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Investigations on bone metabolism in intact and ovariohysterectomised miniature pigs

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This thesis is dedicated to my mother

ALP	Alkaline Phosphatase
Arg	arginine
BAP	Bone specific Alkaline Phosphatase
BMC	Bone Mineral Content
BMD	Bone Mineral Density
BMU	Bone Mineral Unit
BSP	Bone Sialoprotein
cAMP	Cyclic Adenosine Monophosphate
CD_4	Cluster of Differentiation 4
CT	Computed Tomography
CTX	Carboxy-terminal Telopeptide
DLG	Deutsche Landwirtschaftliche Gesellschaft
Dpd	Desoxypyridinoline
DNA	Deoxyribonucleic acid
DXA	Dual X-ray Absorptiometry
EDTA	Ethylen-Diamin-Tetra-Acetat
ELISA	Enzyme-linked Immuno Sorbent Assay
EU	European Union
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor
FSH	Follicle Stimulating Hormone
g Ca/kg	Gram calcium per Kilogram feed
Glu	Glutamic acid
Gly	Glycine
GnRH	Gonadotropine Releasing Hormone
g P/kg	Gram Phosphorous pre Kilogram feed
hCG	Human Chorionic Gonadotropine
His	Histidine
ICTP	Pyridinoline cross-linked carboxy-terminal telopeptide of type I collagen
IL	Interleukin
Leu	leucine
LH	Lutinising Hormone

LHRH	Lutinising Hormone Releasing Hormone
LPS	Lipopolysaccharides
Micro-CT	Micro-Computed Tomography
mmol/l	Millimol/ litre
nm	nanomol
NTX	N-terminal Telopeptide
OCIF	Osteoclastgenesis Inhibiting Factor
OPG	Osteoprotegerin
OVX	Ovariohysterectomised
PDGF	Platlet Derived Growth Factor
PICP	Carpoxy-terminal propeptide of type I collagen
PINP	Amino-terminal propeptide of type I collagen
pQCT	Peripheral Quantitave Computed Tomography
Pro	Proline
PTH	Parathyroid Hormone
Pyd	Pyridinoline
QCT	Quantitave Computed Tomography
RANK	Receptor Activator of Nuclear factor Kappa B
RANKL	Receptor Activator of Nuclear factor Kappa B Ligand
RIA	Radioimmunoassay
SPARC	Secreted Protein, Acidic and Rich in Cysteine
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor
Trp	Tryptophan
Tyr	Tyrosine
VEGF	Endothel growth factor
WHO	World Health Organisation

1 Introduction and aim of this study

Animal models provide uniform experimental material and allow extensive testing of potential therapies (medicinal and operative therapies such as prosthetic devices). The osteoporosis research in particular is one of the most common areas where animal models are necessary. The FDA, Food and Drug Administration, recommends two preclinical animal models, the ovariectomised (OVX) rat and a second non-rodent model, to demonstrate the efficiency and safety of new agents for the therapy of different diseases such as osteoporosis (Thompson et al., 1995).

The goal of an animal model is to replicate the human condition as closely as possible. This has proved to be difficult (Turner et al., 2001). Bellino, (2000) described the usefulness of pig models for humans and the limitations due to the lack of information available on the ovariectomised mature pig in cardiovascular studies, bone studies and sensitivity of bone density to dietary calcium.

Pigs can therefore be considered as appropriate animal models for the use in studies on osteoporosis. The most common form of osteoporosis is the post-menopausal osteoporosis. This condition can be simulated in animals by means of ovariohysterectomy or ovariectomy. The operation should cause an increased bone turnover, which results in a change in bone structure, due to estrogen deficiency, similar to the structure changes of human bones during menopause. Minipigs are not only useful as animal models for studying osteoporosis, but are also useful for the study of other diseases that influence bone metabolism. Bone metabolism, estrus cycle and gastrointestinal functions of the swine are positive features concerning them as an animal model (Turner, 2001).

Throughout adult life the skeleton undergoes continuous renewing (bone remodelling). Bone remodelling is often referred to as coupling, which is a link between bone formation and bone resorption. Biochemical markers of bone formation and resorption allow the assessment of bone turnover. These may assist in the study of pathophysiological growth of the skeleton and in the study of diseases such as osteoporosis, which are characterised by disorders in bone mass and may have disturbances in both formation and resorption. These markers are determined in serum or urine and can be influenced by a number of factors such as age, cycle stage, weight loss, nutrition, seasonal and day- to -day changes.

These factors could cause misinterpretation of bone marker activity which leads to incorrect conclusions with reference to bone metabolism.

The objective of this study was to examine the dependence of bone marker activity (osteocalcin, a bone formation marker and crosslaps, a bone resorption marker) on the estrous cycle of the miniature pig. Studies in human medicine have confirmed bone marker fluctuations during the menstrual cycle, where hormone levels vary. These fluctuations are also possible in the animal model minipig.

Furthermore the behaviour of biochemical markers, osteocalcin and crosslaps were examined at and after ovariohysterectomy to determine the effect of OVX (ovariohysterectomy) on these specific bone markers in the miniature pig. The present study also determined the influence of calcium ration in feed on bone metabolism before and after castration.

To accomplish these objectives a pilot study is performed with 16 Dresdner minipigs. These are divided into three groups; control, OVX (ovariohysterectomised) group and a second control group. The control group receives a 0.99% calcium ration, whereas the OVX and second control group receive a 0.7% calcium ration. All animals are to be synchronised at the start of the study. The study consists of two periods; pre-castration and post-castration. The OVX group will be ovariohysterectomised at the beginning of the post-castration period. Blood samples will be extracted in particular intervals pre-castration and post-castration. Formation marker osteocalcin, resorption marker crosslaps, progesterone and estradiol concentrations are to be determined from collected serum samples.

2 Literature

2.1. Definition

In 1993, the Consensus Development Conference reached the currently acceptable definition of osteoporosis: a systemic skeletal disorder characterised by low bone mass and micro-architectural deterioration of bone tissue, thus causing an increase in bone fragility and susceptibility to fracture risk (Consensus Development Conference, 1993). Characteristic for this disease are low- traumatic fractures. The disease has become a major public health problem. The costs for fracture treatment are staggering and many patients everyday life are affected seriously.
In 2000, this definition was modified to osteoporosis being defined as a skeletal disorder characterised by compromised bone strength that predisposes to an increased fracture risk. (NIH Consensus Development Panel on Osteoporosis Prevention and Therapy, 2001).
In addition to this definition, two main features to bone strength were added:
Bone density and bone quality. Bone density is expressed in grams of mineral per area volume. Bone quality refers to architecture, turnover, damage on bone (fractures) and mineralization.

2.2 World Health Organisation (WHO) definition of osteoporosis

Bone density correlates well with bone strength (NIH Consensus Development Panel on Osteoporosis Prevention and Therapy, 2001). The WHO defines osteoporosis on the basis of bone mineral density (BMD). It was assumed that the relative risk for fracture must be lowest when the BMD is highest (WHO, 1994). The lowest fracture risk would be between the ages of 20 and 40 in healthy people. The fracture risk in this group of people was set at 1.0. It was agreed to that the relative fracture risk doubles for each standard deviation (SD) decrement in BMD.
With this concept in mind, the WHO developed the following definitions:

Normal is defined as BMD less than, or equal to, 1.0 SD below the mean for peak bone mass.

Low bone mass (osteopenia) is defined as more than one, but less than, or equal to, 2.5 SD below the mean.

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Osteoporosis is defined as more than 2.5 SD below the mean.

These definitions are often related to in T-scores. T-score refers to amount of standard deviations of the mean of healthy persons between the age of 25 and 40 years. Z-scores are scores, which are obtained from the mean bone density of persons in a comparative population.

Table 1: WHO definition of osteoporosis, based on bone mineral density and T-scores using dual x-ray absorptiomerty of the proximal femur and spine (WHO, 1994)

Normal	T-score > -1
Osteopenia	T-score < -1 > -2.5
Osteoporosis	T-score< -2.5
Severe osteoporosis	T-score< -2.5 and osteoporotic fractures

2.2.1 Post- menopausal osteoporosis

Osteoporosis represents a bone mass loss unassociated with other chronic diseases and is related to an estrogen deficiency (Type I) and aging (Type II) (Gambacciani and Ciaponi, 2000; Riggs and Melton, 1986; Melton and Riggs, 1988). At the age of 45-50 years woman reach the stage were the ovieran function ceases. This causes a decline in the estrogen concentrations in the body. In women the most frequent type of osteoporosis is due to estrogen deprivation after menopause (Consensus Development Conference, 1993).

Many studies have shown that estrogen intervention reduces the rate of bone loss among postmenopausal women (Ettinger et al., 1985; Lindsey, 1989; Ham and Carlson, 2004; Qi et al., 2004; Carlsten, 2005; Biglia et al., 2005). Ettinger et al. (1985) did a retrospective study comparing subjects using estrogen replacement

therapy and matched controls. Quantitive bone mineral assessments were obtained. Estrogen users showed significantly greater bone mineral. Lindsay, (1989) states that bone mass in females is fairly constant up until the 5th decade and then decline at all skeletal sites. Estrogen therapy prevents bone loss that follows menopause. Ham and Carlson, (2004) measured the trabecular bone volume in ovariectomised untreated, estrogen treated and soy phytestrogens treated monkeys. Estrogen decreased the bone turnover indices. Soy phytestrogens were protective against loss in bone volume. Some studies do not only measure the changes in bone, but also the changes in bone markers due to estrogen therapy. Watts et al. (2000) determined the relationships among bone mineral density changes and bone marker changes in post-menopausal women receiving estrogen and found that bone mineral density changes correlate significantly with bone marker changes at 6 months esterified estrogen therapy. Warming et al. (2004) evaluated the effectiveness of 17β-estradiol and drospirenone combination for the prevention of post-menopausal osteoporosis. Bone mineral density increased at lumber spine hip, and total body, while bone markers decreased (osteocalcin, serum bone alkaline phosphatase, serum Crosslaps and urinary Crosslaps) from base line.

Type II Osteoporosis is a result of aging. This affects all skeletal sites, both cortical and cancellous bone. It is a result of senile decline in bone mass (Riggs and Melton, 1986; Melton and Riggs, 1988).

Type III Osteoporosis is an idiopathic osteoporosis, which affects pre-menopausal women as well as middle aged men (Riggs and Melton, 1986; Melton and Riggs, 1988).

In postmenopausal women the changes in cancellous bone structure are considered to be a consequence of focal imbalances in remodelling processes. This results in perforation and removal of trabecular structures (Boyce et al., 1995). Through the loss in structure the bone is less resistant to pressure and stress, causing fracturing. Three consequences of estrogen deficiency are discussed (Parfitt, 1987). Firstly, the osteoclast activity is elevated, which results in large lacuna due to resorption. Secondly the interplay of bone resorption and formation activity (coupling) is disturbed (Ivey and Baylink, 1981). Thirdly, histomorphometric analyses have given reason to assume that the osteoblast units in bone are insufficient (Lips et al., 1978). Estrogen deficiency causes bone loss. Kameda et al. (1997) was able to show that

17 β-estradiol directly inhibits osteoclastic bone resorption through apoptosis of osteoclasts. An estrogen deficiency would cause an increased generation and activity of osteoclasts, which perforate bone trabeculae, thus causing strength loss and increased fracture risk (Hughes et al., 1996). Hughes et al. (1996) also showed that estrogen causes apoptosis in osteoclasts. In the presence of estrogen, osteoblasts produce transforming growth factor-beta 1 (TGF-beta). Anti TGF-beta antibody inhibited TGF-beta and estrogen. This may indicate that TGF-beta might mediate osteoclast apoptosis. Marie et al. (2000) states that soluble factors, which include bone morphometric proteins, leptin and TGF-beta, can modulate differentiation of mesenchymal stem cells to osteoblasts, chondrocytes or adipocytes.
Interleukin 1 is another factor that is of particular interest in the pathogenesis of osteoporosis. This is a protein, which is known to stimulate bone resorption and perhaps formation. Pacifici et al. (1987) found that monocytes in osteoporotic patients produced more interleukin-1 than subjects in the control group.
Interleukin-1 released from peripheral blood monocytes, appeared to reflect bone formation.
Insulin-like growth-factor (IGF-I) is a regulator of osteoblast activity. A binding protein, insulin-like growth factor–binding protein-4 (IGFBP-4) suppresses biological activity of IGF-I. Parathyroid hormone (PTH) enhances binding activity of IGFBP-4. This is abolished through 17β-estradiol. Kudo et al. (1996) found that IGFBP-4 protease activity, in SaOS-2 cells, which is responsible for cleavage of IGFBP-4, was suppressed in parathyroid hormone-treated (PTH) cells. This suppression was reversed by adding 17β-estradiol. Kudo et al. (1996) explain that the inhibitory effect of PTH on osteoblastic activity occurs by inhibiting PTH-induced suppression of IGFBP-4 protease activity.
O´Shaughnessy et al. (2000) found that nitric oxide plays an important role in the stimulation of osteoblast activity through estrogen. Findings indicated that 17β-estradiol modulates osteoblast function by nitric oxide-dependant mechanisms.

A large number of people are affected by this disease. Osteoporosis causes fractures in more than a million Americans each year (McGarry and Kiel, 2000). Over the past years life expectancy has increased. Most women spend more than one third of their lives after menopause and therefore the consequences of estrogen deficiency have become a major problem for health providers (Gambacciani and Ciaponi, 2000).

This disease is responsible for considerable morbidity as well as deaths in many older women (Gambacciani and Ciaponi, 2000).
During normal aging, the decrease in trabecular bone strength is much more pronounced than the decline in apparent density (Mosekilde et al., 2000). With age the trabecular bone declines with a factor of four, density however declines with the factor of two. Certain factors such as the loss of connectivity in horizontal struts of trabecular network, due to osteoclastic perforations, micro fractures, change in bone mineral quality, influence strength and density, but it is not known how the relationship between the two is influenced (Mosekilde et al., 2000). Furthermore the interplay corticalis, trabecular network, effect of bone marrow as a hydraulic and the effect of soft tissues such as cartilage, connective tissue and muscle on these factors must be investigated further (Mosekilde et al., 2000).

Due to the complications occurring during the progression of the disease, high costs arise. In Europe, in 2000, the number of osteoporotic fractures was estimated at 3.9 million, of which 0.89 million were hip fractures (179000 hip fractures in men and 711000 in women). The total direct costs were estimated at €31.7 billion, which were expected to increase to €76.7 billion in 2050 based on the expected changes in the demography of Europe (Kanis and Johnell, 2005). It is estimated that in Europe, 179 000 men and 611 000 women will suffer a hip fracture each year and that the cost of all osteoporotic fractures in Europe is provisionally €25 billion (Melton et al., 2003). A study in Switzerland showed that the annual costs of hospitalisations (duration of hospital visit), for osteoporotic fractures, were greater than those for myocardial infarction, stroke and breast cancer, and only slightly lower than those for chronic obstructive pulmonary diseases. For women with osteoporosis the costs for hospitalisation were higher than for the other mentioned diseases (Lippuner et al., 1997). In Denmark, the estimated prevalence of osteoporosis in persons aged 50 years or more is 40.8% among women and 17.7% among men (Vestergaard et al., 2005).

2.2.2 Secondary osteoporosis

The National Institute of Health Osteoporosis and Related Bone Diseases- National Reference Centre (NIH ORBD-NRC, 2001) defines secondary osteoporosis as osteoporosis caused by certain medical conditions or treatments that interfere with the attainment of peak bone mass and / or precipitate bone loss. Cushing's disease causes the body to produce excessive amounts of glucocorticoids, causing obesity, muscular wasting and accelerated bone loss. Effective agents that control inflammatory diseases, such as corticosteroids can cause osteoporosis (Sambrook and Lane, 2001). Glucocorticosteroids are commonly prescribed for treating a variety of inflammatory and allergic conditions, such as asthma, rheumatoid arthritis and inflammatory bowl disease. This can cause one of the most common forms of drug induced osteoporosis. Bone loss tends to be greater in individuals using oral forms of medication.

Glucocorticoids exert their negative effects on bone through mechanisms that are not yet completely elucidated (Minetto et al., 2004). An indirect mechanism leading to a decrease in bone mass is the glucocorticoid-induced impairment of gonadal steroid synthesis (Boscaro et al., 2001), which counteracts bone resorption (Canalis, 1996). The loss of muscle mass and strength in patients with Cushing's Syndrome and patients that are treated with exogenous corticosteroids, may contribute to bone loss due to the decrease in tropic action of the muscle contraction on the bone (Lane and Lukert, 1998; Frost, 1987). Corticosteroids have an inhibitory effect on osteoblasts and the differentiation on osteoblast precursor cells. They also decrease the mRNA levels that code osteoblast products such as osteocalcin (Morrison et al., 1989). Glucocorticoid receptors have been identified in cells that reside in the bone microenvironment (Peck, 1984). These cells are able to produce and release cytokines and growth factors that influence bone turnover (Angeli et al., 2002; Kristo et al., 2002). Glucocorticoids can promote osteoclastgenesis by inhibiting osteoprotegerin (OPG) and enhancing RANK-L production by stromal cells (Hofbauer et al., 1999).

Other major effects of glucocorticosteroids are the decrease of intestinal calcium absorption and an increase of urinary calcium and phosphor concentrations (Klein et

al., 1977; Hahn et al., 1981; Morris et al., 1990; Cosman et al., 1994). This leads to a secondary hyperparathyroidism and therefore an increase in bone absorption.

Adequate thyroid hormone levels are essential for normal bone remodelling. Elevated levels of these hormones, also known as thyrotoxicosis, have been linked to bone loss. Thyrotoxicosis can occur naturally from an overproduction by the thyroid gland and can also occur with extensive amounts of thyroid replacement (e.g. treating hypothyroidism). Thyroid hormone has an effect on osteoblasts via its specific nuclear receptor and thereby stimulates bone resorption (Britto et al., 1992; Kim et al., 1999; Allain and McGregor, 1993). The activity of osteoblasts and osteoclasts are both increased, whereas the osteoclast activity predominates, resulting in bone loss. Hyperthyroidism is thus one of the major causes of secondary osteoporosis (Riggs and Melton, 1986). The increased entry of calcium from the bone into the blood (due to increase of bone resorption) suppresses parathyroid hormone (PTH) secretion from the parathyroid gland. This results in a suppression of PTH-induced vitamin D activation in the kidneys, causing a decrease in intestinal calcium absorption and an increase in urinary calcium excretion, resulting in a calcium imbalance. This may accelerate bone loss in hyperthyroidism further (Shafer and Gregory, 1972).

Gonadotropin-releasing hormone agonist administration can cause an increase in bone turnover, due to estrogen deficiency. Stroup et al. (2001) treated cynomolus monkeys with gonadotropin-releasing hormone agonists, and found that bone turnover increased as early as 4 weeks after administration. Bone turnover returned to control levels in two weeks after the administration of estrogen. Medical castration, using gonadottropin-releasing hormone agonists, is widely accepted as androgen deprivation therapy for prostrate cancer. Miyaji et al. (2004) studied the effects of gonadottropin-releasing hormone agonists on bone metabolism markers and bone mineral density in patients with prostate cancer. A decrease in bone mineral density was observed. A functional coupling of bone resorption and bone formation markers suggested that gonadotropin-releasing hormone agonists stimulates bone formation to compensate lower bone mass.

Adults taking antiepileptic drugs have an augmented risk for osteopenia and osteoporosis. This is due to the abnormalities of bone metabolism associated with antiepileptic drugs. The hepatic enzyme-inducing antiepileptic drugs, phenytoin, phenobarbital and primidone have clear association with a decrease in bone mineral

density (Pack and Morrel, 2004). The cytochrome P450 system is induced through these medications, causing an increased conversion of vitamin D into polar inactive metabolites in liver microsomes, causing a reduced bioavailability of vitamin D (Gough et al., 1986; Perucca, 1987). This causes a decrease in intestinal calcium absorption, resulting in hypocalcaemia and an increase in circulating parathyroid hormone. Parathyroid hormone mobilizes calcium stores and subsequent bone turnover (Richens and Rowe, 1970; Hahn et al., 1972; Bouillon et al., 1975; Gough et al., 1986; Stamp et al., 1972; Weinstein et al., 1984).

Rheumatoid arthritis is a systemic autoimmune inflammatory disease that causes osteoporosis (Inaba, 2004). This is associated with three types of bone loss: local subchondral and joint margin bone erosion, paraarticular osteoporosis and generalised osteoporosis (Deodhar and Woolf, 1996). Recently it has been reported that serum TNF-α has a suppressive effect on bone formation. This is increased in rheumatoid arthritis patients. TNF- α may have an important role in the development of osteoblast dysfunction in these patients (Inaba, 2004). Major factors affecting bone loss in rheumatoid arthritis are age, menopausal states, impairment of activity of daily living (Inaba, 2004). In addition, treatment of rheumatoid arthritis with drugs such as glucocorticoid and methotrexate (Buckley et al., 1997) and malnutrition (Inaba et al., 2000) could have harmful effects on bone metabolism.

Bone metabolism is also affected in anorexia nervosa, due to multiple hormonal endocrine and nutritional factors (Munoz and Argente, 2002). Due to amenorrhea in this condition, estrogen deficiency has been described as the main source for bone loss. The mechanisms underlying the estrogen deficiency are not completely understood (Grinspoon et al., 2000). These are only few of the factors that may cause secondary osteoporosis.

In table 2 varies other etiological reasons for the occurrences of secondary osteoporosis are listed.

Other reasons for weak bones may also be smoking, geographical location or genetic inheritance. The cessation of smoking seemed to decrease fracture risk. These findings varied geographically (Vestergaard and Mosekilde 2003). Akhter et al. (2005) studied the influence of smoking on biomechanical properties of bone in female mice and found that the ultimate load and stiffness of the femoral neck was lower in the smoke exposed mice. Micro-computed tomographic scanning of the distal femoral bone volume/total volume and trabecular thickness showed decreased

trends in the smoke exposed group. Mueller and Cievro, (1998) state that smoking has numerous health risks for women, one which includes ovulatory dysfunction and early menopause. Smoking is directly toxic to the ova and it decreases the estrogen secretion (Shulman et al., 1990). Cesar-Neto et al. (2003) found that tobacco smoke had more influence on bone implant contact (in terms of bone/implant contact area) than nicotine treatment alone in the tibiae of adult Winsar rats. This suggests that tobacco smoke constituents other than nicotine may be responsible for compromised biochemical properties of smoker skeleton.

Some studies confirm that osteoporosis is a disease that is inherited. Carbonell Sala et al. (2005) state that it has a polygenetic pattern of inheritance that complicates the identification of genes causing osteoporosis (cytokines, calcitropic hormones, sex hormone pathway synthesis, bone matrix synthesis genes involved in estrogenic metabolism and LDL receptor related protein 5 gene). It is possible to identify associations between candidate gene polymorphisms and disease phenotype in population based and case-control studies. Bone density is an important parameter that is used to detect osteoporotic fracture risk. It is confounded by environmental influences and polygenic inheritance (Campbell et al., 2003). Li et al. (2004) did a study to determine the heritability of low bone mineral density (BMD), at the hip in Ashkenazi Jewish families and found a significant genetic determination in decreased BMD at the femoral neck of these people. Diez et al. (1989) conclude that fractures in the proximal femur due to osteoporosis are common processes in the Mediterranean areas. These reflect the impact of the environment. The Mediterranean areas, however, have a lower incidence compared to the geographical areas in the north. This could be due to the influences of climate and different ethnic groups.

Table 2: Etiology of secondary osteoporosis (Inaba et al., 2004)

Endocrine/ metabolic diseases Hyperparathyroidism (primary, secondary) Thyrotoxicosis Cushing syndrome Hypogonadism Diabetes mellitus Pregnancy Anorexia nervosa
Inflammatory diseases Rheumatoid arthritis Ankylosing spondylitis
Functional Immobilization/weightlessness Chronic obstructive lung diseases Postgastrectomy Hepatic disease (particularly primary bilary cirrhosis) Alcohol abuse Following organ transplantation
Haematopoietic Multiple myeloma Lymphoma/Leucemia Mastocytosis
Congenital Osteogenesis imperfecta Menkes syndrome Ehlers-Donlos syndrome Homocysteinuria Marfan´s syndrome
Drugs Corticosteroid Thyrotoxine Anticonvulsants (barbiturates, phenytion) Anticoagulants (heparin, cumarin), Antimetabolites (metotrexate, cyclosporine)

2.3 Osteoporosis imaging

Osteoporotic fractures may be prevented if the disease is diagnosed soon enough. There are various techniques that enable assessment of osteoporotic bones. In human medicine certain bones have a predisposition to fracture. Important osteoporotic fractures are hip, vertebral and forearm fractures (Lau, 2001).The fracture incidence increases with age in both men and women, but remains higher in women throughout life (Lau, 2001). Conventional radiographs are not suited to diagnose early osteoporotic fractures (Link and Majumdar, 2003). Bone mass measurements are standard techniques in the diagnosis of osteoporosis (Link and Majumdar, 2003).

2.3.1 Osteodensitometry

Several techniques can be used to measure bone density (BMD) and bone mineral content (BMC). BMD measures bone density is the amount of bone tissue in a certain volume of bone, reflecting the amount of calcium in the bone. The strengths of bone mineral density are: it changes slowly, so it provides a statistic measurement of skeletal status; the variability within persons is very low and measurements are very precise; and it can be used to diagnose osteoporosis using the definitions proposed by the World Health Organisation (WHO) (Looker et al., 2000). BMC measures the mineral content in bone. In adult mammals, approximately 20% of the bone wet weight is water, 45% ash, 35% organic matter (Carter and Spengler, 1978). Calcium contributes 37% of the ash content and phosphor contributes 18.5%. On dry weight basis mineral content is 65-70% and organic matter is 30-35% (Keene et al., 2004).

2.3.1.1 Dual X-ray Absorptiometry

This is an established method of the WHO for diagnosing osteoporosis (Looker et al., 2000). The principle behind Dual X-ray Absorptiometry (DXA), is measuring the transmission of x-rays of two different photon energies, through the body. Depending on the attenuation coefficient on the atomic number, photon energy and measurement of transmission factors at two energies, an “areal “density is measured retrospectively. Scans are taken of two tissues, bone mineral and soft tissue. The areal BMD is measured in mg/cm² and includes trabecular and cortical bone.

Accuracy is limited due to the differences in soft tissue composition in which the x-ray beams pass through (Blake et al., 2002). The preferred anatomic sites for DXA are the measurement of bone mineral in lumber spine, proximal femur and whole body. Peripheral sites can also be scanned (Njeh et al., 1999).

The first generation of DXA scanners used pinhole collimator that produced a pencil beam coupled to a single scintillation detector in the scanning arm. More advanced systems introduced a slit collimator to generate a fan beam coupled to a linear array of solid-state detectors. This shortens scan time and produces a higher resolution (Njeh et al., 1999). The latest DXA systems have two- dimensional detector arrays developed for digital radiography systems. They possess a cone-beam system. Measurements are done within seconds (Boudousq et al., 2003).

The main advantages of X- ray systems such as these are the shortened examination time due to an increased photon influence of the X-ray tube, greater accuracy and precision, which results in higher resolution and removal of errors due to source decay (Kelly et al., 1988). Another advantage of DXA is the low radiation exposure (Njeh et al., 1999).

DXA, however, is unable to differentiate between trabecular and cortical bone. The effects of bone loss are more prevalent in trabecular bone, due to the much higher surface area. Bone mineral content is much greater in cortical bone, and can therefore conceal small changes in trabecular bone (Brismar et al., 2001). Osteoporosis is a disease where the micro-architectural deterioration of bone tissue takes place. This method is not able to display this accurately, because bone mineral content is greater in the cortical bone and conceals changes in the trabecular bone.

While density (quantity) does correlate with strength (Keaveny et al., 2001), about 30% of the variability in bone strength remains unexplained (National Institutes of Health, 2000; Hans et al., 1997).

2.3.1.2 Quantitative computer tomography

This technique measures volumetric measurements (mg/ml) of trabecular bone. Quantitative computer tomography (QCT) may be performed at any CT system. All that is required is a calibration phantom and dedicated software to improve precision. Measurements are done on spine and appendicular skeleton (spine and peripheral QCT).

Peripheral quantitave computed tomography (pQCT)

The purposes of these scanners are to measure the bone mineral content (BMC) and bone mineral density (BMD) of the peripheral skeleton. This is a method that enables true volumetric density measurements of the appendicular bone without other tissue overlapping. It provides exact three dimensional localisation of the target volume. Assessing cortical and trabecular bone separately is possible (Njeh et al., 1999). Peripheral scanning is used in the distal radius (Glüer et al., 1995). The patient lies on a phantom. Between patient and phantom, a water or gel filled cushion is placed. This avoids artefacts (Link and Majumdar, 2003). These are performed at one site with a single axial slice of 2.5 mm thickness. This is located at the level that represents 4% of the ulna length from the distal radial cortical endplate. Some pQCT are able to incorporate multi-slice data acquisition and cover a larger amount of bone (Kaisel and Rüegsegger, 1991). Measuring more slices enables an accurate reflection of the bone status and potentially measures more representative changes in the distal radius. Riggs et al. (2004) did scans with a single energy CT. The principle of the scans is as follows: the joint space is visualized and a reference point is set electronically, from a digital image (scout view) of the forearm, at the intersection of joint space with radius-ulna junction for the forearm. From here, an automatic program is selected, which scans a more distal and more proximal site of the radius. A computerised surface detection algorithm peels the bone pixel-by-pixel until a certain percentage of core areas remain. The outer 10% of the bone is excluded to avoid partial volume effects from the bone edge. Depending on the program, certain areas in mg/cm^3 are considered to be cortical and certain areas are considered as trabecular (Russo et al., 2006).

Spinal QCT

An external bone mineral reference phantom is used to calibrate the computed tomography measurements of bone-equivalent values. This is used as a control device to characterize the scanner and to perform a quality assurance test. During the scanning process, the spine and phantom should appear as a single object (Yoganandan et al., 2006). For the regions of interest in the vertebral bodies, special software is required to analyse the image. The most commonly examined vertebra range from the first to the third lumbar vertebral bodies. 8-10 mm thick sections are obtained by a lateral scout image. A low-dose technique and a gantry parallel to the vertebral endplates are required. To improve precision, reduce acquisition and analysis time, the placement of the region of interest and the sagital location of mid vertebral slices can be automated (Njeh et al., 1999; Glüer et al., 1988, Kalender et al., 1988).

Bone marrow fat increases with age. This may decrease BMD falsely (Glüer and Genant, 1989). Kuiper et al. (1996) conclude that bone mineral measurements of the femoral neck with QCT and DXA are highly correlated with chemically determined bone mineral mass. Both techniques are influenced by femoral fat content. While DXA is susceptible to accuracy errors, due to overlying calcified structures and degenerative joint diseases, other studies show that QCT are dependent on the status of degenerative joint diseases (Yu et al., 1995). The ease of use, the possibility to assess cortical and trabecular bone separately, measuring BMD and bone mineral content (BMC) make this method an interesting alternative to other methods. A comparative study using DXA and QCT was done by Angelopoulos et al., (2006). In comparison to DXA, QCT T-and Z-scores were higher. QCT T-scores correlated more closely to age than DXA T-scores. QCT T-scores correlate closely with osteocalcin, urinary N-telopeptide cross-links of type I collagen and deoxypyridinoline, without statistical significance and DXA T-scores correlated only with C-telopeptide cross links of type I collagen, without statistical significance. Which one of the two techniques determine the overall strength of vertebrae more precisely, must still be further investigated.

2.3.1.3 Micro-computed tomography

Micro-computed tomography (micro-CT) is a technique for the non-destructive assessment and analysis of the three-dimensional trabecular bone structure (a network of plates and rods, arranged in a lattice-like network). A compact miniature CT-scanner system has been developed by Rüegsegger et al. (1996). This CT-scanner, also referred to as desktop μCT, allows non-invasive imaging and quantitative morphometry of bone specimens of various sizes. It is therefore likely that this technique will allow characterisation of the effects of diseases and therapeutic agents on trabecular bone architecture in humans and in a variety of animals. Eckstein et al. (2002) examined the body composition, bone mineral states and architecture in Growth Hormone-transgenic mice. The femora of these mice were examined with DXA and micro-CT. A comparison of these two methods confirmed a high consistency between these two methods. Abe et al., (2000) observed the morphological characteristics of the femur in osteopetrotic (op/op) mice, using micro-computed tomography. The accuracy of the reconstructed 3-D images was confirmed by comparing an original femur. The arbitrary cross-section could be displayed on a screen and a detailed examination of the internal structures was possible. The volume percentage of the trabecular bone could be measured in three dimensions. It was confirmed that micro-CT can be applied to 3-D morphometric analysis.

The effect of ovariectomy in rats on the changes in trabecular structure in alveolar bone was investigated by Tanaka et al. (2003), using micro-CT. The alveolar trabeculae in the sham operated group had network structures, whereas rats, that were ovariectomised, had fragmented structures. Trabecular bone volume, number and thickness of ovariectomised rats were significantly lower than those found in the sham operated rats.

The micro-CT is capable of acquiring 3-D X-ray images made up of 2000^3 voxels on specimens up to 5 cm in extent with resolutions down to 2 μm. This allows the 3-D structure of tissue-engineered materials to be imaged across orders of magnitude in resolution. The micro structural features in regenerating bone was studied by Jones et al. (2004).Tissue-engineered bone material based on polycaprolactone scaffold and autologous marrow cells were examined. Imaging occurred at a scale of 1 cm and resolutions of 10 μm. The complex ingrowth of bone into polymer scaffold was visualised. Theoretically, the voxel data may be used to calculate expected mechanical properties of tissue-engineered implants.

The ability to evaluate fracture risk at an early point of time is essential for the prognostics and the treatment of osteoporosis. Mittra et al. (2005) used sheep femoral trabecular bone, to investigate the interrelationship of trabecular mechanical and micro structural properties. These were analysed using mechanical testing and micro-CT. Findings showed, that micro-CT indices are structural in nature, e.g. structural model index (SMI) (Hildebrand and Ruegsegger, 1997), which expresses the structure of the trabeculae, is as good as density orientated indices in predicting strength in the trabecular region. The analysis also showed that a loss of bone primarily affects the connectedness and the number of trabeculae, while increased strength results in an increase of thickness of trabeculae while not improving connectedness.

Teo et al. (2006) studied the relationship between image intensity CT, cancellous bone micro-architecture (using micro-CT) and mechanical properties. It was found that the predictive power of bone strength and stiffness was improved with the combination of bone density and micro architecture information.

Other than displaying the micro architecture of bone, micro-CT can be used to assess emphysema in mice. Postnov et al. (2005) induced emphysema in mice by intratracheal installation of different amounts of porcine pancreas elastase. This emphysema could be clearly detected by micro-CT seven weeks later. Watz et al. (2005) reconstructed terminal airspaces of lung specimens with micro-CT. This made virtual endoscopy of the alveolar ducts possible. Watz et al. (2005) investigated the appearance of lung parenchyma at the structural level of alveoli. A virtual endoscopy of the alveolar ducts was made possible after a 3-D reconstruction of the terminal airspaces. Badea et al. (2005) demonstrated a non-invasive imaging method for the in vivo characterization of cardiac structure and function in mice using 4-D micro-CT.

2.4 Biochemical parameters

Bone metabolism is a dynamic and continuous remodelling process that is normally maintained in a tightly coupled balance between resorption of old or injured bone and formation of new bone. Bone metabolism occurs on focused sites on the surface of the bone called bone remodelling units or bone metabolism units (BMU). Osteoclasts and osteoblasts are the cells that carry out bone metabolism at the specific BMU site. Osteoclasts resorb existing bone. Osteoblasts are responsible for bone formation. These replace the surfaces resorbed by the osteoclasts with a replacement bone matrix, termed osteoid. Some osteoblasts are entrapped in bone matrix. Here they become osteocytes. Osteoporosis is a condition in which an imbalance appears to be between bone resorption and formation, with bone resorption exceeding formation. Biochemical techniques have been developed to measure products of bone resorption and bone formation, and thus the degree of bone turnover is able to be measured. Markers of formation assess either osteoblastic synthetic activity or post release metabolism of procollagen. Resorption markers reflect osteoclast activity and or collagen degradation (Christenson, 1997).

2.4.1 Markers of bone formation

2.4.1.1 Bone alkaline phosphatase

Alkaline phosphatase (ALP) is a membrane bound protein that is synthesized by cells in a variety of tissues. In humans 4 isoenzymes have been identified. 3 are expressed in tissue specific distributions in the intestine, placenta, and germ cells (Prince, 1993). The fourth isoenzyme is tissue non-specific and is distributed throughout the body, but especially abundant in the liver, kidney and bone (Allen, 2003).

This enzyme is also produced by other tissues (liver, placenta, intestine) (Moss, 1987), which contributes to the total amount of alkaline phosphatase in the blood. The bone isoenzyme is situated in the membrane of the osteoblasts and is then released into the circulation. The condition hypophosphotasia lacks this enzyme, suggesting that it has a role in the mineralization of newly formed bone (Watts, 1999). In children and adolescents up to 80 % ALP measurable in serum, derives from bone, mirroring growth. In adults, however, about 50% is bone derived. The remainder comes mostly from the liver (Eberling and Åkesson, 2001).

Four variants of ALP have been identified in animals: bone alkaline phophatase, intestinal ALP, liver ALP and in dogs the corticosteroid induced ALP. Liver and bone ALP are products of the same gene. They only differ in location numbers of posttranslational glycosylation sites, making them isoforms, rather than isoenzymes (Sanecki et al., 1990).

In young animals that still have an immature skeleton, the bone ALP will tend to be predominant. In mature and older animals, medication and disease could cause an increase in liver and corticosteroid- induced ALP (Saini and Saini, 1978).

Different tests have been developed to differentiate between different isoenzymes. Bone ALP can be isolated by heat denaturation or gel electrophoresis (Saini and Saini, 1978). More recently radioimmunoassays (RIA) and enzyme-linked-immuno-asorbert-assays (ELISA) (Price et al., 1997) have been developed for human use, but exhibit an acceptable cross reactivity with bone ALP from a number of animal species (Allen et al., 2000).

2.4.1.2 Osteocalcin

This is a major noncollagen protein of the bone matrix. It consists of 49 amino acids and is rich in glutamic acid (Catherwood et al., 1985). Osteocalcin is vitamin K dependant and is synthesized in osteoblasts and megakaryocytes (Thiede et al., 1994). Vitamin K is a cofactor for the synthesis of blood coagulation factors II, VII, IX, and X and inhibitors such as Protein C and S and bone matrix protein. Its active form is a coenzyme in glutamic acid carboxylation (Mijares et al., 1998; Olson, 1984). When vitamin K is insufficient, a small amount of uncarboxylated osteocalcin is released from osteoblasts into the circulation. So the serum concentration of uncarboxylated osteocalcin is considered as a sensitive marker of vitamin K status in the bone (Sokoll et al., 1997). High levels of uncarboxylated osteocalcin have been associated with low bone mineral density and an increased hip fracture risk (Booth et al., 2003, Booth et al., 2004). Benyahu et al. (1997) reports that osteocalcin mRNA and protein is also expressed in vitro in adipocytes. It is known, that the osteocalcin synthesis is regulated by vitamin D, 1, 25(OH) ² D3 (Cantatore and Pipitone, 1999).

The active metabolite of 1, 25(OH) ² D3 enters the cell and binds with a vitamin D receptor. This complex forms a heterodimer with the retinoid receptor and binds to a vitamin D responsive element on a responsive gene e.g. on that of osteocalcin. Transcription and translation follows and proteins are formed such as osteocalcin (Lips, 2006).

Structurally, the mature osteocalcin peptide consists of two anti-parallel α-helical domains connected by a β turn. At the C-terminal end, there are two further β turns and a β sheet. The structure is stabilized by a Cys_{23}-Cys_{29} disulphide bond. (Hauschka 1982, 1986). During the matrix synthesis, some osteocalcin is released into the bloodstream. This has a short half-life and is determined mainly by renal clearance. No intact osteocalcin is released during resorption, but fragments of osteocalcin are released in vitro and during resorption and formation. Osteocalcin may serve as a site for hydroxyapatite crystals (Delmas, 1993; Gamero et al., 1994; Taylor et al., 1990). Ivaska et al. (2004) discovered that intact and fragmented osteocalcin can also be released during bone resorption. Furthermore in addition to the conventional use of bone formation, osteocalcin can also be used as a marker of bone resorption *in vitro* and bone matrix derived osteocalcin may contribute to the circulating osteocalcin levels, suggesting that serum osteocalcin should be seen as a marker of bone turnover, rather than a marker of bone formation.

Osteocalcin in serum is susceptible to rapid enzymatic cleavage. Thus both intact and fragmented, N-mid-molecular fragments are found in serum. The C-terminal fragment is most susceptible to cleavage, while the N-terminal mid fragment shows apparent greater stability (Diaz Diego et al., 1994). In addition to this, degradation occurs rapidly at room temperature. If the analysis of intact osteocalcin is wanted, specimens should be frozen as soon as possible after collection (Blumsohn et al., 1995). Takahashi et al. (2000) found that the variability of N-mid osteocalcin was less than that of intact osteocalcin. For stability of osteocalcin after storage, the mean value of N-mid osteocalcin was 94.3% of the initial value after 7 days at 4°C whereas intact osteocalcin was 73.4%. The reduction of intact osteocalcin values was significantly larger than N-mid osteocalcin after 2.5 and 7 days. Furthermore at -30°C, N-mid values did not change up to 10 weeks. Blood samples should be collected on ice, and plasma or serum should be kept at -20°C for short term storage, and at -70°C for long term storage (Lee et al., 2000). Samples should only be thawed

once to prevent degradation and generation of fragments. Osteocalcin is also reduced in lipemic serum, because it binds with lipids (Delmas et al., 1990).

A number of sensitive RIA and ELISA kits have been developed for measuring osteocalcin in humans. These assays may be used in some animals (these include pigs, goats, sheep, cows, horses, rabbits and primates). These are not appropriate for others such as dogs, rats, and mice. Sensitive and specific RIAs are now available commercially for rats and mice (Allen, 2003).

Carter et al. (1996), suggests that serum osteocalcin and 1, 25 dihydroxyvitamine D3 are better predictors of bone mineralization and or bone turnover in pigs than serum alkaline phosphatase. Brown et al. (1984) suggested that there are three different groups of postmenopausal osteoporotic women, those with normal, high and low bone formation. In support to this, osteocalcin concentrations have been reported to be similar, higher or lower than the normal age-match controls.

2.4.1.3 Propeptides of collagen type I

During the formation of new bone, collagen type I is synthesized by osteoblasts. The collagen molecule is synthesized as a much larger precursor procollagen molecule. This is secreted into the extra cellular space, where it is attacked by extra cellular protease, releasing free peptide sequences, amino-and carboxy termini of the procollagen type I molecule (Allen, 2003).

The serum concentrations of carboxy-terminal propeptide of type I collagen (PICP), reflect the synthesis of new collagen, not only by osteoblasts in bone, but also by fibroblasts in other connective tissues (Melkko et al., 1990). PICP as a marker of bone formation is appealing, since the synthesis should be directly proportional to the amount of type I collagen produced. PICP, however, appears to lack sensitivity for identifying subtle alterations in bone turnover (Charles et al., 1994). PICP is stable in serum and can be reliably measured with an immunoassay, in specimens which have been frozen and thawed a number of times. It has also been shown that the molecules are stable at room temperature for 15 days (Swaminathan, 2001).

The serum concentrations of amino-terminal propeptide of type I collagen (PINP) also reflect changes in the synthesis of new collagen, by both osteoblasts in bone and by fibroblasts in other connective tissues (Eberling et al., 1992; Melkko et al., 1996). Unlike PICP, a portion of non-dialyzable hydroxyproline is incorporated into bone. This component of the measured fragment may represent bone resorption. PINP, however appears to be a more dynamic and sensitive marker for changes in bone formation, than PICP (Eberling and Åkesson, 2001).
Commercial RIA and ELISA kits are available for both PINP and PICP in humans. Some of these kits show cross reactivity in animals e.g. in sheep and pigs (Allen, 2003).

Palmieri et al. (2000) found that in the second phase of osteogenesis *in vitro*, rat osteoblasts secrete inducers of chemo taxis and chemo invasion of endothelcells and tumors cells. The protein was isolated and identified. It had two sequences coincident with amino-terminal amino acids, of the α_1 and of the $\alpha_2$carboxyl propeptides of type I collagen, as physiologically produced by procollagen C proteinase. This could illustrate the unknown function for the carboxyl-terminal trimer as possibly relevant in promoting endothelial cell migration and vascularisation of tissue producing collagen type I.
Hernandez et al. (2004) studied the effects of glucocorticosteroids on DNA synthesis and cellular function in human osteoblastic cells by using indirect peroxidase staining with type I anti procollagen antibodies and by measuring PICP and PINP. Supraphysiological doses of glucocorticsteroids caused a direct inhibition on osteoblastic function through their effect on type I procollagen synthesis.

2.4.2 Markers of bone resorption

The organic matrix of bone consists of up to 90% collagen type I. Type I collagen is rich in the amino acid hydroxyproline and has a triple helix structure. The strands of the triple helix are connected by cross-links between lysine or hydroxylysine residues, which join the non-helical amino-and-carboxy-terminal ends of one collagen molecule to the helical portion of an adjacent molecule (Eyre, 1987). The amino-and-carboxy-terminals of these collagen type I molecules are linked to the helical region of the adjacent collagen molecules through pyridinium cross-links, deoxypyridinoline (Dpd) and pyridinoline (Pyd). During bone resorption, hydroxyproline and pyridinium cross-links can be released with or without fragments of the collagen molecule. These are not reutilised.

2.4.2.1 Telopeptides of collagen type I

Collagen type I break down is mediated by acid proteases. This is an enzyme derived from osteoclasts. The break down leads to a release of free and peptide bound metabolites of collagen type I (Coleman, 2002). Fragments released from amino-and-carboxy-terminals of collagen type I molecules are termed N-terminal telopeptides (NTX) and C terminal telopeptides (CTX). These consist of short peptide sequences from helical region of the collagen type I molecule and the telopeptide region of the adjacent collagen molecule linked to the pyridinium cross-link (Pyridinoline (Pyd) or deoxypyridinoline (Dpd)). A portion of the telopeptides released from the bone is excreted in the urine, the remaining portion is metabolised in the liver and kidney (Hanson et al., 1992). These markers can be measured in the urine and serum.

A third telopeptid, pyridinoline cross-linked carboxy-terminal-telopeptide of type I collagen (ICTP), can be measured in serum. This is a peptide, which is bound by hydroxypyridinium bridge proteins. These pyridinoline cross-links bind two telopeptid regions of one collagen molecule to another collagen molecule. When collagen is broken down, ICTP is set free into the circulation (Charles et al., 1994). This marker is not significantly affected by renal clearance in healthy individuals (Risteli et al., 1993).

Eriksen et al. (2004) isolated and characterised carboxy-terminal telopeptide structures of collagen type I from human bone. Analysis of collagen cross-links within purified structures indicated the presence of trivalent ICTP structures, divalently

cross-linked peptides and uncross-linked telopeptide chains. Estimations suggest that more than half of the trivalently cross-linked pool of carboxy-terminal telopeptides represent cross-linked structures other than pyridinolines and pyrroles. This suggests the presence of unknown, uncharacterised mature trivalent cross-links.

CTX, NTX and ICTP are commonly used to monitor skeletal effects of biphosphonate therapy for osteoporosis. All three markers have been used in animals. Different species have variable cross reactivity. NTX is measured by an immunoassay using an antibody to the α-2 chain of the NTX fragment (Hanson et al., 1992). CTX concentrations are also measured with an immunoassay. There is a cross reactivity for ICTP in commercial assays for dogs, cats, horses, pigs, rats and primates (Allen, 2003). NTX measurements have been done in growing minipigs kept in metabolic cages (Bollen et al., 1997). As shown in human studies, the reliability of measurements in urine is lower than those in plasma. It is of importance to choose appropriate biochemical markers to demonstrate effects of calcium on bone resorption. Guillemant et al. (2003) discovered that, for this purpose, CTX was more sensitive than NTX, while ICTP was not sensitive to acute changes in osteoclastic activity due to calcium intake.

2.4.2.2 Pyridinium cross links (Pyridinoline and deoxypyridinoline)

The modification of lysine and hydroxylysine produces non reducable pyridinium cross-links, pyridinoline (Pyd) and deoxypyridinoline (Dpd). These stabilize mature collagen. Both markers are released from bone in an approximate ratio of 3:1. Dpd is relatively specific for bone. Pyd is found in articular cartilage and soft tissue (ligaments and tendons). About 60% of the cross-links are bound to proteins. The rest are non protein bound. Pyridinium cross-links are not metabolised or absorbed from the diet (Colwell et al., 1993).

Urinary concentrations of Pyd can be measured using high performance liquid chromatography. Sensitive and specific ELISAs have now replaced this technique. These assays measure free Dpd in urine specimens. Pyd can be measured in both serum and urine. All commercially available kits have wide spread cross-reactivity with animal specimens. The cross-reactivity for Pyd measured in serum is found in dogs, horses, cows, sheep, pigs rabbits and primates (Allen, 2003).

The elevation of pyridinoline is caused by hydroxylation of lysine residues within the collagen telopeptides. Not only processes in bone, but also fibrosis processes can cause an increase in hydroxylation. Cytokines such as transforming growth factor-ß induce substantial increase in lysyl hydroxylase 2b mRNA levels, which induces the hydroxylation of lysine residues and therefore an increase in pyridinoline. Similar effects were observed for Interleukin-4, activine A, and Tumor Necrosis Factor-α (van der Slot et al., 2005).

2.4.2.3 Osteopontin

Osteopontin is a non-collagenous protein. It is very abundant in bone matrix and is synthesised by osteoblast, osteoclasts and osteocytes (Yoshitake et al., 1999). Osteoclasts express αvβ3 integrin, which is a receptor for osteopontin (Flores et al., 1992). Yamate et al. (1997) reported that the expression of osteopontin by osteoclast and osteoblast progenitors in murine bone marrow increased after ovariectomy, and that osteopontin stimulated the interaction between osteoclast progeniters and stromal/osteoblastic support cells that generated tartrate-resistant acid phosphatase-positive cells. Yoshitake et al. (1999) report that osteopontin knock-out mice are resistant to ovariectomy-induced bone resorption compared to wild-type mice. It is speculated that the bone loss observed may be related to the requirement of efficient signalling through the αvβ3 integrin for optimal osteoclast function. So if osteopontin is deficient, as it is in knock-out mice, there is no high-affinity interaction between osteopontin and αvβ3 integrin and therefore less bone resorption activity by osteoclasts. Other non-collagenous proteins such as bone sialoprotein (BSP) can interact with αvβ3 integrin, but with less affinity. Whether osteopontin is required for human postmenopausal osteoporosis requires further investigation.

Parathyroid hormone (PTH) is often used to increase bone mass in osteoporosis patients. It has, however, diverse effect on cortical bone mass. PTH regulates the expression of osteopontin in osteoblasts and therefore osteopontin could be one of the targets of PTH in bone. Kitahara et al. (2003) examined the role of osteopontin in PTH actions and found that osteopontin deficiency induced PTH enhancement of whole-bone bone mineral density as well as cortical bone mass.

Bone osteopontin has properties of an extra cellular matrix protein promoting osteoblast and osteoclast attachment via αvβ3 integrin. The attachment of osteoclasts, but not osteoblasts requires that the protein be appropriately

phosphorylated (Katayama et al., 1998). In a recent study were bone discs (wild-type, osteopontin-null) were implanted subcutaneously or intramuscularly in mice (wild-type, osteopontin-null), revealed the involvement of osteopontin in bone angiogenisis and osteoclast recruitment. The vascularisation and bone resorption were significantly impaired in the absence of osteopontin (Asou et al., 2001). As a molecule that mediates the attachment of bone cells to the bone matrix, osteopontin is poised to tranceduce any stress that would move the cell relative to the matrix (Denhardt et al., 2001). It can bind to hydroxylapitite, to osteocalcin (Ritter et al., 1992), and to type I collagen, especially after being cross-linked by tissue transglutaminase (Kaartinen et al., 1999). It is speculated that osteopontin released by the osteocytes and delivered to the bone surface, might attract osteoclast precursor cells and promote osteoclast mobility and that it could stimulate osteoclast differentiation in marrow, possibly by synergizing with a RANK/RANKL-generated signal (Denhardt et al., 2001).

Substances such as nicotine can regulate the expression of osteopontin. Walker et al. (2001) presented evidence that the nicotinic receptor α4 subunit is present in primary bone cells. To determine the in situ bone cell responses within their trabecular matrix, cores of human bone were isolated and perfused with different dosages of nicotine. An increase in osteopontin was found, suggesting that nicotine has direct effect on the synthesis of osteopontin. Sakamoto et al. (2005) investigated the effect of homocystein on the expression of osteocalcin and osteopontin in MC3T3-E1 preosteoblastic cells and found that osteocalcin decreased in the presence of homocystein and osteopontin was activated, compared to the control levels. This suggests that hyperhomocysteinenmia appears to be a risk factor for osteoporosis by disturbing osteoblast function.

2.4.3 Markers of bone turnover

2.4.3.1 Receptor activator of nuclear factor kappa B, receptor activator of nuclear factor kappa B ligand, osteoprotegerin

RANK, Receptor Activator of Nuclear factor Kappa B, is a protein, which is crucial for all calciumtropic hormones and proresorptive cytokines, to increase calcemia and multiplication of osteoclasts in bone. This is found in the membrane of cells in osteoclast line and in dendritic cells (Suda et al., 1999). RANK is seen as a hematopoietic surface receptor controlling the calcium metabolism and the osteoclastgenesis (Kaneda et al., 2000). RANK ligand (RANKL), is a protein produced by the osteoblasts, cells of bone stroma and by activated T lymphocytes. This protein may promote osteoresorption by induction of cathapsin K gene expression (Suda et al., 1999; Corisdeo et al., 2001). It binds on the surface of preosteoclasts and stimulates their differentiation into mature osteoclasts, thus resulting in osteoabsorption. RANKL is a member of the Tumour Necrosis Factor (TNF) family. It is a crucial cytokine for the differentiation, activation and survival of the osteoclasts. It also acts as a regulator between osteoblasts and osteoclasts (Lacey et al., 1998). Osteoprotegerin or OCIF (OsteoClastogenesis InhibitingFactor) is a protein, which also belongs to the TNF family. Osteoprotegerin inhibits differentiation of progenitors into osteoblasts through binding with RANKL, thus inhibiting maturation of osteoclasts. It therefore competes with RANK. It displays hypocalcemic and antiresorptive effects (Suda et al., 1999). Osteoprotegerin is expressed by a variety of other organs and tissues such as the heart, lungs, kidney, bone and vessel wall (Simonet et al., 1997).

Imbalances in the ratio between Osteoprotegerin and RANKL or RANK underlie the pathology of many skeletal disorders which exhibit excessive bone loss, excessive bone formation or bone remodelling disorders, e.g. rheumatoid arthritis, periprosthetic osteolysis, tumour associated osteolysis, various osteopetosis or Paget´s disease (Hofbauer and Heufelder, 2001; Walsh and Choi, 2003; Sattler et al., 2004; Hofbauer and Schoppet 2004; Schoppet et al., 2002).
RANKL is up regulated by a number of substances .i.e.1α25-dihydroxyvitamin D_3 , parathyroid hormone, glucocorticoids, prostaglandin E2, Interleukin(IL)α, TNFα, IL6,11,17,calcium and immunosuppressants (Cyclosporin A). A down regulation

occurs through Transforming Growth Factor (TGF) β. Osteoprotegerin is stimulated by some of the same factors, such as vitamin D_3, ILα, TNFα, IL6,11,17, calcium and estrogen ,TGF β and decreased by PTH, glucocoticoids, prostaglandin E2, insulin-like-growth factor 1 and immunosuppressants (Collin-Osdoby, 2004).

In vascular diseases, alterations in the RANK, RANKL, Osteoprotegerin system, have been implicated. Bone loss caused by osteoporosis has been prevented through blocking RANKL. Osteoprotegerin, RANK fusion proteins or RANKL antibodies may be used as a blockage. Other conditions such as chronic inflammatory disorders and malignant tumours in animal models have been prevented through a RANKL blockade. This may emerge as a therapy in humans for postmenopausal osteoporosis, myoloma and osteolytic metastasis (Hofbauer and Schoppet, 2004).

Up to now some studies have been performed that have produced contradictory results. Bone remodelling parameters showed only sporadically clear association with osteoprotegerin e.g. deoxypyridinoline and osteoprotegerin, C-terminal collagen I propeptide (Browner et al., 2001). While in other studies no correlations between bone markers and osteoprotegerin were found (Szulc et al., 2001). Browner et al. (2001) proved a slight negative correlation between calcium and osteoprotegerin and slight positive correlations between osteoprotegerin and parathyroid hormone. Osteoprotegerin has, however, had a favourable effect in individuals with osteoporosis or destructive bone diseases. It minimised the development of osteoporosis caused by ovariectomy and reduced development of experimental bone metastasis (Kostenuik and Shalhoub, 2001). These results indicate that the RANK, RANKL, Osteoprotegerin system can be used in diagnostics and therapy in osteoporosis and other conditions such as vascular diseases and tumours.

2.4.3.2 Bone Sialoprotein (BSP)

Bone Sialoprotein is a major constituent of bone matrix. It accounts for approximately 10% of the non-collagenous proteins. BSP mRNA is almost exclusively synthesised in mineralised tissues by osteoblasts, osteoclasts and osteocytes (Franzen and Heinegard, 1985). It therefore appears to be involved in the process of bone formation and resorption. BSP is a highly sulphated, phosphorylated, and glycosylated protein, which is characterised by its ability to bind

hydroxyapatite through polyglutamic acid, sequences (Oldberg et al., 1988). The deposition of BSP into the extra cellular matrix (Chen et al., 1991, 1992) and the ability to nucleate hydroxylapatite crystal formation indicate a potential role in the initial mineralization of bone (Hunter and Goldberg, 1993). BSP has been reported to be able to promote the differentiation of pre-osteoblasts into osteoblasts (Zhou et al., 1995). Valverde et al. (2005) suggest that BSP contributes to RANKL mediated bone resorption, by inducing osteoclastgenesis and osteoclast survival and deceasing osteoclast apoptosis.

Studies have been performed and have demonstrated that BSP is a specific biochemical marker of bone metabolism in post-menopausal women and valuable for the quantitative monitoring of the skeletal response to hormone replacement therapy in postmenopausal women (Störk et al., 2000). Ruf, (2001) studied the value of sialoprotein as a biochemical marker related to screening and diagnosing osteoporosis. The study showed that bone sialoprotein reacted the same way as the known bone formation parameters (BAP, osteocalcin, PICP) and concluded that BSP is a sound and convincing supplement parameter for diagnosing osteoporosis. BSP is expressed in breast, thyroid and prostate cancers (Ibrahim et al., 2000; Waltregny et al., 2000). Recently scientists have, however, recognised that BSP is elevated in the tumours and blood of people that are developing certain other cancer forms.

The National Institute of Dental and Craniofacial Research (NIDCR) reports for the first time (2004) that BSP forms a complex with two other proteins, and therefore possibly enables cancer cells to degrade the tissue that surrounds them and break free from the tumour, causing metastasis. This could mark the protein complex as a potential target to prevent metastasis. Therefore, the regulation of the BSP gene expression is potentially important for the regulation of osteoblast differentiation, bone matrix mineralization and in the tumour metastasis. Kato et al. (2006) showed that lipopolysaccharides (LPS) suppressed BSP gene transcription through protein kinase A and tyrosine kinase-dependant pathways. LPS effects were mediated through cAMP response elements and fibroblast growth factor response elements in the proximal BSP gene promoter. The studies of Shimizu et al. (2006) indicate that the combination of fibroblast growth factor and forsolin act through protein kinase, tyrosine kinase and mitogen-activated protein kinase dependent pathways and target certain elements of the BSP gene, to synergistically increase the BSP expression. Nakajima et al. (2006) showed that chlorpromazine suppresses the BSP

transcription. This occurs through tyrosine and mitogen-activated protein kinase dependent pathways.

2.4.3.3 Osteonectin

Osteonectin is also known as Secreted Protein, Acidic and Rich in Cysteine (SPARC), which is a phosphorylated, acidic, glycine-rich protein, which binds calcium. It may, however, be involved in cell proliferation processes such as modelling of extracellular matrix. Osteonectin binds selectively to hydroxyapatite and to collagen fibrils at distinct sites in bone. It is responsible for the calcification of bone collagen (Termine et al., 1981). SPARC belongs to the matricellular group of proteins. It modulates cellular interaction with an extra- cellular matrix by binding structural matrix proteins such as collagen and vitronectin. It inhibits cellular proliferation by stopping cells of the G1 phase of the cell cycle. Other functions of this protein are regulating the activity of growth factors such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF)-2 and endothelial growth factor (VEGF). In adult animals, osteonectin is expressed largely in remodelling tissue such as bone, gut mucosa, healing wounds, tumours and disorders associated with fibrosis (Brekken and Sage, 2000). It is also secreted by human osteoblasts (Hunzelmann et al., 1998).

Osteonectin can be expressed by endothelium, in response to certain injuries. As a result of certain studies, it appears to regulate endothelial barrier functions, through F-actin-dependent changes in cell shape. The appearance of intercellular gaps changes and a paracellular pathway for extravasation of macromolecules is provided (Goldblum et al., 1994).

Fujita et al. (2002), suggest that SPARC may play an important role in the remodelling and repair of periodontal tissue by promoting proliferation and matrix metallopproteinase-2 production. It may also regulate osteoclast formation through a osterprotegerin/ osteoclastgenesis inhibitory factor in periodontal ligament cells.

In particular, it is clear that although matricellular proteins are not required for bone development and function, the proteins act to modulate post-natal bone structure in response to aging, ovariectomy, mechanical loading and bone regeneration (Alford and Hankensen, 2006).

2.4.4 Bone marker variability

Bone remodelling is often referred to as coupling, which is a link between bone formation and bone resorption. The resorption process takes approximately 7-10 days, whereas the formation process takes 2-3 months. Resorption markers respond to therapy faster (2-12 weeks) than formation markers (3-6 months) (Bernardi et al., 2004). Serum and urinary markers are used as diagnostic and prognostic indicators of skeletal health and disease. The utility of these markers is critically dependent on the ability of the assay to detect significant variation from the normal (healthy) state. The variability within the assay technique (analytical variability) and the variability that is inherent within the study population (biological variability) determine the expressiveness of individual assays. Factors affecting biological variability include age, sex, nutrition, exercise, and systemic diseases (Watts, 1999; Souberbielle et al., 1999). In addition to these, pre-analytical variability can be caused by marked diurnal, day-to-day and seasonal variation or incorrect storage.

Pre-analytical and biological factors

Diurnal variation

The underlying basis for these temporal rhythms has not been explained, but links to activity, food and water intake, renal function and hormonal changes have been implicated (Mühlbauer and Fleisch, 1995; Nielson et al., 1991).
In humans bone alkaline phosphatase has been showed to increase in the morning to the afternoon. Osteocalcin increases at night (Nielson et al., 1990a). NTX, Pyd, Dpd increase at midnight (Schlemmer et al., 1994).
Osteocalcin and bone alkaline phosphatase showed the same patterns in miniature pigs as in humans (Tsutsumi et al., 2004a). Many studies in human medicine have concluded that osteocalcin has a large diurnal variation. Peaks were found in the early morning 0400h (Grundberg et al., 1985; Tracy et al., 1990). Seasonal variations have also been seen. Bone marker concentrations decline from January to July and then increase in winter (Thomsen et al., 1989; Nielsen et al., 1990b). Jackson et al. (2003) studied the circadian variation in biochemical markers of bone cell activity in two year old horses. He found no circadian variation in the analysed

markers, but implied that the circadian variation may come at a later age when the skeleton has reached maturity.
Ostrowska et al. (2003) found that lighting conditions can influence daily fluctuations of biochemical bone markers in rats. The mechanism seems to be dependant on the changes of endogenous melatonin concentrations. Woitge et al. (2000) suggested that biochemical markers BAP, Pyd, Dpd, NTX and PTH, 25(OH) D_3, 1.25$(OH)_2$ D_3 showed significant seasonal variation. Levels of biochemical markers and of PTH were highest in winter and most predominant in premenopausal women under fifty years of age and in subjects who showed a significant individual rhythm in 25$(OH)D_3$. These changes are related directly to variations in the hormonal regulation of skeletal homeostasis.

Age, size, gender

Intra-individual variation is related to age. High values occur in childhood (2-10 fold) of that in adulthood. The metabolism is very active in infants and slows down at the age of 3 years. Bone markers stabilise their activity in prepubertal children and increase at the beginning of the pubertal growth spurt. After a final height is reached marker levels decrease to the levels seen in adulthood (Szulc et al., 2000). Nielson et al. (1990c) discovered that the osteocalcin concentrations reached their highest levels during the luteal phase of the menstrual cycle. High values occur again in the postmenopausal period. Tsutsumi et al. (2004a) studied the age-related changes in bone markers in Göttinger minipigs. The ages 3-76 months, corresponded roughly to the period of childhood to adulthood. Tsutsumi et al. (2004a) discovered that bone alkaline phosphatase, osteocalcin, NTX, Pyd and Dpd followed the same patterns in minipigs as they did in humans at the same hypothetical age. Chailurkit et al. (2005) followed the progression of serum bone alkaline phosphatase (BAP) and serum osteocalcin in boys and girls and found that BAP levels increased with age, peaking at about 12-13 years. There was a progressive decline in BAP levels with advancing age in girls older than 9 years. Serum osteocalcin levels also increased with age and reached a peak at the ages 12 and 13 in girls and boys. Serum BAP and osteocalcin levels varied with pubertal stages. Van der Sluis et al. (2002) did a cross-sectional study on biochemical parameters of bone turnover in healthy Dutch children and young adults and found peak concentrations for collagen type I cross-linked N-

telopeptides, cross-linked telopeptide of type I collagen, carboxy-terminal propeptide of type I procollagen, N-terminal propeptide of type I procollagen, alkaline phosphatase and osteocalcin during puberty. In girls this is approximately 2.5 years earlier than in boys. Children with growth hormone deficiency have reduced bone marker concentrations. These increase with the administration of growth hormone. Federico et al. (2003) showed that there were no changes in serum osteocalcin, carboxy-terminal propeptide of type I procollagen, and cross-linked carboxy-terminal telopeptide of type I collagen during puberty in growth hormone treated, growth hormone deficient children in a longitudinal study. The study, however, failed to demonstrate an association between short and long term changes in bone markers and the long term rate of growth or final height of individuals. Baroncelli et al. (2000) showed that children with a growth hormone deficiency had reduced bone turnovers at baseline. Long-term growth hormone therapy stimulated bone turnover. Osteocalcin, PICP, and ICTP, however, do not predict growth rate during long term treatment in children with growth hormone deficiency.

The larger or bulkier skeleton of a man may increase the levels of bone markers (Bernardi et al., 2004). Breur et al. (2004) investigated the markers of bone metabolism in dog breeds of different size. The results of this study showed that serum bone alkaline phosphatase and ICTP concentrations in larger breeds and toy breeds do not differ from those of the concentrations found in beagles. A lot of studies show a higher concentration of osteocalcin in adult males than in adult females (Duda et al., 1988; Epstein et al., 1984). Zanze et al. (1997) found age and sex related differences in the urinary excretion of collagen degradation markers, suggesting that unlike boys, girls maintain a high degree of collagen degradation up to the age of 24 months despite a decrease in their rate of collagen formation. Gundberg et al. (2002), found that mean values of serum osteocalcin and BAP were lower in women than in men <50 years old. In individuals ≥ 50 years of age, osteocalcin was significantly higher in women than in men. Fares et al. (2003) examined the effect of gender, puberty, and vitamin D status on biochemical markers of bone remodelling and found that they were most affected by gender.

Nutrition

Bone metabolism is dependent on nutritional status. Malnutrition causes a disturbed bone metabolism, therefore causing changes in bone marker concentrations.
With the exception of hydroxyproline, substances contained in diet do not affect serum and urinary levels of bone markers (Bernardi et al., 2004). Extreme fasting over 4 days had no significant effect on pyridinium cross-link concentrations (Grinspoon et al., 1995). In severely malnourished children, however, concentrations were decreased by approximately one-third of those after recovery from malnourishment (Branca et al., 1992).
A cross-sectional study considered the influence of diets in women between the ages of 45-55 with regard to fruit and vegetable intake. The potassium, magnesium and phosphate intake correlated negatively with pyridinoline excretion. Potassium, magnesium, ß-carotine and fibre intake were negatively correlated with deoxypyridinoline excretion. Statistical calculations indicated that magnesium intake accounted for 12% of the variability (New et al., 2000). Vitamin D deficient diets or malabsorption reportedly caused an increase in pyridinium cross-links excretion. Concentrations were two to three times higher in deficient postmenopausal women than in healthy subjects (Kamel et al., 1994).
MacLeay et al. (2004) studied the effect of dietary induced metabolic acidosis and ovariectomy on bone mineral density and bone turnover in ewes, and found that body weights, serum bone alkaline phosphatase and urinary deoxypyridinoline were not influenced by either diet or ovariectomy. Mullins and Sinning, (2005) concluded that high resistance training and subsequent maintenance of a high protein intake for 10 days have no effect on bone metabolism. Strength, lean tissue and deoxypyridinoline increased significantly after a time in the trained group and the high protein group, whereas bone alkaline phosphatase decreased. No significant changes were found in osteocalcin. Arjmandi et al. (2002), treated postmenopausal women who were not on hormone replacement therapy, with dried plums and dried apples for three months, both of which contain similar amounts of calories, fat, carbohydrates and fibres. Dried plums caused an increase in insulin-like growth factor-I and bone alkaline phosphatase activity. Serum and urinary markers of bone resorption were not affected by this dietary regime.

Exercise

Karlsson et al. (2003) studied the effect of exercise as a regulator of bone turnover. There were no differences in remodelling markers within the three groups of active soccer players. The active groups had higher bone markers than the controls. Former players had no differences in bone remodelling markers compared to matched controls. In this study it seemed as if the bone turnover, which was evaluated by serum bone remodelling markers, adapted to the current activity needed to maintain bone strength. Duration of exercise above that level seemed to confer no additional benefits.

Weight bearing and physical activity are important stimuli to bone growth and metabolism. Kim et al. (2000) indicated that bed rest immobilisation may influence the circadian rhythm due to no mechanical loading with the loss of daily activity. The study, however, showed that immobilisation on day 14 does not disrupt the basic diurnal rhythm of bone resorption.

Some studies confirm that exercise increases markers of collagen formation. The effect of whole body vibration training on hip density muscle strength and postural control was studied in postmenopausal women by Verschueren et al. (2004). The vibration training increased BMD significantly in the hip. This suggests that this may be useful for the prevention of osteoporosis.

Maimoun et al. (2004) determined the effect of physical activities (cycling swimming, triathlon), that induced moderate external loading on the skeleton, on bone remodelling. Osteocalcin was higher in swimmers and triathletes, whereas urinary type I collagen C-telopeptide was only higher in swimmers. Bone alkaline phosphatase was lower in cyclists than in other groups. An osteogenic effect was only found at bone sites under higher mechanical stress. This may suggest that bone turnover is sport dependent. Giangregorio et al. (2005) found that body-weight-supported treadmill training did not alter the expected pattern of change in bone biochemical markers over time. Maimoun et al. (2006) found an increase in osteocalcin and type I.-C telopeptid breakdown products in response to exercise performed above ventilatory threshold. Bone alkaline phosphatase increased both under the ventilatory threshold and above the ventilatory threshold. All markers of bone turnover returned to initial values during recovery. Shibata et al. (2003) examined the influences of long-term walking, and walking and jumping training on

bone mineral density and bone metabolism and found no significant differences in osteocalcin, parathyroid hormone and type I collagen cross-linked N-telopeptide. Bone alkaline phosphatase increased significantly after one year. The increase rate of this marker was greater in the walking and jumping group. The conclusion was that long-term walking training is beneficial for the promotion of bone formation. Yamazaki et al. (2004), demonstrated that moderate walking exercise in postmenopausal women with osteopenia/osteoporosis suppresses bone turnover and that an early change of cross-linked N terminal telopeptide of type I collagen may be a useful predictor of long term response to exercise.

The effect of prolonged immobilisation was investigated by Fiore et al. (1999). The results showed that an imbalance between bone formation and resorption occurred during bed rest. A significant increase in the markers of resorption, urinary pyridinoline, deoxypyridinoline and serum type I collagen cross-linked C-telopeptide was observed. The positive correlation between resorption marker increase and length of immobilisation suggests that the resorption rate did not decrease with time. This was confirmed in Sato´s study (Sato et al., 2001). Smith et al. (2005) demonstrated that bone loss is of significant concern for astronauts on long duration missions. The study confirmed that after longer duration in space, bone resorption was increased with an increase in urinary calcium concentrations, and an increase in collagen cross-links (N-telopeptide, pyridinoline and desoxypyridinoline). Formation markers were unchanged at landing, but two to three weeks later bone alkaline phosphatase and osteocalcin increased significantly above pre-flight values.

Donahue et al. (2003) state that bone loss also occurs during hibernation. The study consisted of the analysis of serum bone markers of five black bears. It was shown that the resorption markers remained elevated throughout the entire hibernation period compared to the period before hibernation. Bone formation was not affected by hibernation, but was accelerated after hibernation.

Marker correction

Bone markers can be measured in urine or in serum. Urine provides a higher concentration of analyses and has a less complex matrix, due to the fact that serum constituents are filtered in the kidney. The filtration or modulation of the kidney may change the concentrations of conjugated cross-links compared to free cross-links. Urine values require correction for dilution using creatinine. Many life style and pathological conditions, such as exercise, stress, menstrual cycle or renal insufficiency may influence the creatinine concentrations (Gowans and Fraser, 1987). Serum values however do not require correction.

Storage

A lot of markers are very unstable, particularly proteins (Delmas et al., 2000). Levels of urine deoxypyridinoline and urine N-telopeptide of type I collagen have been reported to change during storage (Schober et al., 2002; Schneider et al., 2002). Qvist et al. (2004) confirmed the stability of C-telopeptides of type I collagen in serum and plasma samples for up to 3 years. Due to the stability at elevated room temperature in EDTA plasma, this might be the preferred matrix.

Intact osteocalcin is only stable for a few hours at room temperature. Specimens should, therefore, be frozen within an hour after collection (Noonan et al., 1996). Serum storage at room temperature caused no significant loss of osteocalcin during the first six hours. After longer storage at room temperature, osteocalcin decrease depends on which test kit is used and which fragment of osteocalcin is measured (C-terminal fragments are more susceptible to enzyme cleavage, N-mid fragments are more stable, see 2.4.1.2) (Knapen et al., 1996).

Analytical factors

This can be described in terms of coefficient variation. It can be determined from replicate analyses of specimens. Quality control materials are however used more often, to determine the acceptability of an analysis.

Different tests have been developed for the same bone marker e.g. osteocalcin, where different antibodies and method have been used. This leads to misinterpretation of results (Diaz Diego et al., 1994). Different antibodies may be directed against different epitopes of the analyte and therefore different analyte measurements may be obtained.
Manufacturers inform users that the analytical variability ranges from approximately 10%-3%. The analytical performances of bone markers are often focused within standardised laboratory settings. Inter-laboratory variation must be taken into account. The source of the analytical variation is relevant, when the results of an analyte are provided from different laboratories (Seibel et al., 2001; Kleerekoper, 2001; Vesper et al., 2001).

How to avoid pre-analytical variability

Keeping in mind that the bone markers fluctuate in a diurnal rhythm, and bone markers are highest in the morning, one should collect specimens (serum, plasma, urine) at the same time each day of collection.
The replicate collection, e.g. collecting of multiple samples over 3-5 days, should have more effect than replicate analyses on analytical variability.

Factors affecting creatinine excretion should be identified and excluded.
Schober et al. (2002) found that urinary NTX and creatinine concentrations decreased significantly respectively after storage at -20°C, whereas deoxypyridinoline and deoxypyridinoline/creatinine increased after storage at -20°C. It was concluded that storage at such low temperatures frees deoxypyridinoline and the epitope used for quantification of NTX is denaturated. These changes are often masked through centrifugation. In this process the free deoxypyridinolines are removed. It is suggested that an additional quotient deoxypyridinoline/NTX should be evaluated as an alternative to normalising via creatinine excretion.

The use of serum assays minimises the variability occurring in urine markers.
Samples should be analysed shortly after collection. If not, they should be stored correctly (Bernardi et al., 2004). Pre-analytical variables, such as the use of anticoagulants and protease inhibitors could decrease the instability of osteocalcin.

Minimal changes were found in heparin-treated plasma with or without aprotinin, stored at -20°C or -70°C for up to 90 days (Noonan et al., 1996). If no appropriate storage is available, then the analysis of more stable regions of osteocalcin should be chosen. Rosenquist et al. (1995) found that mean serum concentrations of intact osteocalcin decreased by 63% after 7 days at 4°C, whereas N-mid osteocalcin was only reduced by 10%.

2.5 Animal models used to study osteoporosis

Animal models make a contribution to the enhanced knowledge of the aetiology of osteoporosis. The preclinical evaluation of efficiency and safety of interventions that should prevent or reverse bone fragility can be studied. The FDA, Food and Drug Administration, “Guidelines for preclinical and clinical evaluation of agents used in the treatment or prevention of osteoporosis (1994)”, recommends two preclinical animal models, the ovariectomised (OVX) rat and a second non-rodent model, to demonstrate the efficiency and safety of new agents for osteoporosis therapy (Thompson et al., 1995).

The goal of an animal model is to replicate the human condition as closely as possible. This has proved to be difficult. Fracture risk has not been reproduced in animals. Other questions such as the investigation of the degree of correspondence between mechanisms, which lead to bone changes in animals and in humans, have proved to be difficult. Species differences in cellular and biochemical levels influence the usefulness negatively (Turner et al., 2001).

There are many things that influence bone mass and these vary in different species. It is, however, possible to evaluate an animal model objectively. This can be done by evaluating to what extent similar events, such as hormone deficiency or aging, lead to similar metabolic, cellular and architectural changes in humans and in animals (Turner et al., 2001).

2.5.1 Small animal models

2.5.1.1 Rodents

Rodents provide the most commonly used model for the study osteoporosis. They have many advantages. They are inexpensive, easy to house and genetically specific strains can be acquired easily. The short life span enables studies on the effect of age on the bone. Rodents, however, do not experience natural menopause, but an ovariectomy can produce an artificial menopause (Wronski et al., 1985). OVX (ovariectomy) results in significant bone loss, but the use of this model has limitations, because young rats have limited naturally occurring Bone Mineral Unit based remodelling. Older rats, however, have a Haversian system and laminar bone (fine fibred), trabecular remodelling and secondary osteonal remodelling. Rats are therefore poor models for the effect of ovariectomy on cortical bone, because of the lack of a Haversian system and the absence of osteoblast activity in the late stages of estrogen deficiency (Wronski and Yen, 1991). Egrise et al. (1999) suggests that the extracellular fluid of bone marrow, which is also a medium surrounding the osteobasts and their precursor cells, may negatively regulate osteogenic cells and may contribute to the fact that older animals are unable to supply osteoblasts to the bone in proportion to demand. Barry et al. (2002) found that older rats have bone mineral which is less soluble and more crystalline than the bone mineral of younger rats. Aronson et al. (2001) suggests that 24-month old rats have a relative deficit in endosteal bone formation which may not be related to cell proliferation but rather to cell organisation. Chen et al. (2003) concluded that with an increase in age, the ability of osteogenesis of rat marrow stromal cells decreases.

Findings of different investigators are not always consistent. This may be due to differences in the age of animals at ovariectomy, and time after ovariectomy (Yamazaki and Yamaguchi, 1989). Patlas et al. (2000), oophorectomised rats at different ages, compared the histomorphometric findings (bone size, bone trabecular, and cartilage volume in different areas) and found that younger, rapidly growing rats exerted significant changes after oophorectomy. No significant changes were found in older 10 month old rats after oophorectomy. Fukuda and Iida (2004) found that total bone mineral density of metaphysic and diaphysis of long bones in rats increased until 12 months and decreased after 15-24 months. Cortical and trabecular bone mineral density were not always similar to total bone mineral density.

2.5.2 Large animal models

2.5.2.1 Dogs

The dog has not been widely used as a model to study osteoporosis. Some investigators detected bone loss after ovariectomy, while others detected no changes (Turner et al., 1994). Kern, (2000) studied the influence of phytoöstrogens, polyunsaturated fatty acids, vitamin D and K on bone metabolism and micro architecture of bone, in intact and ovariohysterectomised beagles, and found that these substances caused a prophylactic increase in bone mineral content. Bone turnover increased significantly after ovariohysterectomy, but bone microarchitecture was only altered to a small extent. Faugere et al. (1990) also did a study with ovariohysterectomised beagles and found cancellous bone volume, trabecular density and wall thickness decreased after ovariohysterectomy. Ovariectomy produced no significant changes in bone composition or loss of cortical area, but a significant bone loss occurred in the spine (Martin et al., 1987). Boyce et al. (1990) describe the increase in bone remodelling after ovariohysterectomy as transient and add that at no time after the intervention was a reduction of bone volume detected.
Wilson et al. (1998) demonstrated that rib cortical bone is a responsive site for the effects of ovariectomy in aged female dogs. A transient increase in cortical bone formation occurred with a six fold increase over sham-operated dogs at 4 months after the intervention. Fukuda and Iida, (2000) orchidectomised male beagles and studied the effects it had on the bone metabolism. 1-6 months after the intervention osteocalcin, alkaline phosphatase total calcium and parathyroid hormone values increased, indicating a high bone turnover. Mean trabecular thickness increased significantly 3 months after orchidectomy. These results argue for an appropriate model to study osteoporosis.
The insensitivity and inconsistency of response of the dog's skeleton may be due to the six month interval between periods of luteal activity. The large size and the long life span of a dog, make it less useful as a model. Costs for the maintenance of the animal and for the administration of larger amounts of chemicals would be a consequence of longer experimental periods (Turner et al., 2001). Not only has the effect of hormone deficiency been evaluated in dogs, but also the effect of alendronate therapy in healthy male dogs. Biphosphonate treatment does not weaken the properties of bone. The trends indicate a slight positive overall effect on the mechanical properties of healthy canine bone (Fischer et al., 2006).

2.5.2.2 Sheep

Sheep are livestock. They are food animals, and society may tolerate the use of such animals as models more than that of dogs or cats. Sheep are docile and therefore easier to handle. They are flock animals and are less stressed when they are housed outdoors, with minimal supervision. Other advantages are the similarities of hormone profiles of ewes to those of women. Sheep have a seasonal polyestrous cycle. Cycle varies with breed and amount of light. Sheep, like rats, do not have a natural menopause that is characterised by bone loss (Newman et al., 1995). A disadvantage of this model is the gastrointestinal tract. In studies where there is an interest in the absorption of drugs in the gastrointestinal system, a surgical insertion of an abomasel fistela is required to bypass the micro flora.

Turner et al. (1995a) studied the bone density in the calcaneus and the distal radius of ovariectomised sheep and a control group. No differences were found between both groups. A further study was done by Turner et al. (1995b), where the bone mineral density was measured twelve months after ovariectomy. No differences were found between control group and operated group. Schorlemmer (2002) showed that a combination of ovariectomy and glucocorticoid therapy, led to osteopenia in sheep. Newton et al. (2004) determined the effects of ovariectomy on the trabeculae of ovine ilac bone. One year after the operation the trabecular volume decreased and the trabecular separation increased. Osteocalcin and urinary deoxypyridinoline were significantly higher at the end of the experiment than at the beginning. Chavassieux et al. (2001) studied the effect of a new selective estrogen receptor modulator (MDL) in comparison with ovariectomised ewes and ovariectomised ewes associated with Lentaron, on bone remodelling in ewes. MDL reduced bone turnover and increased bone mineral density, whereas ovariectomy increased bone turnover. Lantaron tended to amplify the effect of ovariectomy. MDL could therefore be an effective agent to prevent postmenopausal bone loss. Lill et al. (2001) did a pilot study comparing four groups of sheep; control group; ovariectomised, vitamin D/calcium restricted; ovariectomised, glucocorticoid treated and ovariectomised vitamin D/calcium restricted and glucocorticoid treated. The largest decrease in bone mineral density was seen in the ovariectomised vitamin D/calcium restricted and glucocorticoid treated group. Structural changes in the vertebral body were pronounced. These changes were less pronounced in the femoral head. The study

showed that the combined therapy group was the most effective model for inducing osteoporosis.

2.5.2.3 Primates (non -human)

Non-human primates, such as macaques and baboons, demonstrate many advantages. Their organ systems such as gastrointestinal tract, endocrine system and bone metabolism resemble those of humans. Female macaque monkeys have a monthly cycle and have hormonal patterns similar to those of humans (Hodgen et al., 1977). A response to ovariectomy is a significant reduction in vertebral cancellous bone volume (Miller et al., 1986). Jerome et al. (1986) demonstrated that bone turnover is increased following ovariectomy in the baboon. The seemingly parallel endocrine system of primates and human is a notable advantage. Female primates must be of sufficient age, because the extent of osteoporosis is not only linked to rapid bone loss, but also to a sufficient peak bone mass (Rigotti et al., 1991). An important feature of the non-human primate is the presence of harverian osteonal remodelling in the cortical bone (as observed in humans) (Burr, 1992).
In cynomolgus monkeys, the peak bone mass is not reached until 9 years of age (Champ et al., 1996). Many studies, however, have used ovariectomised monkeys aged 4-7 years (Jerome et al., 1993). The ovariectomy of skeletally immature monkeys therefore seems to be an inappropriate model.

To acquire such aged female primates is difficult and costly. Furthermore, once acquired, the handling of such animals is an issue of concern. Aggression can be controlled by skilled technicians.
It was frequently the case, that primates used in studies were captured in the wild. These monkeys were potential reservoirs of zoonotic diseases such as Marburg virus disease, Eloda virus disease, viral hepatitis (Herpesvirus simiae), and tuberculosis. For these reasons and because of legal requirements related to how these animals must be housed and kept; there is public resistance to their use as models. These days, however primates are especially breed and reared under laboratory conditions for the use in studies.

2.5.2.4 Pigs

A commercial farm pig weighs approximately 150kg which limits its use as a research model. Minipigs have eliminated this problem. They weigh up to 60kg when mature and are therefore better to handle and less housing space is required. The reproductive cycle of the pig is similar in duration to that of the human (18-21 days). Like humans the cycle in pigs is continuous. Furthermore, pigs, like humans are omnivore and the anatomy of the gastrointestinal tract is very similar.

Bourchard et al. (1995) found that Sinclair minipigs possess a definable peak bone mass at the age of 2.5 to 3 years. Tsutsumi et al. (2004b) obtained standardised data, established a relationship between growth and bone metabolism in female Göttinger minipigs and observed that the point of peak bone mass was not reached until 76 months of age. Mosekilde et al. (1993a, 1993b), discovered that pigs have a much higher bone mass and a denser trabecular network than humans. They also discovered that they have a different loading pattern of the skeleton. The combined effects of ovariectomy and a mild dietary calcium restriction (0.75%) resulted in excessive resorptive cell function at the level of the remodelling unit, which resulted in vertebral cancellous osteopenia and a reduced biochemical competence of the vertebral cancellous bone. The reduction of the cancellous bone appeared to be due to the trabecular perforation (Mosekilde et al., 1993a, 1993b). These findings suggest that the ovariectomised, calcium-restricted (0, 75%) Sinclair S1 minipig has potential as a model to study bone remodelling in humans and perimenapausal bone loss in women.

Boyce et al. (1995) did an unbiased estimation of vertebral trabecular connectivity in calcium-restricted ovariectomised minipigs. The connectivity density was increased twofold when compared with sham-operated minipigs fed a standard diet. The results of this study suggested that in calcium-restricted ovariectomised minipigs, trabecular plates are transformed into rods by perforation. The changes in topography appeared to be due to the excessive resorptive cell function at the level of the bone remodelling unit. This data suggests that calcium-restricted ovariectomised Sinclair minipigs may be a useful model of cancellous osteopenia and trabecular plate perforation, remodelling, 3D architecture and strength in response to estrogen deficiency.

Minipigs are large enough to receive prosthetic implants and withstand repetitive bone biopsies and large volumes of blood sampling. Bone removal and manipulation

or disposition of trabecular and cortical bone occurs at a rate comparable to that of humans and pigs possess laminar bone (Mosekilde et al., 1987).
Bone metabolism, estrous cycle and gastrointestinal functions of swine are features commending them as animal models. There are, however, also negative factors. The acquisition costs are high and in some areas these animals are rare. Larger farm pigs require more space and handling is more difficult. Regardless of breed, pigs are loud and can be aggressive. This makes them inconvenient in the use of projects, which require a large amount of handling and interaction (Turner, 2001).

2.6 Induction and simulation of post- menopausal osteoporosis

The post-menopausal condition is caused by the absence of sexual hormones. To simulate the absence of these hormones different procedures can be performed.

2.6.1 Oophorectomy, ovariohysterectomy

The removal of the ovaries (oophorectomy) may be required to facilitate research or for pet pigs, such as miniature pigs. The miniature pig is increasingly being kept indoors, as a house pet. This has caused problems with female pigs when they become sexually mature. They tend to become sexually hyperactive. The hyperactivity is expressed through urinating and sometimes aggressiveness. Many pet owners have tried to treat the unwanted urinating with vasopressin, but have not been happy with the results. A method of treating this hyperactivity is to ovariectomise or ovariohysterectomise these animals.
The removal of the ovaries alone is easier and faster, and the risk of fatal haemorrhaging is less than with the ovariohysterectomy. The blood vessels of the broad ligaments of the uterus require ligation when ovariohysterectomised. Ovaries may be removed from the paralumbar flank, ventrolateral, paramedian or the ventral midline. The ventral midline and the paralumbar access to the abdomen are often preferred because the risk of post- operative infection and complications, such as herniation is less. After the ovary removal an uterus atrophy is expected (St-Jean and Anderson, 1999).
In pet pigs it is recommended to perform this procedure before the normal estrus cycle has begun.

In sexually mature pigs, an ovariohysterectomy is recommended, due to the risk of pyometra. The uterus and the ovaries may also be removed via paralumar, ventrolateral, paramedian or ventral midline incision. Like the ovariectomy, it is preferred to remove the uterus and ovaries via the ventral midline or paralumbar (St-Jean and Anderson, 1999).

2.6.2 Immunocastration

Immunocastration refers to a vaccination, which stimulates the development of sufficient antibodies to block the actions of hormones that are necessary for a successful reproduction. Delves (2004), differentiates between immunocontraception and immunocastration. Thus the sexually mature individuals become infertile, as long as certain antibody titres are maintained (immunocontraception). Immunisation to prevent the development of hormones that are needed to reach sexual maturity can be seen as immunocastration.
The target hormone for immunocastration and immunocontraception is gonadotropin releasing hormone (GnRH).As well as this hormone, follicle stimulating hormone (FSH) and human chorionic gonadotropin (hCG) can also be targets for immunocontraception. Immunisation against FSH would suppress the spermatogenesis, whilst not interfering with the libido (LH being responsible for the induction of testosterone, which is necessary for the libido), only producing anti-FSH antibodies. Westhoff et al. (1996), show that, when whole FSH is used for vaccination, in addition to biological activity against FSH, anti-LH activity is also induced.

GnRH, a decapeptid hormone, is produced in the hypothalamus. It consists of 10 amino acids, which have a uniform sequence in mammals. When released, it simulates the anterior pituitary to secrete FSH and luteinizing hormone (LH). In male individuals these two gonadotropines act upon the gonads to stimulate testis growth and steroidogenisis. Testicular steroids such as testosterone are then released into the circulation where they perform certain functions and a feedback regulation of GnRH. Another steroid, androstenon, which is not androgenic, is stored in fat tissue and is in part responsible for the unpleasant boar taint. The immunisation against GnRH, therefore, will disrupt the hypothalamic-pituitary gonad axis and inhibit testis

growth and subsequently reduce boar odour, this being the reason why the immunisation has been used in male pigs.

Bonneau et al. (1994) studied the effect of a vaccination against luteinizing hormone-releasing hormone (LHRH), on performance of sexual development and levels of boar taint related compounds. Male pigs were allocated to three groups: a group of castrates, a group of intact males and an immunised group. Growth performance and carcass traits did not differ significantly between immunised and intact males. Genital tract weight was decreased by immunisation. Testosterone concentration was seventimes lower than that of the intact controls. Fat androstenone levels were reduced in those immunised, compared to the intact controls. Fat skatole, however, did not differ significantly in all three groups. The immunisation had limited effect on performance, but reduced androstenone, a boar taint-related compound, which is unwanted at slaughter.

The effect of immunocastration in gilts has been studied only in few cases. Esbenshade and Britt (1985) studied the effects of the immunisation on gonadotropin secretion, reproductive functions and response to gonadotropin-releasing agonists. Zeng et al. (2002a) studied the effect on serum LH, Inhibin A and the sexual development and growth rate in Chinese female pigs and found that out of 12 immunocastrated female pigs two did not respond to the immunisation. Ovarian and uterus weights of the immunocastrates were lower than intact controls. Non-responders and intact controls had similar ovarian and uterus weights. Antibody titres were significantly lower in non-responders than in immunocastrates. No follicular structures were found in ovaries of immunocastrated, while large follicles and corpora lutea were found in the ovaries of controls and nonresponders. These results showed that immunocastration can be used as an alternative to surgical castration.

The GnRH molecule in its physiological occurrence is small and is not able to induce antibody formation. A substance needs the size of over 5kDa, to have antigenic character. The GnRH-vaccination has been developed in recent years. At first the complete Freund´s aduvant was used, to obtain an immunological response, which was not very high (Talwar, 1985). This is a very effective adjuvant, but it causes unwanted tissue reactions (Thompson, 2000).

Good antibody titres were achieved when the GnRH molecule was linked to a large carrier protein molecule. This linkage can occur at the C-and N-terminals of the GnRH molecule and at the Ser^4 or Tyr^5 hydroxyl groups (Talwar, 1985).

(pyro) Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

Amino acid sequence of GnRH in mammals (Metz, 2003)

The linkage should not, however, obstruct the immunogenic structures of the molecule, which would decrease the antigenity. Many sequences of the GnRH molecule have been said to be antigenic epitops. Talwar´s, (1985) hypothesis preposed the amid form at the C-terminal (Gly^{10}-NH_2) as a binding position for specific antibodies.

An additional improvement of the immune reaction was achieved by conjugating two GnRH molecules (Tandem) (Meloen et al., 1994). The use of the Freund´s adjuvant was necessary here for the first immunization. Oonk et al. (1998) managed to develop a GnRH-tandem-dimer with incomplete Freund´s adjuvant and achieved good results. In further studies, the structure of the GnRH molecule was modified and the function of each amino acid as an antigen was evaluated. G6k-GnRH-Tandem-Dimer (Lys^6 instead of Gly^6) could still bind a carrier, ovalbumin, at the ε-amino group of lysine and showed good immunisation results in a mild oily adjuvant. Turkstra et al. (2002) found that four ($pGly^1$, Ser^4, Arg^8, Gly^{10}) out of nine amino acids can be replaced by alanine, without negatively effecting the immunocastration efficiency. If Tyr^5, Leu^7 and Pro^9 were replaced, a partial decrease in efficiency occurred, while the replacement of His^2 and Trp^3 completely negated immunocastration activity. The conclusion was that two positions in the basic unit of G6k-GnRH-Tandem-Dimer do not allow substitution by alanine, indicating that effective immunocastration can be achieved with modified GnRH peptides.

Zeng et al. (2002b) demonstrated that synthetic lipoproteins as self-adjuvanting immunocontraceptives can be highly immunogenic. Peptides used consisted of a collinear $CD4^+$T helper cell epitope from the L-chain of influenza hemagglutanin and LHRH. LHRH had B cell epitopes but no T cell epitopes in its sequence. Lipides were attached to the N-terminus or between T cell epitope and LHRH. The solubilities and

immunological properties of the lipoproteins depended on the lipid content and the position of attachment of the lipids. The most effective vaccinations were highly soluble and had a lipid attached at the centre of the molecule.
Fromme et al. (2003) investigated the immunological reaction caused by retro-inverse Gonadotropin-Releasing Hormone. They found that retro-inverse GnRH conjugated to ovalbumin as well as unconjugated retro-inverse GnRH elicited high anti-GnRH antibodies in rabbits and mice. The immunisation of mice occurred without Freund´s complete adjuvant. Retro-inverse peptides are resistant to cleavage of proteolytic enzymes and are potentially orally active, which is a positive aspect for the future application in animals.

There are vaccinations available commercially: Vaxstrate® (Arther Webster Pty Ltd, Australia) (Hoskinson et al. 1990) and Improvac® (CSL Animal Health, Victoria, Australia) (Dunshea et al. 2001). Vanxtrate® is used in cattle, while Improvac® is authorized for the use in boars.

3 Material and Methods

3.1 Animals used in this experiment

16 Dresdner miniature pigs were penned in separate stalls in a Danish system. Each animal had eye contact with the neighbouring animal. This enabled a feed ration control. Each animal received the same ration of feed allocated to each specific experimental group, which enabled steady weight gain. The enclosures allowed an individual observation of health status. During the first weeks after arrival animals were weighed regularly.

3.1.1 Animals care during the experiment

Pigs were fed twice a day. The daily ration increased with age. The feed consisted of a commercially obtainable miniature pig pellet feed from Firma Zimmererwerke, Landshut, Germany.
Two different diets were fed. The control group received a diet with a 0.99% calcium concentration. The rest of the pigs received 0.7% calcium in the diet.

3.1.1.1 Feed ration

Table 3: Age and daily feed ration of all three test groups

Age	**- 9 months**	**9 months – 10 months**	**10 months-end of trial**
Ration per day	440g	500g	550g

3.1.1.2 Energy ration

The energy content of both feed types was determined with a bomb calorimeter (C 2000 Basic, IKA, Staufen). This method measures the heat involved or absorbed during a chemical or physical process. The principle of this apparatus is the burning of a hydrocarbon in high pressure oxygen in a stainless steel container, maintaining a constant volume. The stainless steel container is surrounded with water. The heat produced by the reaction is absorbed by the surrounding water. By measuring the temperature changes in the water, the heat measured for the burned sample can be determined. This is the change in internal energy from the initial to the final states.

Sample preparation:
The net weight of a small plastic sample bag is determined. The scale should be tarred with the sample bag on it and then 0.270 grams of grinded feed is weighed and placed into the plastic bag. The energy content of the plastic sample bag should be taken into account before feed is bombed. Feed sample in the plastic bag is then fixated to the small piece of fuse wire and placed into an ignition cup in the bomb. This should be done with great care; to be sure the sample will ignite when the bombing process is started. The net weight of the feed sample and the plastic bag is to be recorded in the digital apparatus. The bomb should then be sealed by screwing the cap on and then filled with high pressure oxygen. The electrical connections are made at the top of the bomb. The bomb is then lowered into a stainless steel bucket containing water. A stirrer that is driven by a motor outside the calorimeter sticks in the water. A thermometer is also found in the water. This enables the water temperature to be monitored during the reaction. Two electrical leads connect the top of the bomb from the outside. These deliver the current that initiates the reaction. After approximately 15 minutes, the bombing process is completed and the energy content appears on the screen (MJ/kg).
Table 4 represents the gross energy ration fed during the trial. Each group of animals received constant energy amounts throughout the trial.

Table 4: Gross energy ration per kg feed measured by means of bomb calorimetery

Feed I 0.99% calcium	Feed II 0.7% calcium
16.5 MJ/kg	15.6 MJ/kg

3.2 Feed Analysis

3.2.1 Weender Analysis

To determine the nutritional content of feed used in this study, a Weender analysis was performed. Each analysis was performed in duplicate. Dry, homogenised feed samples were used for the analysis.

- Determining crude dry substance

The dry substance of each feed sample was determined by drying the sample in a cabinet desiccator type T12 Function Line, Firma Heraeus (Hanau, Germany) until a constant weight was reached. A defined amount of sample material was weighed in porcelain containers. This was placed in a cabinet desiccator, at 103°C for at least 4 hours or more, until a constant weight was reached. This withdraws all crude water (pure water, volatile fatty acids and other substances that are volatile from samples. The samples are placed in an exiccator with silica gel to cool. This prevents samples absorbing air humidity. Dry samples are then weighed. The crude dry substance can be calculated as follows:

$$\text{Crude dry substance (\%)} = \text{(Sample weight after drying/ sample weight before drying)} \times 100$$

- Determining crude Water

Crude water comprises all volatile substances at 103°C. It can be calculated as follows:

Crude water (%) = sample weight - dry substance

- Determining crude ash

Feed samples are incinerated in the muffle furnace P 320, firma Nabertherm (Lilienthal. Germany). Defined amounts of samples are weighed in porcelain containers. These are placed in the muffle furnace for six hours to 3 days at 550°C, until completely incinerated. This process eliminates all organic constituents from samples. Only inorganic substances and mineral nutrients remain. The samples are left to cool in an exiccator and are weighed again. The percentage of crude ash in the samples can be calculated as follows:

Crude ash = (sample weight after incineration / sample weight before incineration) X 100

- Determining crude fat

The crude fat concentration was determined by extracting samples with petroleum ether in a Soxhlet apparatus after an acidic pulping procedure. The appliance used was a Soxtec Avanti 2050, Firma FOSS (Höganäs, Sweden).
After weighing 1-2g of sample, an acidic pulping was done with concentrated hydrochloric acid. Then an extraction or leaching with petroleum ether at 135° C was performed. The attained ether extract consists of neutral fats, lipoids and other ether soluble substances. Each sample was weighed after an hour´s drying in the cabinet desiccator. The amount of crude fat in % can be calculated as follows:

Crude fat (% dry substance) = (weight of extraction/ weight before extraction) X 100

- Determining crude protein

The crude protein analysis was conducted by means of the Kjeldahl technique. An automatic analysis apparatus was used, Kjeltec 2400, Firma FOSS (Höganäs, Sweden).
0, 5-1g sample was weighed for the analysis. Firstly, samples were boiled with concentrated sulphuric acid in a digester. This caused the nitrogen to oxidate and form ammonium. Based on the Kjeldahl technique, the addition of sodium hydroxide solution causes the release of ammoniac. The nitrogen content in samples was then determined by means of titration. The concentration of crude protein in samples was calculated by multiplying the nitrogen concentration with the factor 6.25 because the average nitrogen concentration in proteins is 16%. Other substances containing nitrogen, besides proteins, are included in this analysis.

- Determining crude fibre

This analysis consisted of boiling the samples alternately in acids and bases in a Fibertec-Hotextractor, Firma FOSS (Höganäs, Sweden). Approximately 1 g of sample was boiled in 1. 25 % sulphuric acid and then washed in hot water. This was followed by boiling the samples in caustic potash solution for 30 minutes and renewed washing in hot water. Subsequently samples were washed with acetone. The residue was placed in the cabinet desiccator to dry at 103°C. The dry residue was then weighed. After weighing it was placed in the muffle furnace and weighed again. Crude fibre concentration can be calculated as follows:

Crude fibre (% dry substance) = ((weight after muffle furnace - residue after drying)/sample weight) X 100

- Determining nitrogen free extract NfE

This can be calculated as follows:

NfE (% dry substance) = Dry substance - (crude ash + crude fat + crude fibre + crude protein)

Table 5: Nutritional content of the 0.99% calcium diet and 0.7% calcium diet analysed with the Weender analysis. 0.99% calcium diet was allocated to the control group n=5. 0.7% calcium diet was allocated to the OVX n=5 and second control groups n=6 respectively. The nutritional content is displayed in % of the original substance (oS %) and % of the dry matter (dS)

Nutrients	Control n=5 0.99% Ca diet		OVX n=5	Second control n=6
			0.7% Ca diet	
	oS %	dS%	oS%	dS%
Dry matter	89.2		89.2	
Humidity	10.8		10.8	
Rp	14.7	13.1	14.1	12.6
Rfe	2.2	2	2.6	2.3
Ra	8.1	7.2	8.3	7.4
Rfa	12.1	10.8	12.4	11
NfE	51.8	46.2	48.4	43.2

3.2.2 Calcium and phosphorus determination

Preparations for determining calcium and phosphorus concentration in feed

To determine the calcium and phosphorus concentrations an ashing of the feed samples is required. Crude ash is the result of a 48 hour ashing at 550°C, (M 110 thermicon P®, Heraeus, Hanau). This contains minerals (macro elements and trace elements) and other inorganic substances. To determine calcium and phosphorus concentrations it is necessary to mix crude ash with hydrochloric acid (37%), which

brings minerals into solution. This solution can be used directly to determine levels of calcium and phosphorus.

- Calcium determination

The calcium concentration in the prepared solution was determined with a flame photometer (Elex 6361, Eppendorf, Hamburg) at 622nm. If the measurements turned out too high, the solution was diluted. This was done with the help of a Duel Diluter (Diluter 5213, Eppendorf, Hamburg), with 1% Lithiumchloride solution.
The principle of the flame photometer is as follows: the solution is drawn into the machine and distributed through pressure. It is then injected into a mixture of acetylene. A flame emerges, due to the stimulation of atoms. The strength of light produced is dependent on the number of atoms present. Through measuring the light intensity conclusions can therefore be drawn on the concentration of an element in a solution. The flame photometer reads the concentrations in mmol/l. A calculation is required, to determine the amount of calcium in grams per kilogram feed. The atomic weight of calcium is required for this calculation (40,08g/mol).

$$gCa/kg = \frac{40.08[g/mol]*Reading[mmol/l]*Dilution}{1000 * \text{weight of feed before ashing}[g]}$$

- Phosphorus determination

The phosphorus concentration in the prepared solution was determined by measuring a colour spectrum with a photometer (Spectral photometer, GENESYS 10 UV, Thermo Spectronic, Rochester, New York, U.S.A).
The prepared solution was mixed with trichlor acidric acid, molybdate and vanadate. Two blank values consisting of trichlor acidric acid, molybdate and vanadate and a standard solution, consisting of trichlor acidric acid, molybdate and vanadate and potassium hyrogenphosphate were prepared.

Photometry determines the translucence of a solution in monochromatic light that has a certain wavelength. Determining the blank values adjusts the translucence to 100%. All readings were done at 366nm.

A calculation is required to determine the amount of phosphorus in grams per kilogram feed.

$$\text{g P/kg} = \frac{\text{X} * 10.5 * \text{Dilution}}{\text{Factor} * 100 * \text{weight of feed before ashing}}$$

Factor = 0. 34

Table 6: Calcium and phosphorus concentrations in g/kg feed and percent (%) in feed I and II

Feed I		Feed II	
Calcium	**Phosphorus**	**Calcium**	**Phosphorus**
9.89 g/kg	7.01g/kg	6.98 g/kg	7.00 g/kg
0.99%	0.7%	0.7%	0.7%

3.2 Design of the experiment

This trial was authorised by the government of Bavaria (Regierung von Oberbayern). Animals were divided into three groups. The control group and the OVX group both consisted of 5 animals, while the second control group consisted of six animals.
Blood samples were taken at intervals of seven weeks up until the first ovulation synchronisation, which occurred at a sexually mature age of one year and one month. The first blood samples were taken in week -65 followed by week -58 and -51. The first ovulation synchronisation ended in week -47. Blood samples were taken at this point of time, two weeks later (week -45) in the calculated diestrus cycle and one week after the calculated diestrus cycle (week -44). The same pattern of extraction followed in the following calculated cycle (week -42, week-41); week -42 being the calculated diestrus cycle stage in the following cycle and week -41 being the beginning of the third calculated cycle. The last two blood samples were taken in week -31 and week -19 pre-castration (diagram 2).
A second synchronisation began seven weeks before the operation and ended four weeks before the operation. This was done as described in 3.3.1.
Each animal in the OVX group received 2ml (8 mg) Altrenogest, Regumate®Janssen, Neuss orally two weeks before OVX, daily to suppress the estrus cycle stage. The animals in the OVX group were ovariohysterectomised at week 0. The control and second control group were not manipulated. Both OVX and second control group continued to receive 0.7% calcium diet until the end of the experiment, while the control group received a 0.99% calcium diet. Blood samples were drawn in all groups at week 0 followed by regular intervals of 2 weeks up until eight weeks post-castration. The samples taken from the OVX group in week 0 were takewn before the operative intervention.

3.3.1 Ovulation synchronisation

In order to coordinate the animals´state of cycle, an ovulation synchronisation was performed. On days one to eighteen, 2ml (8 mg) Altrenogest, Regumate®Janssen, Neuss, synthetic progesterone was applied orally. 24 hours after the last Regumate®, Janssen, Neuss, application, 750 IU (3.7ml) Intergonan®, Intervet, Unterschleißheim, a horse serum Gonadotropine (PMSG), was injected im. in the m. brachiocephalicus.

78-80 hours after this application, 500 IU (1.6ml) Ovogest®, Intervet, Unterschleißheim, a choriongonadotropin, was applicated i.m. A day after the last hormone application, blood samples were drawn ("day one of the cycle") (see diagram 1). Two to three days after the last hormone application, animals were observed for estrus signs. Twelve days after first blood sampling, and nine days after the second blood sampling, further blood samples were taken. During the second cycle, the same procedure was repeated. Blood samples were drawn in the estrus, in diestrus and in estrus cycle stages (diagram 1).

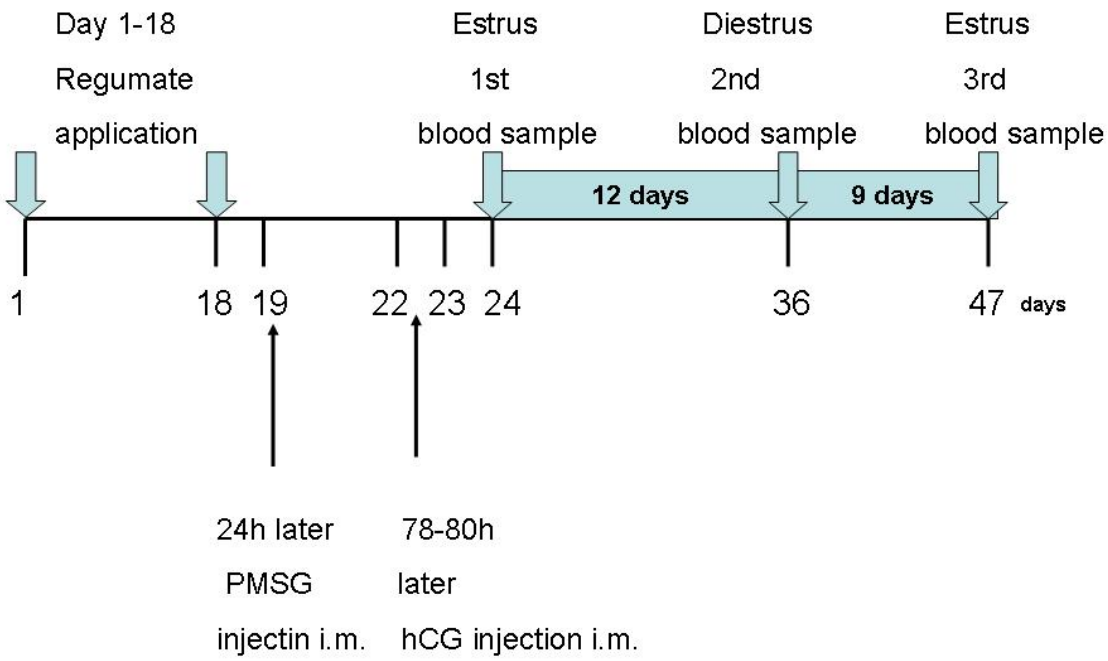

Diagram 1: Time schedule for the ovulation synchronisation and blood sampling. Day 1 indicates the first application day of Regumate®

3.3.2 Ovariohysterectomy

The uterus blood supply was minimised through estrus suppression with Regumate® Janssen, Neuss, which decreased the operation risk.

3.3.2.1 Anaesthesia and preparation

The ovariohysterectomy was performed in the clinic for pigs, Oberschleißheim, under the observation of Professor K. Heinritzi und Dr. M. Ritzmann.
The animals fasted 24 hours before anaesthesia.
Approximately 30 minutes before the operation began, an antibiotic, penicillin, (Procainpenecillin®, Animedica, Senden-Bösensell) 10000IU/kg, Meloxicam (Metacam® Boehringer, Ingelheim) 0.4mg/kg and Metamizol (Vetalgin®Intervet, Unterschleissheim), 20-50mg/kg were applied i.m.

Premedication consisted of Azaparone (Stresnil®, Janssen, Neus), 2mg/kg and Ketaminhydrochlorid (UrsoKetamin®, Bernburg AG, Serumwerk), 10 mg/kg. The medications were applied simultaneously i.m. in M brachiocephalicus. This was followed by inhalation anaesthesia, Isofloran (Forene®, Abbott, Wiesbaden).

An intra- venal catheter was laid in the ear vein, V. auricularis intermedia. During the operation the animals obtained an i.v. drip.
During anaesthesia oxygen saturation and carbon dioxide expiration were controlled regularly. Reflexes (skin, eyelid, interdigital) were tested every ten minutes. Bulbus rotation was observed.
Each animal was laid in dorsal recumbency. Ventral stomach bristle hair was removed. Skin was washed and disinfected.

3.3.2.2 Operation

After the disinfection with alcohol, a laparotomie in the linea Alba was performed.
The incision started about 1cm caudal of the navel and ended 2 to 3 fingers cranial at the edge of the pubic bone. The incision could be widened in direction of the navel. Bleeding was stopped and the rest of the lig. Vesicae medianum was removed.

The uterus, uterus horns and ovaries were exteriorised out of the abdominal cavity. Ligatures were placed at both mesovars. Due to the strong blood supply in the ligamentum latum uteri in swine, two additional ligatures were needed to stop the blood supply. A further ligature was placed in the vaginal region. The uterus, uterus horns and ovaries were then removed through an incision caudal at the cervix, at the mesovars, freeing both ovaries, and at the ligatures that stopped the blood supply in the Ligamentum latum uteri.
The uterus stump mucosa was removed and a diagonal ligation was placed on the stump.
The closing of the abdominal cavity followed in layers. For the mesovar, ligamentum latum uteri, uterus stump ligatures and the inner layers of the abdominal cavity, absorbable sutures were utilised. The surgical skin suture was done with nonabsorbable thread.

3.3.2.3 Postoperative care

On the following two consecutive days, each ovariohysterectomised animal received 10000 IU/kg penicillin, (Procainpenicllin®, AniMedica, Bösensell), and 20-50mg/kg Metamizol, (Vetalgin®, Intervet, Unterschleisheim). Meloxicam 0.4mg/kg (Metacam®, Boehringer, Ingelheim) treatment was repeated one day after the operation. Sutures were removed ten days after the intervention.
Despite the fact that each animal in the OVX group received 8mg (2ml) Regumate® Janssen, Neuss to suppress the cycle stage causing strong blood supply in the uterus and ligamentum latum uteri, one animal did not survive the intervention. The OVX group post operation therefore consisted of four animals.

3.4 Collection of blood samples

Fixation occurred with the help of a pig catcher. Blood was drawn from the v. jugularis with monovettes (Monnovette®, Sarstedt, Nümbrecht). Samples were directly centrifuged, serum separated and frozen at -80°C.

3.5 Analysing blood samples

3.5.1 Determining progesterone concentrations

The progesterone concentrations were determined with a commercial radioimmunoassay, (Coat- A-Count, progesterone kit, Diagnostic Products Corporation, Los Angeles, CA.) validated for the use as a direct assay with porcine plasma and serum (Novak et al., 2003; Smith et al., 1992).

This is a solid phase radioimmunoassay, where progesterone labelled with ^{125} I competes with progesterone in the patient's sample, for a certain amount of time. The antibody is immobilised through fixation to the wall of a polypropylene tube. Simple decantation allows a termination of the competition and an isolation of antibody-bound, labelled progesterone. Counting is done with the aid of a gamma counter. A number is yielded and converted, with the help of a calibration curve, to measure progesterone present in the patient sample.

Before use, all test components are to have room temperature (15-28°C). Four uncoated polypropylene tubes are to be labelled. Two with T (total counts) and two NSB (non specific binding)

The tubes coated with antibodies are labelled A to G in duplicate, followed by the same coated tubes for the patient's samples in duplicate. 100µl of zero calibrator is placed into tubes A and NSB. The rest of the calibrators B to G are placed into the corresponding tubes. 100µl of serum from each patient are pipetted into the coated tubes for each patient.

1mL of ^{125} I Progesterone is now placed into each tube and mixed. After an incubation period of 3 hours, the contents of all tubes, except the T tubes, are decanted thoroughly. The gamma counter should be programmed according to the calibrator concentrations. Tubes are then placed into the gamma counter. The progesterone concentrations are read from the gamma counter. Each reading lasts a minute per tube.

3.5.2 Determining estradiol concentrations

Serum estradiol concentrations are determined with the Elecsys® and estradiol reagents, calibrators and controls (Fa. Seidel medipool, Buchendorf). The Elecsys® 1010 analyser is a fully automatic analyser system for determination of immunological tests, using an electrochemiluminescent process. The

chemiluminescent reactions that lead to the emission of light are initiated electronically, rather than chemically, which enables the precise control over the entire reaction. Serum samples are transferred into bar-coded sample tubes which are recognised by the machine. Reagents are also bar-coded and recognised. All components, samples and reagents for routine analysis are integrated in the analyser. Calibration occurs before each test run. The test runs automatically.

3.5.3 Determining bone markers

Each test was performed with the help of the epMotion 5070®, Eppendorf, Hamburg. The epMotion 5070® enables performing dispensing processes automatically. The fluid is taken up in pipette tips (epT.I.P.S.Motion), transported and then discharged from these at the dispensing position (destination). To perform the dispensing process, it is necessary to program each test, which is to be performed, step by step. Processes are performed according to programming, with tips, as well as tubs (30ml and 100ml, for wash solution, enzyme conjugate, substrate solution, antibody solution, reagents, stop solution, waste etc.) or tubes (for samples, standards and controls) in racks and plates with 6, 12, 24, 96, or 384 wells. These are placed in four positions of the area and three external positions. It is possible to exchange the tips, cups, tubs and tubes from external to internal positions and within internal positions, according to program. A waste box must also be installed. The required dispensing tool is set in place manually (50 µl, 300 µl, 300 µl- 8 channel tool). The 50 µl tool has a volume ranging from 1-50 µl and 300 µl tools have volumes ranging from 20-300 µl. The dispensing tools function as piston stroke pipettes with an air cushion. Dispensing tools can be used with or without filter. This is to be determined in the program. These tools are coded, so they are recognised by the workstation. If necessary the optical sensor checks for the correct selection and positioning of the tubes, the supply of tips in the tip racks and also the filling of the tubs and tubes.

3.5.3.1 C-terminal telopeptides of collagen type I

Serum Crosslaps® ELISA (Nordic Bioscience Diagnostics, Herlev, Denmark) is an enzyme immunologic test, which quantifies the dismantling products of C-terminal telopeptides of type I collagen in serum and plasma of humans. Allen (2003) confirms

the cross-reactivity in this test for the following animals: dogs, horses, sheep and pigs.

Serum Crosslaps® ELISA is based on two highly specific monoclonal antibodies which target the amino acid sequence EKAHD-β-GGR. Two amino acid chains with this sequence must be cross-linked to one another, to enable the serum Crosslaps® ELISA to measure a specific signal.

Before beginning with the test procedure, all solutions and test components should have reached room temperature. The antibody solution should be prepared approximately 30 minutes before test beginning.

Test procedure begins with the addition of standards, controls and samples into the provided microtitre wells. This is followed by pippetting the prepared antibody solution into the mictrotitre wells. A complex is formed between the Crosslaps® antigen, and antibodies, which bind to the surface of the wells after an incubation period of 120 minutes. The wells are then decanted and washed five times. Subsequently chomogen substrate solution is added and the colour reaction is stopped with a stop solution. The absorption is measured at 450 nm, with a reference of 650nm.

3.5.3.2 Osteocalcin

Metra Osteocalcin immunoassay (Metra Osteocalcin EIA®) (Quidel Corpartion, San Diego, CA, USA), allows a quantitative measurement of intact (*de novo*) osteocalcin in serum. This is a competitive immunoassay, which uses osteocalcin- coated strips, a mouse anti-osteocalcin antibody, an anti-mouse IgG-alkaline phosphatase conjugate and a (p-Nitrophenyl phosphate) pNPP substrate to quantify the osteocalcin in serum. Due to the high concentration of osteocalin in porcine serum, it was necessary to dilute the samples (1:10) with 1x wash buffer. Allen, (2003) confirms the cross-reactivity of this test for pigs. The cross-reactivity has been validated (Allen, 2003). Standards and controls are to be dissolved with 1x wash buffer, within an hour of use. Enzyme conjugate should be prepared within two hours of use. Each required vial is reconstituted with 10ml of 1x wash buffer. Working substrate solution should be prepared within an hour of use. One substrate tablet is placed into each required bottle and left to dissolve. The test procedure begins with the addition of 25 µl standards, controls and samples into each well of the coated strips. This is followed by 125 µl of anti osteocalcin and an incubation period of 2

hours (+/- 10 minutes) at 20-25°C. After a washing step consisting of a total of three washes and vigorous blotting on dry paper towels, 150 µl of enzyme conjugate is added to each well. This is followed by an incubation period of 60 minutes. A second washing step is now required. After three washes, coated strips are again vigorously blotted dry on paper towels. Before the last incubation period, 35-40 minutes at 20-25°C, starts, 150 µl of working substrate solution are added to each well. The last step consists of the addition of 50 µl of stop solution. The test should be read at an optical density of 405 nm.

4 Results

4.1 Weight gain prior to castration

During the first sixteen weeks after arrival of pigs and study began, weight remained constant in all three groups, ranging from 9.0kg to11.1kg. After this time a steady weight gain proceeded, reaching 27.0kg±1.8, 27.0±1.7 and 28.5kg±3.5 in the control, OVX and second control groups respectively. No significant differences were found at the different weighing times. No significant differences were found between the groups (table 7).

Table 7: Weight development of miniature pigs over a period of 70 weeks in control n=5, OVX n=5 and second control n=6 groups in kilograms; mean ± standard deviation

Weighing time point in weeks	0.99% Ca ration	0.7% Ca ration	
	Control Group n=5 weight in kg	OVX n=5 weight in kg	Second control group n=6 weight in kg
0	9.0±1.6	8.4±1.3	8.7±1.5
3	9.2±1.7	8.7±1.3	9.1±1.7
8	9.2±1.6	8.7±1.4	9.1±1.6
10	9.2±1.7	8.7±1.4	9.1±1.6
11	9.4±1.6	8.8±1.2	9.2±1.6
12	9.5±1.6	9.2±0.9	9.5±1.6
14	10.3±1.5	9.9±0.8	10.3±1.8
16	10.7±1.5	10.8±0.9	11.1±1.8
22	14.5±1.9	14.5±1.2	14.1±2.7
25	17.5±1.6	17.9±1.6	17.7±2.5
29	19.4±1.2	19.7±1.6	19.8±2.6
33	20.7±1.2	20.7±1.5	20.4±2.2
35	21.3±1.3	22.4±1.9	21.9±1.9
39	23.2±1.4	22.3±1.5	22.5±2.2
49	26.7±0.7	25.5±1.7	25.7±2.2
61	26.1±1.6	25.7±2.2	26.7±4.2
70	27.0±1.8	27.0±1.7	28.5±3.5

Different letters [a, b] characterise the significant differences ($p < 0,05$) between different blood sampling points of time

Study procedures

The pre-castration period consisted of a period of 65 weeks. During this time 10 blood samples were extracted and two ovulation synchronisations were performed, the first synchronisation beginning in week -50 and ending in week -47. The second synchronisation was performed shortly before the castration period, beginning in week -7 and ending in week -4. The post-castration period began at week 0, at ovariohysterectomy. This period consisted of 5 blood sample extractions, each with an interval of 2 weeks (diagram 2).

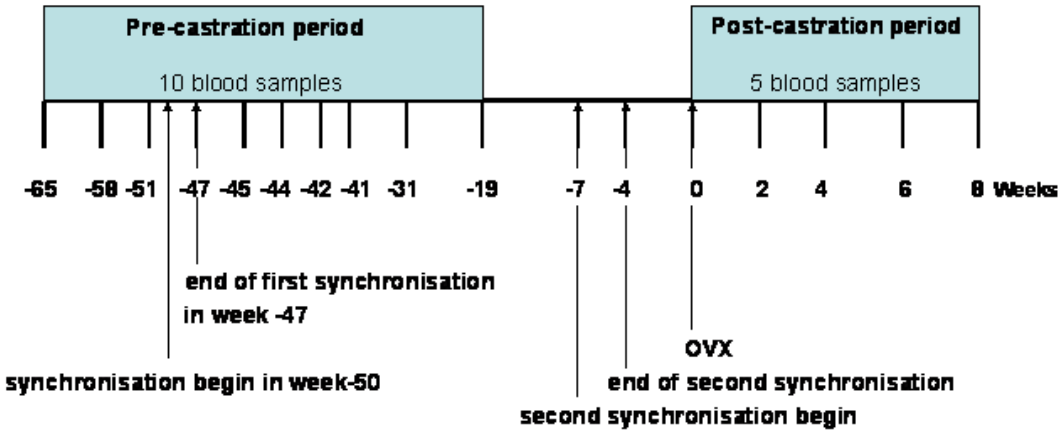

Diagram 2: schematic diagram of procedures during the experiment; 10 blood samples were taken pre-castration (week -65,-58,-51,-47,-45,-44,-42,-41,-31,-19); 5 blood samples were taken post-castration (0, 2, 4, 6, 8); 0 being at OVX

4.2 Pre-castration period

4.2.1 Bone marker progression

At the beginning of the study: blood samples were drawn every seven weeks. Each extraction took place in the morning, at 9:00am. Samples were centrifuged directly and frozen at -80°C. For each week of extraction pre-castration and post-castration see diagram 2.

4.2.1.1 Osteocalcin

The osteocalcin concentration development was very similar in all three experimental groups during the pre-castration period. All three groups started with low concentrations at study begin (80.2±25.7ng/mg in control group, 89.4±33.1 ng/mg in OVX group and 93.7±38.4 ng/mg in the second control group). This was followed by a peak in both the control groups. The OVX group reached a maximum seven weeks after both control groups. All further concentrations in the OVX group decreased constantly. In both control groups one slight increase can be seen in the descending concentrations, this being 31 weeks pre-castration in the control group and 41 weeks pre-castration in the second control group. Minimal concentrations were measured at the last blood analysis before castration.
No statistical differences were found among the experimental groups pre-castration (p=0,684), considering the area under the curve. The statistical significances within the different groups at various sampling periods for the osteocalcin concentrations are displayed in table 8.

Table 8: **Osteocalcin concentrations (ng/ml) pre-castration, in control n=5, OVX n=5 and second control n=6 groups at certain blood sampling points of time; mean values ± SD**

Osteocalcin concentrations ng/ml			
Blood sampling periods in weeks before castration	**0.99 %Ca ration**	**0.7% Ca ration**	
	Control n=5	**OVX n=5**	**Second control group n=6**
-65	80.2±25.7 ^{bc}	89.4±33.1 ^{ab}	93.7±38.4 ^{ab}
-58	169.6±38.8 ^{a}	151.5±39.4 ^{ab}	176.0±65.3 ^{a}
-51	154.9±18.4 ^{ab}	184.7±149 ^{ab}	170.5±44 ^{a}
-44	140.1±54 ^{ab}	171.1±57.4 ^{b}	122.3±20.2 ^{ab}
-41	95.9±48.5 ^{bc}	112.7±19.6 ^{ab}	127.0±40.4 ^{ab}
-31	106.5±40.9 ^{b}	98.7±22.6 ^{ab}	99.8±18.3 ^{ab}
-19	60.3±20.3 ^{bc}	71.6±10.6 ^{a}	84.6±28.7 ^{b}

Different letters $^{a, b}$ characterise the significant differences ($p < 0,05$) between different blood sampling points of time

Table 9: **Comparing osteocalcin concentrations among the experimental groups control n=5, OVX n=5 and second control n=6 pre-castration using area under the curve. Mean area under the curve ± SD is represented for each group**

Area under the curve		
0.99% Ca ration	**0.7% Ca ration**	
Control n=5	**OVX n=5**	**Second control group n=6**
1069.3±247.2	1209.8±230.4	1168.9±303.9

The significance between the three groups was p=0.684

4.2.1.2 Crosslaps

Each group had similar concentration progression during the pre-castration period. All groups reached peak concentrations seven weeks after the first analysis. This was followed by a simultaneous decrease. The OVX increased after this, while both control groups declined further. In week -41 the second control group showed an increase in concentrations. The trend that followed was a decrease in concentrations, followed by an increase reaching the last peak nineteen weeks before castration.
Considering the area under the curve, no statistical differences were found among the experimental groups (p=0.866). The significances within the groups at the demonstrated sampling times, for the crosslaps concentrations can be seen in table 10. No statistically significant differences were found in the OVX group at demonstrated sampling times pre-castration (p=0.292).

Table 10: Crosslaps concentrations (pg/ml) pre-castration, in control n=5, OVX n=5 and second control n=6 groups at certain blood sampling points of time; mean values ± SD

Crosslaps concentrations pg/ml			
Blood sampling periods in weeks before castration	**0.99% Ca ration**	**0.7% Ca ration**	
	Control n=5	**OVX n=5**	**Second control group n=6**
-65	164.5±118.3 [a]	435.4±261.6	209.8±123.99 [b]
-58	590.4±245.9 [b]	448.2±244.3	483.02±204.4 [a]
-51	398.6±119.4 [ab]	333.8±129.1	419.3±253.8 [ab]
-44	364.7±63.7 [ab]	436.3±118.6	310.96±149.5 [ab]
-41	310.3±154.0 [ab]	324.2±128.4	345.8±105.7 [ab]
-31	338.3±226.8 [ab]	371.5±145.5	360.7±98.5 [ab]
-19	546.3±281.2 [b]	544.9±188.9	411.9±247.8 [ab]

Different letters [a, b] characterise the significant differences ($p < 0.05$) between different blood sampling points of time

Table 11: Comparing crosslaps concentrations among the experimental groups control n=5, OVX n=5 and second control n=6 pre-castration using area under the curve. Mean area under the curve ± SD is represented for each group

Area under the curve		
0.99%Ca ration	**0.7% Ca ration**	
Control n=5	**OVX n=5**	**Second control group n=6**
3450.2±992.8	3665.6±896.5	3419.6±828.6

The significance between the three groups was p=0.866

4.2.2 Hormone analysis

4.2.2.1 Progesterone concentrations

Blood sample collection in the pre-castration period stretched over a period of 65 weeks or calculating with the first drawn blood sample as day one, 315 days. Calculating with a 21 day cycle of a pig, this period could contain approximately 15 cycles. Three clear progesterone peaks were demonstrated, in the control group, in the total analysis time pre-castration, (the first being in week -45 and the second in week -42). This is an interval of 21 days. Taking an approximate deviation of two to three days in a cycle into account, the high progesterone concentrations in week -42 seem to indicate the following cycle in this analysis period. The third peak was found in week -31, 77 days after the second peak. This is an interval of approximately three cycles, again taking a two to three day cycle fluctuation into account. The OVX and the second control group also showed a definite peak in week -45.The second peak was reached in week -42 in the OVX group, whereas the second control group reached the second peak in week -41. Progesterone concentrations started increasing in week -42 in the second control group. Taking a two to three day cycle fluctuation into account, these two groups could be in approximately the same stage of cycle. The peak found in week -31 could not clearly be represented. These two groups reached higher progesterone concentrations in week -19, 84 days later, which would be the equivalent of four cycles later.

The significant differences between the three groups were calculated using the area under the curve (p=0.274). The differences in the median values among the groups is greater than would be expected to occur randomly when comparing the delta

values between the three groups (p=0.045) at sampling time point -41 and -31. The significance in the groups at the different sampling points of time is demonstrated in table 12.

Table 12: Progesterone concentrations in ng/ml in control n=5, OVX n=5 and second control n=6 groups mean ± SD. Blood sampling time points are demonstrated in weeks and days in the pre-castration period; progesterone peaks are coloured grey

Progesterone ng/ml				
Blood sampling time point in days	**Blood sampling time point in weeks**	**0.99% Ca ration**	**0.7% Ca ration**	
		Control n=5	**OVX n=5**	**Second control group n=6**
1	-65	0.1±0.1 ^{b}	0.2±0.1 ^{ab}	0.2±0.1 ^{b}
49	-58	0.1±0.02 ^{b}	0.1±0.04 ^{b}	0.2±0.1 ^{b}
98	-51	0.2±0.1 ^{ab}	0.5±1.0 ^{ab}	7.4±18.0 ^{b}
126	-47	3.5±2.3 ^{ab}	4.8±4.9 ^{ab}	2.5±1.7 ^{ab}
140	**-45**	**116.0±70.9 ^{a}**	**35.5±47.4^{ab}**	**31.7±60.1 ^{ab}**
147	-44	0.2±0.1 ^{ab}	1.0±1.7 ^{ab}	4.7±8.0 ^{ab}
154	**-42**	**28.2±18.3 ^{ab}**	**27.6±27.6 ^{ab}**	5.1±12.0 ^{ab}
161	-41	3.7±7.6 ^{ab}	5.0±7.4 ^{ab}	**15.3±6.8 ^{ab}**
231	-31	**19.8±22.1 ^{ab}**	15.0±14.8 ^{ab}	7.9±11.3 ^{ab}
315	-19	0.8±0.6 ^{ab}	**34.9±32.2 ^{a}**	**38.8±34.7 ^{a}**

Different letters $^{a, b}$ characterise the significant differences ($p < 0.05$) between different blood sampling points of time

Table 13: Comparing progesterone concentrations in control n=5, OVX n=5 and second control n=6 groups pre-castration using area under the curve. Mean area under the curve ± SD is represented for each group

Area under the curve		
0.99%Ca ration	**0.7% Ca ration**	
Control n=5	**OVX n=5**	**Second control group n=6**
172.1±72.2	107.0±72.8	101.7±99.4

The significance between the three groups was p=0.274

4.2.2.2 Estradiol concentrations

The estradiol concentration development in the analysed weeks -47,-45,-44,-41 was very similar in each group. The first measured concentrations were minimal concentrations of 22.0±4.1pg/ml, 16.5±5.3 pg/ml and 26.7±8.1 pg/ml respectively in control, OVX and second control group. These concentrations increased in week -45 and reached peak concentrations in week -44. Thereafter concentrations decreased. The significance in the groups for estradiol concentrations at certain analysis points of time pre-castration are displayed in table 14. No significant differences were found in the OVX group and the control group pre-castration. The statistical significance between the groups for estradiol calculated using the area under the curve is shown in table 15. p=0.125 which characterises a trend between the groups.

Table 14: Estradiol concentrations (pg/ml) measured in week -47,-45,-44 and -41 pre-castration in control n=5, OVX n=5 and second control groups n=6; mean ± SD

Estradiol concentrations pg/ml			
Blood sampling periods in weeks before castration	**0.99% Ca ration**	**0.7% Ca ration**	
	Control n=5	**OVX n=5**	**Second control group n=6**
-47	22.0±4.1	16.5±5.3	26.7±8.1 [b]
-45	30.3±1.2	19.5±13.6	29.0±7.6 [b]
-44	33.8±20.9	29.2±19.1	38.5±7.1 [a]
-41	23.4±3.1	21.0±6.3	31.9±11.7 [ab]

Different letters [a, b] characterise the significant differences ($p < 0.05$) between different blood sampling points of time

Table 15: Comparing estradiol concentrations in control n=5, OVX n=5 and second control n=6 groups pre-castration using area under the curve. Mean area under the curve ± SD is represented for each group

Area under the curve		
0.99% Ca ration	**0.7% Ca ration**	
Control n=5	**OVX n=5**	**Second control group n=6**
98.4±22.1	77.9±29.4	113.6±29.0

The significance between the three groups was p=0.125

4.3 Post- castration period

Osteocalcin and crosslaps concentrations were determined from the blood samples drawn at ovariohysterectomy and 2,4,6,8 weeks after the operation.

4.3.1 Bone marker progression

4.3.1.1 Osteocalcin

The concentration development of osteocalcin at castration and two weeks after castration was very similar in all three groups. These concentrations increased in the OVX group four weeks after the ovariohysterectomy, and stayed higher until eight weeks post-castration, when comparing concentrations with the two control groups. The post-castration statistical differences within the various groups at different points of sampling time can be seen in table 16. No significant differences were found in the second control and the OVX group post-castration. A comparison of osteocalcin concentrations post-castration among the experimental groups, surveying the area under the curve, is displayed in table 17. There were no statistically significant differences found in this analysis (p=0.93).

Table 16: Osteocalcin concentrations (ng/ml), at castration and after castration in control n=5, OVX n=4 and second control n=6 groups; blood sampling periods displayed in weeks; mean values ± SD

Osteocalcin concentrations ng/ml			
Blood sampling periods at castration and weeks after castration	**0.99% Ca ration**	**0.7% Ca ration**	
	Control n=5	**OVX n=4**	**Second control group n=6**
0	75.6±39.9 [a]	79.6±31.0	86.6±12.4
2	86.8±42.6 [ab]	84.5±25.6	88.6±21.2
4	84.3±22.2 [ab]	142.5±11.9	90.0±23.6
6	86.4±26.6 [ab]	141.6±16.3	89.3±26.7
8	113.6±53.1 [b]	139.4±13.4	115.1±13.3

Different letters [a, b] characterise the significant differences ($p < 0,05$) between different blood sampling points of time

Table 17: Comparing osteocalcin concentrations among the experimental groups control n=5, OVX n=4 and second control n=6 at castration and after castration using area under the curve. Mean area under the curve ± SD is represented for each group

Area under the curve		
0.99% Ca ration	**0.7% Ca ration**	
Control n=5	**OVX n=4**	**Second control group n=6**
409.3±156.8	446.3±234.2	410.8±43.7

The significance between the three groups was p=0.93

4.3.1.2 Crosslaps

All groups had similar concentration development post-castration. The OVX and the control groups had increased concentrations two weeks after ovariohysterectomy. This increase was followed by a peak in the fourth week post- operation in the OVX group. The control group and the second control group reached a peak two weeks later in the sixth week post-operation. Eight weeks after the intervention all concentrations in all groups decreased.

The statistical significances for the post-operative period of eight weeks during the different sampling periods are displayed in table 18 for each group. No statistically significant differences were found in the second control group (p=0.308). The area under the curve for each group is shown in table 19. No statistically significant differences were found between the three groups in this analysis (p=0.916). A statistical significance was found when comparing the delta values of each group with one another. The delta values calculated from the 6^{th} and 8^{th} week post-operation gave a significant difference of p=0,016 between the OVX and the control group.

Table 18: Crosslaps concentrations (pg/ml), at castration and after castration in control n=5, OVX n=4 and second control n=6 groups; blood sampling periods displayed in weeks; mean values ± SD

Crosslaps concentrations pg/ml			
Blood sampling periods in weeks at castration and after castration	**0.99% Ca ration**	**0.7% Ca ration**	
	Control n=5	**OVX n=4**	**Second control group n=6**
0	492.4±62.1 ^{a}	640.8±183.1 ^{b}	587.6±210.3
2	584.9±236.2 ^{ab}	727.7±321.7 ^{ab}	536.4±177.6
4	588.5±123.5 ^{ab}	1037.0±253.9 ^{a}	574.8±202.9
6	965.1±364.7 ^{a}	809.3±120.4 ^{ab}	702.5±282.7
8	539.2±279.5 $^{b/y}$	715.6±331.0 $^{ab/x}$	633.8±175.6 ^{xy}

Different letters $^{a, b}$ characterise the significant differences ($p < 0.05$) between different blood sampling points of time

Different letters ^{xy} characterise the Δ values of each group which show the significant differences($p<0.05$) between the groups

Table 19: Comparing crosslaps concentrations among the experimental groups control n=5, OVX n=4 and second control n=6 at castration and after castration, using area under the curve. Mean area under the curve ± SD is represented for each group

Area under the curve		
0.99% Ca ration	**0.7% Ca ration**	
Control n=5	**OVX n=4**	**Second control group n=6**
2923.9±921.6	2952.1±1708.4	2692.3±604.7

The significance between the three groups was p=0.916

4.3.2 Hormone analysis

Blood samples from each sampling point of time at castration and after castration were used to determine progesterone concentrations. The estradiol concentrations were determined from samples up until and including the 6th week post-castration.

4.3.2.1 Progesterone concentrations

At ovariohysterectomy progesterone concentrations in the OVX group were at 31.4 ±0.7 ng/ml. These reached a peak two weeks later. Thereafter the concentrations decreased (11.4 ±0.3 ng/ml) and stayed almost constant in the last four weeks of the examination period. Both control groups, however, showed high concentrations in week 0. The concentrations in both groups decreased two weeks later. In the 4th week the second control group increased, while the control group declined. In week six, the second control group reached a second peak. Minimum concentrations were found in the control group at this time. In the last examination week, the control group reached a second peak, while the second control group declined (table 20).

No significant differences were found between the sampling times in the control group (p=0.549). In the OVX group the differences in the mean values among the sampling periods was greater than expected randomly (p=0.031). No significant differences were found in the paired multiple comparisons in this group. No significant differences were found in the second control group between the different sampling periods in the post-castration period.

All groups were compared with one another, surveying the area under the curve. No significant differences were found between the groups (p=0.502) (table 21). The delta values calculated from the different blood sampling time points were compared between the three groups. No significant differences were found between the groups when examining this value.

Table 20: Progesterone concentrations (ng/ml), at castration and after castration in control n=5, OVX n=4 and second control n=6 groups; blood sampling periods displayed in weeks; mean values ± SD

Progesterone concentrations ng/ml			
Blood sampling periods in weeks at castration and after castration	**0.99% Ca ration**	**0.7% Ca ration**	
	Control n=5	**OVX n=4**	**Second control group n=6**
0	36.21±1.4	31.4±0.7	23.8±1.2
2	21.0±0.5	34.3±1.1	11.6±0.3
4	19.6±0.2	11.4±0.3	23.9±0.9
6	8.9±0.3	15.5±0.4	57.1±0.6
8	30.8 ±1.4	14.7±0.3	43.1±1.4

Different letters [a, b] characterise the significant differences ($p < 0.05$) between different blood sampling points of time

Table 21: Comparing progesterone concentrations among the experimental groups control n=5, OVX n=4 and second control n=6 at castration and after castration, using area under the curve. Mean area under the curve ± SD is represented for each group

Area under the curve		
0.99% Ca ration	**0.7% Ca ration**	
Control n=5	**OVX n=4**	**Second control group n=6**
82.1±52.5	63.6±64.7	78.8±88.6

Different letters [a, b] characterise the significant differences ($p<0.05$) between the experimental groups

4.3.2.2 Estradiol concentrations

Both control groups have similar concentration gradient development 0, 2, 4 weeks after castration. In the sixth week after ovariohysterectomy the control group estradiol concentrations decrease, while the concentrations in the second control group increase. The OVX group has higher concentrations at castration. These decrease 39% after the operation and stay more or less constant there after.

No significant differences were found in control, OVX and second control groups in the post-castration period when comparing the blood sampling time points. A statistical significance was found between the groups using the area under the curve $p<0.001$ (table 23). A significant difference of $p<0.001$ was found when comparing the area under the curve in the second control group with the OVX group $p<0.05$ when comparing the control and OVX group. No significant difference was found between second control group and the control group. Comparing the delta values between the groups, statistical significances were found in the first calculated delta value post-operation; a statistical significance was found when comparing the control group with the OVX group ($p<0.01$) and when comparing the second control group and the OVX group ($p<0.05$). No significances were found between both the control groups for this delta value.

Table 22: Estradiol concentrations (pg/ml) measured in week 0, 2, 4 and 6 during the post-castration period in control n=5, OVX n=4 and second control groups n=6; mean ± SD

Estradiol concentrations pg/ml			
Blood sampling periods in weeks at castration and after castration	**0.99% Ca ration**	**0.7% Ca ration**	
	Control n=5	**OVX n=4**	**Second control group n=6**
0	19.2±3.9	38.2±16.9	30.2±19.9
2	36.9±14.3 ^{x}	14.7±16.7 ^{y}	35.3±8.1 ^{x}
4	26.7±14.7	16.6±3.2	34.2±14.6
6	20.6±3.2	15.3±0.4	40.6±10.9

Different letters $^{a, b}$ characterise the significant differences ($p < 0.05$) between different blood sampling points of time

Different letters ^{xy} characterise the Δ values of each group which show the significant differences($p<0.05$) between the groups

Table 23: Comparing estradiol concentrations among the experimental groups control n=5, OVX n=4 and second control n=6 at castration and after castration, using area under the curve. Mean area under the curve ± SD is represented for each group

Area under the curve		
0.99% Ca ration	**0.7% Ca ration**	
Control n=5	**OVX n=4**	**Second control group n=6**
93.7±15.6 ^{b}	56.3±25.6 ^{a}	130.0±21.6 ^{b}

Different letters $^{a, b}$ characterise the significant differences ($p<0.05$) between the experimental groups (control, OVX and second control groups). The significance between the three groups was $p<0.001$

4.4 Comparing cycle stage, calculated cycle day, progesterone and estradiol concentrations in all three groups

One day after synchronisation and hCG application, the estrus cycle stage was reached. Calculating with a time span of three days in the estrus cycle stage, each group reached the diestrus cycle stage two weeks later. The progesterone concentrations reached higher concentrations in this cycle stage in all groups. In week -31 and -19 the calculated day of cycle was day 9, the diestraus cycle stage. No samples were extracted on day one of these two cycles during these extraction times. Estradiol concentrations were not measured in week -31 and -19.

4.4.1 Pre-castration

4.4.1.1 Control group

Progesterone and osteocalcin concentration parameters reacted inversely in the control group. Where progesterone concentrations were at a minimum, osteocalcin concentrations were at a maximum (week -58 and -51). In week -47 osteocalcin concentrations decreased to 137.7ng/mg, while progesterone still had low concentrations of 3.5ng/ml. In week -45, the calculated day 14 of the cycle, diestrus, progesterone concentrations reached a peak. Simultaneously osteocalcin concentrations decreased. The same pattern was found in the following calculated estrus and diestrus cycle stages. Osteocalcin reaching higher concentrations in the estrus cycle stage (week -44) and lower concentrations in the diestrus cycle stages (week -43) (table 24).

This inverse behaviour was not clearly seen when comparing the crosslaps and progesterone concentration development. Crosslaps had low concentrations on the first day of analysis (164.5 ±118.3 pg/ml) when progesterone concentrations were low (0.1 ±0.01 ng/ml) in week -65. An increase in the crosslaps concentrations was seen when viewing week -58. A steady decrease was seen until week -45, where progesterone concentrations peaked in the calculated diestrus cycle stage. This inverse behaviour was not followed up at the second progesterone peak in week -42, where crosslaps concentrations increased (441.5 ±286.6 pg/ml). Each parameter had similar development thereafter up until week-19. Here progesterone again had lower concentrations 0.8 ±0.6 ng/ml, while crosslaps seemed to reach a third peak.

Osteocalcin and crosslaps had similar development during most of the pre-castration analysis. The only difference in concentration behaviour was seen in week -42 and

-19, where osteocalcin decreased while crosslaps increased.

Comparing estradiol and progesterone concentrations, estradiol concentrations increased at the calculated day one of the cycle in week -44, where progesterone concentrations were low (0.2 ±0.1ng/ml). This increase was not clearly seen in week -47 and -41 where progesterone concentrations were also low, but not at a minimum (table 24). Both crosslaps and osteocalcin concentrations increased in week -44, where estradiol had high concentrations.

Table 24: Blood sampling point of time displayed in weeks; calculated day of cycle in a 21 day cycle of a pig; mean± SD of estradiol in pg/ml, progesterone in ng/ml, osteocalcin in ng/mg and crossLaps in pg/mg in the control group n=5 pre-castration

Blood sampling time point in weeks	Calculated day of cycle	Estradiol pg/ml	Progesterone ng/ml	Osteocalcin ng/ml	Crosslaps pg/ml
-65			0.1±0.1	80.2±25.7	164.5±118.3
-58			0.1±0.02	169.6±38.8	590.4±245.9
-51			0.2±0.1	154.9±18.4	398.6±119.4
-47	1	22.0±4.1	3.5±2.3	137.7±27.1	388.6±214.7
-45	14	30.3±1.2	116.0±70.9	81.7±30.6	262.4±133.4
-44	1	33.8±20.9	0.2±0.1	140.1±54	364.7±63.7
-42	15		28.2±18.3	112.7±29.2	441.5±286.6
-41	1	23.4±3.1	3.7±7.6	95.9±48.5	310.3±154.0
-31	9		19.8±22.1	106.5±40.9	338.3±226.8
-19	9		0.8±0.6	60.3±20.3	546.3±281.2

4.4.1.2 OVX group

As in the control group, progesterone and osteocalcin concentration development in the OVX group also reacted inversely.
Comparing crosslaps and progesterone, the concentration development was similar to that of the control group. At the beginning of the analysis, both parameters reacted inversely. Progesterone peaked in week -45 and crosslaps reached a minimum. The opposite behaviour continued until week -41. The following concentration course was similar.

Comparing week -65 and -58, osteocalcin and crosslaps concentrations had the same concentration development. This was followed by an inverse reaction of osteocalcin and crosslaps in week -57 and -47. Both parameters analysed in the weeks that followed reacted in the same way. During the last analysed weeks -31 and -19 parameters reacted inversely.
Similar to the estradiol concentrations in the control group, estradiol reached high concentrations in week -44 on the first calculated day of cycle. Here progesterone concentrations are low (1.0 ±1.7 ng/ml). On both the other calculated day one of the cycle, week -47 and -41, estradiol peaks were not seen. Progesterone concentrations, however, are not at a minimum at these analysis times. Both osteocalcin and crosslaps increased their concentrations in week -44 (see table 25).

Table 25: Blood sampling point of time displayed in weeks; calculated day of cycle in a 21 day cycle of a pig; mean± SD of estradiol in pg/ml, progesterone in ng/ml, osteocalcin in ng/ml and crosslaps in pg/ml in the OVX group n=5 pre-castration

Blood sampling time point weeks and days	Calculated day of cycle	Estradiol pg/ml	Progesterone ng/ml	Osteocalcin ng/ml	Crosslaps pg/ml
-65			0.2±0.1	89,4±33,1	435.4±261.6
-58			0.1±0.04	151.5±39.4	448.2±244.3
-51			0.5±1.0	184.7±149	333.8±129.1
-47	1	16.5±5.3	4.8±4.9	207.7±77.6	315.98±115.2
-45	14	19.5±13.6	35.5±47.4	88.5±32.0	283.6±32.8
-44	1	29.2±19.1	1.0±1.7	171.1±57.4	436.3±118.6
-42	15		27.6±27.6	114.5±14.7	336.9±114.6
-41	1	21.0±6.3	5.0±7.4	112.7±19.6	324.2±128.4
-31	9		15.0±14.8	98.7±22.6	371.5±145.5
-19	9		34.9±32.2	71.6±10.6	544.9±188.9

4.4.1.3 Second control group

Like the control group and the OVX group, the second control group showed the opposite behaviour of osteocalcin and progesterone at many time points in the analyses.

Comparing the concentration gradient development of crosslaps and progesterone, up until week -44 these parameters reacted oppositely. Thereafter each parameter increases simultaneously with exception of week- 41.

Crosslaps and osteocalcin concentrations reacted the same way up until week -44. After this they reacted inversely.

As in the control and OVX groups analysed, estradiol concentrations were highest in week -44. Both osteocalcin and crosslaps concentrations had increased in week -44.

Table 26: Blood sampling point of time displayed in weeks; calculated day of cycle in a 21 day cycle of a pig; mean± SD of estradiol in pg/ml, progesterone in ng/ml, osteocalcin in ng/ml and crosslaps in pg/ml in the second control group n=6 pre-castration

Blood sampling time point weeks and days	Calculated day of cycle	Estradiol pg/ml	Progesterone ng/ml	Osteocalcin ng/ml	Crosslaps pg/ml
-65			0.2±0.1	93.7±38.4	209.8±123.99
-58			0.2±0.1	176.0±65.3	483.02±204.4
-51			7.4±18.0	170.5±44	419.3±253.8
-47	1	26.7±8.1	2.5±1.7		334.8±208.2
-45	14	29.0±7.6	31.7±60.1	104.7±50	287.5±196.95
-44	1	38.5±7.1	4.7±8.0	122.3±20.2	310.96±149.5
-42	15		5.1±12.0	120.2±43.1	414.3±58.2
-41	1	31.9±11.7	15.3±6.8	127.0±40.4	345.8±105.7
-31	9		7.9±11.3	99.8±18.3	360.7±98.5
-19	9		38.8±34.7	84.6±28.7	411.9±247.8

4.4.2 Post-castration

4.4.2.1 Control group

The osteocalcin concentrations reacted inversely with the progesterone concentrations in week 0, 2 and week 6. An opposite behaviour was not seen in week 4, concentrations decreased in both parameters, and in week 8, concentrations increased in both parameters.

Comparing crosslaps and progesterone at each analysis point in time, concentration development was opposite in each week. One parameter increased while the other decreased.

Both crosslaps and osteocalcin concentrations increased from week 0 to week 2 post-castration. In week 4 crosslaps reached a peak, while osteocalcin decreased. Both parameters showed increases in week 6. Osteocalcin reached peak concentrations in week 8, while crosslaps´ concentrations fell.

The calculated day of cycle was day 14, at ovariohysterectomy. This would be the diestrus cycle stage. Progesterone concentrations peaked at this time, while estradiol concentrations were at a minimum. Two weeks later, the calculated day 5 of the cycle, metestrus, estradiol concentrations peaked, while progesterone concentrations decreased in comparison to previous concentrations. In week 4 progesterone concentrations had decreased further. Estradiol concentrations were at 26.7pg/ml on the 21st day of cycle (last day of cycle, proestrus). In week 6, day 14, diestrus, progesterone concentrations were at a minimum. Estradiol decreased to 20.6pg/ml.
In week 2 estradiol concentrations peaked, osteocalcin and crosslaps concentrations increased in this week. Week 4 showed a decrease in estradiol concentrations, while osteocalcin decreased slightly, and crosslaps increased slightly. Week 6 showed another decrease in estradiol concentrations, whereas crosslaps and osteocalcin increased (see table 27).

Table 27: Blood sampling point of time displayed in weeks; calculated day of cycle in a 21 day cycle of a pig; mean± SD of estradiol in pg/ml, progesterone in ng/ml, osteocalcin in ng/mg and crosslaps in pg/mg in the control group n=5 at castration and after castration

Blood sampling time point in weeks	Calculated day of cycle	Estradiol pg/ml	Progesterone ng/ml	Osteocalcin ng/ml	Crosslaps pg/ml
0	14	19.2±3.9	36.2±1.4	75.6±36.9	492.4±62.1
2	5	36.9±14.3	21.0±0.5	86.8±42.6	584.9±236.2
4	21	26.7±14.7	19.6±0.2	84.3±22.2	588.5±123.5
6	14	20.6±3.2	8.9±0.3	86.8±26.6	965.1±364.7
8	5		30.8±1.4	113.6±53.1	539.2±279.5

4.4.2.2 OVX group

Osteocalcin and progesterone concentrations increased simultaneously from week 0 to week 2. Week 4 and 6 showed an inverse pattern of these parameters. Each parameter decreased simultaneously from week 6 to week 8.

Comparing crosslaps and progesterone, crosslaps showed the same parameter development as osteocalcin.

Osteocalcin and crosslaps had similar concentration development at all analysis times.

Estradiol concentrations peaked at ovariohysterectomy (38.2 ±16.9 pg/ml). Week 0 also showed high progesterone concentrations 31.4ng/ml. Two weeks later estradiol concentrations decreased to 14.7pg/ml and stayed almost constant at the rest of the analysis points. Progesterone, however, reached a peak 2 weeks after ovariohysterectomy. This parameter also declined, 4 weeks after the operation (11.4ng/ml). Week 6 and 8 showed almost constant progesterone concentrations.

Where estradiol concentrations were high in week 0, both markers showed the lowest concentrations. In week 2, 4, 6 where estradiol concentrations were almost constant, both markers reached maximum values in week 4. These decreased in week 6 (table 28)

Table 28: Blood sampling point of time displayed in weeks; calculated day of cycle in a 21 day cycle of a pig; mean± SD of estradiol in pg/ml, progesterone in ng/ml, osteocalcin in ng/ml and crosslaps in pg/ml in the OVX group n=5 at and after castration

Blood sampling time point in weeks	Calculated day of cycle	Estradiol pg/ml	Progesterone ng/ml	Osteocalcin ng/ml	Crosslaps pg/ml
0	14	38.2±16.9	31.4±0.7	75.6±31.0	640.8±183.1
2	5	14.7±1.68	34.3±1.1	84.5±25.6	727.7±321.7
4	21	16.6±3.2	11.4±0.3	142.5±11.9	1036.9±253.9
6	14	15.3±0.41	15.5±0.4	141.6±16.3	809.3±120.4
8	5		14.7±0.3	139.4±13.4	715.6±331.1

4.4.2.3 Second control group

Osteocalcin and progesterone concentrations developed inversely in week 0, 2, 6, 8. Week 4 showed the same concentration development in both parameters.
Both crosslaps and progesterone showed the same behaviour in concentrations at all analysis times.
Osteocalcin and crosslaps concentrations had inverse development at all times other than week 4. Here both parameters increased simultaneously.
On the calculated day 14 of the cycle estradiol concentrations were low, compared to the other concentrations measured in this group. Progesterone concentrations were at 23.8 ±1.2 ng/ml. Cycle day 5, metestrus showed higher estradiol concentrations. On this day progesterone showed minimum concentrations. Week 4, day 21 of the cycle estradiol concentrations were at 34.2 ±14.6 pg/ml, while progesterone was at 23.9 ±0.9 ng/ml. Week 6 showed a progesterone peak. Estradiol also showed high concentrations at this time 40.6±10.8 pg/ml (table 29).

Table 29: Blood sampling point of time displayed in weeks; calculated day of cycle in a 21 day cycle of a pig; mean± SD of estradiol in pg/ml, progesterone in ng/ml, osteocalcin in ng/ml and crosslaps in pg/ml in the second control group n=6 at castration and after castration

Blood sampling time point in weeks	Calculated day of cycle	Estradiol pg/ml	Progesterone ng/ml	Osteocalcin ng/ml	Crosslaps pg/ml
0	14	30.2±19.9	23.8±1.2	86.6±12.4	587.6±210.3
2	5	35.3±8.1	11.6±0.3	88.6±21.2	536.4±177.6
4	21	34.2±14.6	23.9±0.9	90.0±23.6	574.8±202.9
6	14	40.6±10.8	57.1±0.6	89.3±26.7	702.5±282.7
8	5		43.1±1.4	115.1±13.3	633.8±175.6

4.5 Correlations

4.5.1 Pre-castration

During the pre-castration period a total of 50 variables for each parameter (osteocalcin, crosslaps and progesterone) were analysed in the control and OVX group and 60 in the second control group. Due to the fact that estradiol was only analysed at four sampling points of time, only 20 variables (control and OVX group; 24 variables in the second control group) were analysed for this parameter. The correlations between osteocalcin, crosslaps and progesterone could therefore be examined with 50 variables (60 in the second control group). The correlations for these three parameters and estradiol were examined with 20 variables (24 in the second control group). Each correlation was performed with Pearson's correlation.

4.5.1.1 Correlations in the control group

A positive significant connection was found between osteocalcin and crosslaps with a correlation coefficient of 0.373 (see table 30). No other significant correlations were found in this analysis.

Table 30: Correlations for the parameters crosslaps, osteocalcin, progesterone and estradiol at all blood sampling points of time in the control group pre-castration (correlation coefficient R; significance p; n=number of samples analysed)

	Osteocalcin	Progesterone	Estradiol
Crosslaps	**0.373** **p<0.01** **n=50**	-0.173 p=0.231 n=50	-0.187 p=0.429 n=20
Osteocalcin		-0.235 p=0.101 n=50	-0.701 p=0.769 n=20
Progesterone			0.128 p=0.592 n=20

Due to the fact that no definite correlations were found when analysing all blood sampling time points together, each sampling time was analysed separately and the following correlations were found; in week -42 a correlation was found between

osteocalcin and progesterone, with a significant negative correlation coefficient. One variable increased while the other decreased. In weeks -47 and -41 significant correlations ($p<0.05$ and $p<0.01$) were found between osteocalcin and crosslaps. The correlation coefficients for week -47 and -41 were 0.917 and 0.965 respectively. In week -41 a significant correlation was also found between estradiol and osteocalcin. In the same week a correlation was also found between estradiol and crosslaps. A negative correlation coefficient was found for each parameter. In this week one variable seemed to decrease while the other increased. A significant ($p<0.001$) correlation was found for crosslaps and progesterone in week -42 with a correlation coefficient of -0.886 (table 31). No correlations were found between estradiol and progesterone at the analysed blood sampling points of time.

Table 31: correlations for osteocalcin, crosslaps, progesterone and estradiol at certain blood sampling time points during the pre-castration period in the control group (correlation coefficient R; significance p; sampling time point in weeks)

	Osteocalcin	Progesterone	Estradiol
Crosslaps	0.917 p<0.05 **week-47** 0.965 p<0.01 **week-41**	-0.886 p<0.01 **week-42**	-0.971 p<0.001 **week-41**
Osteocalcin		-0.934 p<0.05 **week -42**	-0.905 p<0.05 **week-41**
Progesterone			**-**

4.5.1.2 Correlations in the OVX group

No correlations were found for the pre-castration period when all parameters were analysed together at all blood sampling time points.

At separate analysis of each blood sampling time point the following correlations were found; a significant ($p<0.05$) positive correlation coefficient (0.892) was found between osteocalcin and crosslaps in week -58; there was a significant negative correlation between crosslaps and progesterone in week -44 (table 32); a negative correlation coefficient was calculated in week -47 for estradiol and crosslaps

Table 32: correlations for osteocalcin, crosslaps, progesterone and estradiol at certain blood sampling time points in the pre-castration period in the OVX group (correlation coefficient R; significance p; sampling time point in weeks)

	Osteocalcin	Progesterone	Estradiol
Crosslaps	0.892 p<0.05 **week-58**	-0.881 p<0.05 **week-44**	-0.879 p<0.05 **week-47**
Osteocalcin		-	-
Progesterone	-		-

4.5.1.3 Correlations in the second control group

A significant connection ($p<0.05$) was found between osteocalcin and crosslaps in this group, when analysing all blood sampling points of time (table 33). The correlation coefficient for these two parameters was R=0.290. No correlations were found for any of the parameters in this analysis.

No correlations were found in this group for any of the parameters at different blood sampling points of time in the pre-castration period.

Table 33: Correlations for the parameters crosslaps, osteocalcin, progesterone and estradiol at all blood sampling points of time in the second control group pre-castration (correlation coefficient R; significance p; n=number of samples analysed)

	Osteocalcin	Progesterone	Estradiol
Crosslaps	**0.290** **p<0.05** **n=60**	0.0296 p=0.825 n=60	0.318 p=0.130 n=24
Osteocalcin		-0.0936 p=0.481 n=60	-0.0811 p=0.706 n=24
Progesterone			-0.277 p=0.190 n=24

4.5.2 Post-castration

During the post-castration period 25 variables for each parameter osteocalcin, crosslaps and progesterone were analysed in the control group. In the second control group, which consisted of six animals, at five blood sampling points of time, a total of 30 variables were used to calculate the correlations for these three parameters. Estradiol, however, was only analysed at four extraction times, therefore 20 variables were used in the control group and 24 variables in the second control group. The OVX group had one animal less (n=4) two weeks after the ovariohysterectomy and at the rest of the analysis time, therefore only 21 variables for osteocalcin, progesterone and crosslaps and 17 variables for the estradiol were included in the correlations tests.

4.5.2.1 Correlations in the control group

No significant correlations were found in this group between the different parameters at all sampling time points.
Due to the fact that no correlations were found when analysing all blood sampling time points together, each extraction time was analysed separately. No correlations were found for any of the analysis time points in the control group post-castration.

4.5.2.2 Correlations in the OVX group

A positive weak significant connection was found between the parameters osteocalcin and crosslaps(R: 0.449; $p<0.05$). Estradiol and crosslaps showed a negative weak significant connection (R:-0.522; $p<0.05$). No other significant connections or correlations were found in this analysis (table 34)

Table 34: Correlations for the parameters crosslaps, osteocalcin, progesterone and estradiol at all blood sampling points of time in the OVX group in the post-castration period (correlation coefficient R; significance p; n=number of samples analysed)

	Osteocalcin	Progesterone	Estradiol
Crosslaps	**0.449** **p<0.05** **n=21**	-0.428 p=0.0529 n=21	**-0.522** **p<0.05** **n=17**
Osteocalcin		-0.268 p=0.239 n=21	-0.357 p=0.160 n=17
Progesterone			0.293 p=0.254 n=17

Each blood sampling point of time was analysed separately for correlations in the OVX group. A strong negative significant connection was found between estradiol and crosslaps in week 0 (R: -0.935; p<0.05). This strong negative significant correlation was found again in week 2 (R:-0.957; p<0.05). No other significant correlations were found at any analysis time points (table 35).

Table 35: correlations for osteocalcin, crosslaps, progesterone and estradiol at certain blood sampling time points in the post-castration period in the OVX group (correlation coefficient R; significance p; sampling time point in weeks)

	Osteocalcin	Progesterone	Estradiol
Crosslaps	-	-	-0.935 p<0.05 **week 0** -0.957 p<0.05 **week 2**
Osteocalcin		-	-
Progesterone			-

4.5.2.3 Correlations in the second control group

No significant connections were found between any of the analysed parameters at all sampling points of time in this group post-castration.
A significant negative correlation was found in the second control group for estradiol and progesterone in week 4 post-castration. No other connections or correlations were found between any of the parameters in this group (table 36).

Table 36: correlations for osteocalcin, crosslaps, progesterone and estradiol at certain blood sampling time points in the post-castration period in the second control group (correlation coefficient R; significance p; sampling time point in weeks)

	Osteocalcin	Progesterone	Estradiol
Crosslaps	-	-	-
Osteocalcin		-	-
Progesterone			-0.959 p<0.05 **week 4**

5 Discussion

The objective of this study was to examine the dependence of bone marker activity (osteocalcin and crosslaps) on the estrous cycle of the miniature pig. This was done by comparing the development of marker concentrations with progesterone and estradiol concentrations in the respective cycle stages of each pig after synchronisation and at particular sampling points of time.
Furthermore the behaviour of biochemical markers, osteocalcin and crosslaps were examined at and after ovariohysterectomy to determine the effect of OVX on these specific bone markers in the miniature pig. A further purpose of this thesis was to determine the influence of a calcium deprived feed ration on bone metabolism before and after castration.
This was a pilot study, which should reduce uncertainties in, and gather data for future studies in this area; therefore the animal numbers were very small.
Sixteen animals were divided into three groups, namely control, second control and OVX group. The control group received a 0.99% calcium ration in the feed, while the OVX and the second control group received a 0.7% calcium ration.
The experiment consisted of two periods; the pre-castration period, lasting approximately 65 weeks and a post-castration period lasting 8 weeks. Blood samples were drawn and analysed at selected time points.

5.1 Weight gain during the experiment

Weight control was practised from the arrival of the pigs up until 70 weeks after arrival. Weight showed a constant gain until peaking and levelling at 70 weeks. This corresponded to age and growth of the animals. The weight controlling was done to avoid over- weight or under-weight animals. Over-weight could have been followed by weight loss, which influences markers of bone metabolism during the estrous cycle pre-castration and post-castration. Thus far no consistent abnormal findings have been described with regard to bone remodelling markers in obese persons (Seibel, 2002). There is, however, evidence that weight loss is associated with changes in BMD and bone remodelling. Ricci et al. (2001) suggested that the reduction of body fat mass was directly associated with a decrease in serum oestrone, and therefore estrogen is a major determinant of high bone turnover during weight loss. Adipocytes produce Interleukin 6. 30% of the circulating cytokines

derrive from fat cells (Mohamend et al. 1997). Factors such as interleukin 1 and interleukin 6 are known to play an important role in bone resorption (Cannon and Dinarello, 1985; Angstwurm et al., 1997). Adipocyte- produced cytokines are the pathogenic link between obesity and its metabolic consequences, such as bone resorption (Friebe and Peters, 2005). Further weight development was not followed in the present study. The reason for this was because each animal was in a steady state of health and remained constant in weight, so there was no risk of weight loss or weight gain.

5.2 Parameter determination, test validation, sample extraction and sample handling

Biochemical measures of bone formation and resorption allow the assessment of bone turnover. These may assist in the study of the pathophysiological growth of the skeleton and in the study of diseases such as osteoporosis, which are characterised by disorders in bone mass and may cause disturbances in both formation and resorption. The most common example of loss in bone balance (formation and resorption are balanced during remodelling) occurs after menopause when estrogen loss leads to increased bone resorption with low bone mass as a consequence of this (Allen, 2003). At least one formation and one resorption parameter should be measured to determine the status of bone metabolism. Most of the tests for these parameters found on the market are human specific. The use of these tests in animals is possible if a validated cross reactivity for the specific parameter exists.

Osteocalcin is a sensitive and specific marker of bone formation (Szulc et al., 2000). Concentrations were determined with the Metra Osteocalcin immunoassay (Metra Osteocalcin EIA® Quidel Corpartion, San Diego, CA, USA). This assay measures quantitative intact osteocalcin in serum. It is a competitive immunoassay, which uses osteocalcin coated strips, a mouse anti-osteocalcin antibody, an anti-mouse IgG-alkaline phosphatase conjugate and a (p-Nitrophenyl phosphate) pNPP substrate to quantify the osteocalcin in serum. Allen (2003) confirms the cross reactivity of this test for pigs and a number of other animals, which has been validated. The serum concentrations of osteocalcin, however, are lower in humans than in pigs; therefore porcine serum had to be diluted 1:10. This test could be used in the present study due to the validation. The degree of dilution was considered when calculating the final osteocalcin concentrations.

Serum crosslaps is a marker of bone resorption. The breakdown of collagen type I is mediated by osteoclast- derived acid proteases. This leads to a release of free peptide- bound metabolites of collagen type I molecules (amino terminal telopeptides or carboxy terminal telopeptides) (Coleman, 2002). Serum Crosslaps® ELISA (Nordic Bioscience Diagnostics, Herlev, Denmark) is an enzyme immunologic test, which quantifies the dismantling products of C-terminal telopeptides of type I collagen in serum and plasma of humans. Allen (2003) confirms the cross- reactivity in this test for the following animals: dogs, horses, sheep and pigs. Serum Crosslaps® ELISA is based on two highly specific monoclonal antibodies which target a certain amino acid sequence. Two amino acid chains with this sequence must be cross-linked to one another, to enable the serum Crosslaps® ELISA to measure a specific signal. It was not necessary to dilute porcine serum samples in this test. This test was easy to perform and the cross reactivity and validation for pigs made it useful for the present study.

The formation marker, osteocalcin and the resorption marker, crosslaps showed changes that were due to age, cycle and ovariohysterectomy. These markers are therefore parameters that can be used in further studies on bone metabolism in minipigs.

To determine the dependence of bone marker activity on the estrous cycle of the pig, hormone concentrations (progesterone and estradiol) were measured. These parameters assisted in the approximate cycle stage classification of each animal. The progesterone concentrations were measured with a commercial radioimmunoassay, (Coat- A-Count, progesterone kit, Diagnostic Products Corporation, Los Angeles, CA.) validated for the use as a direct assay with porcine plasma and serum (Novak et al., 2003; Smith et al., 1992). This is a solid phase radioimmunoassay, where progesterone labelled with ^{125}I competes with progesterone in the patient's sample, for a certain amount of time. The antibody is immobilised through fixation to the wall of a polypropylene tube. Simple decantation allows a termination of the competition and an isolation of antibody bound, labelled progesterone. Counting was done with the aid of a gamma counter. Again the validation for porcine serum and plasma made this test useful for the present study. Progesterone concentrations showed the expected peaks in the appropriate calculated cycle stages, which was of assistance in determining the cycle dependence of the bone markers in minipigs.

Serum estradiol concentrations were determined with the Elecsys® and estradiol reagents, calibrators and controls (Fa. Seidel medipool, Buchendorf). This test is a test for humans and is not specifically validated for porcine serum. The concentrations measured in the experiment were an aid and support to the measured progesterone concentrations for the cycle stage determination. The course of the hormonal concentration development can be determined from these test results. No statement can be made about each single hormone concentration at each point in the analysis time. This test was, however, used in the present study. The course of parameter development was used together with the progesterone concentrations to determine the approximate cycle stage of each animal and group. Estradiol did show high concentrations as expected at calculated ovulation or follicular cycle stages. This test, therefore, can be used as a backup in determining the cycle stage of minipigs. Determining two hormonal parameters allows precise cycle stage determination.

Each blood sample was centrifuged directly after extraction, serum separated and frozen at -80°C to prevent further degradation of parameters. The samples were kept frozen until all specimens from one trial period (pre-castration or post-castration) were complete. Only then were they thawed for the use in different ELISAs and the RIA. This was done to prevent degradation of bone markers such as intact osteocalcin.

Levels of urine deoxypyridinoline and urine N-telopeptide of type I collagen have been reported to change during storage (Schober et al., 2002; Schneider et al., 2002). Osteocalcin degradation occurs rapidly at room temperature therefore if the analysis of intact osteocalcin is wanted specimens should be frozen soon after collection (Blumsohn et al., 1995). Lee et al. (2000) state that samples should be collected on ice, plasma and serum stored at -20°C for short term storage and at -70°C for long term storage. Furthermore samples should only be thawed once to prevent degradation and degeneration of osteocalcin fragments. If parameter degradation in serum specimens of minipigs was the same as in humans, the handling of these specimens would have excluded larger degradation of osteocalcin and crosslaps.

Biochemical markers are known to have diurnal variations. Studies in human medicine have concluded that osteocalcin concentrations peak in the early morning

0400h (Grundberg et al., 1985; Tracy et al., 1990). In humans bone alkaline phosphatase has been showed to increase in the morning to the afternoon. Osteocalcin increases at night (Nielson et al., 1990a). NTX, Pyd, Dpd increase at midnight (Schlemmer et al., 1994). Osteocalcin and bone alkaline phosphatase showed the same patterns in miniature pigs as in humans (Tsutsumi et al., 2004a). To avoid diurnal variations in the present study, all samples were extracted at the same time of day at 9 a.m.

Qvist et al. (2002) examined the diurnal variation in serum crosslaps and found maximum levels in the morning at 5 a.m. and minimum levels in the afternoon at 2 p.m. The study showed that the only parameter which had a pronounced influence on the circadien variation was fasting, which reduced the circardian variation to about one fourth. All animals in the present study fasted before sample extraction.

Tsutsumi et al. (2004a) found highest values for osteocalcin in miniature pigs at 18:00h and the lowest were found at 06:00h. In the present study bone formation marker, osteocalcin, therefore did not have maximum concentrations at extraction times. Tsutsumi et al. (2004a) found different results for resorption parameters in pigs, in comparison to those of humans. In this study, however, the parameter NTX was measured. If serum crosslaps react the same way in pigs as they do in humans, concentrations were also not at a maximum at extraction time in this study. The present study did not determine the diurnal variations of each analysed parameter. The constant extraction times in the morning, would have excluded the variation of each marker, so if both markers were not at maximum concentrations at each extraction time, they were never at a maximum at any point of extraction, which would indicate a uniform concentration analysis, which was welcome in the present study. The errors in concentration deviation caused by diurnal variations were therefore avoided by keeping each extraction time at the same time of day.

5.3 Development of biochemical markers in the pre-castration period

5.3.1 Osteocalcin

Osteocalcin age-related

The first blood samples were drawn at an approximate age of 9 months. The samples that followed were collected approximately at monthly intervals. Osteocalcin concentrations increased from 9 to 13 months and decreased at 14 months (diagram 3). The increase in osteocalcin concentrations at the beginning of the study was age-related. Both male and female miniature pigs reach sexual maturity at 4-6 months of age (Panepinto and Phillips, 1986). This would be the equivalent of the beginning of human puberty. Tsutsumi et al. (2004a) investigated the age-related changes in female Göttinger minipigs where the pubertal stage was estimated at 3-11 months. So, calculating with Tsutsumi´s age estimations, 12 months would be the approximate age of an adult miniature pig. As a result of measuring vertebral diameter, Inui et al. (2004) found that skeletal growth ended at about 20 months in male miniature pigs and at the age of 25 months in female miniature pigs. This, however, is restricted to the vertebral bone development. Bone markers stabilise their activity in pre-pubertal children and increase at the beginning of the pubertal growth spurt. After a final height is reached marker levels decrease to the levels seen in adulthood (Szulc et al., 2000). Tsutsumi et al. (2004a) confirm that the changes in osteocalcin concentrations in minipigs at this age are the same as in humans. The prepubertal stage was not included in the present study, so there is no evidence that the miniature pig has the same marker development at this age as they do in humans at the same age. At puberty it was, however, clear that osteocalcin increased by 91%. Puberty is characterised by noticeable changes in linear growth, modifications in body composition and reproductive capacity, which are regulated by changes in the endocrine milieu. Growth hormone increases two-fold during puberty and insulin-like growth factor I and its binding protein also increase during puberty. The rises in sex steroids, growth hormone and insulin-like growth factor I are crucial for linear growth, bone mass and muscle bulk (Federico et al., 2003; Mauras, 2001). Baroncelli et al. (2000) observed that growth hormone treatment caused the increase of osteobalst and osteoclast activity. The osteocalcin activity at puberty in minipigs could, therefore, be due to increased growth hormone concentrations and a rise in

sex hormones at this age. Fares et al. (2003) confirmed that all markers of bone turnover, which included osteocalcin, increased in girls during puberty and decreased toward adult levels in the last Tanner stage. A large increase from 9 to 10 months was seen in the present study, followed by an increase of 1.4% and then 1.5% at 12 and 13 months. If 10 months is seen as a late Tanner stage in the minipig, osteocalcin reacts more slowely than it does in humans because concentrations are still high at this time. High osteocalcin concentrations are found in the second and third Tanner stages in girls (mid-puberty), while boys showed a higher marker at the beginning of puberty (Fares et al., 2003). Assuming that female minipigs in the present study can be compared with female humans during puberty, the present study shows that osteocalcin concentrations are still high at late puberty, unlike those in humans, which are high at midpuberty.

At 12 and 13 months concentrations were still high. If the age assumption of Tsutsumi et al. (2004) is correct this would be early adulthood. In humans, however, after a final height is reached marker levels decrease to the levels seen in adulthood (Szulc et al., 2000). The levels did not decrease in the equivalently aged minipigs. Osteocalcin stayed at high concentration at 12 and 13 months and decreased 47% in the 14th month. This study shows that osteocalcin concentrations are still high in early adulthood, if age estimations are correct. It could, however, be possible that the Göttinger minipig used in Tsutsumi´s study had an earlier growth development compared to the minipigs used in the present study. If this is the case, the osteocalcin concentration development in Dresdner minipigs is equivalent to the concentration development in humans.

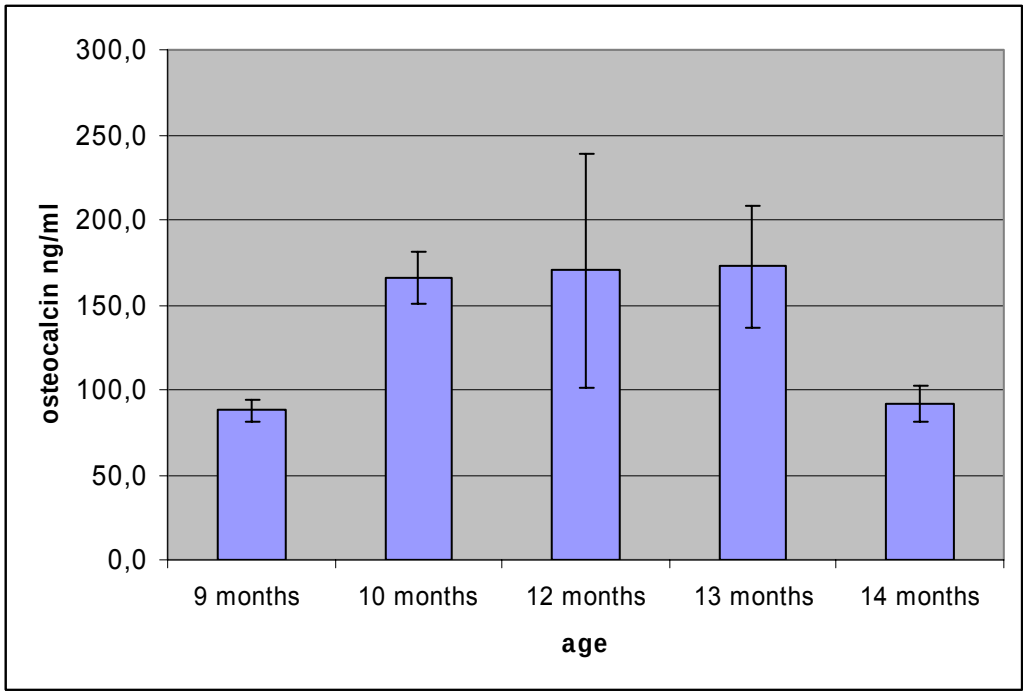

Diagram 3: **osteocalcin concentrations (ng/ml) represented in mean and SD calculated from mean and SD of control, OVX and second control group at 9, 10, 12, 13 and 14 months of age**

Osteocalcin estrous cycle-related

The pig typically has an estrous cycle duration of 21 days. This can vary from 17-25 days. The cycle can be divided into four phases: estrus (sexual receptivity; ovulation; day 1-3)), metestrus (end of ovulation and beginning of the luteal phase; days 4-7), diestrus (luteal phase; day 8-17) and proestrus (follicular phase; day 18-21). Knox et al. (2003) measured plasma progesterone during the estrous cycle and found baseline concentrations 3 days after the LH peak (LH peak = estrus). Progesterone concentrations increased, reaching a peak on day 10 and remained elevated until day 14 (mid-late diestrus). Progesterone concentrations decline to a base line over a period of 2 days, at luteolysis. Knox et al. (2003) also measured estradiol concentrations in pigs. A base line was found during the progesterone elevated period. Concentrations increased 5 days before estrus, at estrus and a day after estrus.

All sixteen animals included in this experiment were synchronised during the pre-castration period. After synchronisation the pigs were all approximately in the same stage of cycle which eased working conditions. Blood samples were drawn a day after the last hCG application, in the assumed diestrus cycle two weeks later,

followed by another extraction a week after this (estrus). The same pattern was followed in the following cycle: one extraction in the diestrus cycle stage and one in the estrus cycle stage. This pattern of sample extraction was selected to determine the cyclic changes of sex hormones (progesterone and estradiol) during the estrous cycle of the pig and to determine related changes in bone formation and resorption.

These samples showed that osteocalcin was cycle stage dependant in minipigs. Progesterone and osteocalcin concentrations reacted inversely for four extractions after the first synchronisation (see diagram 4 and 5).

Diestrus

Week -45 represented the calculated diestrus cycle stage with high progesterone concentrations in all three groups (116.0ng/ml, 35.5ng/ml, and 31.7ng/ml). Osteocalcin had lower concentrations at this time of extraction in all three groups (81.7ng/ml, 88.5 ng/ml and 104.7ng/ml). Week -42 again represented the calculated diestrus cycle stage, with high progesterone concentrations in control and the OVX groups (28.2ng/ml and 27.6ng/ml). At this extraction time, osteocalcin concentrations were again low in all three groups compared to the previous concentrations (112.7ng/ml, 114.5ng/ml and 120.2ng/ml) (diagram 4 and 5).

Estrus

Week -47 showed high osteocalcin concentrations in control and OVX groups (137.7ng/ml and 207.7ng/ml) where progesterone concentrations had low levels in conrol, OVX and second control groups respectively (3.5 ng/ml, 4.8ng/ml and 2.5ng/ml).
Week -44 represented the estrus cycle stage, again with low progesterone concentrations (0.2ng/ml, 1.0ng/ml, and 4.7ng/ml) and higher osteocalcin concentrations in all groups (140.1ng/ml, 171.1ng/ml, and 122.3ng/ml) (diagram 4 and 5).
Estradiol and osteocalcin had the same concentration development in the first cycle after syncronisation (diestrus) and in the following estrus cycle stage in all three groups (see diagram 4 and 6). Week -45 represented the first calculated day of cycle

where higher estradiol concentrations were expected. This was not the case here (22.0pg/ml, 16.5pg/ml, and 26.7pg/ml). Week -45 and -44, diestrus and estrus respectively showed the expected concentration development, high concentrations in the estrus cycle stage (33.8pg/ml, 29.2pg/ml, and 38.5pg/ml) and lower concentrations in the diestrus cycle stages (30.3pg/ml, 19.5pg/ml, and 29.0pg/ml).

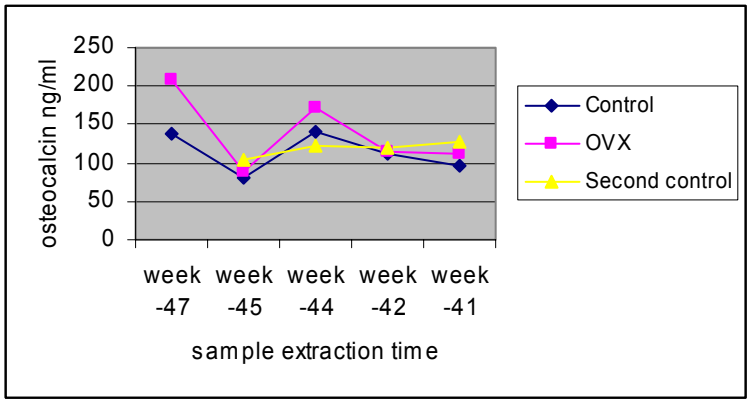

Diagram 4: **mean osteocalcin concentrations (ng/ml) in control, OVX and second control, pre-castration, sample extraction time in weeks**

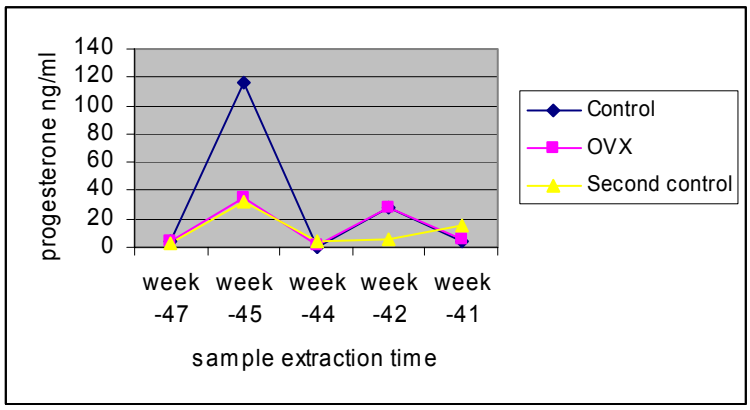

Diagram 5: **mean progesterone concentrations (ng/ml) in control, OVX and second control, pre-castration, sample extraction time in weeks**

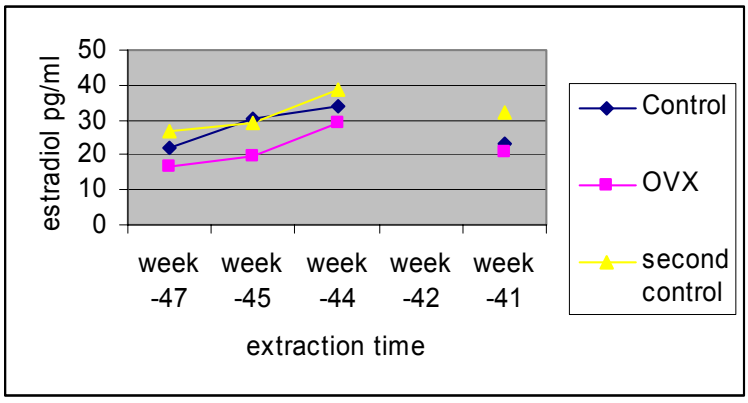

Diagram 6: **mean estradiol concentrations (pg/ml) in control, OVX and second control, pre-castration, sample extraction time in weeks**

Chiu et al. (1999) found that osteocalcin did not vary substantially across the cycle in humans. No significant correlations were found for estradiol and progesterone with osteocalcin in this study. Schlemmer et al. (1993) observed no cyclic changes in urinary parameters of resorption or in biochemical markers of bone formation, which included osteocalcin. This could not be confirmed in the present study. Osteocalcin showed steady cycle fluctuations in the miniature pig (diagram 4, 5, and 6). These studies, however, may be contraversial, because other studies have confirmed the opposite in humans. Studies have been performed to confirm that markers of bone metabolism vary during the menstrual cycle. De Souze et al. (1997) discovered that disturbances in the progesterone production as a result of exercise are not associated with a decrease in bone mass. Long follicular phases, are associated with decreased estrogen production in the early follicular phase, which are both associated with a decline in bone mass. Bone mass was not determined in this study, but according to the development of the formation marker osteocalcin, during the estrous cycle, an increase in concentrations seemed to be dependent on the estradiol concentrations. In the present study no correlations were found for osteocalcin and estradiol in the OVX and in the second control group. This could be due to the small number of animals included in this study. The control group, however, showed an inverse significant correlation in week -41 for osteocalcin and estradiol ($R=-0.905$; $p<0.05$). This correlation shows that osteocalcin concentrations increased, where estradiol concentrations decreased. Each osteocalcin concentration measurement is a snapshot of the actual bone metabolism at extraction time. Osteocalcin may have been on the verge of decreasing, and was measured exactly during the process, whereas estradiol had already reached lower concentrations. This could be an explanation for such a correlation. Week -41 represented the calculated day 1 of the estrous cycle, which was not mirrored in the estradiol concentrations, whereas progesterone concentrations showed low concentrations, which may represent the estrus stage or the late metestrus early diestrus cycle stage, where estradiol may have slightly higher concentrations. It is therefore possible that the animals in this group were in the metestrus cycle stage and the estradiol concentrations were not high enough to influence the osteocalcin concentrations.

Progesterone concentrations showed distinct inverse development with osteocalcin (see table 4 and 5). However no correlations were found for osteocalcin and progesterone in the OVX and in the second control group. This could also be due to

the small animal number included this study. Osteocalcin and progesterone however showed a definite significant inverse correlation in week -42 in the control group (R=-0.934; $p<0.05$). Haruyama et al. (2002) found a significant positive correlation between progesterone and osteocalcin, which suggested that bone forming activity also varies depending on the progesterone level during the estrous cycle in rats. Although the presence of progesterone receptors and the anabolic effects of progesterone have been demonstrated in cells of osteoblastic linage (Eriksen et al., 1988; Scheven et al., 1992), the role of this sex hormone in bone metabolism is less clear than that of estrogen. The present study, however, shows inverse correlations between progesterone and osteocalcin, which does not indicate that progesterone, is needed for an increase in bone formation. Rats have a different sexual cycle compared to that of pigs and the importance of age and bone metabolism in the rat should not be forgotten. Haruyama´s study used 10 week old rats. The formation marker development during the experimental period could have been due to bone growth activity and not due to cycle dependency. This would explain the simultaneous high osteocalcin concentrations with high progesterone concentrations.

Unlike the results found in the present study Nielsen et al. (1990c) found the highest osteocalcin levels in the luteal period of the menstrual cycle and a significant variance of osteocalcin in the menstrual cycle in humans. Hotchkiss and Brommage, (2000) however found osteocalcin peaks in the early follicular phase in cynomlgus monkeys.

In the present study the highest osteocalcin concentration were found in the estrus cycle stage or in the late follicular period, which confirm the effect of estradiol on bone metabolism. Estradiol increases osteoblast activity.

The high osteocalcin concentrations in week -47 and week -44 (calculated estrus; ovulation period or late follicular period) are due to the high estradiol concentrations at these times. Hughes et al. (1996) also showed that estrogen causes apoptosis in osteoclasts. In the presence of estrogen, osteoblasts produce transforming growth factor-beta 1 (TGF-beta). Anti TGF-beta antibody inhibited TGF-beta and estrogen. This may indicate that TGF-beta might mediate osteoclast apoptosis. The presence of estradiol allows an increased osteoblast activity. These high concentrations may be a result of a direct effect of estradiol on bone formation. The production of collagen type I extra-cellular matrix occurs primarily within the first 10 days of the

osteoblast development sequence (Stein et al., 1990). Because osteoblasts exhibit estrogen receptors and estrogens are able to enhance collagen synthesis (Komm et al., 1988; Eriksen et al., 1988), high osteocalcin concentrations may be due to the direct effect of estrogen on bone formation. Zitterman et al. (2000) confirmed that the normal menstrual cycle in young women is associated with monthly fluctuations of bone turnover which are most probably estradiol related.

The estradiol concentration in week -47 could have been higher in all three groups. The reason for its not reaching the expected peak could be the fact that the sample extraction in week -47 was the first, directly after the last hormone application at synchronisation. Estradiol peaks could have been reached two to three days later. For more precise hormone concentration development samples should have been extracted at lesser intervals.

Week -45 and -42 each represent a clear progesterone peak in the control and OVX group (diestrus) (see diagram 5). These confirm the cycle course during the experiment. The second progesterone peak in the second control group was found a week later. This could be due to cycle fluctuations after synchronisation. Each animal can deviate approximately 2 to 3 days from one another. The longer the interval after the last the hormone application, the greater the cycle deviation between the animals.

Taking this into consideration, the last three extractions showed clear inverse behaviour between osteocalcin and progesterone in the OVX and the second control group. The cycle-related changes for osteocalcin are maintained even though the extraction intervals were not as regular and had longer intervals compared to the weeks before. The concentration development of the control group during this time cannot be explained.

These results confirm that osteocalcin concentrations increase with an increase in estradiol concentrations, so estradiol stimulates formation in miniature pigs.

The bone formation marker osteocalcin has age-related changes and cycle-related changes in miniature pigs. This should be considered in further studies which include measuring the bone metabolism marker osteocalcin in the miniature pig. Bone turnover is higher at a younger age (puberty, 3-11) and decreases in adulthood, which was the equivalent of 14 months in this study. Additional fluctuations of this

marker occur during puberty and adulthood. Fluctuations during adulthood are cycle-stage dependant

5.3.2 Crosslaps

Age-related changes

In the pre-castration period of the present study an increase in the crosslaps concentrations was found between the ages of 9 and 10 months (puberty). At the age of 12 months these high concentrations decreased and stayed at low concentrations in all three groups (see diagram 7).

Many studies in humans have confirmed that the marker crosslaps, a bone resorption marker has age-related changes; Van der Sluis et al. (2002) found significant differences between three pubertal stages for cross-linked telopeptides of type I collagen in humans. The concentrations were higher in the earlier Tanner stages than they were here in the equivalently aged miniature pigs. Peichl et al. (2001) found rather poor associations between age of patients and markers of bone turnover, which include serum crosslaps. This could have been due to the fact that most of the patients included in the study were adults with fully developed bones. Gajewska et al. (2005) found peak concentrations for serum crosslaps and bone specific alkaline phosphatase in Polish children during puberty. After puberty a gradual lowering in both markers was observed. The data collected in this study confirms that the miniature pig also shows age-related changes for the resorption marker crosslaps. Crosslaps increased up until the age of 10 months and then showed a constant decrease in concentrations (see diagram 7). To determine the status of the bone, however, it is necessary to determine formation markers with resorption markers. Formation and resorption are coupled processes in the bone metabolism. If osteocalcin and crosslaps are determined at the same time, as they were in this study, it is conspicuous that osteocalcin and crosslaps have shifted responses to age. Osteocalcin had a lethargic reaction and stayed at higher concentrations for a longer period of time (12 to 13 months), while crosslaps decreased rather quickly at the third extraction at the age of 12 months. The decrease in concentrations for this marker at this point of time were very clear (diagram 7), whereas osteocalcin concentrations showed only slight increases at 12

and 13 months (diagram 3). Normally, a temporal and spatial coupling exists between resorption and formation in the skeleton, with resorption preceding formation during the remodelling sequence (Parfitt, 1982). The increase in bone formation can therefore be caused by an increase in bone resorption.

Limitations in serum markers such as tissue specificity should, however, be kept in mind. Type I collagen is not only present in bone, but it is also found in skin, ligaments, cartilage and synovia. Cross-linked telopeptides of type I collagen is released when collagen type I undergoes degradation. This could be a product deriving from sources other than bone. Huber et al. (2003) compared the established urine marker deoxypyridinoline, related to creatinine with plasma cross-linked telopeptides of type I collagen and found that if plasma was extracted from fasting patients in the morning, cross-linked telopeptides of type I collagen seemed to indicate bone resorption in women on hormone replacement therapy correctly to normal. According to these results plasma (or serum) seemed to be preferable. The test used in this study was validated for porcine serum and results showed fluctuations expected. This could indicate that serum as a medium for examination and crosslaps as a parameter for bone resorption are useful to express features of the bone metabolism in miniature pigs.

Almost all bone markers show diurnal variation. Qvist et al. (2002) examined the diurnal variation in serum crosslaps and found maximum levels in the morning at 5 a.m. and minimum levels in the afternoon at 2 p.m. The study showed that the only parameter which had a pronounced influence on the circadian variation was fasting, which reduced the circadian variation to about one fourth. This was taken into account in this study. During the present study, all animals fasted before sample extraction. Each extraction was performed at 9 a.m. in the morning, so the maximum levels in the diurnal rhythm were not expected, if the diurnal rhythm of the miniature pig were the same as that in humans. Each extraction was performed at the same time of day. If there was a definite diurnal rhythm in the miniature pig for this marker, concentration differences due to this circadian rhythm would have been excluded, due to the constant pattern of extraction. A circadian rhythm for cross-linked telopeptides of type I collagen was not only found in humans but also in dogs (Ladlow et al., 2002). If other animals have a circadian rhythm for this marker, the possibility exists that miniature pigs could also have a circadian rhythm. To determine

this, further studies have to be done with the minipig. Blood samples should be taken in repeated hourly intervals and daily to determine a rhythm of this marker.

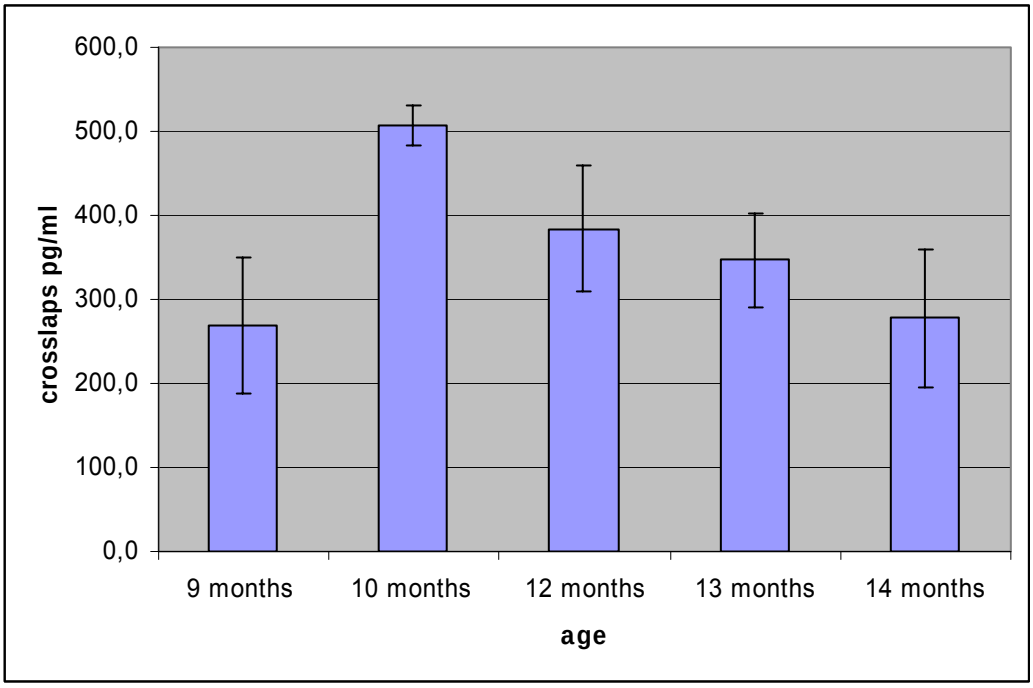

Diagram 7: crosslaps concentrations (pg/ml) represented in mean and SD calculated from mean and SD of control, OVX and second control group at 9, 10, 12, 13 and 14 months of age

Crosslaps estrous cycle-related

Crosslaps concentrations decreased from week -47 to week -45 in all groups. These increased two weeks later in week -44 (see diagram 8, 9, and 10). Like osteocalcin, crosslaps concentration development was opposite to that of progesterone at these extraction times.

The first synchronisation ended in week -47. The expected high estradiol concentrations in the estrus cycle stage were not found in week -47 in any of the groups. The reason for not reaching the expected peak could be due to the fact that the sample extraction in week -47 was the first, directly after the last hormone application at synchronisation. Estradiol peaks could have been reached two to three days later. For more precise hormone concentration development, samples should have been extracted in smaller intervals. The sample extraction in very short intervals was not possible, due to the fact that so many samples had already been extracted during this trial. Each extraction was done directly from the V. jugularis, which caused

haemorrhaging after each extraction and difficulty at the next extraction, when intervals were too short.

Estrus

With these missing high estradiol concentrations in week -47 (diagram 10), higher resorption markers (diagram 8) were expected if the resorption marker, crosslaps has the same cyclic fluctuations in the minipig as other resorption markers have in humans. Chiu et al. (1999) stated that deoxypyridinoline, a resorption marker, has higher concentrations in the follicular phase of the cycle. This could also be the case with crosslaps concentrations. Week -47 was the calculated day one of the estrous cycle. Cycle fluctuations of two to three days should be taken into account. High crosslaps concentrations could, therefore, occur at the end of the follicular phase or the beginning of ovulation in minipigs. This behaviour was confirmed in the following calculated cycle stages in week -44, calculated day 1 of the cycle (ovulation or with cycle fluctuations late follicular stage), low progesterone concentrations and high estradiol concentrations, with higher crosslaps concentrations (diagram 8, 9, 10)

Diestrus

Low crosslaps concentrations were found in week -45 in the diestrus cycle stage (calculated day 14). Progesterone concentrations were high at this time in all three groups (see diagrams 8, 9, and 10). This confirmed the calculated cycle stage. Gorai et al. (1995) found low concentrations of urinary resorption marker N-telopeptide in the luteal phase in humans. This was confirmed in this study using minipigs. Day 14 represents the beginning of the corpus luteum degradation period in the cycle of the pig where progesterone concentrations are still high. The estradiol concentrations however also seemed to be high at this time, but did not peak (see diagram10). These can be high at approximately day 7-9 of the diestrus cycle of a pig. This data confirms the early luteal phase. Crosslaps concentrations are therefore low in the luteal phase in pigs. Week -42 diestrus calculated day 15, luteal phase, with a decrease in crosslaps concentrations in the OVX group and simultaneous high

progesterone concentrations (see diagram 8, 9 and 10). Serum crosslaps therefore do have cyclic fluctuations in minipigs.

Osteocalcin and crosslaps seem to have cyclic fluctuations at the same time. It is important to remember that bone formation rate changes follow changes in bone resorption, due to the coupling of formation and resorption in bone remodelling units. As seen in the age-related change, crosslaps is a marker that reacts rather promptly to changes, whereas osteocalcin reacts lethargically. Keeping this in mind and the fact that samples were taken in weekly and larger intervals, it could be possible that osteocalcin peaks represented in this study were not final peaks. The osteocalcin concentrations could have reached higher concentrations after or between the examined blood sampling points of time, which were not represented in this study. To confirm this further studies are needed, with shorter intervals between extraction times.

Hughes et al. (1996) showed that estrogen causes apoptosis in osteoclasts. In the presence of estrogen, osteoblasts produce transforming growth factor-beta 1 (TGF-beta). Anti TGF-beta antibody inhibited TGF-beta and estrogen. This may indicate that TGF-beta might mediate osteoclast apoptosis. Marie et al. (2000) state that soluble factors, which include bone morphometric proteins, leptin and TGF-beta, can modulate differentiation of mesenchymal stem cells to osteoblasts, chondrocytes or adipocytes. This would be one of the reasons why formation and resorption would not occur at the same time.

Other factors such as interleukin 1 and interleukin 6 are known to play an important role in bone resorption. These factors also fluctuate during the menstrual cycle in humans (Cannon and Dinarello, 1985; Angstwurm et al., 1997). Interleukin 1 is known to stimulate bone resorption and perhaps formation. Pacifici et al. (1987) found that monocytes in postmenopausal osteoporotic patients (estrogen concentrations are low) produced more interleukin-1 than subjects in the control group. This could also be possible in minipigs. Interleukin 1 and 6 were not analysed in this study, but this would be an interesting prospect to evaluate in further studies.

Week -47 showed inverse correlations for crosslaps and estradiol in the OVX group ($R= -0.879$; $p<0.05$). Zittermann et al. (2000) demonstrated that resorption marker deoxypyridinoline was inversely associated with estradiol, especially when estradiol was in the lower physiological range. Uebelhart et al. (1991) demonstrated that

estrogen had profound effects on collagen cross-link excretion. Pyridinoline and deoxypyridinoline concentrations in postmenopausal women returned to premenopausal values after 6 months of estrogen containing hormone replacement therapy. This may also be the case in minipigs, but definite evidence in not displayed in the present study. The estradiol concentrations in week -47 were expected to be high after synchronisation. The estradiol test used in this study was a test for human serum and was not validated for porcine serum and results should be evaluated with caution. The animals in the OVX group could have reached the expected higher estradiol concentrations two to three days later. To confirm the inverse correlation of these two parameters, further studies are needed, with larger animal numbers, and smaller intervals between extraction times, to demonstrate the resorption processes in bone metabolism and its relationship to estradiol concentrations in the minipig. Week -44 showed significant inverse correlations in the OVX group for crosslaps and progesterone ($R=-0.881$; $p<0.05$). This was the calculated day 1 of the estrous cycle. Progesterone concentrations were low, with simultaneous high crosslaps concentrations. If progesterone has a definite function in bone resorption, it is not clear. The question of whether progesterone stimulates formation as a result of increased resorption must be evaluated further in follow up studies.

The lower progesterone concentrations in week -42 (diagram 9) in the second control group could indicate the beginning of the follicular stage, which would explain the high crosslaps concentrations. Simultaneous high crosslaps and high progesterone concentrations in the control group could be due to the representation in mean concentrations. The standard deviation in this group at this extraction time was very high for crosslaps.

The last three extraction weeks in the pre-castration period showed that the crosslaps and progesterone seemed to have the same concentration development in the OVX and the second control group. Week -41 showed a decrease in estradiol concentrations with progesterone concentrations that did not peak. This was also calculated as day 1 of the estrous cycle. The hormone concentrations however indicate the metestrus stage (approximate day 3-4) or day 16-18, the end of the diestrus beginning of the proestrus cycle stage (late luteal phase or beginning of the follicular phase). At this extraction time in the control group, estradiol and crosslaps showed a significant inverse correlation ($R= -0.971$; $p<0.001$). Crosslaps

concentrations were between 300-400 pg/ml. Comparing these concentrations to the previous concentrations, no definite conclusion can be made about cycle-related concentration changes. The last two extraction points of time were very far apart. This could cause imprecise cycle stage assumption without estradiol concentration backup. In week -19 progesterone peaks in OVX and second control group suggest that these groups were in the diestrus cycle stage. Crosslaps concentrations seemed to be at a peak. To interpret definite cycle fluctuations in markers, extraction times should be closer to one another and more frequent.

This study showed that crosslaps had cyclic fluctuations in minipigs. High crosslaps concentrations were found in the estrus cycle stage or late follicular cycle stage; whereas low crosslaps were found in the luteal phase. This study did not demonstrate a chronological difference between formation and resorption marker cyclic fluctuations. Remodelling is a temporal and spatially constrained process in which bone is subsequently removed and then replaced by new bone. In skeletally mature animals, bone resorption and bone formation are balanced during remodelling so there is no net change in bone mass (Allen, 2003). The data of this study does not show a precise separation of remodelling and formation processes. This could be due to the large intervals between sample extractions and the reactivity of each marker measured. The formation and resorption processes, however do seem to be balanced, which was expected in adult animals.

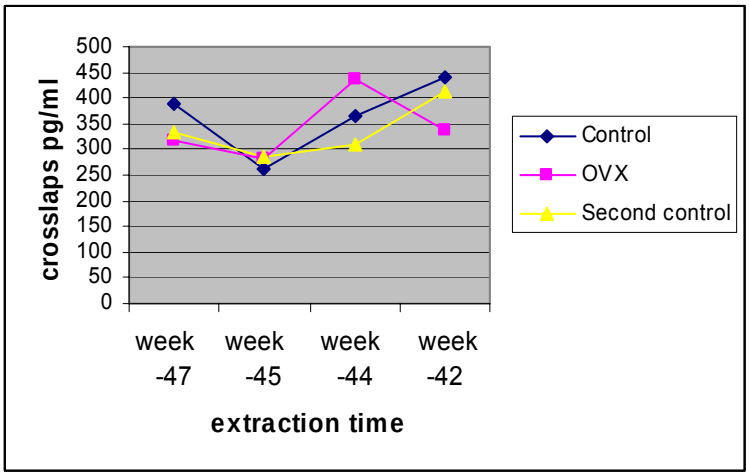

Diagram 8: **mean crosslaps concentrations (pg/ml) in control, OVX and second control groups pre-castration, sample extraction time in weeks**

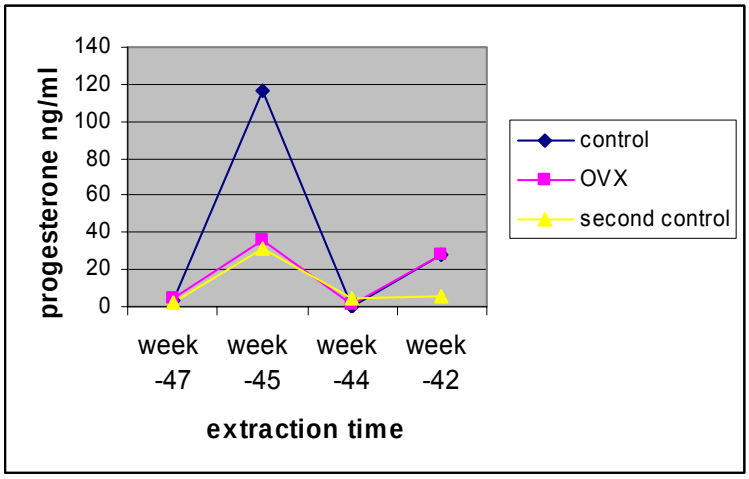

Diagram 9: **mean progesterone concentrations (ng/ml) in control, OVX and second control groups pre-castration, sample extraction time in weeks**

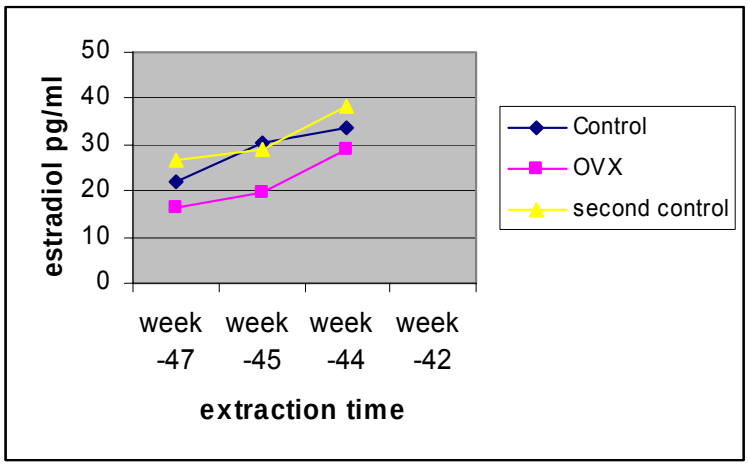

Diagram 10: **mean estradiol concentrations (pg/ml) in control, OVX and second control pre-castration, sample extraction time in weeks**

Correlations between crosslaps and osteocalcin in the pre-castration period

Peichel et al. (2001) found correlations between osteocalcin and crosslaps in humans. Analysing correlations over all groups for all extraction times, these correlations were not found. Analysing correlations in each group for each extraction time, a significant positive connection was found in the control ($p<0.001$; $R=0.373$) and second control groups ($p<0.05$; $R=0.290$) during the pre-castration period. These results confirm that the formation and resorption processes are coupled processes, which are balanced in the physiological bone metabolism. This was expected in healthy adult minipigs. Further correlation analysis was done at each extraction time and additional connections were found for these two parameters. The control group showed significant correlations in week -47 and -41 respectively ($p<0.05$; $R=0.917$); ($p<0.01$; $R=0.96$) and the OVX group in week -58 ($p<0.05$; $R=0.892$). The reason why the correlations between these parameters were only found at these specific times could be; the fact that the crosslaps and osteocalcin concentrations measured at each extraction time are a snapshot of the actual bone metabolism at the particular time of extraction. Formation and resorption processes continued changing during extraction between each animal.

5.4 Development of biochemical markers in the post-castration period

5.4.1 Osteocalcin

The biochemical marker, osteocalcin was examined at and after ovariohysterectomy to determine the effect of ovariohysterectomy on this marker. Blood samples were extracted at ovariohysterectomy and at two week intervals up to and including eight weeks after operation in all groups. The osteocalcin concentrations showed an increase 4 weeks after the intervention in the OVX group. These high concentrations stayed constant up until 8 weeks (diagram 11). The osteocalcin concentrations in the control and second control groups stayed almost constant from week 0 to week 6. Week 8 showed a slight increase in both groups, which did not exceed the concentrations found in the OVX group.

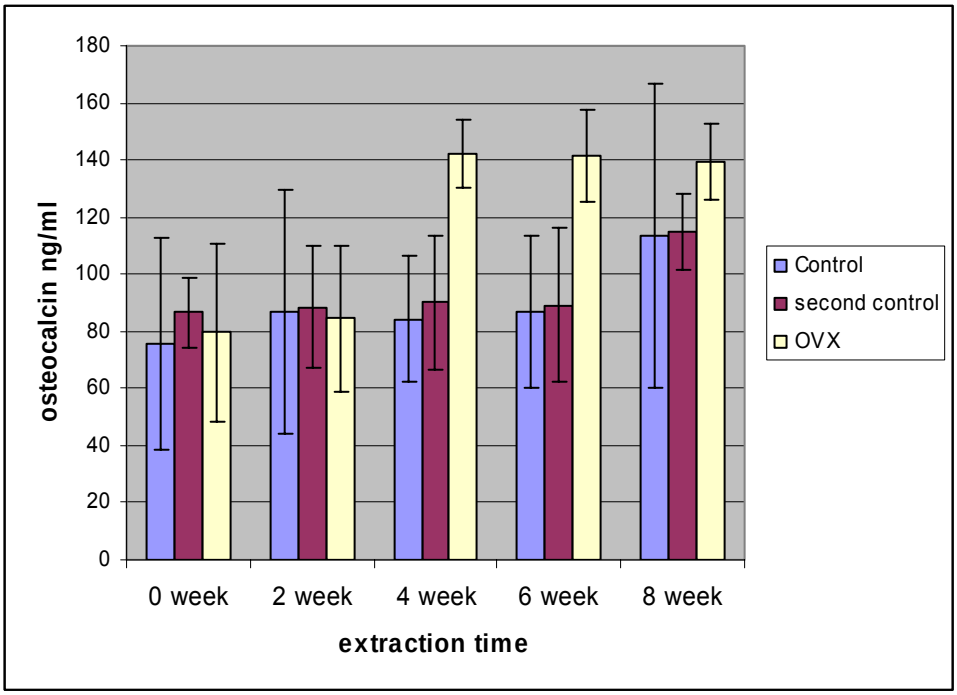

Diagram 11: Osteocalcin concentrations ng/ml (mean and SD) at extraction times 0, 2, 4, 6, 8 weeks; post-castration period in control, second control and OVX group

Dresner-Pollak et al. (1996) found negative correlations between formation, resorption markers and bone mass. An increase in bone turnover in humans can cause a loss in bone mass. Conclusions can therefore be drawn from bone markers about bone density and fracture risk (Looker et al., 2000).

Many studies have been done in humans and animals, which confirm the increase of bone turnover after ovariohysterectomy and ovariectomy; Peris et al. (1999) determined biochemical markers of bone metabolism after surgical menopause in humans. These markers included formation marker osteocalcin. Three months after the operation all formation and resorption markers determined in this study increased. Itoh et al. (2002) did a similar study with mature cynomolgus monkeys. These were ovariectomised and evaluated over a period of 16 months. Serum osteocalcin values increase 168% compared to sham operated animals. The ovariectomised cynomolgus monkey is a recognised animal model used as a model in the human osteoporosis research. Legrand et al. (2003) ovariectomised seven old cynomolgus monkey and used thirteen age matched sham controls to demonstrate that reliable biochemical parameters are available to determine formation and resorption activity in the bone. Osteocalcin correlated highly with a decrease in bone mineral density induced by ovariectomy. Newton et al. (2004) determined the effects of ovariectomy on the trabeculae of ovine iliac bone. Blood samples were draw at

ovariectomy and in three month intervals up until twelve months. Osteocalcin concentrations were significantly higher in the OVX groups compared to the controls. Lukacs et al. (2006) demonstrated that women in early menopause years (40-52 years) had elevated osteocalcin values.

In this study the increase of the formation marker osteocalcin 4 weeks after ovariohysterectomy indicates that osteoblasts may have been activated, due to an imbalance in formation and resorption processes. The ovariohysterectomy caused an estrogen depletion (diagram 12), which triggered an increase in resorption and bone loss. This was followed by higher bone formation rates. The same scenario is found in the post menopausal osteoporosis.

Diagram 12 shows a decrease in estadiol concentration two weeks post operation. This decrease was significant $p< 0.05$ for the delta values among the groups for the extraction time week 0 and week 2. The area under the curve in the OVX group showed a significant difference $p< 0.001$ compared to the control and the second control group. Estradiol concentrations stayed at a minimum after the 2nd week in the OVX group, which confirmed the effect of the intervention. The estradiol concentrations continued to alternate at higher concentrations in the control and second control groups. This showed that the rest of the animals continued their estrous cycle.

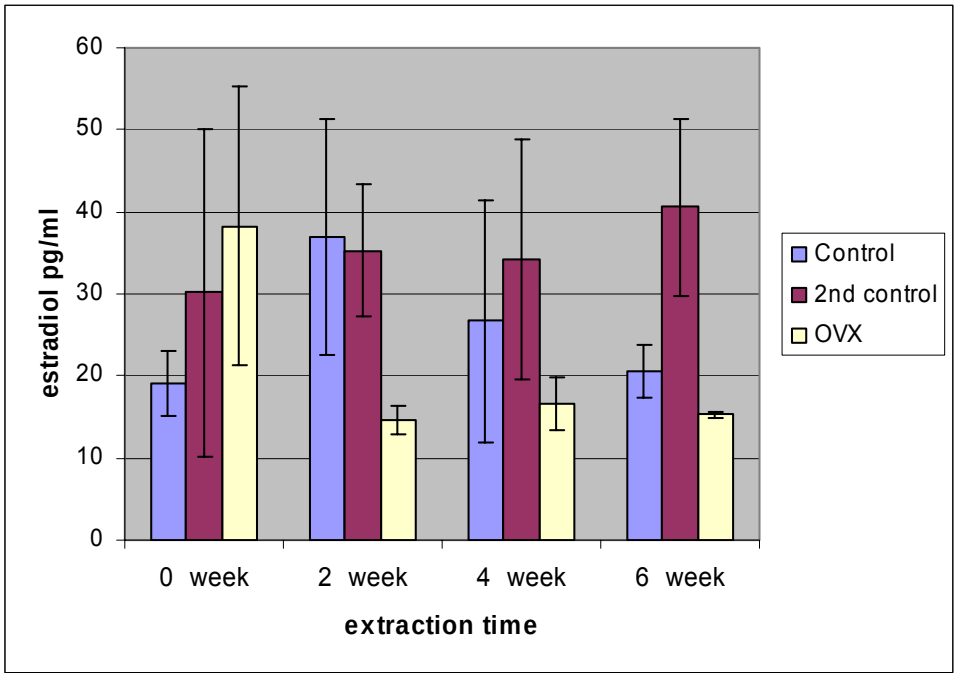

Diagram 12: estradiol concentrations (pg/ml) mean and SD at extraction times 0, 2, 4, 6, 8 weeks in the post-castration period in control, second control and OVX groups

Progesterone was a further parameter determined, which confirmed the effect of castration on the cycle of the animals (diagram 13). Two weeks after OVX progesterone concentrations still seemed to be high in the OVX group. This could be due to a slow breakdown of progesterone in pigs after anaesthesia. This could have been caused by the application of Regumate®, synthetic progesterone, in this group before castration, to suppress the estrus cycle stage. In the fourth week after operation, progesterone declined and stayed at low concentrations in this group, as expected. The control and second control group continued to show fluctuations in progesterone concentrations, which were also expected in the cyclic occurrences of the pig.

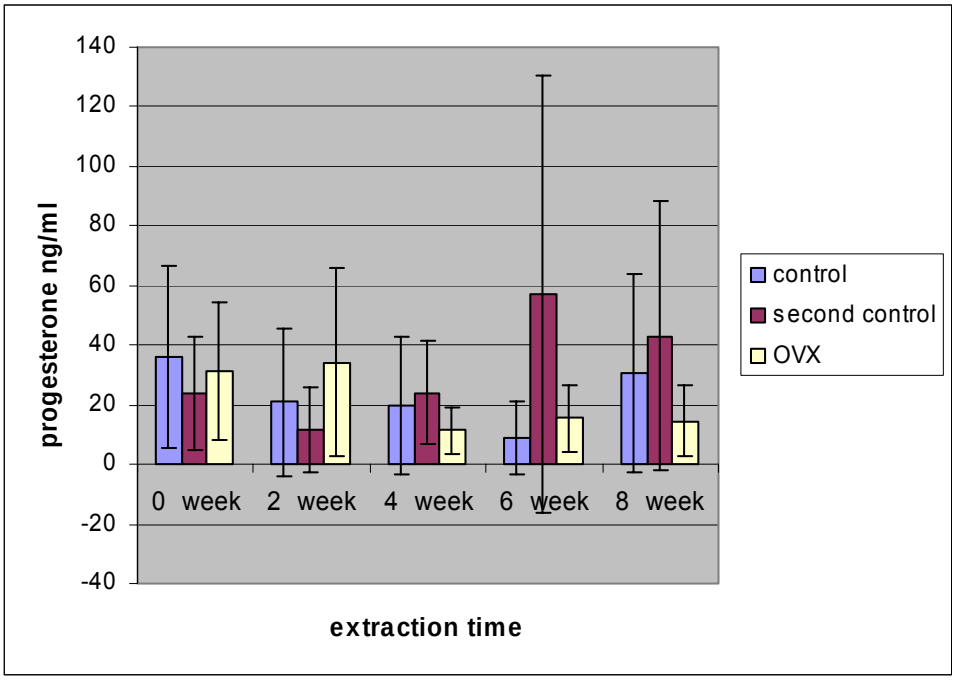

Diagram 13: progesterone concentrations (ng/ml) mean and SD at extraction times 0, 2, 4, 6, 8 weeks in the post-castration period in control, second control and OVX groups

There is an effect of OVX on osteocalcin in minipigs. The absence of significances between the two control groups and the OVX group for osteocalcin was due to the small number of animals included in this experiment. To determine the effect of ovariohysterectomy on the bone mineral density and bone quality, further studies have to be performed including other forms of analysis such as DXA and micro-CT.

5.4.2 Crosslaps

Like osteocalcin, serum crosslaps were analysed at castration and in two week intervals after castration up to and including 8 weeks post-castration (diagram 14). Crosslaps concentrations increase 2 and 4 weeks post operation in the OVX group. Thereafter concentrations decreased in this group.

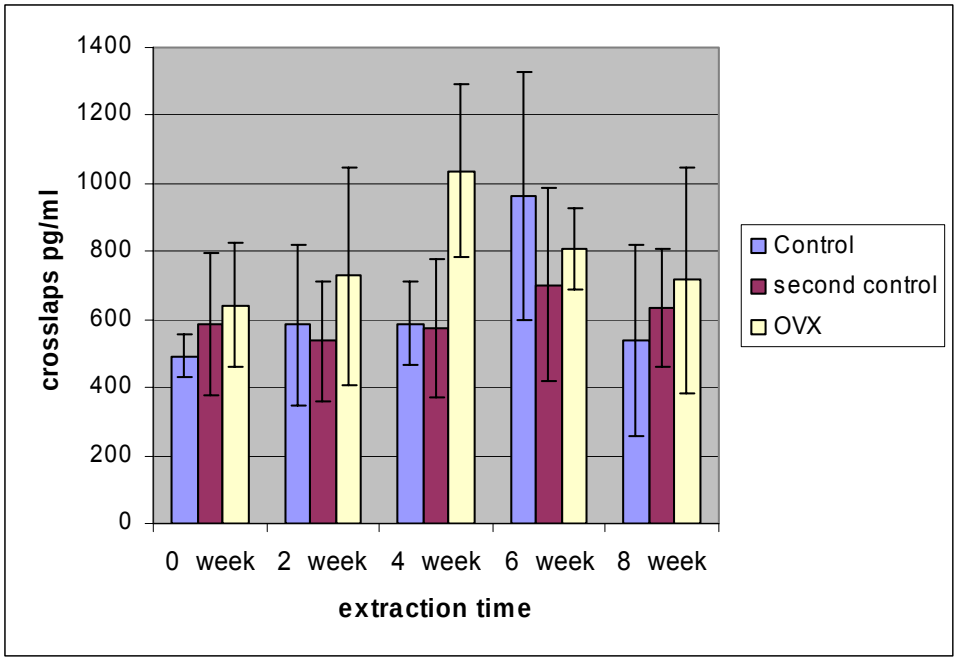

Diagram 14: Crosslaps concentrations pg/ml (mean and SD) at extraction times 0, 2, 4, 6, 8 weeks; post-castration period in control, second control and OVX groups

Gaumet et al. (1996) studied the influence of ovariectomy on bone metabolism in very old rats and found an increase in bone resorption markers pyridinoline and deoxypyridinoline seven weeks after OVX compared to the controls. These rats were mature, and therefore had slower bone growth (Patlas et al., 2000). The changes in crosslaps concentrations after OVX in the minipig were very prompt and clear in comparison to those of the resorption markers in the mentioned study using rats. The minipigs were at a mature age, which rules out a high bone turnover due to growth. This aspect makes the minipig a convenient model to study postmenopausal osteoporosis.

The absence of estrogen caused an increase in osteoclast activity, post operation. Diseases characterized by disorders in bone mass may have disturbances in both formation and resorption. These circumstances cause the balance that typifies bone remodelling in adult animals to be lost. i.e. increased osteoclastic activity in post

menopausal osteoporosis may be accompanied by an increase, decrease or unchanged osteoblastic activity (Civitelli et al., 1988). This increase in osteoclast activity was portrayed by the increase in crosslaps activity after operation. This was accompanied by an increase of bone formation, osteoblast activity (osteocalcin concentrations increased 4 weeks after the intervention) (see diagram 11). Together these results show a higher bone turnover after ovariohysterectomy, which makes the minipig an interesting model for further studies on osteoporosis.

5.5 The effect of calcium ration on bone metabolism markers

To evaluate this effect both control groups were examined in the pre and post-castration period. The control group received a calcium ration consisting of 0.99% calcium, while the second control group received a 0.7% calcium diet.
The calcium metabolism is regulated by parathyroid hormone, calcitriol (vitamine D hormone) and calcitonin. Bone resorption and intestinal calcium absorption are regulated by calcitriol (vitamine D hormone) and parathyroid hormone. An increase in serum calcium concentrations would result in a decreased production of these two hormones and an increase in calcitonin. Calcitonin is the antagonist of parathyroid hormone in the bone, but acts synergistically with parathyroid hormone in the kidney, causing calcium resorption and phosphorus excretion. One would not expect that the calcium in the diet would change the serum calcium values; therefore calcium was not determined in the serum in the present study.

In the 2, 4 and 6 week post operation period, serum crosslaps concentrations showed a tendency to higher concentrations in the control group, which received 0.99% calcium diet. Assuming that higher calcium concentrations caused an increase in calcitonin concentrations and therefore stopping the effect of parathyroid hormone and calcitriol, which would have decreased resorption, which was not the case in week 2, 4, and 6. The resorption marker had increased concentrations (diagram 15). There was however a decrease in resorption in week 0 and week 8 in this group. No definite influence could be seen in the concentration development of this parameter in connection with the calcium ration when comparing crosslaps concentrations in both control groups in the pre-castration period. The feed was fed during the whole

experimental period, so the calcium ration had sufficient time to influence the bone metabolism.

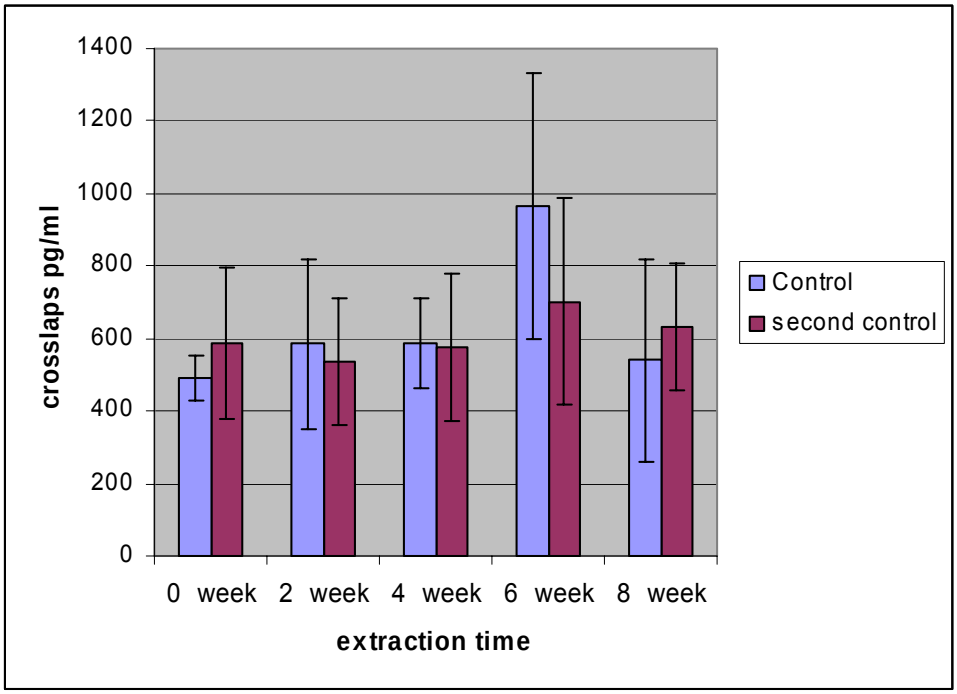

Diagram 15: Crosslaps concentrations pg/ml (mean and SD) at extraction times 0, 2, 4, 6, 8 weeks; post-castration period in control and second control groups

Liesegang and Risteli, (2005) investigated the influence of different calcium diet in growing dairy goats and sheep. This study showed that serum crosslaps had the tendency towards lower concentrations in the groups of animals fed 17.7g calcium per day and 18.5g calcium per day. In the present study the control group received a 0.99% calcium ration, which is equivalent to 9.89g/kg feed. Each animal received a daily ration of 550g of feed. The actual calcium ration in the control group was 5.49g per day and 3.84g in the second control group. These were very low concentrations in comparison to the study described above. This could be the reason for the missing or minimal reaction of the resorption markers during the present study.

Liesegang et al. (1998) found reduced bone formation markers in a group of cows that were fed a low calcium diet. High calcium rations however had no effect on resorption markers in this study. In the present study osteocalcin showed slightly more activity in the second control group (0.7% calcium ration) (diagram 16). This was also the case, for the majority of the time in the pre-castration period. Lower calcium concentrations would increase in parathyroid hormone and cacitriol secretion. This would lead to an increase in bone resorption and intestinal calcium

absorption. An increase in formation markers does not indicate increased bone resorption. The slight increase in osteocalcin in the second control group could be a result that occurred by chance. The difference between the calcium concentrations in both control groups was very small. In future studies performed to demonstrate the effect of calcium rations on markers of bone metabolism, calcium ration differences between the groups should be greater to achieve the expected results.

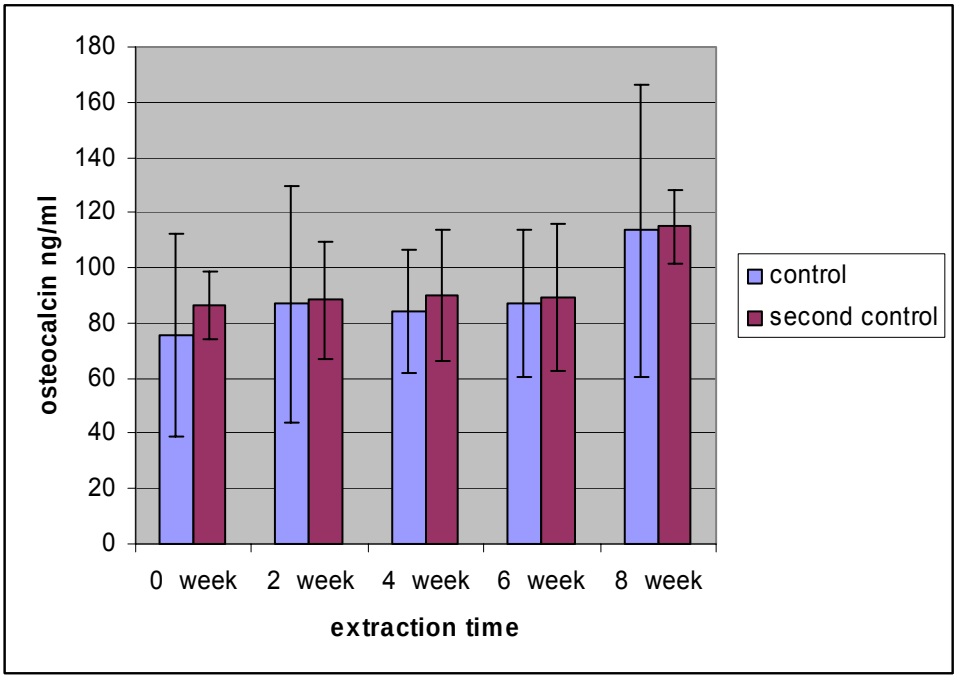

Diagram 16: Osteocalcin concentrations ng/ml (mean and SD) at extraction times 0, 2, 4, 6, 8 weeks; post-castration period in control and second control groups

Studies such as Mosekilde´s show that calcium restriction in diets can cause bone loss; showed that the OVX-minipig on 0.75% calcium showed significant changes in remodelling parameters and lead to alterations in bone structure (Mosekilde et al. 1993). In this study not only calcium restriction, but also the lack of estrogen post OVX caused bone loss. In the presented study the difference in calcium rations between the two control groups was too small to show a difference in effect on the bone markers between these groups, while the effect in the OVX group, who were fed the 0.7% ration, was very clear (diagram 11 and 14).

Scholz-Ahrens et al. (1996) studied the effect of ovariectomy on bone metabolism in sows and fed a standard diet of 1.5% calcium. No significant differences were found between the OVX and control animals in bone chemical or histological analysis neither 12 nor 20 months post OVX in young sows. Multiparous sows had increased plasma parathyroid hormone and calcitriol 8 months after operation, while total and

bone alkaline phosphatase increased 12 months post OVX. Bone chemical or histiological analyses were not affected by OVX 12 and 20 months post OVX. Due to the results of this study, it is clear that the changes after OVX with an ample amount of calcium in the daily diet, does not result in osteopenia/osteoporosis. In the present study bone markers do not show definite differences between the control group and the second control group fed with a calcium diet of 0.99% and 0.7% respectively. It is, however not clear if the structure of the bone had changed. No histological analysis or micro-CT was performed from the bone. To determine these changes further studies have to be performed with these calcium rations. The OVX group received a 0.7% calcium diet. Resorption and formation markers showed changes post-castration, which shows that this is a calcium ration that can be used in further studies where induced osteopenia is required.

6 Summary

Brenda Babel: Investigations on bone metabolism in intact and ovariohysterectomised miniature pigs

Biochemical markers of bone metabolism have become a common form of analysis for bone metabolism. A number of factors, however, cause fluctuations in these parameters such as day-to-day changes, seasonal changes and age. In women, studies have confirmed parameter fluctuations during the menstrual cycle (Zittermann et al., 2000). Up until now, the coherence hormone status and bone marker activity have been scanty in animal models.

The objective of this study was to examine the dependence of bone marker activity on the estrous cycle in the Dresdner miniature pig. The present study included the bone formation marker osteocalcin, and bone resorption marker serum crosslaps. The estrous cycle dependency was determined by comparing the development of marker concentrations with progesterone and estradiol in the respective cycle stages of each pig after synchronisation and at particular sampling points of time. Furthermore the behaviour of biochemical markers, osteocalcin and crosslaps were examined at and after ovariohysterectomy (OVX) to determine the effect of OVX on these specific bone markers. Another aspect of this study was to determine the influence of calcium content in the diet on bone metabolism before and after castration.

Sixteen animals were divided into three groups, two control groups and a OVX group. One control group received a 0.99% calcium diet, while the OVX and the second control group received a 0.7% calcium diet. The animals in the OVX group were ovariohysterectomised, while the pigs in the control and the second control groups were not manipulated. The trial consisted of two periods; the pre-castration period, lasting 65 weeks and a post-castration period lasting 8 weeks. Blood samples were drawn and analysed at selected extraction times.

The bone marker development pre-castration showed age-related and cycle-related changes. Osteocalcin showed definite increased concentrations, at the age of 9-13 months. If 10 months were seen as a late prepubal stage (Tanner stage) in the minipig, osteocalcin reacts more slowly in minipigs as it does in humans. Assuming that female minipigs in the present study can be compared with female humans during puberty, the present study shows that osteocalcin concentrations are still high

at late puberty, unlike those in humans, which are high at midpuberty. Crosslaps increased up until the age of 10 months and then showed a constant decrease in concentrations. This would be the equivalent reaction of resorption markers in humans.

Osteocalcin concentrations increased with an increase in estradiol concentrations, so estradiol stimulates bone formation in miniature pigs. The resorption marker serum crosslaps also showed cyclic fluctuations. High crosslaps concentrations were found in the estrus cycle stage or late follicular cycle stage; whereas low crosslaps were found in the luteal phase.

As was often the case in other studies, the effect of a calcium reduced diet was not evident between the control and second control group. Resorption and formation markers showed changes post-castration in the OVX group, which shows that this is a calcium diet that can be used in further studies where induced osteopenia is required.

Osteocalcin showed a definite concentration increase 4 weeks after castration. Serum crosslaps increased 2 weeks post operation. These increased concentrations were not significant, which could be due to the small animal numbers included in this study. Both markers reacted within a short time after ovariohysterectomy, which is a useful aspect for further osteoporosis research.

This study portrayed the usefulness of the minipig as an animal model, not only for further studies on postmenopausal osteoporosis, but also verified the use of osteocalcin and serum crosslaps as biochemical parameters of bone metabolism in this species, to have age-related and cycle-related fluctuations, which should be considered in further studies.

7 Zusammenfassung

Brenda Babel: Untersuchungen zum Knochenstoffwechsel am intakten und ovariohysterektomierten Miniaturschwein

Die Bestimmung von biochemischen Markern des Knochenstoffwechsels stellt bei der Analyse des Knochenstoffwechsels eine weit verbreitete Methode dar. Eine Reihe von Faktoren, wie Tageszeit, Jahreszeit und Alter, beeinflussen dabei diese Parameter. Bei Frauen konnte eine Abhängigkeit vom Menstruationszyklus nachgewiesen werden (Zittermann et al., 2000). Eine Beeinflussung der Knochenmarkeraktivität durch den Hormonstatus wurde für die verschiedenen Tiermodelle noch nicht ausreichend untersucht.

Das Ziel der vorliegenden Arbeit war daher die Untersuchung der Zyklusabhängigkeit der Knochenmarkeraktivität an Dresdener Miniaturschweinen. Als Marker für die Knochenformation wurde Osteocalcin und als Marker für die Resorption wurden Crosslaps im Serum bestimmt. Die Zyklusabhängigkeit wurde durch den Vergleich der Veränderungen der Markerkonzentrationen mit der Progesteron- und Östradiolkonzentration, im zeitgleich gewonnenen Serum an unterschiedlichen Zyklustadien im Anschluss eine Zyklussynchronisation, bestimmt. Zusätzlich wurden Veränderungen der Konzentrationen von Osteocalcin und Crosslaps im Zeitraum nach Ovariohysterektomie (OVX), zur Ermittlung des Einflusses einer Ovariohysterektomie auf die Höhe der Knochenmarker, untersucht. Ein weiterer Aspekt der Arbeit war die Untersuchung der Auswirkung unterschiedlicher Calciumgehalte der Futterrationen auf den Knochenstoffwechsel vor und nach der Kastration.

Sechzehn Tiere wurden in drei Gruppen, zwei Kontrollgruppen und eine OVX Gruppe aufgeteilt. Eine Kontrollgruppe wurde mit einer Futterration die 0.99% Kalzium enthielt, gefüttert, während der zweiten Kontrollgruppe und die OVX Gruppe eine Futterration mit einem Kalziumgehalt von 0.7% erhielten. Die Tiere der OVX Gruppe wurden ovariohysterektomiert, während die Tiere der beiden Kontrollgruppen keinem Eingriff unterzogen wurden. Die Untersuchungen wurden getrennt nach der Vorkastrationsperiode, die einen Zeitraum von 65 Wochen umfasste, und der Nachkastrationsperiode, die einen Zeitraum von 8 Wochen umfasste, ausgewertet. An bestimmten Zeitpunkten wurden Blutproben gewonnen und analysiert.

Die Knochenmarkerkonzentrationen zeigten während der Vorkastrationsperiode eine Alters- und Zyklusabhängigkeit. Die Osteocalcinkonzentration steigt bis zu einem Alter von 9-13 Monaten. Wenn eine Alter von 10 Monaten als spätes präpubertales Stadium (Tanner stage) des Miniaturschweines angesehen wird, scheinen sich die Osteocalcinkonzentrationen beim Miniaturschwein langsamer zu verändern als es beim Menschen beschrieben ist. Die Miniaturschweine in dieser Untersuchung können in ihrem Entwicklungstand mit Mädchen während der Pubertät verglichen werden. Unter dieser Annahme kann man davon ausgehen, dass die Konzentrationen von Osteocalcin beim Miniaturschwein, in der Spätpubertät noch hoch sind, während sie beim Menschen in der Mitte der Pubertät am höchsten sind und dann abfallen. Die Crosslapskonzentration im Serum nimmt bis zum Alter von 10 Monaten zu, im Weiteren konnte dann aber ein konstanter Abfall der Konzentration nachgewiesen werden. Diese Konzentrationsentwicklung entspricht der die beim Menschen beschrieben ist.

Die Osteocalcinkonzentration nimmt mit ansteigender Östradiolkonzentration zu, dies lässt darauf schließen, dass Östradiol die Knochenformation stimuliert. Die Knochenmarker Crosslaps zeigten ebenfalls zyklusabhängige Konzentrations-veränderungen. Hohe Crosslapskonzentrationen konnten im Östrus oder im Proöstrus nachgewiesen werden, während in der Lutealphase niedrige Crosslapskonzentrationen im Serum festgestellt werden konnten.

Wie schon in anderen Studien beschrieben, konnten keine Effekte einer kalziumreduzierten Futterration zwischen den beiden Kontrollgruppen festgestellt werden. Resorptions- und Formationsmarker reagieren in der Postkastrationsperiode in der OVX Gruppe sensitive. Dies zeigt, dass die gefütterte Ration mit einem Kalziumgehalt von 0.7% in Versuchen zur Erzeugung einer Osteoporose in Miniaturschweinen als Futterration geeignet erscheint.

Die Osteocalcinkonzentration stieg in der vierten Woche nach der Kastration an. Die Serum Crosslapskonzentration stieg zwei Wochen nach der Kastration an. Diese Anstiege waren nicht signifikant, was mit der kleine Tierzahl in der vorliegenden Arbeit zusammenhängen könnte. Beide Marker reagierten in einem sehr kleinen Zeitabschnitt nach der Ovariohysterektomie, was für die Osteoporoseforschung sehr nützlich sein kann.

Die vorliegende Arbeit zeigt auf, dass das Miniaturschwein ein geeignetes Modell zur postmenopausalen Osteoporoseforschung darstellt. Die Osteoclacin und

Crosslapskonzentration als Marker für den Knochenstoffwechsel folgt bei dieser Spezies einem alters- und zyklusabhängigen Verlauf. Dies muss bei weiterführenden Studien berücksichtigt werden.

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10 Curriculum vitae

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