Osteoporosis in Chronic Liver Disease

BY

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ABSTRACT

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Osteoporosis is a well-known and frequently reported complication of chronic liver disease (CLD) with a high fracture rate contributing to significant morbidity after liver transplantation. The pathogenesis is unknown and controversy exists about many risk factors for osteoporosis in CLD.

In the present thesis, bone mineral density (BMD) was found to be significantly lower at the lumbar spine ($p<0.01$) in a cohort of patients with CLD compared with age- and gender -matched individuals. Osteoporosis was found in 30% of the patients and 15% of the controls, respectively. Low body mass index (BMI), corticosteroid treatment, prothrombin time, age and female gender were independent risk factors for osteoporosis in the patients.

In a follow-up study, 43 of 72 patients were available for a second BMD measurement 25 months (median) after the first. Bone loss at the femoral neck was $1.5 \pm 2.4\%$ in females and $2.9 \pm 2.0\%$ in males with a significant decrease in BMD Z-score over time ($p=0.005$ and $p=0.02$ for females and males, respectively), indicating increased bone loss at this site. Hyperbilirubinaemia and low circulating levels of 25-hydroxy vitamin D_3 predicted increased bone loss at the femoral neck. These findings suggest that cortical bone, in addition to trabecular bone, may be affected in CLD and bilirubin and vitamin D_3 may be involved in the pathophysiology of osteoporosis in CLD.

In order to elucidate the suggested role of insulin-like growth factors (IGFs) and leptin in the pathophysiology of osteoporosis in CLD, we studied the relationship between these factors and BMD. Levels of IGFs were extremely low ($p<0.0001$ compared with the controls) and related to liver function but no correlation was found between the IGFs and BMD. Serum leptin adjusted for BMI correlated negatively with BMD in female patients ($p=0.003$ and $p=0.04$ at the lumbar spine and the femoral neck, respectively) and in male patients at the femoral neck ($p=0.04$). Thus, the IGFs appear not to be involved in the pathophysiology of osteoporosis in CLD but a role of circulating leptin is possible.

Key words: Chronic liver disease, bone mineral density, osteoporosis, body mass index, corticosteroids, hyperbilirubinemia, vitamin D, insulin-like growth factors, leptin.

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SKALAT MAÐR RÚNAR RISTA

NEMA RÁÐA VEL KUNNI

EGILL SKALLAGRÍMSSON

PAPERS

The thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Ormarsdóttir S, Ljunggren Ö, Mallmin H, Brahm H, Lööf L. Low body mass index and use of corticosteroids, but not cholestasis, are risk factors for osteoporosis in patients with chronic liver disease. **Journal of Hepatology** 1999; 31: 84-90.

II. Ormarsdóttir S, Ljunggren Ö, Mallmin H, Lööf L. Increased rate of bone loss at the femoral neck in male patients with chronic liver disease. **Submitted for publication**

III. Ormarsdóttir S, Ljunggren Ö, Mallmin H, Olofsson H, Blum WF, Lööf L. Circulating levels of insulin-like growth factors and their binding proteins in patients with chronic liver disease. Lack of correlation with bone mineral density. **In press, Liver**

IV. Ormarsdóttir S, Ljunggren Ö, Mallmin H, Olofsson H, Blum WF, Lööf L. Inverse relationship between circulating levels of leptin and bone mineral density in chronic liver disease. **Submitted for publication**

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CONTENTS

ABBREVIATIONS

$1,25(OH)_2 D_3$	1,25-dihydroxy vitamin D_3
$25(OH) D_3$	25-hydroxy vitamin D_3
ALT	alanine transaminase
AST	aspartate transaminase
auCAH	autoimmune chronic active hepatitis
BMD	bone mineral density
BMI	body mass index
Ca	calcium
CLD	chronic liver disease
DXA	dual-energy X-ray absorptiometry
ERT	estrogen replacement therapy
FSH	follicle stimulating hormone
GH	growth hormone
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
PBC	primary biliary cirrhosis
PSC	primary sclerosing cholangitis
PTH	parathyroid hormone
TGF-β	transforming growth factor-β

INTRODUCTION

A. Metabolic bone disease

Bone metabolism

Remodeling of bone continues throughout life in response to mechanical stimuli and other regulatory factors. The normal sequence of bone remodeling involves 4 steps, the first of which is activation of osteoclasts by osteoblasts [1]. The next step is bone resorption, which involves replication of osteoclast precursors and their differentation, migration and fusion into mature osteoclasts. The third phase begins when the osteoclasts have resorbed most of the mineral and matrix. This is the reversal step or coupling, meaning the reversal from bone resorption to formation, the signal for which is not definitely known [2]. The last step is the formation of new bone by osteoblasts filling the resorption cavity. Mineralization then follows within a few days. This sequence of activation, resorption, reversal, formation and mineralization occurs normally on 10 percent of the bone surface and has a duration of several months [3]. The remodeling process is regulated by circulating hormones and by local factors (Figure 1).
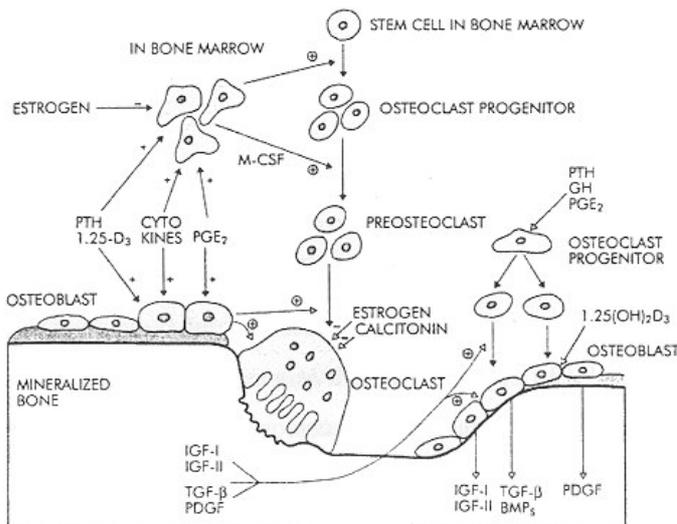

Figure 1. Normal bone remodeling (Ljunghall et al, Doctors Manual, 1995)

Hormones that influence the rate of normal bone remodeling are most notably parathyroid hormone (PTH), vitamin D and calcitonin. Increased PTH levels stimulate bone remodeling by increasing resorption. Vitamin D, as its active metabolite 1,25-dihydroxy vitamin D_3 ($1,25(OH)_2 D_3$) (the 25 hydroxylation step taking place in the liver), is essential for mineralization of new bone and is also a potent bone resorption agent. The full role of vitamin D in normal bone metabolism is not clearly understood [4-5]. Other important hormones in bone metabolism are growth hormone (GH), thyroid hormone and sex steroids. Examples of other systemic and local factors affecting osteoblast and/or osteoclast function are interleukin-1 and -6, transforming growth factor-β (TGF-β), and insulin-like growth factors (IGF).

There are two types of bone tissue in adult life, trabecular and cortical bone. Trabecular bone is concentrated in the spine and at the ends of long bones and constitutes about 25% of the total bone mass. The annual turnover rate in trabecular bone is about 25% and in cortical bone about 2-3% making trabecular bone more vulnerable to factors influencing bone metabolism [3]. Bone mass is determined by the peak bone mass achieved around the age of 20-30 and the subsequent gradual loss of bone by about 0.5-1% per year [6-8]. Bone mass is higher in men than in women throughout adult life, and in women there is an accelerated bone loss the first years after menopause of about 2% per year [9-10]. During the course of their lifetime, women lose about 50% and men 30% of their trabecular bone [11].

Osteoporosis and osteomalacia

Osteoporosis and osteomalacia are two forms of metabolic bone disease. Osteoporosis is defined as a systemic disease of the skeleton, characterized by low bone mass and altered micro-architecture of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture [12]. There is a disintegration of the bone matrix with normal ratio of mineral to matrix [13]. *Primary osteoporosis* includes postmenopausal osteoporosis (type l osteoporosis) and senile osteoporosis (type ll osteoporosis) of elderly women and men. Postmenopausal osteoporosis is characterized by increased bone resorption (high bone turnover) due to estrogen deficiency. There is predominantly a loss of trabecular bone with resulting fractures mainly at the vertebrae.

In senile osteoporosis the loss of trabecular and cortical bone is similar due to the combined effects of increased resorption and decreased bone formation (low bone turnover) and is associated with vertebral or hip fractures [3,14]. Other general risk factors for osteoporosis are low body weight, physical inactivity, smoking, and genetic factors [15]. *Secondary osteoporosis* refers to bone loss caused by a specific defined clinical disorder (Table 1). Secondary osteoporosis can be either high or low bone turnover osteoporosis depending on the cause.

Table 1. Causes of secondary osteoporosis in adults

Endocrine/metabolic	Drugs
Hypogonadism Hyperadrenocorticism Thyrotoxicosis Systemic mastocytosis	Glucocorticosteroids Chronic heparin administration Anticonvulsants
Nutritional	**Other**
Malabsorption/malnutrition Chronic liver disease Vitamin D deficiency Anorexia nervosa Alcoholism Gastric surgery	Osteogenesis imperfecta Ehler-Danlos syndrome Marfan syndrome Myeloma Immobilization/space flight

Osteomalacia is rare in developed countries but is characterized by impaired mineralization of bone matrix, most often due to vitamin D deficiency. Biochemical abnormalities are common in osteomalacia but the diagnosis is best established by use of transiliac bone biopsy in combination with the use of timed tetracycline [16]. The histological examination of the undecalcified bone shows an increase in the amount of unmineralised osteoid and depressed rate of bone formation.

Measurements of bone mineral density

Bone mineral density (BMD;g/cm^2) is measured by non-invasive methods based on radiology [17]. A specified amount of electromagnetic energy, in the form of a gamma- or X-ray beam, is sent through a region of interest and the amount exiting is quantified by a detector. Single photon absorptiometry (SPA), introduced in the 1960s measures BMD reliably only at peripheral sites, having small amounts of surrounding tissue, such as the heel and the wrist. Dual-energy X-ray absorptiometry (DXA) was introduced in the late 80-ies and is now the most widespread technique for evaluating BMD in patients at risk of osteoporosis. With DXA, two distinct energy levels are used to resolve contributions from soft tissue and bone making it possible to measure BMD at central sites such as the spine and the proximal femur. The precision error for DXA is about 1-2% which is important when estimating bone loss in longitudinal studies. If expected bone loss is of the same order, i.e. 1-2% per year, measurements should be performed with not less than 1-2 years interval [18].

Since 1994 the World Health Organization (WHO) has recognized a working definition where osteoporosis in caucasian women is defined as a BMD value of 2.5 SDs below the mean for healthy young women [18] (Table 2). No such generally accepted definition of osteoporosis exists for men at the present.

Table 2. World Health Organization (WHO) working definition.

Bone mineral density	Classification
Above –1 SD[1]	Normal
Between –1 SD and –2.5 SD	Low bone mass or osteopenia
Below –2.5 SD	Osteoporosis
Below –2.5 SD and fractures	Severe (or manifest established) osteoporosis

[1]SD = standard deviation

The comparison with the mean BMD for young adults of the same sex is termed the *T-score* and is expressed as the number of standard deviations from the reference group mean value. Thus, according to the WHO´s definition, a woman with a T-score below

–2.5 has osteoporosis. In clinical practice the use of T-scores has also been adopted for men. A *Z-score* is the number of standard deviations from age-matched and weight-adjusted reference population of the same sex.

B. Metabolic bone disease in chronic liver disease

Chronic liver disease

Chronic liver disease (CLD) can be classified into diseases with primarily *hepato-cellular* damage and *cholestatic* diseases. Examples of hepato-cellular CLD are autoimmune chronic hepatitis (auCAH), chronic viral hepatitis B and C, and alcoholic liver disease. Autoimmune CAH is a disease of unknown etiologi, has a prevalence of about 5-10/100.000, occurs mainly in young women (sex ratio 8:1) and is treated with long-term corticosteroid therapy. Alcoholic liver disease includes steatosis, which is reversible upon abstinence, alcoholic hepatitis and cirrhosis. Alcohol is the most common cause of liver cirrhosis in the European countries. Cholestatic CLD includes primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC). The prevalence of PBC is about 10-15/100.000 and it mainly affects women (sex ratio 9:1) between the ages of 40 and 59. It is characterized by a progressive destruction of intra-hepatic bile ducts leading to cholestasis. The etiology is unknown but profound immunological disturbances are found and PBC is often associated with other autoimmune disorders. There is no specific treatment for PBC. PSC occurs in about 5-10% of those with inflammatory bowel disease, mainly ulcerative colitis. Males are twice as commonly affected as females, usually between the ages of 25 and 45. It is characterized by a chronic, fibrosing, inflammatory process that involves all parts of the biliary tree resulting in its obliteration and ultimately in biliary cirrhosis. The etiology is unknown and there is no specific treatment for PSC.

Liver failure and the Child-Pugh classification

The final stage of chronic inflammation in the liver is cirrhosis. Liver cirrhosis gives rise to portal hypertension and complications such as bleeding esophageal varices, ascites and encephalopathy. Hepato-cellular failure results in hyperbilirubinemia, hypoalbuminemia and prolonged prothrombin time. Child´s grade is used to assess hepato-cellular function in cirrhosis based on these factors. The Child-Pugh classification is a modified grading system shown to be reliable in predicting survival of patients presenting with variceal bleedings but is also widely used as a method of assessing liver function [19] (Table 3).

Table 3. Child-Pugh classification of hepatic functional reserve in cirrhosis

Assessment criteria	Points scored for abnormality			Grade	Scores
	1	2	3		
Encephalopathy grade	None	1-2	3-4	A	5-7
Ascites	None	Slight	Moderate		
Bilirubin (μmol/L)	<35	35-50	>50	B	8-9
Albumin (g/L)	>35	28-35	<28		
Prothrombin index %	>70	40-70	<40	C	10-15

"Hepatic osteodystrophy"

In 1939 a 69 year old woman with long-standing intrahepatic obstructive jaundice and spinal osteoporosis with vertebral compressions was described [20]. Since then it has been firmly established that chronic cholestasis, and also other forms of CLD, are associated with metabolic bone disease [21-29]. In the era of liver transplantation, metabolic bone disease complicating CLD has become a major clinical problem. After liver transplantation the combination of high dose corticosteroids and immobilization accelerates bone loss leading to a high post-transplant fracture rate ranging from 17-65% [30-34]. Decreased BMD pre-transplant, however, is a major risk factor for the development of post-transplant fracture [31].

The term "hepatic osteodystrophy" covers both osteomalacia and osteoporosis [35]. Steathorrhea with malabsorption of fat-soluble vitamins, including vitamin D, accompanies symptomatic cholestatic liver disease. Therefore osteomalacia might be expected to complicate CLD, as in fact was reported in earlier studies [36-37]. Over the last two decades, better histomorphometric techniques (including double-tetracycline labeling for diagnosing osteomalacia) have made it clear that the main bone abnormality in CLD, cholestatic or hepato-cellular, is osteoporosis and that osteomalacia is very rare [38-42]. Most histomorphometric studies have found osteoporosis in CLD to be of a low bone turnover type with reduced osteoblast function, and measurements of biochemical markers of bone metabolism, such as osteocalcin, have confirmed these findings [39-40, 43-46].

The pathogenesis of osteoporosis in CLD is unknown. Advanced liver disease and cirrhosis are associated with an increased prevalence of osteoporosis [25, 31, 41, 46-47]. The way in which liver failure affects osteoblasts and contributes to the development of osteoporosis is unclear. Numerous growth factors, some of which affect osteoblast function, such as IGF-l and TGF-β, are synthesized by the liver. Toxic substances, such as aluminium and copper, which accumulate in liver failure might also affect bone metabolism. In haemochromatosis an increased iron burden might impair osteoblastic activity [48-49]. Bilirubin has been shown to inhibit osteoblast proliferation in vitro [50-51]. Whether cholestasis per se is a risk factor for osteoporosis in CLD is uncertain. Cholestatic CLD has traditionally been considered to be associated with osteoporosis more than other types of CLD and most studies of metabolic bone disease in CLD involve women with PBC [24, 28]. In pre-transplant patients suffering from different liver disorders, the highest prevalence of metabolic bone disease was found in patients with PBC and PSC [31]. Other studies, however, have reported similar prevalence rates for osteoporosis in patients with hepato-cellular CLD as in patients with cholestatic CLD, ranging from 9 to 53% [23-24, 26, 52-55]. Low levels of serum vitamin D_3 metabolites and calcium (Ca) malabsorption are found in CLD [23, 38, 45, 56-58]. Whether vitamin D deficiency is associated with metabolic bone disease in CLD is uncertain. Hyperparathyroidism, despite vitamin D replacement, has been described in PBC [59]. Others have not found evidence of hyperparathyroidism in patients with CLD [60].

Treatment with corticosteroids and hypogonadism in men and women are reported by some as risk factors for osteoporosis in CLD [24, 26, 58, 61]. Others have not found treatment with corticosteroids to be associated with low BMD in CLD [62-63]. Other general factors in patients with CLD such as alcohol consumption, low body weight and physical inactivity have not been reported as independent risk factors for osteoporosis in CLD but can be assumed to be important (Table 4).

Table 4. Potential pathophysiological factors in osteoporosis in chronic liver disease

Lack of growth factors produced by the liver
Accumulation of toxins
Cholestasis
    Hyperbilirubinaemia
    Vitamin D deficiency
    Vitamin K deficiency
    Calcium deficiency
Hypogonadism
Treatment with corticosteroids
Alcohol consumption
Low body weight
Physical inactivity

C. Insulin-like growth factors

Insulin-like growth factors and insulin-like growth factor binding proteins

Somatomedins or IGF-l and IGF-ll, are peptide hormones that play a pivotal role in the regulation of proliferation, differentiation and specific functions of many cell types. IGF-l is the main mediator of the effects of GH on growth and development. IGF-ll has more insulin-like activity than IGF-l, but much less is known about its exact function [64]. The

liver is the major source of the IGFs, which exert negative feedback on the hypothalamus and pituary gland [65-66] (Figure 2).
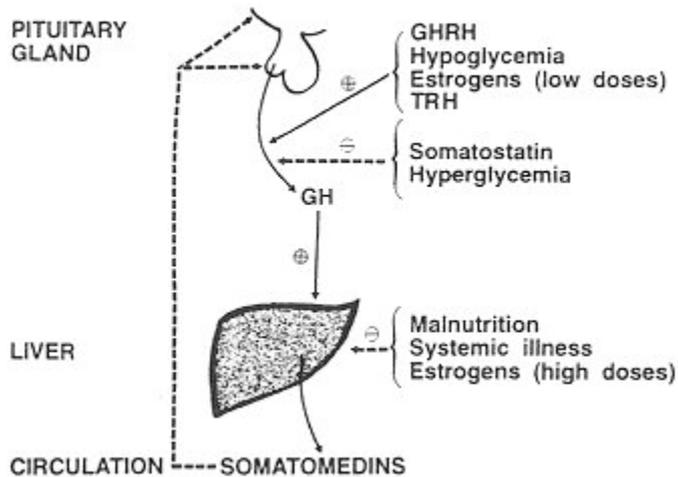

Figure 2. Factors affecting somatomedin (IGF) regulation [66].

The fact that the adult liver virtually lacks receptors for IGF-l, and that the synthesis of IGF-l occurs in many tissues in addition to the liver, has lead to a new concept that the IGFs have both endocrine as well as autocrine/paracrine effects [64, 67-68].

GH and nutrition are the major regulators of the IGFs, IGF-l being more GH dependent then IGF-ll. IGF-l levels in adults decrease with age and IGF-l levels during the seventh decade are only half those in the second and third decades of life. Variations in nutritional status are important in modulating serum IGF-l levels; both acute (days) and long-term fasting cause a reduction, and IGF-1 has therefore been suggested as a useful clinical indicator of nutritional insufficiency [69].

In contrast to other hormone peptides, the IGFs are not stored in endocrine glands but form a circulating reservoir in the plasma. More than 99% of the IGFs in serum are complexed with the binding proteins IGFBP-1 to -6 and at least 95% are bound to IGFBP-3 [70-71]. The functions of the IGFBPs are not completely known but apart from regulating the bioavailability of the IGFs they also seem to modulate the action of the

IGFs at cellular level [70]. The liver is the major source of IGFBP-1 and -3 and probably also of IGFBP-2 [71]. The major regulators of circulating IGFBP-1 are insulin (suppressive effect) and the intracellular glucose availability in the liver [73]. IGFBP-2 has a higher affinity for IGF-ll than for IGF-l but little is known about its regulation and physiological role [74]. IGFBP-2 levels are generally increased in severe illnesses, particularly malignant diseases. Principally, IGFBP-3 serum levels are regulated by the same variables as IGF-l, especially by GH [73].

Insulin-like growth factors in chronic liver disease

In patients with CLD, irrespective of etiology, IGF levels are low and levels of GH high [75-76]. The reduction is most pronounced in those with advanced liver disease and is also related to the degree of portal hypertension and porto-systemic shunting [75, 77-81]. Malnutrition is often seen in cirrhosis and may to some extent contribute to the low IGF concentration in these patients [82] but the liver dysfunction probably is more important [83]. IGF-l has been suggested as an early marker of liver dysfunction in CLD (75-76, 84]. Furthermore, IGF-l has been shown to be an independent marker of survival in patients with liver cirrhosis [85].
IGF-ll and IGFBP-3 levels are reduced in CLD and positively related to the severity of the liver disease [76, 84]. On the contrary, increased levels of IGFBP-1 are found in liver cirrhosis [76, 84, 86]. The reason for this is not clear but may be due to increased hepato-splanchnic generation and/or lower renal extraction [81, 87]. One suggestion is that the role of IGFBP-1 is to limit the bioavailability of IGF-l and hence its potential hypo-glycaemic effects in the state of low substrate availability as seen in liver cirrhosis [76]. As for IGFBP-1, increased levels of IGFBP-2 and a positive correlation with Child-Pugh score has been found in liver cirrhosis [84].

Insulin-like growth factors and bone

There are numerous reports that IGF-l and -ll have prominent anabolic effects on bone *in vitro*, enhancing protein synthesis and osteoblastic proliferation [88-90]. The IGFs have been proposed to participate in the coupling between bone resorption and bone formation.

Synthesis of all IGFBPs has been demonstrated in bone cells and has been shown to modulate IGF-mediated osteoblast proliferation and differentiation [90]. The relative importance of circulating IGF-l versus locally produced IGF-l with respect to the effects on bone formation is unclear.

Clinical studies have reinforced the hypothesis that IGF-l is related to BMD. A positive correlation between serum levels of IGF-l and BMD has been reported in primary and idiopathic osteoporosis [91-94]. Circulating IGF-l has been found to correlate with markers of bone turnover and low levels of IGF-l to correlate with histomorphometric evidence of reduced bone formation [95-96]. Low levels of IGFBP-3 have been reported in osteoporotic men and women [97-98].

In animal studies treatment with IGF-l in osteopenic rats with liver cirrhosis was beneficial with respect to bone density [99]. Systemic administration of IGF-l to humans has been found to increase markers of bone formation and bone resorption but in low doses only of bone formation [100-102]. Positive effects of treatment with IGF-l on bone mass have been described in a case report [103].

D. Leptin

Leptin, a 16 kDa peptide hormone, was initially identified in 1994 as the product of the *ob* gene which is expressed exclusively in adipose tissue [104]. Leptin circulates in rodents and humans in free form and also bound to other proteins yet unknown. The central nervous system (CNS) is the site of action for leptin but the mechanism of leptin transport into the CNS has not been elucidated. The effects of leptin appear to be mediated in part through hypothalamic neuropeptide Y suppression [105]. Four major physiological functions have been identified for leptin: 1) As a satiety factor inhibiting food intake 2) As a stimulator of energy expenditure 3) As a signal to the reproductive system 4) As a factor in haematopoiesis [106]. The major constitutive determinants of serum leptin are body fat mass and androgens. In humans, leptin levels correlate strongly with body fat content and obese humans in general have high leptin levels [107-109].

Leptin expression has been shown to take place in activated hepatic stellate cells grown in cell culture suggesting that locally produced leptin may partly mediate the hepatic response to injury [110-111]. Clinical studies have found serum leptin levels to be elevated in patients with alcoholic liver cirrhosis and it has been suggested that elevated serum leptin levels may be involved in the malnutrition of liver cirrhosis [112-114]. However, decreased levels have been found in chronic viral hepatitis and in post-hepatitis liver cirrhosis [114-115]. In some studies leptin has been found to correlate, negatively or positively, with the degree of liver function, [114-115] but in others no correlation was found [112-113].

Obesity is bone protective and thus leptin has been suggested as a mediator of the increased bone mass in obese individuals. Surprisingly, intra-cerebroventricular infusions of leptin to normal and obese *ob/ob* mice caused low bone density and a central control hypothesis was formed, i.e. that leptin affects bone metabolism via a central neuroendocrine pathway [116-117]. This was further supported by the observations that leptin does not act directly on bone cells in culture. Clinical studies on the relationship between serum leptin and BMD in adult women have not been able to support the hypothesis that circulating leptin influences bone mass [118-120].

AIMS OF THE STUDY

The objective of this thesis was to describe BMD in patients with CLD and furthermore to elucidate possible pathophysiological mechanisms behind osteoporosis in CLD. The more specific aims of the different studies were:

- To study the prevalence of, and risk factors for, osteoporosis in a cohort of patients with CLD (Paper l)

- To prospectively evaluate the rate of bone loss and potential predictors of bone loss in these patients (Paper ll)

- To study the relationship between circulating levels of IGF-l, IGF-ll, IGFBP-1-3, and BMD in patients with CLD (Paper lll)

- To study the relationship between circulating levels of leptin and BMD in patients with CLD (Paper lV)

SUBJECTS

In **Paper l**, 78 patients with CLD hospitalised at the Department of Internal Medicine, University Hospital, Uppsala, were recruited non-consecutively between September 1994 and April 1996. It was not possible to conduct bone densitometry in 6 of the patients (3 refused, 2 were too obese and 1 died before the densitometry was performed) leaving a total of 72 patients for evaluation.

One man and 1 women for each year of birth between 1910 and 1970 were randomly selected from the population register in Uppsala county, (total population of 290.000), to serve as controls with respect to BMD (n=122) [121]. From this group, age- and gender-matching was possible for 67 of the 72 patients.

Information about menopausal status and medication was derived from a questionnaire used for patients and controls as well as from medical records. In those patients for whom information from the questionnaire was lacking or uncertain, the menopausal status was determined by measurements of serum follicle stimulating hormone (FSH) and estradiol.

Seventy six percent of the female patients and 58% of the control females were post-menopausal. Five of 23 male patients (22%) were classified as hypogonadic based on the presence of subnormal serum testosterone levels.

Treatment with corticosteroids was defined as the use of corticosteroids for more than 6 weeks during some period of the patient´s life. The individual cumulative dose of corticosteroids, standardised as milligrams of prednisolone, was expressed as the product of the average maintenance dose per day multiplied by the number of treatment years.

Some individuals in the patient group were on current treatment with vitamin D + Ca supplementation (n=3), Ca supplementation only (n=2), estrogen replacement therapy (ERT) (n=3) or bisphosphonates (n=1), whereas none in the control group received any of the afore-mentioned drugs aside from ERT (n=5).

Demographic and clinical data of the patients and controls are shown in Table 5.

Table 5. Demographic and clinical data of the patients and controls in **Paper l**

PATIENTS		Sex female/male	Age years	Child-Pugh grade A	B + C	Use of cortico- steroids	Body mass index kg/m^2
	(n)	(n)	(mean)	(n)		(n)	(mean $\pm$ SD)
Cholestatic CLD	37	27/10	54.5	30	7	11	24.5 $\pm$ 4.5
PBC	22	21/1	59.5	17	5	3	
PSC	15	6/9	47.1	13	2	8	
Non-cholestatic CLD	35	19/16	53.7	23	12	12	24.1 $\pm$ 4.6
auCAH	10	5/5	57.0	6	4	8	
Alcoholic CLD	10	5/5	57.7	4	6	0	
Viral hepatitis	8	5/3	43.4	7	1	2	
Other	7	4/3	55.1	6	1	2	
All	72	46/26	54.1[1]	53	19	23	24.4 $\pm$ 4.5
CONTROLS	67	41/26	54.2[2]			1	25.7 $\pm$ 3.8

Abbreviations: CLD: Chronic liver disease, PBC: Primary biliary cirrhosis, PSC: Primary sclerosing cholangitis, auCAH: autoimmune chronic hepatitis [1] Range: 21-84 years. [2] Range: 23-83 years

In **Paper ll** the patient cohort was followed-up with an invitation by mail to a second measurement of BMD 2 years after the initial measurement.

The patients in **Papers lll and lV** were 58 of the patients in Paper l, 39 females and 19 males. Matching with regard to age and gender was possible for 54 of the patients with the same controls as in Paper l. Twenty-one patient had primary biliary cirrhosis, 13 had primary sclerosing cholangitis, 10 had autoimmune hepatitis, 8 had alcoholic liver disease and 1 had haemochromatosis. Five patients had other types of non-specified cholangitis or hepatitis. Forty-five patients were classified as Child-Pugh grade A, 9 as grade B and 5 as grade C. Mean age of the patients was 55.9 years (range 23-79) and of the controls 55.6 years (range 23-79). Mean BMI was 24.6 $\pm$ 4.6 and 26.0 $\pm$ 3.9 kg/m^2 in the patients and the controls, respectively.

METHODS

Definition of chronic liver disease

CLD was defined as liver disease with a duration of at least 6 months and with a diagnosis based on biochemical, serological and histopathological investigations. Patients with terminal liver disease and patients with only liver steatosis were excluded from the study.

Body mass index

Body mass index (BMI; kg/m^2) was determined by dividing body weight in kilograms by the square of body height in meters. Reference ranges for BMI were set to 19-24 kg/m^2 for women and 20-25 kg/m^2 for men [122].

Measurements of bone mineral density

Bone mineral density (BMD; g/cm^2) of the lumbar spine and the left proximal femur (if unaffected by disease, otherwise the right proximal femur) was measured by dual energy X-ray absorptiometry (DXA) with the use of a Lunar DPX-L™, Lunar Co, Madison, WI, USA, at baseline (BMD1) and at follow-up (BMD2). The mean BMD value of the second, third and fourth lumbar vertebrae (lumbar spine BMD) and of the femoral neck of the proximal femur (femoral neck BMD) were used in the present analysis.

The long-term precision error (less than 1 %) for the DXA-equipment was evaluated by the use of a spine phantom and expressed as the coefficient of variation.

BMD was expressed in absolute values or as Z-score. The WHO´s definition for osteoporosis was applied to classify individuals as either osteoporotic or not.

Bone loss was expressed as annual change in BMD calculated as follows: Δ BMD = ((BMD2-BMD1)/BMD1) x 100%/interval between measurements in years.

X-ray of the thoracic and lumbar spine

X-ray examinations of the thoracic and lumbar spine for the diagnosis of vertebral compression were performed prospectively, in association with the BMD measurements in 52 of the 72 patients in **Paper l**.

Biochemical tests

All blood samples were drawn after an overnight fast. Analyses of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, bilirubin, prothrombin time, albumin, Ca (corrected for serum albumin), ionised Ca, intact parathyroid hormone (PTH), total testosterone, FSH, sex-hormone binding globulin (SHBG) and estradiol were carried out as part of the clinical routine at the Department of Clinical Chemistry, University Hospital, Uppsala.

Serum levels of 25-hydroxy vitamin D_3 (25(OH) D_3) and 1,25-dihydroxy vitamin D_3 (1,25(OH)$_2$ D_3) were measured at MedScand Diagnostics AB, Malmö, Sweden, with a method based on a high performance liquid chromatography for 25(OH) D_3 (intra-and interassay variation $\leq$ 10%) and by a competitive radioreceptor assay (Incstar Corp, Stillwater, Minnesota, USA) for 1,25(OH) $_2$ D_3 (intra-and interassay variation$\leq$ 10%).

IGF-l levels were measured by an IGFBP-blocked radioimmunoassay (RIA) in the presence of an excess of IGF-ll as described previously [73]. Intra- and interassay coefficients of variation were 3.1 and 7.3%, respectively. IGF-ll was measured in the presence of excess IGF-l using a commercial RIA kit (Mediagnost, Tübingen, Germany). IGFBP-1 and IGFBP-2 were determined by RIA as described previously [74, 123]. Intra- and interassay coefficients of variation were 3.4 and 8.1% for IGFBP-1 and 3.7 and 9.6% for IGFBP-2. IGFBP-3 levels were measured by RIA using a commercially available kit (Diagnostic system Laboratories Inc., Webster, Texas, USA), with intra- and interassay coefficients of variation 4.9 and 7.2%, respectively.

Leptin levels were measured by a sensitive RIA, detecting primarily total leptin levels as described previously [108]. Adjustment of serum leptin for BMI and gender was performed by calculation of standard deviation (SD) scores according to the equation previously described [124].

Statistics

The StatView version 4.5 (Abacus Concepts, Berkeley, CA, USA) and the SAS software (SAS Institute Inc., North Carolina, USA) were used for statistical calculations.

The comparative analyses between patients and controls were performed with the use of Student's paired *t* - test and Chi-square test with a Fishers exact test to adjust for small numbers. The Mann-Whitney non-parametric test was used for comparison of unpaired groups. Unmatched patients were excluded from all comparative analyses.

Simple regression analysis and Spearman correlation statistics were used for analysis of correlations. For dichotomous outcomes, unconditional logistic regression analysis was used. A logistic, stepwise multivariate analysis was used for the evaluation of risk factors for osteoporosis and bone loss (**Papers l and ll**).

Multiple regression analysis was used to examine interactions between age, IGF-l levels and BMD (**Paper lll**).

All results are given as mean values $\pm$ SD if not otherwise stated. A *p* value < 0.05 was considered statistically significant in all analyses.

ETHICS

The study was approved by the Ethics Committee of the Medical faculty at Uppsala University in Uppsala. Patients and controls gave their informed consent to participation in the study.

RESULTS

Prevalence and risk factors for osteoporosis in CLD **(Paper l)**

Table 6 shows the prevalence of osteoporosis in the patient group compared with the control group. In a sub-group analysis, the difference in prevalence was significant for females and patients classified as Child-Pugh B+C. On X-ray examination 8 of 52 (15%) patients had vertebral fractures at the thoracic or lumbar spine.

Table 6 Prevalence of osteoporosis in patients and controls[1]

	Patients	**Controls**	***p* value**
All	20/67 30%	10/67 15%	NS[2]
Females	12/41 29%	6/41 15%	<0.01
Males	8/26 31%	4/26 15%	NS
Steroid treatment	9/21 43%	4/21 19%	NS
No steroid treatment	11/46 24%	6/46 13%	NS
Cholestatic CLD	8/35 23%	5/35 14%	NS
Non-cholestatic CLD	12/32 37%	5/32 16%	NS
Child´s group A	12/48 25%	9/48 19%	NS
Child´s groups B+C	8/19 42%	1/19 5%	< 0.05

[1] localisation either femoral neck or lumbar spine. [2]NS = not significant.

A logistic bivariate analysis was used for the evaluation of risk factors for osteoporosis. Variables that achieved a level of significance in the bivariate analysis as well as those variables considered to be of potential major importance in view of the disease conditions were entered into a multivariate analysis.

Table 7 shows the odds ratios for osteoporosis from a bivariate analysis with demographic, clinical and biochemical parameters in the patient cohort (n=72).

Following variables were shown to be independent risk factors for osteoporosis in the patient cohort: BMI, steroid treatment, prothrombin time, age and sex. In order to simplify the model for clinical use, prothrombin time was excluded from the model, which did not change the predictiveness of the parameters left in the model (Table 8). In

a final regression model for the prediction of osteoporosis the sensitivity was 62% and the specificity 92%.

Table 7 Bivariate relationships on the risk of osteoporosis[1]

	Odds ratio	**95% Confidence Interval**	***p* value**
Body mass index	2.99	1.34-6.67	<0.01
Age	2.21	1.16-4.19	<0.05
Age, males	3.65	1.24-10.72	<0.05
Age, females	4.30	1.47-12.59	<0.01
Prothrombin time	1.88	1.11-3.19	<0.05
Bilirubin	1.06	0.64-1.76	NS[2]
Albumin	1.57	0.94-2.62	NS
Duration of CLD	1.73	0.99-3.00	NS
Testosterone	1.12	0.65-1.93	NS
Cumulative steroid dose	1.46	0.90-2.39	NS
Sex	0.89	0.31-2.54	NS
Steroid treatment	2.66	0.92-7.69	NS
Vitamin D (low/normal)	2.63	0.88-7.90	NS
Child group	2.24	0.74-6.76	NS
Cholestatic CLD	0.47	0.17-1.32	NS

[1] For continuous variables the odds ratio indicates the risk of osteoporosis when the magnitude of the variable changes by one standard deviation. For dichotomous variables the odds ratio indicates the risk when the variable changes from 0 to 1. [2]NS=not significant

Table 8 Independent risk factors for osteoporosis

	Odds ratio	**95% Confidence Interval**	**P-value**
Body mass index (BMI)	14.1	66.6-2.9	<0.01
Steroid treatment	18.9	2.8-125.0	<0.01
Age	3.6	1.2-10.7	<0.05
Sex[2]	0.000	0.0-0.4	<0.05

[1]stepwise logistic regression, prothrombin time omitted from the model [2]0=female, 1=male

*Vitamin D, calcium and PTH in patients with CLD (**Paper l**)*

Serum 25(OH) D_3 levels were subnormal in 8 of 64 patients (12%) (mean=36.8 ± 18.7 ng/L; ref. 15-65 ng/L) and 1,25(OH)$_2$ D_3 levels were subnormal or undetectable in 19 of 66 patients (29%) (ref. 20-60 ng/L). Of those with subnormal 25(OH) D_3 levels all except two, also had subnormal 1,25(OH)$_2$ D_3 levels. A positive correlation was demonstrated between the serum levels of albumin and 25(OH) D_3 (r^2=0.16; *p*=0.0009). Serum Ca levels, adjusted for serum albumin and ionised Ca levels, were within the normal range in all patients except 8 who had borderline increased levels (between 2.60-2.70 mmol/L; ref. 2.20-2.60 mmol/L). None of these had increased PTH. Serum PTH was outside the reference range in 6 patients (ref. 12-55 ng/L). Two had decreased levels, 3 had borderline increased levels and 1 highly increased levels of PTH. This patient had borderline reduced ionised serum Ca and vitamin D_3 levels despite supplementation with vitamin D and Ca.

*BMD and bone loss in patients with CLD (**Papers l and ll**)*

The mean BMD expressed as Z-score was significantly reduced compared with the controls at the lumbar spine but not at the femoral neck. A sub-group analysis found the reduction to be most prominent in patients who had received treatment with corticosteroids and in patients classified as Child-Pugh B+C. Mean lumbar spine and femoral neck BMD were reduced to the same extent both in patients with cholestatic CLD and with non-cholestatic liver disease.

The mean BMD at the lumbar spine and the femoral neck was reduced compared with the controls in the two Child-Pugh classification groups (Child A and Child B+C). The difference was statistically significant for the Child-Pugh A group of patients at the lumbar spine and for the Child-Pugh B+C group at the femoral neck, (Table 9).

Table 9 Bone mineral density (BMD) expressed as Z-score (SD) of patients and controls[1]

	Lumbar BMD			Femoral neck BMD		
	Patients	**Controls**	**_p_ value**	**Patients**	**Controls**	**_p_ value**
All	-0.35 ± 1.36	0.26 ± 1.19	< 0.01	-0.18 ± 1.48	0.17 ± 1.08	NS
Females	-0.38 ± 1.32	0.41 ± 1.17	< 0.01	-0.27 ± 1.39	0.35 ± 0.97	< 0.05
Males	-0.30 ± 1.45	0.02 ± 1.19	NS	-0.03 ± 1.63	-0.11 ± 1.20	NS
Steroid treatment	-0.92 ± 1.23	0.53 ± 1.31	< 0.01	-0.83 ± 1.39	0.17 ± 1.17	< 0.05
No steroid treatment	-0,10 ± 1.35	0.14 ± 1.12	NS	0.12 ± 1.14	0.17 ± 1.05	NS
Cholestatic CLD	-0.35 ± 1.30	0.13 ± 1.20	NS	-0.04 ± 1.43	0.15 ± 1.07	NS
Non-cholestatic CLD	-0.34 ± 1.45	0.40 ± 1.17	< 0.05	-0.32 ± 1.54	0.19 ± 1.11	NS
Child-Pugh A	-0.25 ± 1.44	0.38 ± 1.21	< 0.05	0.05 ± 1.46	0.18 ± 1.14	NS
Child-Pugh B+C	-0.59 ± 1.14	-0.06 ± 1.07	NS	-0.81 ± 1.39	0.15 ± 0.95	< 0.05

[1]Student's paired _t_- test. NS = not significant. Data presented as means ± SD

Forty-three of 72 patients, 31 females and 12 males, were available for a second measurement of BMD with a median of 25 months (range 18-41 months) between the measurements. Thirty-eight patients were classified as Child-Pugh grade A at the follow-up. Eighteen patients (25%) died (n=13) or were transplanted (n=5) during follow-up, 11 patients (15%) did not turn up for the follow-up or could not be measured for geographical or other reasons.

Table 10 shows the BMD Z-score in the patients at baseline and at follow-up. The mean Z-score was significantly reduced at follow-up compared with baseline at the femoral neck, (p=0.005 and p=0.02 for females and males, respectively), but not at the lumbar spine. The mean Z-score was not significantly less than expected either at baseline or at the follow-up.

In patients who died or were transplanted during follow-up, however, the mean Z-score at baseline was significantly less than expected at the lumbar spine but in male patients only (Z-score –0.93 ± 1.1; p=0.01).

Table 10 Bone mineral density expressed as Z-score (SD) at the lumbar spine (LS) and at the femoral neck (FN) at baseline and at follow-up (n=38)[1].

		Baseline	Follow-up
Z-score LS	All	0.01 ± 1.4	0.00 ± 1.4
	females	-0.19 ± 1.3	-0.22 ± 1.4
	males	0.67 ± 1.4	0.53 ± 1.2
Z-score FN	All	0.28 ± 1.1	-0.07 ± 1.0[2]
	females	0.22 ± 1.1	-0.13 ± 1.0[2]
	males	0.41 ± 1.1	0.07 ± 1.3[2]

[1]Patients treated with oestrogen replacement therapy and/or bisphosphonates are excluded from the table. [2] $p<0.01$ when compared with baseline Z-score. Data presented as means ± SD.

Mean annual bone loss at the lumbar spine and the femoral neck, respectively, was 0.6 ± 2.0% and 1.5 ± 2.4% in females and 0.8 ± 1.9% and 2.9 ± 2.0% in males. Mean annual bone loss at the lumbar spine and the femoral neck, in patients classified as Child-Pugh A at follow-up was 0.4 ± 1.9% and 1.6 ± 2.3%, respectively. Annual bone loss in Child-Pugh B+C was 2.4 ± 1.6% and 4.3 ± 2.2% at the lumbar spine and the femoral neck, respectively. The difference in bone loss rate between the two Child-Pugh groups was significant at the lumbar spine ($p=0.04$) but not at the femoral neck ($p=0.06$), (Figure 3).
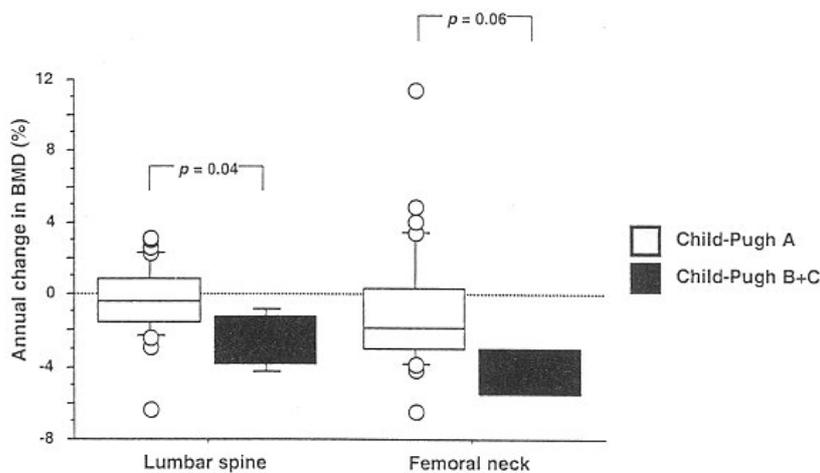

Figure 3. Mean annual change in bone mineral density (BMD)

*Predictors of bone loss (**Paper ll)***

Out of 18 baseline variables, (age, gender, BMI, bilirubin, alkaline phosphatase, AST, ALT, albumin, prothrombin time, testosterone, estradiol, SHBG, 25(OH) D_3, 1,25(OH)$_2$ D_3, menopausal status, treatment with corticosteroids, femoral neck BMD and lumbar spine BMD), prothrombin time, testosterone, 25(OH) D_3 and bilirubin correlated with the rate of bone loss at the femoral neck in the correlation analysis and were entered into the stepwise multiple regression analysis. Serum bilirubin correlated independently and positively (r^2=0.27;p=0.003) and 25(OH) D_3 negatively (r^2=0.11;p=0.03) with the rate of bone loss at the femoral neck. No variable correlated with the rate of bone loss at the lumbar spine.

*IGF-l, IGF-ll and IGFBP-1-3 in CLD (**Paper lll)***

Serum levels of IGF-l, IGF-ll and IGFBP-3 were significantly lower and IGFBP-1 and IGFBP-2 levels higher in the patient group than in the control group (Table 11). There was no significant correlation between the growth factors and BMI in either patients or controls.

Table 11. Serum levels of insulin-like growth factors (IGF;ng/mL) and the IGF-binding proteins (IGFBP;ng/mL) in patients and controls.

	Patients (n=54)	Controls (n=54)	p value
IGF-l	54 ± 31	153 ± 55	< 0.0001
IGF-ll	388 ± 180	827 ± 137	< 0.0001
IGFBP-1	51 ± 23	36 ± 14	< 0.0001
IGFBP-2	664 ± 318	287 ± 188	< 0.0001
IGFBP-3	1945 ± 999	3003 ± 577	< 0.0001

Data presented as means ± SD.

IGF-l, IGF-ll, IGFBP-1 and IGFBP-3 all correlated with parameters of liver function while IGFBP-2 did not correlate with any of the liver function parameters.

Patients classified as Child-Pugh B + C had significantly lower IGF-l (*p*<0.0001), IGF-ll (*p*<0.0001) and IGFBP-3 levels (*p*<0.001) and higher IGFBP-1 levels (*p*<0.05) than patients classified as Child-Pugh A (Figure 4).
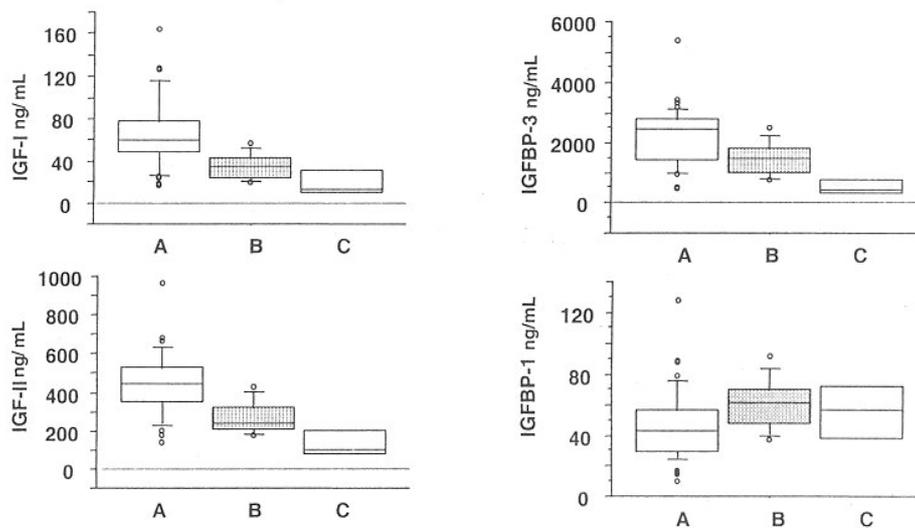

Figure 4. Serum levels of insulin-like growth factors (IGF-l and-ll) and insulin-like growth factor binding proteins (IGFBP-1 and-3) versus Child-Pugh grade.

*Relationship between the growth factors and BMD in CLD (**Paper lll**)*

No significant, independent correlation was found between age-adjusted IGF-l and BMD in either patients or controls.

There were no correlations between IGF-ll or any of the binding proteins and BMD in either patients or controls.

Patients classified as Child-Pugh A and B+C respectively did not differ with respect to the relationship between the growth factors and BMD.

Excluding individuals from the analyses due to bone protective medication (6 patients and 4 controls), and/or corticosteroid treatment, did not change the results.

*Serum leptin in CLD (**Paper lV**)*

Serum leptin levels correlated with BMI in the patients and in controls (Figure 5). Serum leptin and serum leptin adjusted for BMI did not differ significantly between patients and controls (Table 12).
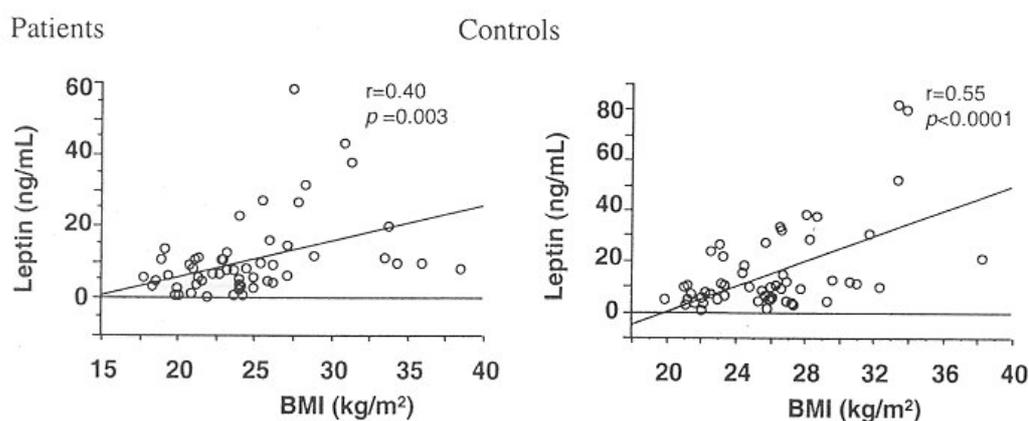

Figure 5. Relationship between serum leptin and body mass index (BMI)

Table 12. Serum leptin and serum leptin adjusted for body mass index (BMI)[1] in the patients and controls [2]

	Patients	Controls	p value
Serum leptin (ng/mL)			
All	10.4 ± 11.3	15.2 ± 17.9	0.1
Females	13.2 ± 12.8	20.7 ± 19.9	0.09
Males	5.1 ± 4.0	4.4 ± 2.4	0.6
Leptin adjusted for BMI (SD)			
Females	-0.41 ± 1.67	0.16 ± 1.15	0.1
Males	-0.06 ± 1.93	-0.04 ± 1.11	1.0

[1] Reference 124

Patients classified as Child-Pugh A did not differ with regard to serum leptin from patients classified as Child-Pugh B+C (11.9 ±13.3 *vs* 9.2 ± 6.7 ng/mL; NS). Leptin

adjusted for BMI in male patients classified as Child-Pugh A, however, was significantly lower when compared with males classified as Child-Pugh B+C (-0.34 ±1.84 *vs* 1.51 ± 0.98 SD; *p*=0.04). In female patients serum leptin levels adjusted for BMI were -0.44 ±1.51 *vs* -0.02 ±1.94 SD (NS) in patients classified as Child-Pugh A and Child-Pugh B+C, respectively. In the male patients only, serum leptin adjusted for BMI correlated significantly with AST (r=0.53;*p*=0.03) but not with other parameters of liver function.

*Relationship between serum leptin and BMD in CLD (**Paper IV**)*

Serum leptin did not correlate with BMD in either the patients or the controls. Serum leptin adjusted for BMI, however, correlated negatively with BMD in female patients. In male patients the same negative trend was observed, but was only significant at the femoral neck (Figure 6).
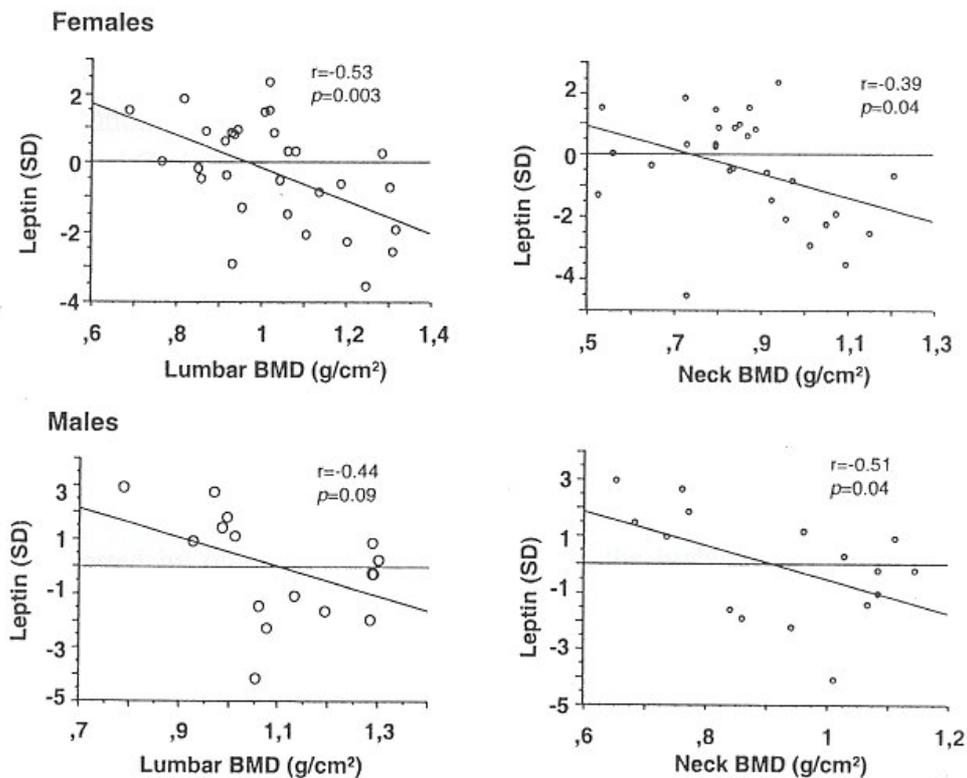

Figure 6. Relationship between serum leptin adjusted for body mass index and bone mineral density at the lumbar spine (lumbar BMD) and the femoral neck (neck BMD) in the patients.

In a sub-group analysis the relationship was only significant in patients classified as Child B+C (r=-0.8;*p*=0.03 and r=-0.96;*p*=0.01 at the lumbar spine and r=-0.96;*p*=0.002 and r=-0.85;*p*=0.07 at the femoral neck in females and males, respectively). In the control group there was no correlation between serum leptin adjusted for BMI and BMD in either females or males.

*Summary of the results (**Papers l-lV**)*

- The prevalence of osteoporosis in our cohort of patients with CLD was twice that of a normal population and the reduction in BMD was most pronounced at the lumbar spine. In addition to age and female gender, low BMI, corticosteroid treatment, and advanced liver disease, were independent risk factors for osteoporosis in these patients.

- The patients had an increased rate of bone loss at the femoral neck, but not at the lumbar spine, that was predicted by hyperbilirubinaemia and low vitamin D levels.

- The patients had low levels of IGF-l, IGF-ll and IGFBP-3 that correlated with liver function. No relationship was found between low levels of growth factors and BMD.

- An inverse relationship between serum leptin and BMD was found.

DISCUSSION

Osteoporosis is a common condition and the highest incidence rates for osteoporotic fractures are found in northern Europe. The prevalence of osteoporosis is highly dependent upon age and gender. Thus, in order to evaluate whether metabolic bone disease is more common in CLD than in the population it is necessary to compare the patient cohort with an age- and gender- matched population. By doing this, we found lower overall BMD in a cohort of patients with CLD when compared with the controls. This reduction was statistically significant at the lumbar spine, which is in accordance with previous reports on metabolic bone disease in CLD affecting mainly trabecular bone [43, 46, 54]. The prevalence of osteoporosis in the cohort was 30%, or twice that in the control group. This difference, however, was not significant possibly due to type ll error. The size of our patient cohort was limited by several factors. The prevalence of CLD is low in Scandinavia and our patient cohort was not a randomised sample from the population. These limitations, however, can also be found in previously published studies. The reported prevalence figures for osteoporosis in patients with CLD range from 9 to 53% [23, 25-26, 52-54]. These differences in reported prevalence can probably be explained by different patient selection factors, different techniques of bone mass measurement and definition of osteoporosis.

We found a high prevalence rate, 42%, in patients classified as Child-Pugh B and C compared with 25% in the Child-Pugh A group and increased prothrombin time (as a marker of liver function) was an independent risk factor for osteoporosis. This is supported by other studies that have found the highest prevalence of metabolic bone disease in patients with advanced liver disease (25, 31, 41, 46, 54) whereas in earlier stages of liver disease no evidence of bone disease has been found [41, 125].

Apart from belonging to Child-Pugh B+C, patients with steroid treatment had the most pronounced reduction in BMD and 43% of these patients had osteoporosis. Furthermore, in the multiple regression analysis, we found treatment with corticosteroids to be an independent risk factor for osteoporosis. It is well known that treatment with corticosteroids give rise to secondary osteoporosis predominantly at trabecular sites

positively correlated with the duration of treatment and the cumulative dose [44, 126-127]. Treatment with corticosteroids in patients with CLD was in some reports associated with decreased BMD [22, 26, 61, 128-129] in others not [25, 53, 62-63]. In one of the studies, with no relationship between bone disease and treatment with corticosteroids, the prevalence of metabolic bone disease, however, was very low, 9%, which may have influenced the results [53].

Low BMI is a known risk factor for osteoporosis and increased rate of bone loss in the normal population [130-135]. Our findings of decreased BMI as an independent risk factor for osteoporosis in CLD has not been reported previously. In one previous study, however, BMD correlated with urinary clearance of creatinine as an indirect measure of muscle mass [23]. Malnutrition is common in CLD but the reported figures range from 15 to 100% in pre-transplant patients [136-139]. Reasons for low BMI in patients with CLD are probably multi-factorial including malnutrition resulting from malabsorption and anorexia, decreased physical activity and increased resting metabolic rate associated with progression of the liver disease. Only 8 patients in our cohort were underweight (data not shown) but by using only BMI as a measure of nutrition, we probably underestimated the frequency of malnutrition. BMI is frequently used when evaluating nutritional status in patients with CLD, but somewhat imprecise as it is influenced e.g. by fluid retention in these patients.

It is unknown whether patients with CLD have an increased rate of bone loss and which potential risk factors for increased rate of bone loss may be present. Since very little longitudinal data exists we went ahead with a follow-up study of our patient cohort. Of 72 patients, 43 were available for a second measurement of BMD, mainly patients with well preserved liver function. In these patients we found that the rate of bone loss was increased at the femoral neck with a significant reduction in Z-score from baseline values compared with the Z-score at follow-up. We did not include a follow-up of the controls, but comparing our results with the published rate of annual bone loss in the normal Swedish, and non-Swedish, population the rate of 2.9% at the femoral neck in the male patients was 2-10 fold higher than expected [6-7, 140]. The rate of bone loss of 0.8% at the lumbar spine was somewhat higher than expected. In the female patients the rate of bone loss of 0.6% at the lumbar spine was not increased but a rate of 1.5% at the femoral

neck was somewhat higher than would be expected [6-8, 131, 141]. Table 13 represents data from 4 different studies on the rate of bone loss in the normal population.

Table 13 Annual relative changes of BMD (%) in females and males

	< 50 years		> 50 years	
	Females	Males	Females	Males
Lumbar spine BMD				
Karlsson et al (n=273) [1]	0.0 ± 0.1	-0.2 ± 0.1	-0.1 ± 0.2	+0.4 ± 0.4
Hannan et al (n=800) [2]			-1.12	-0.87
Femoral neck BMD				
Karlsson et al (n=273) [1]	-0.5 ± 0.2	-1.2 ± 0.2	-0.9 ± 0.2	-1.5 ± 0.3
Hannan et al (n=800) [2]			-0.09	-0.38
Ensrud et al (n=8255) [3]			-0.35 (-0.26_-0.43)	
Jones et al (n=769) [3]			-0.96 (-0.64_-1.28)	-0.82 (-0.52_-1.12)

[1] Data presented as mean ± SEM

[2] Data presented as mean

[3] Data presented as mean (95% confidence interval)

The bone loss in our patients was most prominent at the femoral neck and not at the lumbar spine as would be expected since osteoporosis in CLD has mainly been observed in trabecular bone. However, in the small number of patients classified as Child-Pugh B+C at follow-up an increased rate of bone loss was observed both at the lumbar spine and the femoral neck. Few studies in CLD have included measurements of BMD at the femoral neck. BMD was decreased to a similar degree at the femoral neck and the lumbar spine in patients with end-stage liver disease while in male patients with viral hepatitis the reduction was most prominent at the lumbar spine [42, 54]. In a study on pre-transplant patients, mainly males with alcoholic or post-hepatitis cirrhosis, femoral neck BMD was related to the Child-Pugh score and the lowest values of femoral neck BMD were found in patients with alcoholic CLD [46]. It was suggested that advanced liver disease in combination with alcohol consumption might exert simultaneous damaging effects on cortical bone. Bagur et al studied females with PBC and found the legs to be most severely affected when the total skeleton was measured [142]. Treatment

studies with corticosteroids in females with PBC have reported an increased rate of bone loss at both the femur and the lumbar spine [128-129]. Histomorphometric studies on patients with auCAH treated with corticosteroids found that not only trabecular but also cortical bone was affected [44]. No longitudinal study on the rate of bone loss at the femoral neck in male patients with CLD has been published previously but in post-transplant patients the rate of bone loss at the femoral neck in males was three-fold the rate in female patients [55]. Thus, there is some evidence that in CLD even cortical bone may be affected and most prominent in male patients and in advanced liver disease. It is possible that changes at the lumbar spine dominate early in the disease process because of the greater surface area available, but as trabeculae get thinner, the trabecular surface area decreases with slowing of the rates of bone loss at the spine. Compression fractures, the clinical end-point of osteoporosis, can be misinterpreted as an improvement in bone density when evaluated with bone densitometry only. Since we did not include an X-ray of the lumbar spine (in order to detect new compression fractures) at the follow-up it is possible that some values of lumbar spine BMD were falsely high resulting in a false low rate of bone loss. Other potential biases that could result in false high BMD values at the lumbar spine are aortic calcifications and osteoarthritis but in the relative young osteoporosis population as our cohort this is unlikely to be a problem.

Whereas vertebral fractures are a frequent complication during the first year after liver transplantation, hip fractures are rare. However, there are no data available regarding the long-term effects of liver transplantation on hip fracture rates. As the number of liver recipients increases and with increased survival, the incidence of hip fractures can be expected to emerge if bone loss is accelerated at this site.

In accordance with our results, others have found similar rates of bone loss at the lumbar spine in male patients with CLD [53, 143]. In females with PBC, however, Eastell et al reported a rate of 2% per year or twice that of normal controls [33]. Others, however, could not confirm his findings of an increased rate of bone loss at the lumbar spine [53, 144-145]. It is possible that the overall better liver function in our study as well as those others can explain the discrepancy. This is supported by data from an unpublished study on post-menopausal females with PBC, all Child-Pugh grade A, where we have not found an increased rate of bone loss at either the lumbar spine or the femoral neck.

Hyperbilirubinemia and decreased levels of the 25-hydroxy vitamin D_3 metabolite predicted increased bone loss in our study but were not related to baseline BMD or found to be risk factors for osteoporosis. In previous studies the only factors associated with increased bone loss in patients with CLD have been advanced liver disease and post-menopausal status [33, 53, 128]. However, in cross-sectional studies an inverse relationship between BMD and bilirubin has been found by some [33, 145] but not by others [142]. *In vitro* studies have found unconjugated bilirubin to inhibit osteoblast proliferation in a dose-dependent manner [50-51]. However, when sera from patients was added to the cultures there was no difference between sera from cholestatic and non-cholestatic CLD with regard to inhibition of osteoblast proliferation and no correlation was found between serum levels of bilirubin and proliferation of osteoblasts [50]. Thus, bilirubin may have a pathophysiological role for the metabolic bone disease in CLD but other plasma components that regulate osteoblast activity, such as growth factors or retention of other toxic substances, may also be involved.

Vitamin D deficiency is common in the elderly normal population and associated with increased risk for fractures [146]. Previous reports on the relationship between vitamin D_3 and BMD in CLD have been conflicting. Subnormal levels of vitamin D_3 metabolites in patients with CLD have been reported in several studies and found to be related to BMD by few [40, 52], but in most studies no correlation with BMD has been found [41-42, 46-47, 54, 57, 60]. Treatment with vitamin D has been shown to be effective in improving BMD in alcoholic CLD and viral hepatitis [143, 147], but not in PBC [60, 148]. It has been suggested that low vitamin D_3 levels in CLD merely reflect the lack of vitamin D binding protein associated with liver dysfunction [149]. We found a positive correlation between serum albumin (as a marker of carrier protein status) and vitamin D_3 metabolites supporting this. However, inexplicably low levels of free 1,25-dihydroxy vitamin D_3 have been found in patients with CLD suggesting reasons other than just low vitamin D binding protein as the cause of low levels of vitamin D_3 metabolites [149-150]. Impaired 25-hydroxylation has been reported in severe liver disease, but usually serum levels can be normalised with adequate vitamin D substrate [38, 151-152]. Thus, it is likely that a lack of substrate due to poor diet, lack of exposure to sunlight and

malabsorption of vitamin D due to cholestasis or drugs interfering with uptake also contribute to the low levels.

Hyperbilirubinaemia and vitamin D deficiency are expected in cholestatic CLD rather than hepato-cellular CLD. Whether cholestatic CLD is associated with osteoporosis more than hepato-cellular CLD has been debated. Since the first description of "spondylomalacie dans la cirrhose biliaire" [20] most studies on BMD in CLD represent women with PBC. With few exceptions, however, metabolic bone disease has affected patients with cholestatic and hepato-cellular CLD equally (23, 25, 32, 55). This is supported by our findings, i.e. that cholestatic CLD was not a risk factor for osteoporosis and that patients with cholestatic CLD did not have an increased rate of bone loss when compared with non-cholestatic CLD (data not shown). It is likely that osteoporosis in CLD is related to the jaundice itself and is not a specific hallmark of cholestatic CLD. Osteomalacia as a result of vitamin D deficiency is rare in CLD. Since we did not perform any bone biopsies the possibility that some of our patients had osteomalacia can not be excluded.

In accordance with many others we found no signs of hyperparathyroidism in our cohort of patients [23, 41, 43].

As the IGFs are known to have prominent anabolic effects on bone we raised the question whether low levels of IGFs in CLD could be associated with decreased BMD in these patients. No relationship was found between IGF-l or any of the other growth factors and BMD in either the patient group or the control group. Previous reports on the relationship between circulating IGF-l and BMD have been conflicting. Several studies have reported a positive association between serum levels of IGF-l and/or IGFBP-3 and BMD in primary osteoporosis and in idiopathic osteoporosis in men [91-94, 98]. Only one study has examined the relationship between IGF-l and BMD in patients with CLD. In men with viral hepatitis a positive association between serum levels of IGF-l and BMD at the lumbar spine was found [54]. However, in several of the studies only weak associations have been found that have been attenuated when adjusting for confounding factors such as age and in some of these studies no adjustment for age was made, which is essential since both BMD and IGF-l are strongly influenced by age [73]. In fact, we found a positive association between IGF-l and BMD at both the lumbar spine and the

femoral neck in the control group but when adjusted for age no significant independent correlation between IGF-l and BMD was found. Others have failed to demonstrate a positive association between IGF-l and/or IGFBP-3 and BMD or have found a positive relationship in males or females only when analysed separately [93, 96, 153-154]. Studies on the relationship between IGF-l or IGFBP-3 and biochemical markers of bone turnover or histomorphometric parameters in bone biopsies have been conflicting [95-96, 155]. Thus, evidence for the importance of circulating IGF-l or IGFBP-3 with respect to bone metabolism is weak, and locally produced growth factors may be of more importance. The relative importance of circulating IGF-l towards locally produced IGF-l in bone has been a matter of debate. In a recent study on knock-out animals where the liver production of IGF-l was eliminated, no negative effect on longitudinal growth of bone was found, supporting the concept that IGF-l acts in a paracrine/autocrine fashion rather than an endocrine one [156]. The results of our study support this since we found no relationship between circulating levels of IGFs and BMD despite extremely low levels of the growth factors.

The liver produces IGF-l, IGF-ll and IGFBP-3 and in accordance with our findings, low levels have been reported in up to 90% of patients with liver cirrhosis with significant associations to liver failure [54, 77, 84, 85]. In alcoholic and viral CLD, IGF-l and IGFBP-3 are good predictors of survival as well as early markers of liver dysfunction [83, 85]. The liver also produces IGFBP-1 and in liver failure levels are elevated and correlate inversely with liver function as in our study [76, 84, 86]. The reason for this is unknown but may be related to the role of IGFBP-1 in glucose homeostasis [76, 86]. Less is known about the origin and regulation of IGFBP-2 [74, 81, 84]. Serum levels of IGFBP-2 are increased whenever the organism is severely ill and there is evidence that the major regulator of IGFBP-2 is IGF-ll [74]. We found elevated levels of IGFBP-2 in our patients compared with the controls, however, no relationship was found between IGFBP-2 and parameters of liver function. Others have reported normal or moderately elevated levels in liver cirrhosis (81, 84).

We did not find a correlation between IGF-l and BMI or weight in either the controls or the patients. In malnourished patients IGF-l was found to be a sensitive marker of malnutrition [157]. IGF-l in patients with CLD, however, is more strongly related to

41

liver function than to nutritional status [83]. A weak association between IGF-l and anthropometric indices, such as BMI, has also been reported in normal individuals [158-159].

In order to elucidate the pathophysiology of osteoporosis in CLD further, we studied the relationship between serum leptin and BMD. Since serum leptin is highly dependent on BMI and gender, (this physiological correlation between serum leptin, BMI and gender was maintained in our patients with CLD as well), calculations to adjust for BMI were made separately for males and females and used in the analyses [108]. We found that serum leptin adjusted for BMI correlated significantly and inversely with BMD at the lumbar spine and the femoral neck in female patients and the femoral neck in male patients when analysed as a whole group, while no relationship was found in the controls. In a sub-group analysis this inverse relationship in the patients was only significant in patients with advanced liver disease (Child-Pugh B+C) even if the same trend could be observed in Child-Pugh A group of patients (data not shown). Serum leptin is reported high in patients with liver cirrhosis, and since BMD is known to decrease with advanced liver disease it is possible that this relationship is indirect. However, the possibility of a pathophysiological role of serum leptin in the osteoporosis of CLD can not be excluded. In a study in ob/ob mice, leptin was found to be a potent inhibitor of bone formation when given intracerebrally [116]. Treatment of bone cells in culture with leptin, however, did not influence bone formation and a central, neuroendocrine pathway for the effect of leptin on bone mass was suggested. Clinical studies in adult females have not given support to the hypothesis that circulating leptin influences bone metabolism [118-120]. Thus, there are no current data, apart from ours, suggesting that elevated levels of leptin in the circulation can inhibit bone formation.

Serum leptin and serum leptin levels adjusted for BMI did not differ between the patients and the controls. Liver function, however, seemed to influence leptin levels since male patients classified as Child-Pugh B+C had significantly higher levels of leptin adjusted for BMI than males classified as Child-Pugh A, and a positive association between AST and serum leptin was found. In female patients the same trend was observed but was not significant. Both high and low serum leptin levels have been reported in patients with CLD [112-115]. One explanation for these discrepancies could be that no adjustments

for BMI was made and that the results are confounded by various combinations of malnutrition in these patients (resulting in low serum leptin) and liver dysfunction (resulting in high serum leptin). In one study, however, serum levels of leptin expressed per kilogram of fat mass were elevated in 28 patients with alcoholic liver cirrhosis compared with a control group [112]. The cause of high levels of serum leptin in CLD is unknown but could be a result of impaired renal clearance or stimulation of leptin secretion [113]. Androgens have suppressive effects on leptin expression and high levels are found in hypogonadic men [108, 160]. Clinical and sub-clinical hypogonadism in male patients with CLD could therefore result in high levels of serum leptin. Only 5 of our male patients were hypogonadic. They had higher serum leptin adjusted for BMI which was nearly significant when compared with eugonadic males (p=0.05) (data not shown). As increased levels of leptin have mainly been found in alcoholic cirrhosis it has been suggested that alcohol per se may influence serum leptin levels [112-114].

CONCLUSIONS

Patients with CLD have decreased BMD at the lumbar spine and increased rate of bone loss most prominent at the femoral neck, which suggests that even cortical bone may be affected in CLD. Risk factors for osteoporosis were low BMI and treatment with corticosteroids. In a follow-up study including mainly patients with well preserved liver function factors related to increased bone loss were hyperbilirubinemia and low vitamin D levels. The pathogenesis of osteoporosis in CLD is probably multifactorial including inhibitory effects of hyperbilirubinemia and corticosteroid treatment on osteoblasts, effects of malnutrition and low vitamin D levels and hormonal changes. Furthermore, patients with CLD are a heterogeneous group and it is possible that different types of CLD affect bone by different mechanisms. IGFs probably are not related to osteoporosis in CLD but are strongly associated with liver function suggesting that they can be used as early markers of liver function and may be helpful in the future selection of patients for liver transplantation. Serum leptin correlated with the nutritional status (BMI) of patients with CLD and was inversely related to BMD. However, its role in the pathophysiology of osteoporosis is possible but unclear.

Advanced liver disease is highly associated with osteoporosis, presenting new hazards in the post-transplant era. As it can be expected that more transplants will be performed in the future it is important to prevent the development of osteoporosis at an early stage. The results in this report suggest that the combination of subnormal BMI and corticosteroid medication warrants special attention with regard to osteoporosis. Measurements of BMD should include measurements of both the lumbar spine and the femoral neck. Vitamin D levels should be measured in patients with CLD, cholestatic or not, and if low, the patients should receive supplementation. Furthermore, post-menopausal females with CLD should be evaluated with respect to osteoporosis and receive preventive treatment.

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