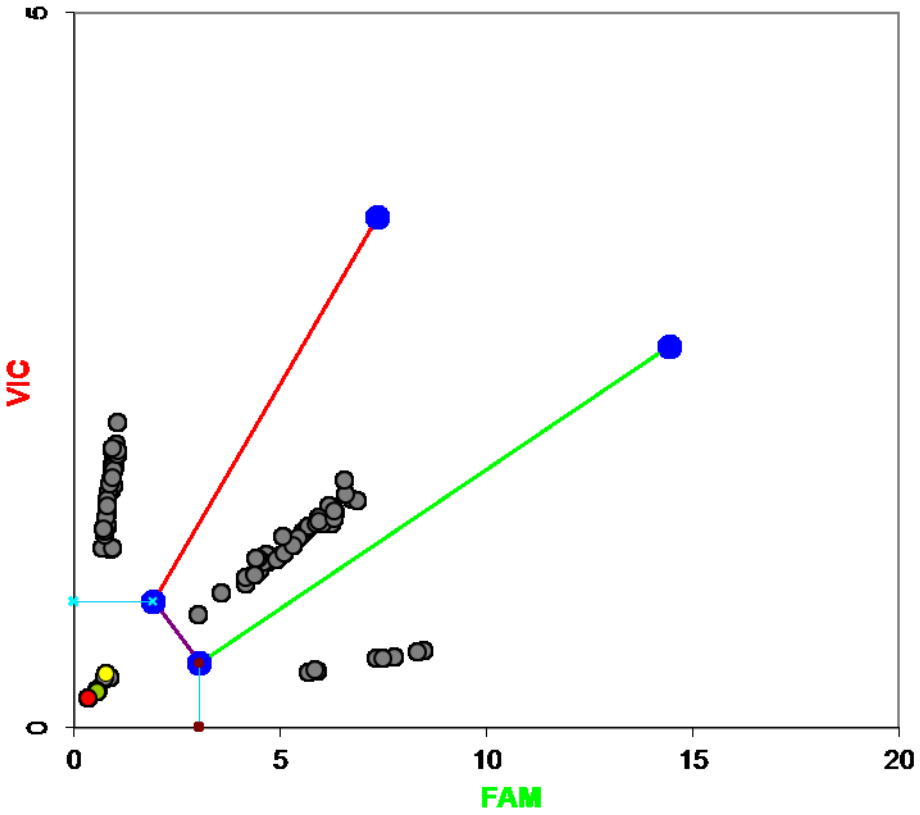


Figure 12: Genotypes of OC polymorphism rs2241106.

The cluster closest to the y-axis represents CC genotype carriers, the middle cluster represents CG genotype carriers, the cluster closest to the x-axis displays GG genotype carriers.

7 OVERALL CONCLUSION

OC, released by osteoblasts and known as a marker of bone turnover, has hormonal characteristics and is involved in exerting the endocrine role of the skeleton. In murine models it influences both energy metabolism as well as male fertility. Results from clinical studies have shown many associations with the carboxylated and undercarboxylated fraction of OC and parameters of lipid and glucose metabolism as well as testosterone. A haplotype of three SNPs in the OC gene is associated with BMI.

Our results support the proposed association of OC with glucose metabolism as we have shown decreases in OC and cOC upon glucose load in non-insulin-resistant women. The extent of the decrease is associated with insulin resistance and sensitivity in premenopausal women. As in insulin-resistant women OC and cOC are suppressed we conclude that there are different osteoblast responses to oral glucose depending on HOMA-IR index. OC reflects this finding.

As for male fertility, our results are less enthusiastic about the role of OC in influencing testosterone levels and sperm count. As the association between OC and testosterone is weak and parameters of OC do not predict oligozoospermia, we conclude that with the evidence given to date, OC cannot be considered to play a strong role in male fertility.

Regarding the influence of polymorphisms in the OC gene on body composition phenotypes, our results in a cohort of PCOS women support the findings of one of the rare studies on this topic: the association of OC SNPs with BMI.

All in all, we conclude that a lot of evidence has accumulated in recent years regarding the association of OC with glucose metabolism. Our results underline this suggested association and highlight the role of the carboxylated fraction of OC as opposed to the undercarboxylated fraction in humans. Questions remaining are whether OC is merely reflecting the connection between bone and glucose metabolism as a marker or whether OC is the mediator itself. Cell culture experiments and interventional studies are needed to elucidate this association.

Likewise, evidence has compiled reflecting an association between OC and testosterone. Our data merely show a weak connection. That parameters of semen analysis are influenced by OC can currently not be supported. Further studies are therefore needed to fully answer this question.

OC polymorphisms are associated with BMI in a cohort of healthy individuals. As shown by our results, this is reproducible in women with polycystic ovary syndrome who have an

adverse metabolic profile. More cohorts need to be investigated to understand the extent of this association also in patients with diabetes or other diseases.

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