Linköping University Medical Dissertations No. 1427
Linköping 2014

Bone alkaline phosphatase isoforms in chronic kidney disease – mineral and bone disorder

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Cover:

Overall structure of the tissue-nonspecific alkaline phosphatase molecule illustrated as a homodimer. The active site phosphate (PO_4^{3-}), one magnesium and two zinc ions are indicated in one subunit for reference, as well as the fourth metal binding site likely occupied by calcium. Printed and modified with permission (1).

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Printed in Sweden by LiU-Tryck, Linköping, Sweden, 2014

ISBN: 978-91-7519-204-8

ISSN: 0345-0082

"All true science begins in the love, not the dissection of your fellow creatures"

John Ruskin, 1819 - 1900

Dedicated to Erika and Friedrich

ABBREVIATIONS

AAC	Abdominal aortic calcification
ABD	Adynamic bone disease
ALP	Alkaline phosphatase
ANK	Progessive ankylosis protein
ASBMR	American Society for Bone and Mineral Research
AUC	Area under the curve
BALP	Bone alkaline phosphatase
B/I, B1x, B1, B2	Bone alkaline phosphatase isoforms
BMD	Bone mineral density
BMI	Body mass index
BSP	Bone sialoprotein
CKD	Chronic kidney disease
CKD-MBD	Chronic kidney disease – mineral and bone disorder
CORD	Calcification Outcome in Renal Disease
CRP	C-reactive protein
CTX	Carboxy-terminal cross-linking telopeptide of type I collagen
CV	Cardiovascular
DKK1	Dickkopf-related protein 1
DMP1	Dentin matrix acidic phosphoprotein 1
DXA	Dual-energy X-ray absorptiometry
FGF23	Fibroblast growth factor 23
GPI	Glycosylphosphatidylinositol
GPI-PLC	Glycosylphosphatidylinositol-specific phospholipase C
GPI-PLD	Glycosylphosphatidylinositol-specific phospholipase D
HA	Hydroxyapatite
HASMC	Human aortic smooth muscle cell
HD	Hemodialysis
HPLC	High-performance liquid chromatography
HPT	Hyperparathyroidism
KDIGO	Kidney Disease Improving Global Outcome
KDOQI	Kidney Disease Quality Outcomes Initiative

LALP	Liver alkaline phosphatase
LRP	Low density lipoprotein receptor-related protein
MAR	Mineral apposition rate
MEPE	Matrix extracellular phosphoprotein
MGP	Matrix γ-carboxyglutamic acid (Gla) protein
MI	Myocardial infarction
MICS	Malnutrition inflammation cachexia syndrome
MSC	Mesenchymal stem cell
MSX2	Muscle segment homeobox 2
MUO	Mixed uremic osteodystrophy
MV	Matrix vesicle
NPP1	Nucleoside pyrophosphohydrolase-1
OC	Osteocalcin
OF	Osteitis fibrosa
OM	Osteomalacia
OPG	Osteoprotegerin
OPN	Osteopontin
PD	Peritoneal dialysis
PINP	Type I procollagen intact amino-terminal propeptide
PKA	Protein kinase A
PPi	Inorganic pyrophosphate
PTH	Parathyroid hormone
PWV	Pulse wave velocity
ROC	Receiver operator characteristic
ROD	Renal osteodystrophy
ROS	Reactive oxygen species
RUNX2	Runt-related transcription factor 2
sHPT	Secondary hyperparathyroidism
SOX2	Sex determining region Y box 2
SOX9	Sex determining region Y box 9
TNALP	Tissue-nonspecific alkaline phosphatase
TNFα	Tumor necrosis factor-α
TRACP5b	Tartrate-resistant acid phosphatase isoform 5b
TSALP	Tissue-specific alkaline phosphatase

ULN	Upper limit of normal
UTR	Untranslated region
VSMC	Vascular smooth muscle cell
Wnt	Wingless-type mouse mammary tumor virus integration site

In this thesis, CKD staging is based on the suggestion by the KDIGO initiative from 2009 (2):

CKD stage	Description	GFR (ml/min per 1.73m^2)	Treatment
1	Kidney damage with normal or ↑ GFR	>90	1–5T if kidney transplant recipient
2	Kidney damage with mild ↓GFR	60–89	
3	Moderate ↓GFR	30–59	
4	Severe ↓ GFR	15–29	
5	Kidney failure	<15 (or dialysis)	5D if dialysis (HD or PD)

CKD, chronic kidney disease; GFR, glomerular filtration rate; ↑, increased; ↓, decreased

ABSTRACT

Chronic kidney disease (CKD) is associated with increased mortality and cardiovascular complications. Disturbances in mineral metabolism occur early during the course of CKD and several components of the CKD-mineral and bone disorder (CKD-MBD) are independent predictors of mortality. Alkaline phosphatase (ALP) is necessary for skeletal mineralization and is also involved in the process of vascular calcification. In recent years, ALP has evolved as a strong predictor of mortality in the CKD population. The significant role of ALP in the mineralization process renders it a putative target for the treatment and prevention of vascular calcification. Three circulating isoforms of bone ALP (BALP) have been identified (B/I, B1, and B2). A fourth isoform, B1x, has been identified exclusively in serum from patients with CKD. The aim of the present thesis was to further elucidate the role of the BALP isoforms in CKD with respect to bone abnormalities and vascular calcification.

In **study I** we identified the novel BALP isoform B1x in 20% of patients with mild to moderate CKD. B1x was associated with lower glomerular filtration rate and higher serum phosphate and calcium × phosphate product, which are risk factors for cardiovascular mortality in CKD. We also identified the BALP isoforms B/I, B1 and B2 as predictors of total hip bone mineral density.

Study II was an experimental study, investigating the role of the BALP isoforms in phosphate induced calcification of human aortic smooth muscle cells (HASMCs). We found that the ALP expressed in HASMCs is exclusively BALP. Phosphate induced calcification of HASMCs was associated with increased BALP isoforms B/I, B1x, and B2 activities, which implies functional differences between the BALP isoforms in HASMC calcification.

In **study III** we investigated the association of BALP isoforms in serum and histomorphometric parameters of bone in patients on chronic hemodialysis. We identified the BALP isoform B1x as a novel marker for reduced osteoblastic activity.

Study IV was a prospective cohort study of the association of serum BALP isoforms with aortic calcification and vascular stiffness in prevalent chronic dialysis patients. B1x was associated with baseline and time varying vascular stiffness, determined by pulse wave velocity, but not with calcification of the abdominal aorta. We also found an association of B1x with better event-free survival.

In conclusion, these studies demonstrate that the BALP isoforms, especially isoform B1x, are involved in different aspects of CKD-MBD. This opens up for further research to identify the BALP isoforms as diagnostic markers and possible treatment targets in CKD-MBD.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Kronisk njursvikt (CKD) är ett folkhälsoproblem. Omkring 5 % av befolkningen har nedsatt njurfunktion och förekomsten ökar kraftigt hos den äldre befolkningen. Störningar av mineralbalansen sker redan i tidiga stadier av CKD hos de allra flesta patienter. Dessa störningar kan orsaka skelettrubbningar samt förkalkningar av artärer och hjärtklaffar, vilket leder till en ökad hjärt-kärl sjuklighet och dödlighet. Ben alkalisk fosfatas (BALP) är ett enzym som spelar en viktig roll vid mineraliseringen av skelettet, men som även är involverad i kärlförkalkningen. Hos patienter med CKD är en ökad BALP aktivitet i serum förenad med ökad dödlighet. Genom dess centrala roll i kärlförkalkningsprocessen är BALP intressant för utvecklingen av framtida behandlingar mot kärlförkalkning. En bättre förståelse av den roll BALP spelar för skelettmineralisering och kärlförkalkning kan därför bereda väg för utveckling av nya behandlingar mot kärlförkalkning och skelettrubbningar vid CKD. Tre isoformer av BALP har beskrivits i serum hos friska försökspersoner och olika patientgrupper med metabola bensjukdomar. En fjärde isoform, B1x, har enbart hittats i serum hos dialyspatienter. Syftet med denna avhandling var att kartlägga de olika BALP isoformernas samband med skelettrubbningar och kärlförkalkningar hos patienter med CKD.

I **studie I** hittade vi den nya BALP isoformen B1x i serum hos 20 % av patienter med lätt till måttlig njursvikt. Patienter med B1x hade sämre njurfunktion, högre fosfat och en förhöjd kalcium × fosfat produkt i serum. Högre fosfat och kalcium × fosfat produkt är riskfaktorer för ökad dödlighet i hjärt-kärlsjukdomar. Vi fann också att BALP isoformerna B/I, B1 och B2 kunde förutsäga bentätheten i höften, ett område som består till större del av trabekulärt ben.

Studie II var en experimentell studie där vi undersökte vilka isozyrmer av ALP som förkommer i mänskliga glatta aortamuskelceller. Våra resultat visar att all ALP aktivitet i mänskliga glatta aortamuskelceller utgörs av BALP. Vi kunde också visa att förkalkning av dessa celler genom tillsats av kalcium och fosfat i cellmediet är förenat med ökade aktiviteter av BALP isoformerna B/I, B1x och B2, men inte B1. Resultaten tyder på funktionella skillnader mellan isoformerna i kärlförkalkningsprocessen.

I **studie III** undersökte vi sambandet mellan BALP isoformerna i serum och skelettrubbningar, diagnostiserade genom benbiopsi hos hemodialyspatienter. BALP isoformen B1x förekom i serum hos 53 % av patienterna. Vi fann ett samband mellan serum B1x och en störd osteoblastfunktion i benbiopsierna.

I **studie IV** följde vi 68 dialyspatienter under två år. Patienterna genomgick röntgen av bukaortan för mätning av aortaförkalkningen vid studiens början och efter två år. Dessutom mättes kärlstyvheten vid studiens början, efter ett och efter två år. Bestämning av BALP isoformerna i serum skedde i början av studien och efter 2 år. Vi fann ett samband mellan isoformen B1x och kärlstyvheten, men inte kärlförkalkningen. Patienter med B1x hade en något mindre risk att dö eller utveckla en första hjärt-kärlsjukdom under uppföljningstiden.

Sammanfattningsvis visar dessa studier att BALP isoformerna, ffa B1x, har betydelse för flera manifestationer av störningen av mineralbalansen som förekommer redan i tidiga stadier av CKD. Våra resultat öppnar för framtida studier, som kan förtydliga BALP isoformernas roll för diagnostik och behandling av mineralrubbningar och efterföljande komplikationer vid CKD.

LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I-IV):

I **Haarhaus M**, Fernström, A, Magnusson M, Magnusson P. Clinical significance of bone alkaline phosphatase isoforms, including the novel B1x isoform, in mild to moderate chronic kidney disease. Nephrol Dial Transplant 2009;24:3382–3389.

II **Haarhaus M**, Arnqvist HJ, Magnusson P. Calcifying human aortic smooth muscle cells express different bone alkaline phosphatase isoforms, including the novel b1x isoform. J Vasc Res 2013;50:167–174.

III **Haarhaus M**, Monier-Faugere MC, Magnusson P*, Malluche HH*. Bone alkaline phosphatase isoforms in CKD patients on hemodialysis with low and high bone turnover. Submitted to Am J Kidney Dis. * Shared senior authorship

IV **Haarhaus M**, Fernström A, Magnusson P. A multicenter prospective study of bone alkaline phosphatase isoforms and arterial calcification in chronic kidney disease patients on dialysis. Manuscript

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1. BACKGROUND

1.1 History of alkaline phosphatase in bone mineralization

Phosphorus was detected as early as 1669 by the German alchemist Henning Brand while attempting to convert urine into gold. In 1769 the Swedish chemist Carl Wilhelm Scheele described a method to derive phosphate from bone. During the 19th century it became known that phosphorus existed in biological organisms in organic and inorganic forms. Around 1900 several researchers investigated the conversion of organic phosphates to inorganic phosphates by different biological tissues and enzymes. This effort was elegantly summarized by Plimmer (3) and led to the finding of a phosphatase with reaction optimum at pH 7-9. In 1923, Robert Robison developed the original concept of the importance of alkaline phosphatase (ALP, EC 3.1.3.1) for skeletal mineralization (4). In 1970, the role of ALP in bone mineralization was further elaborated by HC Anderson's group, who demonstrated that cartilage mineralization was driven by ALP rich matrix vesicles (5). Robison (4) hypothesized in his original concept that ALP rendered inorganic phosphates by hydrolyzation of organic phosphate esters, thereby providing a substrate for mineralization. Later, Fleisch and Neuman found the predominant role of ALP in the calcification process to inactivate inhibitory polyphosphates (6), more specifically the calcification inhibitor pyrophosphate (PPi) (7). Further putative mechanisms are under investigation, among them the inactivation of another calcification inhibitor, osteopontin (OPN), through dephosphorylation.

1.2 ALP – from gene to protein

There are four different genes coding ALP isozymes in humans. Of these, three are tissue-specific (TSALP), the *ALPI*, coding the intestinal ALP, *ALPP*, coding the placental ALP, and *ALPPL*, coding the germ-cell ALP. The fourth gene locus, *ALPL*, is expressed in different tissues and therefore termed tissue-nonspecific ALP (TNALP) (8). The human TNALP gene was sequenced by Harris group in 1988 (9). It is located on chromosome 1p (10), whereas the TSALP genes are located on chromosome 2. Several different alleles have been detected for *ALPP* and *ALPPL*, and a considerable number of phenotypes of these ALP isozymes have been described (11-13). On the other hand, no physiological genetic or phenotypic variation has been observed for *ALPL* or *ALPI* (13).

The *ALPL* gene consists of 12 exons, but exon 1, which is part of the 5' untranslated region (UTR) exists in two variants, which respond to different promotors (14). The transcription of the two variants of exon 1 is species- and cell type-specific (15). The transcripts are called "bone *ALPL* transcript" when exon 1A is transcribed and "liver *ALPL* transcript" when exon 1B is transcribed (16). In mouse cardiac muscle, a high transcription of 1B containing mRNA is found, although almost no translation takes place and protein concentration is very low (15).

TNALP is expressed ubiquitously, but the highest expression is found in osteoblasts (bone specific ALP, BALP), hepatocytes (liver-specific ALP, LALP) and proximal renal tubulus cells (kidney-specific ALP). The translational product is a glycoprotein, which is comprised of 485 amino acids in its mature form and approximately 1/3 of its molecular weight of 60-90 kDa consists of carbohydrates (9, 13, 17-20). It functions as an ectoenzyme, attached as a homodimer to the outer cell membrane by a glycosylphosphatidylinositol (GPI) anchor (16, 21-23). It can also be found in plasma as a circulating soluble homodimer (21).

Our knowledge of the three dimensional structure of ALP comes from studies of human PLAP (24) and ALP from E. coli (25). In 2002, Magnusson *et al.* presented a putative three dimensional structure of human TNALP (**Figure 1**) (1). In the homodimeric constellation the N-terminus of one monomer wraps around the second monomer conferring allosteric properties of the dimer (26, 27). Each monomer contains one active site, which contains two zinc, one magnesium, and a fourth metal ion, likely to be calcium (1, 28-31). The importance of zinc for the proper function of BALP in the mineralization process has repeatedly been demonstrated (32-35). Some evidence also points towards the influence of magnesium on ALP activity in vivo (36, 37).

1.3 TNALP isoforms – a matter of protein glycosylation

Placental and germ cell ALP demonstrate considerable genetic polymorphism (13). Reports concerning TNALP gene polymorphisms are rare, but at least one has been described in a Japanese cohort (38). Still, different TNALP isoforms have been detected in serum, and differences in glycosylation as well as remaining GPI anchor fragments, with or without cell membrane fragments, have been suggested as explanations for these findings (39-42). In 1992, Magnusson *et al.* (43) separated and quantified six different TNALP isoforms in serum

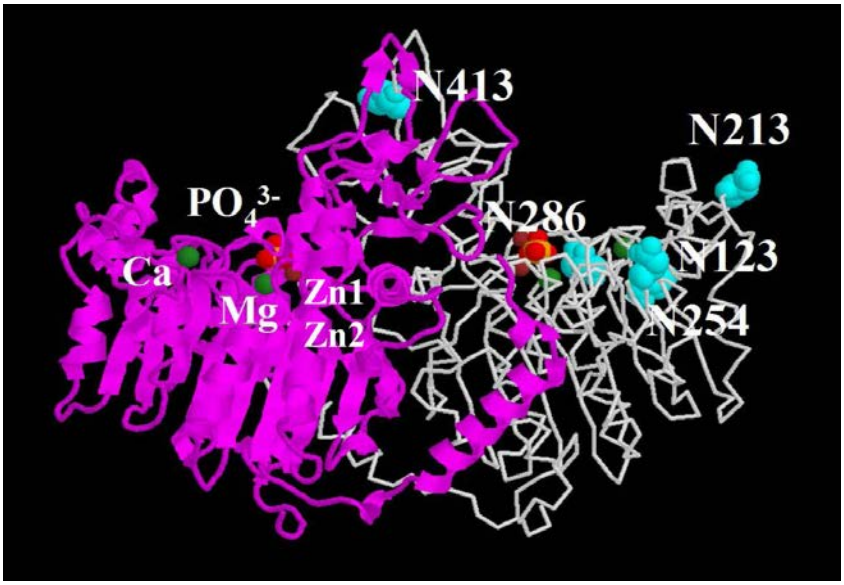

Figure 1. Overall structure of the TNALP molecule illustrated as a homodimer, i.e., the same form that is present in the circulation. Subunit A is shown in white with backbone representation and subunit B is shown in magenta with ribbon representation. The five putative complex-type N-glycosylation sites (i.e., Asn residues) with terminal sialic acid residues are highlighted in subunit A with space-filling models in cyan. The active site phosphate (PO_4^{3-}), Zn and Mg ions are pointed out in subunit B as well as the fourth metal site likely to be occupied by calcium. Printed and modified with permission (1).

by high-performance liquid chromatography (HPLC) in serum from healthy individuals: one bone/intestinal (B/I), two BALP (B1 and B2), and three LALP isoforms (L1, L2, and L3) (**Figure 2 (A)**). The B/I isoform co-elutes with circulating intestinal ALP and is not only of bone origin, instead, it is composed of approximately 70% BALP and 30% intestinal ALP. Serum BALP isoforms differ only in glycosylation without any detectable GPI anchor fragment attached (44, 45), whereas differences between serum LALP isoforms are more complex, possibly due to both glycosylation differences and attached GPI anchor or cell membrane fragments (42, 46-48). At least five putative N-glycosylation sites have been described for LALP and BALP (9, 13). In addition, BALP, but not LALP is O-glycosylated (19). The N-glycosylation sites are located around the active site of the TNALP molecule, and differences in glycosylation between the BALP isoforms affect their enzymatic activity (45, 49).

All three BALP isoforms found in serum have also been identified in bone tissue. In addition, a fourth isoform, B1x, was detected in bone tissue extracts (44, 50). This isoform was later also found in serum from some (60%) dialysis patients (**Figure 2 (B)**) (51) and in children with chronic kidney disease (CKD) (52), but not in serum from healthy adults (51). Even though the circulating levels of BALP isoforms can vary independently during adolescence (53) and in different metabolic bone diseases (54-56), B1x has never been detected in serum from any non-CKD population studied. Previous characterization studies with monoclonal antibodies, heat-inactivation and various inhibitors, confirm that B1x is of bone origin (44). B1x has also been detected in serum from a bilaterally nephrectomized patient, thus ruling out that B1x could be a possible kidney ALP isoform (44). The clinical significance and physiological role of B1x is, however, still unclear. The isoforms B1 and B2 are the most abundant, both in serum and in bone tissue, whereas B/I and B1x represent only minor fractions. In bone, the ratio of B1 and B2 differs between trabecular and cortical bone; while a

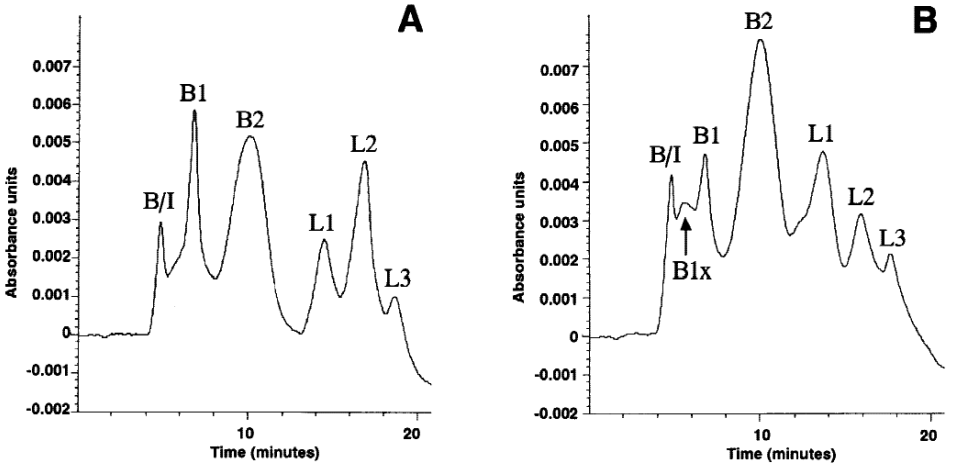

Figure 2. (**A**) A serum ALP isoform profile from a healthy female, 53 years of age, with total ALP 2.8 µkat/l. Peaks, retention times, and activities, in order of elution, are: B/I, 4.85 minutes, 0.11 µkat/l; B1, 6.82 minutes, 0.51 µkat/l; B2, 10.07 minutes, 0.97 µkat/l; L1, 14.45 minutes, 0.42 µkat/l; L2, 16.85 minutes, 0.64 µkat/l; L3, 18.56 minutes, 0.15 µkat/l. (**B**) A serum ALP isoform profile from a female patient with severe renal insufficiency and on chronic dialysis therapy, 70 years of age, with total ALP 3.6 µkat/l. Peaks, retention times, and activities, in order of elution, are: B/I, 4.88 minutes, 0.16 µkat/l; B1x, 5.40 minutes, 0.19 µkat/l; B1, 6.85 minutes, 0.45 µkat/l; B2, 10.02 minutes, 1.25 µkat/l; L1, 13.92 minutes, 0.79 µkat/l; L2, 16.06 minutes, 0.49 µkat/l; L3, 17.72 minutes, 0.27 µkat/l. Printed with permission (44).

higher activity of B1 is found in cortical bone (B1/B2 ratio 1.72), B2 dominates at trabecular sites (B1/B2 ratio approximately 0.7 at different trabecular sites) (44). Interestingly, the B1/B2 ratio was higher in serum from dialysis patients compared to healthy controls, which could be due to higher cortical bone turnover associated with hyperparathyroidism (HPT) in these patients (51).

1.4 BALP expression during the differentiation of mesenchymal stem cells to osteoblasts

Osteoblasts, as well as chondrocytes, adipocytes and myocytes, belong to a large family of cells, originating from the same type of mesenchymal stem cell (MSC). The osteogenic differentiation of MSCs is stimulated by two groups of growth factors, wingless-type MMTV integration site family (Wnt) and bone morphogenetic proteins (BMP). The canonical Wnt pathway stimulates osteoblastic differentiation through the activation of frizzled receptors, whose co-receptor low density lipoprotein receptor-related protein (LRP5/6) is inhibited by dickkopf-related protein 1 (DKK1) and sclerostin. The non-canonical Wnt pathway stimulates osteoblastic differentiation by a tumor necrosis factor α (TNFα) dependent process. BMPs stimulate osteoblastic differentiation mainly through the Smad pathway.

The activation of several transcription factors is associated with the differentiation of MSCs into osteoblasts. Among them, Runt-related transcription factor 2 (RUNX2) plays a central role (57). RUNX2 is stimulated in the MSC mainly by the canonical Wnt pathway. It is necessary for the early differentiation of preosteoblasts and immature osteoblasts. In contrast, RUNX2 seems to inhibit late maturation of osteoblasts, keeping them in an early developmental phase. RUNX2 stimulates the transcription of ALP, collagen type I alpha 1, and OPN (**Figure 3**).

1.5 BALP – biological function in tissue mineralization

BALP is essential for skeletal mineralization. This is illustrated by the genetic disorder of hypophosphatasia, a rare inherited metabolic bone disease, characterized by a deficiency of

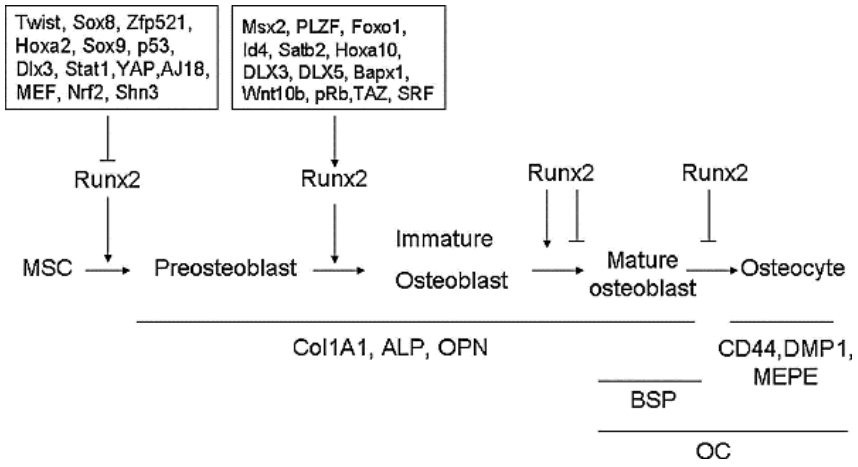

Figure 3. Role and delicate regulation of RUNX2 during osteoblast differentiation. RUNX2 is essential for skeletal development. In osteoblast differentiation, RUNX2 expression is detected in preosteoblasts expressing type I collagen weakly and is upregulated in immature osteoblasts expressing OPN. However, RUNX2 expression is downregulated in mature osteoblasts expressing osteocalcin (OC). As the osteoblast transitions to an osteocyte expressing secreted proteins CD44, dentin matrix acidic phosphoprotein 1 (DMP1), and matrix extracellular phosphoglycoprotein (MEPE), ALP is reduced, whereas OC is elevated. The expression and transcriptional activity of RUNX2 are tightly regulated by multiple proteins during osteoblast differentiation. Printed with permission (57).

TNALP. Patients have low serum ALP activities and defective bone and tooth mineralization. (58). The clinical picture is characterized by a high variability, ranging from lethal perinatal forms to mild forms, manifesting as stress fractures of the lower extremities or tooth abnormalities in adults (59).This variability can probably be attributed to different mutations in the *ALPL* gene, and even a single missense mutation can lead to the most lethal form of perinatal hypophosphatasia (60). The recent development of mineral targeting TNALP (61) has led to a treatment trial with impressive effect on skeletal mineralization in severe perinatal hypophosphatasia (62), thereby underlining the essential role of TNALP in skeletal mineralization.

Tissue mineralization is initiated in matrix vesicles (MVs), shed by mineralization competent cells, such as osteoblasts or calcifying vascular smooth muscle cells (VSMCs) (63). These MVs are actively loaded with BALP on their outer membrane, a process that involves microvilli and actin (64, 65). The initial step of mineralization is the formation of

hydroxyapatite (HA) crystals in these matrix vesicles (**Figure 4 A-C**). This process requires PHOSPHO1, an intravesicular phosphatase, that reduces the ratio of the mineralization inhibitor PPi to phosphate (66), thereby generating a procalcific environment, that promotes

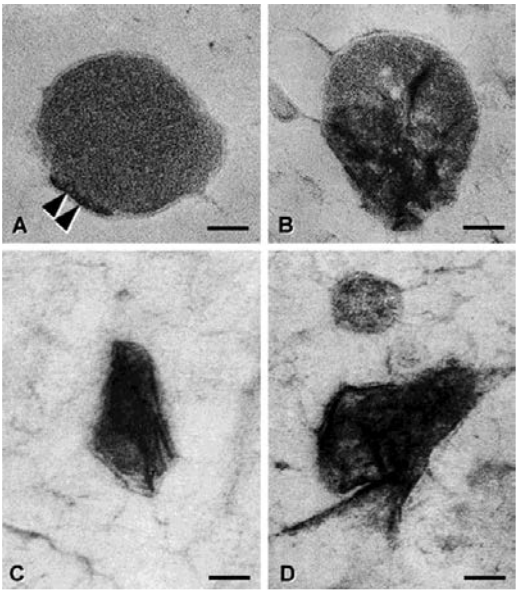

Figure 4. Electron micrographs of matrix vesicle in the rat epiphyseal plate obtained by freeze-substitution methods at liquid helium temperature. Electron dense materials (arrowheads, **A**) seen along the inner leaflet of the investing membrane of the matrix vesicle (MV) at the early stage of mineralization. Matrix vesicles contain numerous crystal-like structures (arrows **B**, **C**). The intravesicular crystal-like structures are exposed to the extravesicular environment, with accumulation and growth of crystals (**D**). Bar, A-D: 50 nm. Printed with permission (67).

HA synthesis. When the HA crystal grows in size, it perturbs through the MV membrane (**Figure 4D**). In the extracellular space BALP, either membrane bound, or attached to HA crystals, stimulates crystal growth similar to PHOSPHO1, by influencing the PPi/Pi ratio through the hydrolyzation of PPi. Yadav *et al.* (68) have elegantly shown that the elimination of both, PHOSPHO1 and TNALP leads to a complete absence of skeletal mineralization.

Pyrophosphate is produced locally from ATP by the membrane bound enzyme ectonucleotide pyrophosphatase phosphodiesterase 1 (NPP1) in osteoblasts and VSMCs (66), and the tightly regulated interaction of the PPi transporter progressive ankylosis protein (ANK) and the

enzymes NPP1 and ALP controls mineralization under physiologic conditions (16) (**Figure 5**). Pyrophosphate inhibits mineralization by binding to hydroxyapatite crystals and thereby inhibiting further growth of these crystals (142). NPP1 is found in MVs whereas ANK is not; NPP1 plays, therefore, a more important role in PPi production than ANK. By a negative feedback loop PPi inhibits the expression of the *Enpp1* and *Ank* genes, thereby limiting its own production and transport through the cell membrane. In addition to its direct inhibitory effect on mineralization, PPi induces the production of OPN, another mineralization inhibitor.

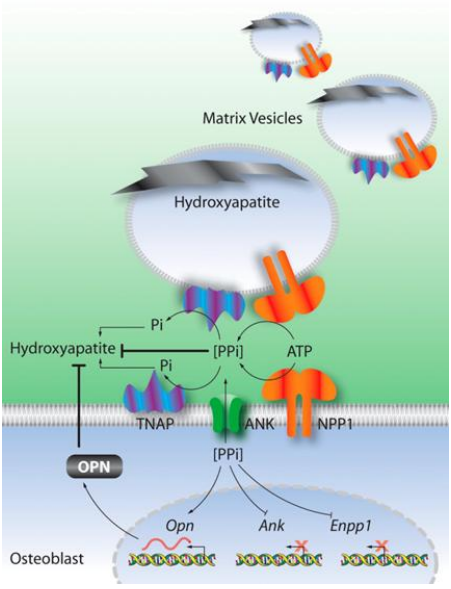

Figure 5. Diagrammatic representation of the roles of TNALP, ANK, NPP1, PPi, and OPN in the regulation of hydroxyapatite deposition. Both NPP1 and ANK raise extracellular levels of PPi while TNALP is required for depletion of the PPi pool. Both TNALP and NPP1 are functional in MVs whereas ANK is not; NPP1 plays, therefore, a more crucial role in PPi production than ANK. As a result, the absence of NPP1 in *Enpp1*$^{-/-}$ mice results in a more severe phenotype than in *ank/ank* mice. A negative feedback loop exists in which PPi, produced by NPP1 and transported by the channeling action of ANK, inhibits expression of the *Enpp1* and *Ank* genes. In addition, PPi induces expression of the *Opn* gene and production of OPN, which further inhibits mineralization. In the absence of TNALP, high levels of PPi inhibit mineral deposition directly and also via its induction of OPN expression. The combined action of increased concentrations of PPi and OPN causes hypomineralization. In the absence of NPP1 or ANK, low levels of PPi, in addition to a decrease in OPN levels, leads to hypermineralization. This model clearly points to NPP1 and ANK as therapeutic targets for the treatment of hypophosphatasia. Similarly, targeting TNALP function can be useful in the treatment of hypermineralization abnormalities caused by altered PPi metabolism. Adapted from Harmey *et al.* (70) with permission.

OPN acts as inhibitor of mineralization in a similar way as PPi, by binding to hydroxyapatite crystals and inhibiting their further growth (69).

In addition to the hydrolysis of PPi, a further, more speculative, stimulatory effect of ALP on mineralization is the hydrolysis of phosphorylated OPN (70). Finally, the hydrolysis of polyphosphates by BALP results in an increased phosphate concentration, with a possible stimulatory effect on mineralization. The concerted actions of BALP, ANK, NPP1, PPi, and OPN are summarized in **Figure 5**. Our group has demonstrated differences in the enzymatic activities of BALP isoforms with respect to different substrates, among them PPi, suggesting putative different roles for the BALP isoforms in the mineralization process (71).

1.6 Circulating BALP – release from mineralizing cells and clearance

Circulating BALP is associated with bone turnover (72-74), mortality (75, 76), cardiovascular (CV) disease (77, 78), and fractures (79, 80). In osteomalacia and Paget's disease exceptionally high BALP activities can be detected in the circulation. It is therefore of interest to understand the mechanism by which BALP is released into circulation. The release of ALP rich MVs by mineralizing cells, such as osteoblasts, chondrocytes, and calcifying vascular smooth muscle cells (VSMCs), is an important step in the mineralizing process (5, 81). Even though MVs can be detected in the circulation (82), no reports on circulating ALP-rich MVs have, until now, been published. While ALP at sites of mineralization is attached to the cell membrane by a GPI-anchor, Van Hoof *et al.* (46) described different forms of circulating ALP, membrane bound ALP, attached to membrane fragments by the GPI-anchor, anchor bearing ALP, and anchor free soluble ALP. ALP is cleaved from the GPI-anchor by GPI-specific phospholipases C and D (GPI-PLC and GPI-PLD). GPI-PLD is abundant in serum (83). Circulating BALP is almost exclusively anchor free (84, 85) and only very low activities of anchor bound BALP can be detected in serum from adolescents and patients with osteoblastic bone disease (46). All ALP isozymes are cleared by the liver via the galactose receptor (86). Clearance rates are influenced by protein glycosylation. The half-life of BALP is reported as 1.1-4.9 days (87, 88). It can be speculated, that this variety of clearance rates could be explained by differences in glycosylation between the different BALP isoforms.

1.7 CKD – MBD

1.7.1 Disturbance of mineral metabolism

For more than 100 years ago, Lucas described an influence of CKD on bone and introduced the term renal rickets (89). The term renal osteodystrophy was first used by Liu and Chu in 1942 (90) to describe osseous changes associated with CKD, that resembled "rickets, osteomalacia or osteitis fibrosa cystica". At that time, disturbed vitamin D metabolism, hyperphosphatemia, hypocalcemia, and HPT were discussed as possible causes of renal osteodystrophy. Treatment options consisted mainly of native vitamin D with little effect on bone, and parathyroidectomy with high complication rates. The introduction of chronic dialysis treatment, with greatly increased survival of patients with CKD stage 5, has resulted in an increased prevalence of patients with advanced disorders of mineral balance.

In 2009, the international Kidney Disease Improving Global Outcome (KDIGO) initiative summarized the multitude of known complications and pathophysiological processes related to mineral dysregulation in CKD by introducing the new concept of CKD – mineral and bone disorder (CKD-MBD) (2). In the early phase of CKD-MBD, phosphate retention and elevated circulating klotho stimulate fibroblast growth factor 23 (FGF23) excretion by osteocytes. FGF23 has a phosphaturic effect in the renal tubule and inhibits the activation of vitamin D by the kidneys, thereby keeping phosphate concentrations low. The reduced vitamin D activation leads to secondary hyperparathyroidism (sHPT) and, at more advanced stages of CKD, even to reduced intestinal calcium uptake, which further exacerbates the sHPT (91-94). Sclerostin, another peptide produced by osteocytes, is also increase in early CKD and counteracts the stimulatory effect of PTH on bone turnover by inhibiting Wnt signaling (95). In more advanced CKD, these disturbances lead to manifestations in bone and the vasculature, with vascular calcification being almost always present, in addition, a high frequency of bone disturbances with both pathologically high and low turnover occurs.

1.7.2 Renal osteodystrophy

Bone disorders in CKD are multifactorial and comprise changes of bone turnover and mineralization, often also affecting bone volume and bone material properties (96, 97). Changes in bone occur early during the progression of CKD (98, 99). The only reliable

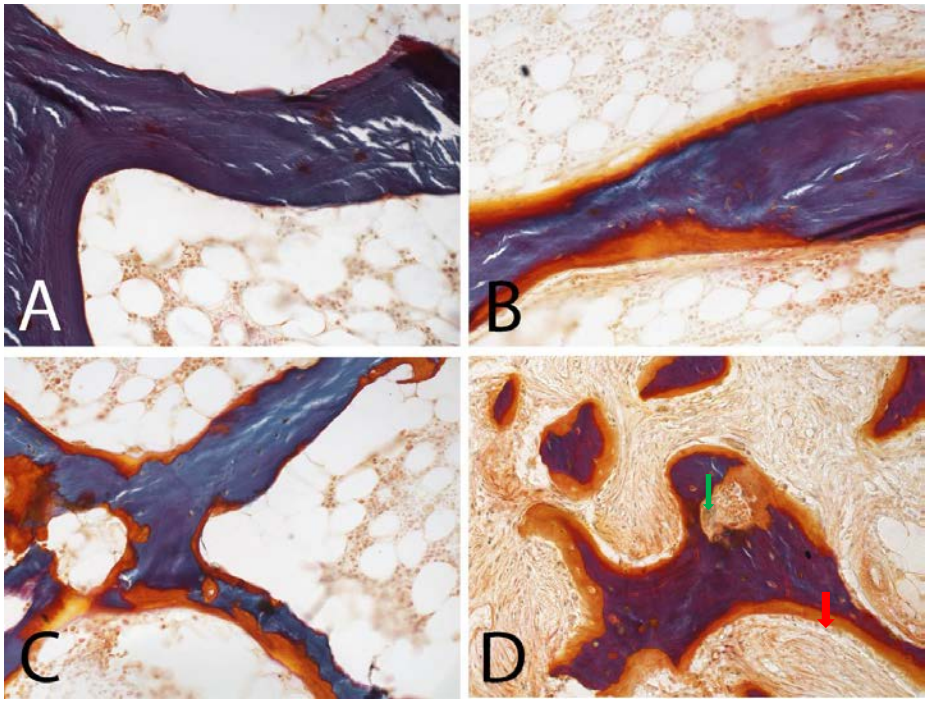

Figure 6. Histologic images of undecalcified bone, demonstrating different types of ROD. Modified Goldner stain, showing mineralized bone stained blue, osteoid stained red-orange. Cellular cytoplasm is stained pink-orange while cell nuclei are stained dark blue-black. Original magnification: 200x (**A**) Adynamic bone disorder (ABD). This bone is completely acellular, with the exception of some osteocytes. Osteoblast and osteoclast activity is virtually absent, osteoid parameters are also greatly reduced and only faint layers of osteoid can be suspected. (**B**) Mixed uremic osteodystrophy (MUO). This bone has some features of high bone turnover, such as increased number of osteoblasts, some fibrosis, but also some features of low bone turnover, and defective mineralization, as shown by the increased osteoid parameters. (**C**) Osteomalacia (OM). Osteoid perimeter and osteoid width are greatly increased, but the number of bone cells is clearly reduced, indicative of low bone turnover and impaired mineralization. Also note the scalloped mineralization front (interface mineralized bone-osteoid), indicative of a mineralization defect. (**D**) Osteitis fibrosa (OF). This picture demonstrates very high bone turnover. Almost all bone surface is covered with osteoid and osteoblasts (red arrow), or actively resorbing osteoclasts (green arrow). Osteoid depth is minimally increased, indicative of normal mineralization. Some osteocytes are recently incorporated into osteoid. There is extensive marrow fibrosis. For the determination of dynamic parameters of turnover and mineralization, fluorescent images of tetracycline labeled bone is mandatory (not shown). Images courtesy of Prof. D'Haese, Laboratory of Pathophysiology, University of Antwerp, Belgium.

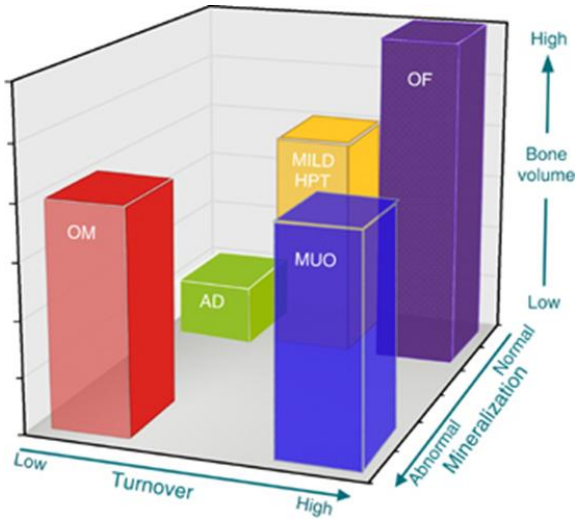

Figure 7. The turnover, mineralization, and volume (TMV) classification system for bone histomorphometry. The figure is a graphical example of how the TMV system provides more information than the present, commonly used classification scheme. Each axis represents one of the descriptors in the TMV classification: turnover (from low to high), mineralization (from normal to abnormal), and bone volume (from low to high). Individual patient parameters could be plotted on the graph, or means and ranges of grouped data could be shown. For example, many patients with renal osteodystrophy cluster in areas shown by the bars. The red bar (OM, osteomalacia) is currently described as low-turnover bone with abnormal mineralization. The bone volume may be low to medium, depending on the severity and duration of the process and other factors that affect bone. The green bar (ABD, adynamic bone disease) is currently described as low-turnover bone with normal mineralization, and the bone volume in this example is at the lower end of the spectrum, but other patients with normal mineralization and low turnover will have normal bone volume. The yellow bar (mild HPT, mild hyperparathyroid-related bone disease) and purple bar (OF, osteitis fibrosa or advanced hyperparathyroid-related bone disease) are currently used distinct categories, but in actuality represent a range of abnormalities along a continuum of medium to high turnover, and any bone volume depending on the duration of the disease process. Finally, the blue bar (MUO, mixed uremic osteodystrophy) is variably defined internationally. In the present graph, it is depicted as high-turnover, normal bone volume, with abnormal mineralization. In summary, the TMV classification system more precisely describes the range of pathologic abnormalities that can occur in patients with CKD. Printed with permission (104).

method to determine the type of bone disorder in CKD is by histomorphometric analysis of bone biopsies (100). The histomorphometric method uses a standardized nomenclature to describe static and dynamic parameters in histologic sections of non-decalcified bone biopsies (101, 102). The introduction of this standardized nomenclature, initiated by the American Society for Bone and Mineral Research (ASBMR) in 1987 (101), and updated in 2012 (102), improved the communication between bone histomorphometric centers, facilitated furthermore by the classification of renal osteodystrophy, suggested by Sherrard *et al.* (103) in 1993, who described five different forms of renal osteodystrophy: Mild osteodystrophy, osteitis fibrosa, mixed osteodystrophy, osteomalacia, and aplastic osteodystrophy (**Figure 6**). These categories were mainly defined based on bone formation rate and osteoid parameters. In 2006, the KDIGO initiative (104) suggested a further development of this classification, requiring that any type of ROD should be classified using parameters of turnover, mineralization, and volume (TMV classification, **Figure 7**).

Several bone markers have been suggested as substitutes for bone biopsies, but until today no non-invasive method can reliably differentiate between different types of ROD (105, 106). In clinical routine PTH is often used as turnover marker, but recently BALP has been suggested instead, due to lower variability and better outcome predictability (107-109).

1.7.3 Vascular calcification

The phenomenon of CV calcification was known already to the ancient Greeks. Aristotle described cardiac ossification in ageing compared to young animals and found an association with diminished physical performance in affected animals (110). In 1575, Fallopius described a degenerative ossification of arteries; and during the 16th and 17th century this process was commonly seen as a natural part of ageing. Rudolph Virchow made a distinction between vascular calcification and the atheromatous process, both of which he saw as consequences of inflammation (111). In 1903, Johann Georg Mönckeberg published a detailed description of 130 autopsies of patients with vascular calcification (112). He was not the first to describe the calcification of the arterial muscular tunica media, but he was the first to make a clear distinction between media calcification and intimal calcification as two separate processes. In this pioneering publication he also described different distributions of intimal arteriosclerotic calcifications, mainly located in the aorta and proximal arteries of the lower extremities, and

media calcification, located in more distal arteries of both lower and upper extremities. Especially in the upper extremities, he reported a predominance of media calcification and the almost complete absence of intimal calcifications. About 60 years later, Haimovici *et al.* (113) gave experimental support to Mönckebergs clinical observation. They demonstrated that the propensity of arteries to undergo intimal calcification is tissue-specific, rather than determined by localization, thus, autotransplants of thoracic aorta into abdominal aorta do not develop intimal calcification under atherogenic conditions and vice versa (113). DeBakey *et al.* (114) identified different patterns of atherosclerosis specific to different localizations in the arterial tree in a large number of surgical cases and the authors hypothesized this to be an effect of "genetic differences of the vessel wall". In 2007, Majesky summarized the embryologic diversity of the arterial tree in an elegant review (115). Leroux-Berger *et al.*(116) provided evidence that differences in the propensity of VSMCs from different aortic locations to calcify depend on their embryologic origin. Interestingly, they found a higher propensity of VSMCs from the neural crest derived aortic arch to calcify in response to phosphate in comparison with VSMCs from the mesodermal derived abdominal aorta. The difference in calcification was paralleled by a difference in ALP activity, which increased earlier in the neural crest derived VSMCs compared to the mesodermal derived VSMCs.

Histologic studies confirm the difference in the propensity of arteries from different locations to develop intimal or medial calcification. A Japanese autopsy study of 117 patients with coronary artery disease and different stages of CKD identified only six cases with media calcification of the coronary arteries (117). All of the affected patients had CKD stage 4-5D, but they represented only 15% of all patients with CKD stages 4-5D in that study. On the other hand, 61 – 96% of patients with CKD stage 5D had intimal calcification of coronary arteries, and the prevalence in some or all coronary arteries was significantly higher in CKD stages 3-5D compared to patients without CKD. Reduced kidney function, age and diabetes were risk factors for intimal calcification. Gross *et al.* (118) found medial calcification in distal coronary arteries of CKD patients whereas intimal calcifications were present in more proximal coronary arteries. Moe *et al.* (119) found media calcification of the inferior epigastric artery in 12 of 39 CKD stage 5D patients, whereas intimal calcification was present in only one patient. Sakata *et al.* (120) found exclusively medial calcification in the internal thoracic artery in 13 of 17 diabetic and in 2 of 12 non-diabetic patients without CKD, undergoing coronary artery bypass grafting.

Medial as well as intimal calcification is increased in patients with CKD (118, 121) and is associated with increased CV morbidity and mortality (122). For many years, the deposition of calcium salts in vessels and soft tissue of CKD patients was thought to be a passive process, driven by an elevated calcium × phosphate product (123-125). Recent research efforts have widened our understanding of the pathophysiology of vascular calcification. Today we are aware of the high complexity of the calcification process and its active control both in tissues where calcification is functional and in tissues where it needs to be inhibited. A number of cellular, regional and systemic stimulators and inhibitors of vascular calcification have been identified (**Figure 8**), many of them are reported to be independent predictors of mortality and CV morbidity. We also know that the calcification processes in the intimal layer and the muscular media of arteries are not identical with respect to pathophysiologic mechanism (126).

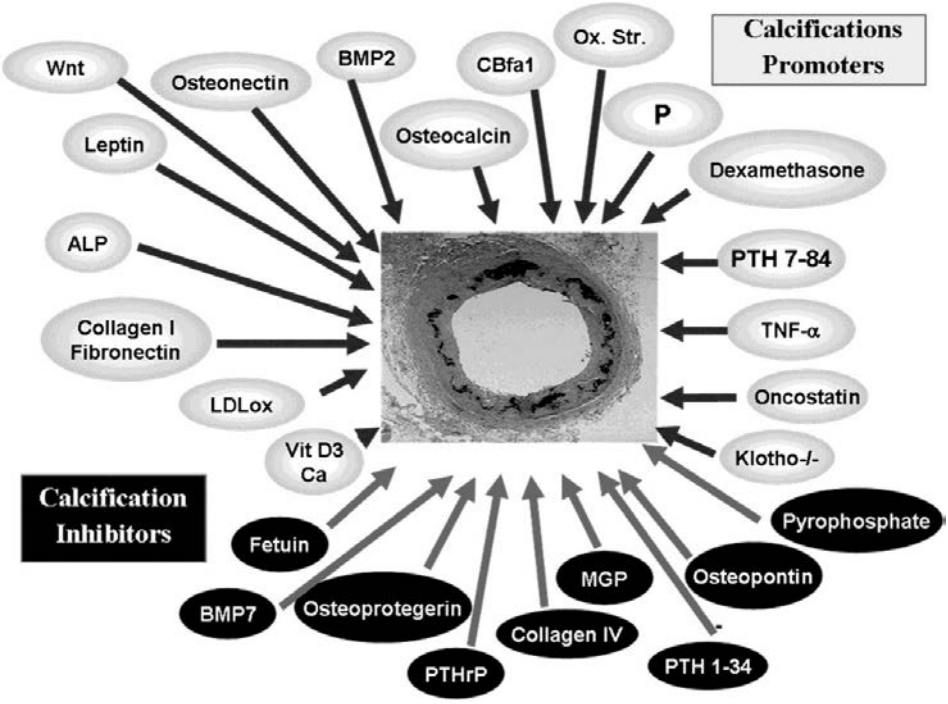

Figure 8. Promoters and inhibitors of vascular calcification . ALP, alkaline phosphatase; Ca, calcium; LDLox, oxidized low-density lipoprotein; MGP, matrix GLA protein; P, phosphorus; PTHrP, parathyroid hormone-related protein; TNF-α, tumour necrosis factor-alpha; Vit D3, calcitriol. Printed with permission (127).

Intimal calcification is associated with the formation of atherosclerotic plaques. In addition to the above mentioned tissue-specific predilection for atherosclerotic lesions, locations of low or oscillatory endothelial shear stress, such as abdominal aorta, coronary arteries, ilio-femoral arteries, and carotid bifurcations are typically the most affected (128). The atherosclerotic lesion develops from an early intimal lipid accumulation through an inflammatory phase of macrophage infiltration to the development of a fibroatheroma with a lipid-rich necrotic core and a fibrous cap (129). The extracellular matrix of atheromas is rich in collagen type I, produced by VSMCs, which have undergone transition from a contractile to a synthetic phenotype (130). Several different origins of these synthetic VSMCs have been suggested, among them VSMCs (131) or multipotent stem cells (132) which have migrated from the medial or adventitial layers of the artery and circulating smooth muscle progenitor cells (133), although the existence of the latter has been disputed (134). Synthetic VSMCs play a central role in atheroma calcification, not only through the production of extracellular matrix and proteins that actively regulate calcification, but also by shedding ALP-rich MVs, which are the nidus for early hydroxyapatite formation (135). VSMCs that undergo apoptosis release apoptotic bodies, which also can initiate matrix calcification (136). Recently, New *et al.* (137) described a calcification process which involves macrophage-derived matrix vesicles, much like the processes seen in osteoblast or VSMC associated calcification. These could be responsible for microcalcifications in thin cap fibroatheromas (TCFA), which contain few or no VSMCs, but are heavily infiltrated by phagocytes. Microcalcifications in TCFAs are associated with an increased risk of plaque rupture (138).

Medial calcification is, in contrast to intimal calcification, not associated with lipid accumulation or macrophage infiltration (139). Instead it is associated with elastin disruption and increased collagen type I synthesis in the medial layer (140). It is predominantly found in diabetes, CKD and old age (141-145). Medial calcification can be experimentally induced by CKD and high calcium and phosphate diet (140), but several clinical studies did not find associations between histologically proven medial calcification with laboratory parameters of CKD-MBD in humans (119, 146). On the other hand, London *et al.* (147), who classified the type of calcification (medial vs. intimal) based on a combination of X-ray and ultrasound examinations, found an association of arterial intimal calcification in dialysis patients with older age, diabetes, hypertension, smoking, more frequent history of CV disease, and a higher mortality rate, whereas medial calcification was associated with younger age, longer dialysis vintage, higher frequency of parathyroidectomy and lower BMI. The diagnostic accuracy of

non-invasive methods to differentiate between medial and intimal calcification can be questioned, since no non-invasive method can directly identify intimal or medial calcifications. London's elaborate method was not validated by histologic studies, and when Schlieper *et al.* (146) compared plain X-rays of the pelvis with histologic analysis of pelvic arteries they found a discrepancy between both methods with respect to the diagnosis of medial calcification.

1.7.4 The role of the vascular smooth muscle cell in vascular calcification

VSMCs play a central role in both medial and intimal calcification. As mentioned above, this involves the transition of VSMCs from a contractile to a synthetic phenotype, with the down-regulation of muscle specific proteins, such as α-actin and the up-regulation of osteoblast-specific proteins (139, 148). This transition is driven by osteoblast-specific transcription factors RUNX2 and muscle segment homeobox 2 (MSX2), which both seem to be stimulated synergistically by phosphate (149), but also can be activated independently of each other (150-153). Msx2 is further stimulated by oxidized LDL (149), TNFα, and BMP2 (150), RUNX2 by glucose (152). Tyson *et al.* (154) detected MSX2, but not RUNX2 in the intima of human endarterectomy specimen, using *in-situ* hybridization.

In addition to the osteoblastic transition, VSMCs can adapt a chondrocyte-like phenotype in both intimal and medial calcification (155, 156). This endochondral ossification is initiated by the chondrocyte-specific transcription factor SOX9 in VSMCs and is associated with the synthesis of a typical cartilage matrix consisting of collagen II and proteoglycans (157). Mice lacking the mineralization inhibitor matrix GLA protein (MGP), which is highly expressed in VSMCs of the arterial media and in proliferating chondrocytes, demonstrate a spontaneous endochondral mineralization of the medial layer of arteries (158). Also the deficiency of another mineralization inhibitor, PPi, which is the main substrate for ALP, is associated with an increase of SOX9 and chondrocyte specific proteins in arterial medial calcification (159). Finally, Neven *et al.* (160) hypothesized, that the transdifferentiation of VSMCs to chondrocytes, rather than to osteoblasts, is the dominating pathophysiologic process in arterial medial calcification of CKD.

The mineralization process is orchestrated by numerous promotors and inhibitors (**Figure 8**). In CKD, the balance of these factors is disturbed towards a predominance of promotors over inhibitors of mineralization. ALP is an important promotor of mineralization, and ALP activity increases early in the transition of VSMCs towards an osteblastic phenotype (139, 161, 162). Shioi *et al*. demonstrated that the ALP type expressed in calcifying VSMCs is BALP (163). The mechanisms by which BALP promotes VSMC calcification are the same as in bone mineralization; i.e. the hydrolysis of the calcification inhibitor PPi (164, 165) and, more speculative, the hydrolysis of the calcification inhibitor phosphorylated OPN (166). Lomashvili *et al.* demonstrated recently that circulating PPi has an inhibitory effect on vascular calcification (167). Circulating PPi levels are reduced in CKD (168), and systemic treatment with PPi has an inhibitory effect on uremic vascular calcification (169). The enzymatic differences of the BALP isoforms with respect to PPi and other substrates, described by our group (71), suggest putative differences in the significance of the BALP isoforms for the process of vascular calcification.

1.8 ALP and BALP – clinical relevance

1.8.1 Mortality

ALP has in recent years emerged as a risk factor for all-cause mortality in different populations. This focus on ALP and mortality is new. A meta-analysis of the relationship of total ALP, mortality and cardiovascular disease in non-CKD patients, published in 2014, identified 24 studies in PubMed and Embase between inception and December 2013 (170). All of these studies were published during the last decade. The authors of this meta-analysis found an independent positive relationship of ALP with all-cause mortality with a relative risk of 1.14 and 1.57 for patients with ALP 70-90 UI/L and >90 UI/L respectively, compared with patients with ALP <70 UI/L. A similar relationship existed with CV mortality. The association of ALP with mortality was independent of kidney function and preexisting CV disease (170). Tonelli *et al.* (171) demonstrated that the combination of increased phosphate and increased ALP, compared with each factor alone, was associated with an increased mortality risk in the general population. In a large cohort of the general North-American population, Filipowicz *et al.* (172) described associations of ALP with C-reactive protein (CRP) and mortality. These associations did not exist for BALP. The authors speculated that

ALP derived from non-skeletal sources was responsible for the associations of ALP with CRP and mortality. Since their results were corrected for liver function, they excluded liver ALP and suggested instead activated neutrophil leukocytes as the source for this non-skeletal ALP (172).

In CKD, numerous longitudinal and cross-sectional studies have demonstrated an association of ALP with mortality in different CKD populations (78, 173-188). This association seems to be independent of PTH, phosphate, and calcium (174, 176). The group of Kalantar-Zadeh demonstrated an association of ALP with all-cause mortality in a population of >50 000 North-American dialysis patients (174). This association was found both for baseline ALP, determined at a single time point, and for its trend during the whole observational period of two years, and was independent of a large number of confounders, including liver function, vitamin D treatment, and the malnutrition, inflammation, cachexia syndrome (MICS). The relationship of ALP and all-cause mortality was almost linear, with a low risk for low ALP levels and a high risk for high ALP levels, whereas phosphate, calcium and PTH demonstrated U-shaped associations with higher risk for both low and high ALP levels. When the observational period was extended to three years (175) it became clear, that the increasing survival advantage of baseline ALP below 100 U/L was lost when looking at time varying values over the entire observation period. Furthermore, patients whose ALP levels decreased during the observation period experienced no survival benefit, whereas patients with increasing ALP levels during the 3-year study period had an increased risk of all-cause mortality. The authors hypothesized that an association of low ALP with adynamic bone could explain the loss of survival benefit at low ALP levels. A cut-off limit of ALP > 120 U/L was suggested to be associated with increased mortality risk, but it was pointed out that the formulation of a treatment target should be based on randomized prospective trials (175).

Time-averaged ALP during dialysis treatment was also associated with mortality, but not with graft function, after kidney transplantation in a large (>10 000 patients) cohort of hemodialysis patients (189). No associations of pre-transplant PTH with mortality or post-transplantation graft function were found in that study. In a prospective study in prevalent kidney transplant recipients, ALP and gamma-glutamyl transferase, but not BALP, were independently associated with mortality (190). The authors speculated that their findings could be explained by vascular oxidative stress. No correction was made for liver function tests, but separate analyses of these tests showed no associations with mortality.

Total ALP has been associated with mortality (177, 187) and CKD progression (187) in CKD stages 3-4. In all studied populations, the association of ALP with mortality was found for ALP activities within the high normal range for the analytical method used. The use of different analytical methods for total ALP and BALP has been considered a limitation by many investigators and could be a reason why expert committees until now do not recommend specific therapeutic target levels for ALP (2).

The use of BALP in clinical routine is much less common, but several studies demonstrate an independent association of BALP with mortality in more advanced CKD stages (75, 76). Drechsler *et al.* (75) described a stronger association of BALP than of total ALP with short-term mortality in 800 stable dialysis patients. BALP was also associated with long-term mortality, but the association was weaker. Compared with patients with low BALP and low PTH, patients with high BALP and low PTH had an increased short-term mortality risk. These patients demonstrated signs of wasting. Kobayashi *et al.* (76) found an independent association of BALP, but not PTH or other metabolic bone markers, with all-cause 70-month mortality in male Japanese dialysis patients. BALP showed a much lower variability than PTH and other metabolic bone markers. Total ALP was determined in this study, but was not included in any prognostic analyses. Beige *et al.* (188) found a significant positive association of total ALP with mortality in dialysis patients, which could not be reproduced for BALP. On the contrary, when total ALP was excluded from the Cox regression analysis BALP was positively associated with better survival. Several studies demonstrated a stronger association of ALP or BALP with short-term survival than with long-term survival (75, 173, 179), suggesting that the pathophysiologic process linking total ALP or BALP to mortality is of a rather short-lived nature.

1.8.2 Cardiovascular disease

In addition to the association of ALP and BALP with all-cause and CV mortality in numerous studies, several studies have also demonstrated an association between increased ALP and CV disease. Increased total ALP was associated with acute myocardial infarction (MI) in a recent case control study on patients who experienced a first acute MI (77). Further characterization identified BALP as the main source for the increased ALP activity. Total ALP was associated with CV mortality in a large population of patients with coronary artery disease who

underwent coronary angiography (191). In contrast to the findings by Filipowicz *et al.* (172), no association of total ALP and CRP with mortality was found in this study and the association of total ALP with mortality was independent of CRP and classical CV risk factors. Higher total ALP activities predicted stent thrombosis, and the authors speculated that this could be an effect of impaired endothelialization in calcified coronary arteries. Tonelli *et al.* (171) described an association of increased baseline total ALP with the development of heart failure, but not MI, stroke, or CV mortality, in patients without CKD who had experienced a MI. The above mentioned meta-analysis by Li *et al.* (170) found ambiguous results for the association of total ALP with CV disease in individuals with preserved kidney function. Total ALP was independently associated with CV mortality, but the association with coronary heart disease or all CV events disappeared after full adjustment. Thus, the association of total ALP with CV disease in non-CKD populations is variable and might be influenced by the type of CV disease.

In a prospective study on patients with CKD stages 1-5, not on dialysis, Fahrleitner-Pammer *et al.* (78) described a positive association of BALP with CV events, and a negative association of the bone resorption marker tartrate-resistant acid phosphatase isoform 5b (TRACP5b) with CV events, whereas no such associations was found for PTH. The authors speculated that BALP was derived from vascular calcification rather than from bone, since TRACP5b is not produced in vascular tissue in any quantities that could influence the activities in circulation, thus, low levels of circulating TRACP5b were interpreted as an indication of reduced bone turnover.

1.8.3 Fracture

Strong evidence exists for a link between reduced bone mineral density (BMD) and increased fracture risk in the general population (192), and fracture risk prediction can be further improved by the use of FRAX, the World Health Organization's clinical fracture risk assessment tool (193, 194). The role of bone turnover markers as predictors of fracture risk is less well studied. A recent meta-analysis of the role of different bone markers as predictors of BMD and asymptomatic vertebral fractures found only weak associations, which were best documented for ALP, OC, and the carboxy-terminal cross-linking telopeptide of type I collagen (CTX) (195). The authors suggested that these markers could be used to identify

patients at risk, who should be further examined by dual-energy X-ray absorptiometry (DXA). Tamaki *et al.* (79) studied vertebral fracture risk in postmenopausal women randomly selected from the general population of several Japanese municipalities. The authors found an average yearly fracture incidence of 1.6% and BALP as well as urinary total and free deoxypyridinoline predicted fracture incidence independently of BMD. These results were corrected for several confounders, but not for kidney function.

In CKD, fracture risk prediction is complicated by the influence of CKD-MBD on bone, and the ability of established diagnostic criteria, such as the BMD based WHO definition of osteoporosis or the FRAX algorithm, to predict fracture risk is limited (196). It is therefore of interest to identify additional predictors, such as circulating bone markers, that can improve fracture risk assessment in the CKD population. Unfortunately, several bone markers used in the clinical evaluation of patients with bone disorders are dependent on kidney function and their value in CKD can be questioned. ALP, intact type I procollagen intact amino-terminal propeptide (PINP) (197), and TRACP5b are the only bone markers whose serum concentrations are not influenced by renal excretion and their role as predictors of fracture risk in CKD is therefore of special interest. In a large (N = 180 000) 1 year prospective study of Japanese dialysis patients the incidence of hip fracture was 1% (184). Increased ALP was independently associated with the incidence of hip fractures. Another Japanese study found an average yearly incidence of 2% for any type of fracture in hemodialysis patients during an observational period of 5 years (80). BALP had a higher diagnostic accuracy for the incidence of fracture in comparison with PTH or BMD at any site. In the Dialysis Outcomes and Practice Patterns Study (176) the risk for hospitalization due to fracture was 42% and 67%, respectively, for dialysis patients with >1 to 1.4 × upper limit of normal (ULN) and > 1.4 × ULN of baseline ALP. Mean observational time was 1.6 years. Higher ALP was associated with a history of hip fractures in hemodialysis patients (198), however, the authors did not correct for any confounders. A cross-sectional study by Atsumi *et al.* (199) found a 20.9% prevalence of vertebral fractures in hemodialysis patients. The highest fracture prevalence was found in patients with high ALP and low PTH. Higher ALP was associated with increased fracture risk across all tertiles of PTH.

2. AIM / HYPOTHESIS

2.1 General aim and hypothesis

The general aim of the proposed studies was to further investigate the clinical utility of the BALP isoforms for diagnosis and monitoring of CKD-MBD, and as possible future therapeutic targets for the treatment of vascular calcification, in different stages of CKD.

I hypothesize that the BALP isoforms will be clinically useful to detect and monitor the development of renal osteodystrophy in different stages of CKD. I further hypothesize that BALP isoforms are involved in the calcification of VSMCs and that calcification of the vascular muscle layer is associated with specific changes in the serum BALP isoform pattern.

2.2 Specific aims

1. To study the relationship between the BALP isoforms, other biochemical markers of bone turnover, and BMD in patients with different stages of predialytic CKD.
2. To study the ALP isoforms in calcifying and non-calcifying phenotypes of cultured human aortic smooth muscle cells (HASMCs).
3. To study the relationship between the BALP isoforms and bone pathology assessed by bone histomorphometry in patients with CKD on maintenance dialysis treatment.
4. To study the association between the BALP isoforms, vascular calcification and arterial stiffness in CKD patients undergoing maintenance dialysis treatment.

2.3 Specific hypotheses

1. Changes in BALP isoform pattern in patients with mild to moderate CKD correlate with changes in the expression of other markers of bone metabolism and BMD.

2. Vascular smooth muscle cells demonstrate different ALP isoforms activities, depending on their phenotype.

3. Disturbed osteoblastic function in renal osteodystrophy is associated with changes in serum BALP isoform patterns, including the appearance of the novel BALP isoform B1x.

4. Vascular calcification and arterial stiffness are associated with changes in serum BALP isoform pattern in patients with CKD on maintenance dialysis treatment.

3. MATERIALS AND METHODS

3.1 Ethical considerations

Study I was a cross-sectional study. The study was approved by the regional ethics committee, Linköping University, Sweden, 02-214. Study II was an experimental study using commercially available HASMCs. Ethical approval was not required for this study. Study III was a cross-sectional diagnostic-test study, which was approved by the institutional review board, Lexington, KY, USA, 04-0295-P2B. Study IV was a prospective, multicenter cohort study with approval from the regional ethics committee, Uppsala University, Sweden, 2004:M-326.

3.2 Study design, patients and control subjects

Patients in study I (n=46) were recruited from the nephrology outpatient clinic at Linköping University Hospital between 2003 and 2005. Patients were included during winter semi-annum to avoid potential influence of seasonal variations of vitamin D and PTH. Patients with an estimated creatinine clearance of ≤60 mL/min/1.73 m^2 were eligible for inclusion. Exclusion criteria were age < 18 years, mental retardation and chronic treatment with lithium or systemic corticosteroids. Fifteen females and 31 males, aged 28–86 years, with the following underlying nephropathies were included: Nephrosclerosis (n = 13), diabetic nephropathy (n = 12), chronic glomerulonephritis (n = 11), chronic tubulo-interstitial nephritis (n = 5), polycystic kidney disease (n = 1), and unknown (n = 4). Estimated GFR was between 11 and 59 mL/min/1.73 m^2. Patients were categorized according to the National Kidney Foundation Kidney Disease Outcomes Quality Initiative (KDOQI) classification of CKD (200): Stage 3 (n = 23), stage 4 (n =15), and stage 5 (n = 8). Thirty-eight percent of the patients were treated with alphacalcidol. Patients were compared with a historical control group comprising 153 healthy individuals (108 females and 45 males) with age range 21–90 years (51).

For study III, 20 patients with low and 20 patients with non-low bone turnover were selected from the Kentucky Renal Osteodystrophy Registry. Inclusion criteria for this study were: dialysis duration at least three months, age 18 years or older, and histomorphometric evidence

of low or non-low bone turnover, and exclusion criteria were systemic illnesses or organ diseases that may affect bone (except diabetes mellitus type 1 or type 2), treatment during the six months before inclusion with drugs that may affect bone metabolism (e.g., teriparatide, bisphosphonates, cinacalcet, selective estrogen receptor modulators, and others except for vitamin D), and chronic alcohol and/or drug addiction. Primary kidney diseases were diabetic nephropathy (n = 7), hypertension (n = 6), polycystic kidney disease (n = 4), obstructive nephropathy (n = 3), glomerulonephritis (n = 2), amyloidosis (n = 1), and unknown (n = 17). Patients were hemodialyzed three times a week. The Kentucky Renal Osteodystrophy Registry is in accordance with the Declaration of Helsinki and approved by the institutional review board.

Patients participating in study IV were recruited from four Swedish dialysis centers participating in the Calcification Outcome in Renal Disease (CORD) study. A detailed description of the inclusion process for the CORD study is reported elsewhere (201). Briefly, patients 18 years of age or older and treated by peritoneal dialysis (PD) or maintenance hemodialysis (HD) for at least 3 months were enrolled after selection by a computer algorithm, which guaranteed the selection of a representative sample of all patients screened at each dialysis center with regard to gender, age, diabetes, time on dialysis, and smoking status. Exclusion criteria were life expectancy of less than 6 months by judgment of the local investigator and conditions in which pulse wave analysis was technically impossible or unreliable (severe peripheral vascular disease, cardiac arrhythmias, and amputations). All patients gave their informed consent in writing. The study was in accordance with the Declaration of Helsinki and approved by the local research ethics committee. During the study period of 2 years, patients were followed prospectively for the occurrence of either a first nonfatal CV event (coronary revascularization procedures, MI, stroke, or transient ischemic attack) or death. When multiple CV events occurred in the same patient, only the time to the first event was analyzed.

3.3 Cell culture

In study II, HASMCs (Cascade Biologics Inc., Portland, OR, USA) were cultured to confluence in growth medium (medium 231, Cascade Biologics Inc.), containing 5% smooth muscle growth supplement (Cascade Biologics Inc.), with final concentrations of 4.9% fetal

bovine serum (Sigma, St. Louis, MO, USA), 1.2 mmol/l calcium and 2.0 mmol/l phosphate. For the experiments, cells were cultured for 30 days in calcification medium containing growth medium supplemented with additional $CaCl_2$ to a final calcium concentration of 2.0 mmol/l and β-glycerophosphate (Sigma) to final phosphate concentrations of 5 or 10 mmol/l or in growth medium as above. For the inhibition of ALP, tetramisole (Sigma) was added to the medium at a final concentration of 0.1 mmol/l. Medium was changed three times per week. All experiments were performed three times independently from each other and each experiment was performed in triplicates.

3.4 Visualization of calcification in cell cultures

In study II, calcification of HASMCs was determined by von Kossa staining. Briefly, cells were fixed with 95% ethanol after the removal of culture medium and exposed to 5% silver nitrate for 30 min in darkness. After rinsing twice with distilled water, cells were dyed with eosin (Sigma) for 30 s and rinsed again. Calcification was detected by light microscopy.

3.5 Quantification of calcium content in cell cultures

For the quantification of calcium accumulation in the cell layer of HASMCs in study II, cells were decalcified with 0.6 mol/l HCl for 24 h. The calcium concentration in the supernatant was determined colorimetrically at 650 nm, using the Arsenazo III method (Randox Laboratories Ltd., Crumlin, UK) after the addition of 1 ml of Arsenazo III dye to 15 ml of cell lysate. Calcium concentrations were related to protein content in the cell layer, determined by the BCA protein assay kit (Pierce, Rockford, IL, USA).

3.6 Blood and urine sampling and routine laboratory

In study I, GFR was determined by single-sample iohexol clearance (n = 42) or creatinine clearance calculated from a plasma and 24-h urine sample (n = 4) according to routine laboratory methods. Blood and 24-h urine samples were obtained at the time of measurement of iohexol clearance. In studies III and IV, blood samples were obtained prior to a dialysis

session in hemodialysis patients. In study III, blood samples were obtained at the time for the determination of pulse wave velocity (PWV). GFR was not determined in studies III and IV, since all patients were on maintenance dialysis treatment. Serum samples were stored at −70˚C prior to analysis. In study IV, serum levels for albumin, calcium, phosphorus, and PTH, were determined by local routine laboratory methods.

3.7 Purification of GPI-PLD

The HPLC analysis of BALP isoforms in cell lysates requires pre-treatment with a GPI-phospholipase to separate ALP from its GPI anchor. We therefore purified GPI-PLD from commercially available human serum (Sigma). In order to remove insoluble materials, serum was incubated with 9% polyethylene glycol for 1 h and centrifuged at 2,600 g for 15 min. The supernatant was filtered through a 0.22-micrometer filter, centrifuged through a Vivaspin 20 concentrator, MWCO 300 kDa (Vivascience AG, Hannover, Germany), concentrated with Aquacide (Calbiochem, Merck Millipore, Billerica, MA, USA), and dialyzed in 'Buffer A' (i.e., 50 mmol/l Tris, 10 mmol/l NaCl, 2 mmol/l $CaCl_2$, pH 7.75). The resulting solution was applied to a DEAE Sepharose (Sigma) column, which was eluated with 0.010–0.500 mol/l NaCl (total volume 500 ml) in Buffer A. Each fraction was analyzed for GPI-PLD activity (45). Fractions containing GPI-PLD were pooled and went through a second cycle of concentration with Aquacide and dialysis in Buffer A. For the final purification step the solution was applied to a Concanavalin A (Sigma) column and eluated with 0–1.0 mol/l glucose in Buffer A. Again, fractions containing GPI-PLD were pooled, concentrated with Aquacide, dialyzed with Buffer A and stored at –70°C.

3.8 Preparation of GPI anchor free ALP from cell lysates

After removal of culture medium, cells were washed three times with phosphate-buffered saline, solubilized with 1% Triton X-100 (Sigma) in 0.9% NaCl and centrifuged. Total ALP activity was analyzed in the supernatants by a kinetic assay in a 96-well microtitre plate format (202). For the preparation of GPI anchor-free BALP isoforms supernatants from each triplicate of the cell experiments were pooled. Purified GPI-PLD with an activity of approximately 2,000 U/ml was added to a final concentration of 25% together with 0.01%

NP-40 (nonylphenoxypolyethoxyethanol, Sigma) and 5 µmol/l zinc acetate and incubated for 16 h at 37°C with constant shaking, in order to obtain soluble (anchor-depleted) preparations of ALP.

3.9 Quantification of BALP isoforms by HPLC

The BALP isoforms B/I, B1x, B1, and B2 in serum (study I,III, IV) or in GPI-PLD treated cell lysates (study II) were determined by a previously described HPLC method (43, 203). In brief, the BALP isoforms were separated on a weak anion-exchange column using a gradient of 0.6 mol/L sodium acetate, SynChropak AX300 (250 × 4.6 mm I.D.) (Eprogen, Inc., Darien, IL, USA). For the determination of the BALP activities, the column effluent was mixed online with 1.8 mmol/L p-nitrophenylphosphate in a 0.25 mol/L diethanolamine buffer at pH 10.1. The ensuing reaction was developed in a packed-bed postcolumn reactor at 37°C and the formed product (p-nitrophenol) was directed on-line through a detector set at 405 nm. BALP isoform activities were quantified by integrating the area under each peak in relation to total ALP activity, which was determined by a kinetic assay in a 96-well microtitre plate format (202). This BALP isoform HPLC method has intra- and inter-assay coefficients of variance (CVs) of 5% and 6%, respectively, for each of the BALP isoforms (43). Assay performances are for the B/I isoform (detection limit, 0.01 µkat/L; measuring upper limit, 1.70 µkat/L), B1x isoform (detection limit, 0.02 µkat/L; measuring upper limit, 1.00 µkat/L), B1 isoform (detection limit, 0.02 µkat/L; measuring upper limit, 4.70 µkat/L), and B2 isoform (detection limit, 0.03 µkat/L; measuring upper limit, 9.50 µkat/L. Samples with activities above the assay range were diluted in appropriate assay diluent as necessary. The BALP isoform reference intervals for healthy individuals are: B/I, 0.04-0.17 µkat/L; B1, 0.20-0.62 µkat/L; and B2, 0.34-1.69 µkat/L (51).

3.10 Biochemical determinations of bone related proteins in serum

In study I serum intact PTH was determined by a two-site immunoradiometric assay (Nichols Institute, San Clemente, CA, USA), with a reported reference interval of 12–65 ng/L for healthy adults (204). This method detects the whole PTH molecule (PTH 1-84), as well as at least one known PTH fragment (PTH 7-84). Serum PTH 1–84, was measured with an

immunochemiluminometric assay on a Nichols Advantage analyzer (Nichols Institute) (205). The reported reference interval for healthy adults is 7–50 ng/L. Serum 25(OH) vitamin D was measured by the Nichols Advantage automated chemiluminescence protein-binding immunoassay (Nichols Institute) (206). Serum high-sensitivity CRP was analyzed by an immunoturbidimetric assay on the ADVIA 1650 chemistry system (Siemens Medical Solutions Diagnostics AB, Mölndal, Sweden) with a lower limit of detection of 0.12 mg/L (207). Serum PINP was measured by radioimmunoassay (Orion Diagnostica, Oulunsalo, Finland) (208). Type I collagen degradation was determined by the serum CrossLaps enzyme-linked immunosorbant assay (Nordic Bioscience Diagnostics A/S, Herlev, Denmark, which measures CTX (209, 210). Serum TRACP5b was assessed by a solid-phase immunofixed enzyme activity assay (SBA Sciences, Oulu, Finland) (211).

In Study III serum PTH levels were determined by the electrochemiluminescence Elecsys PTH assay (Roche Diagnostics, Indianapolis, IN, USA) with a reference interval for healthy individuals of 18-74 pg/mL. Serum BALP levels were assessed with the MicroVue BAP enzyme immunoassay (Quidel Corp., San Diego, CA, USA). The reference interval for healthy individuals is 11.6-42.7 U/L.

3.11 BMD measurements

BMD was measured at the dominant hip and at the distal 1/3 radius of the dominant arm by DXA using a Lunar Prodigy densitometer (GE Healthcare, Chalfont St. Giles, UK). Z-scores and T-scores were calculated based on the National Health and Nutrition Examination Survey reference population.

3.12 Bone biopsy

In study III, bone biopsies were obtained from the iliac crest close to the anterior superior spine. Prior to biopsy, patients were given a two day course of oral tetracycline hydrochloride 500 mg twice daily and another course of 300 mg declomycin twice daily for four days, separated by a 10-day tetracycline-free interval. Bone biopsies were performed four days after the last declomycin intake.

3.13 Mineralized bone histology and bone histomorphometry

For the histomorphometric analysis undecalcified bone samples were fixed in ethanol, dehydrated and embedded in methyl methacrylate (212). Serial sections of 4- and 7-µm thickness were cut with a heavy duty microtome (Model HM360, Microm, Walldorf, Germany) and stained with the modified Masson-Goldner trichrome stain (213) for determination of static parameters of bone formation, bone resorption, and bone structure. Aluminum deposition was assessed by solochrome azurine and aurin tricarboxylic acid stains (214, 215). Measurements of wall thickness and dynamic parameters of bone formation and mineralization were performed by phase-contrast and fluorescence light microscopy in unstained sections. Bone histomorphometry was performed using the Osteoplan II system (C. Zeiss, New York, NY, USA) (216, 217). Histomorphometric parameters in study III comply with the recommendations of the ASBMR (101, 102). In cases of labelling escape (patients with very low bone turnover with only single labels present in cancellous bone), mineral apposition rate (MAR) was assigned a value of 0.1 µm/day. This approach is based on recommendations by Hauge *et al*. (218) and the nomenclature committee of ASBMR (102).

3.14 Determination of PWV

In study IV, PWV was measured by sequential recording of electrocardiogram-gated carotid and femoral artery pressure waves using applanation tonometry (SphygmoCor version 7, AtCor Medical, Sydney, Australia). Patients were examined in the supine position, either before a hemodialysis session or after drainage of the abdominal cavity in PD patients. For the determination of PWV the path length (the distance between sternal notch and carotid recording site minus the distance between sternal notch and femoral site) was divided by the transit time of the pulse wave (m/s). Measurements were performed at baseline, and after 1 and 2 years during the follow-up period.

3.15 Determination of abdominal aortic calcification score

In study IV, abdominal aortic calcification (AAC) scores were determined at baseline and after 2 years of follow-up by a semi-quantitative analysis of a plain lateral lumbar x-ray

including the aorta (219). All x-rays were scored by a single investigator who was blinded to patient data.

3.16 Statistical analysis

Statistics were calculated using SPSS 11.5.1 – 21.0.0.0 for Windows (IBM SPSS Inc., Chicago, IL, USA). A critical significance level of 0.05 was chosen. Results are expressed as mean ± standard error (SE) for normally distributed data and as median with 95% confidence intervals (CI) for non-normally distributed data. In study III, data are presented as median and interquartile. For normally distributed data paired Student's t test was used for paired comparison and multiple comparisons were performed using ANOVA with Fisher's least significant difference test as post hoc analysis. When data was not normally distributed, differences between two groups were calculated using the Mann–Whitney U test for continuous variables and χ^2 statistics for discrete variables. Kendall's tau rank correlation coefficient was chosen for calculation of nonparametric correlations. For the multiple regression analyses in study I, diabetes and gender were treated as dummy variables. In study II, differences between groups for the effect of calcification medium on BALP isoform activities were calculated by Friedman's two-way analysis of variance by ranks. In study III, receiver operator characteristic (ROC) curves and areas under the curve (AUC) were obtained to compare the ability of B1x, PTH and BALP to diagnose low osteoblast activity determined by MAR (cut-off point lower limit of normal 0.36 μm/day) and high bone turnover, i.e., high number of osteoblasts (cut-off point > 200 cells/100 mm bone perimeter). In study IV, independent determinants of baseline AAC score and PWV were identified by multiple regression analyses. Mixed model regression analysis was used to identify determinants of variation of PWV and AAC score over time with visits, smoking status, body mass index (BMI), PTH, ALP, and B1x as covariates. Cox regression analyses were performed to determine independent predictors of the combined outcome of all-cause mortality or a first CV event with age, ALP, B1x, PTH, AAC score, and PWV as covariates.

4. RESULTS AND DISCUSSION

4.1 Study I

Mineral and bone disorders are ubiquitous findings in patients with CKD and are associated with increased mortality, CV disease and fractures. To prevent and treat these serious complications, a number of strategies have been developed; still, complication rates remain high and several large randomized controlled treatment trials in the field of CKD-MBD have failed to improve survival or reduce CV complications (220-223). It can be speculated that this shortcoming of trials aimed at single risk factors could be due to the complexity of CKD-MBD. In recognition of this complexity the KDIGO initiative enquired for intensified research in this field in 2006 (104). The group asked specifically for a clarification of the role of biochemical bone markers in CKD-MBD and if bone markers in combination with BMD could be used to better define CKD-MBD and thereby promote the development of improved treatment strategies (104). In order to address this need we designed the current study to investigate the ability of biochemical bone markers to predict BMD in CKD. We chose to study patients in early stages of CKD, since bone disorders have been reported to occur already early during the course of CKD (98, 99), and since research in CKD patients not on dialysis was sparse. A further aim of the current study was to investigate the BALP isoforms during different stages of pre-dialysis CKD and more specifically to assess, whether the novel BALP isoform B1x, which our group had detected recently in serum from dialysis patients, could be identified in serum from patients with earlier stages of CKD. We studied the bone resorption markers CTX and TRACP5b and the bone formation markers BALP, BALP isoforms, and PINP. With the exception of CTX, none of these markers are renally excreted.

BALP isoforms B/I, B1 and B2 were detected in all patients and accounted for 4%, 13%, and 44%, of the total ALP activity, respectively. Distributions of the BALP isoforms in different CKD stages are shown in **Figure 9**.

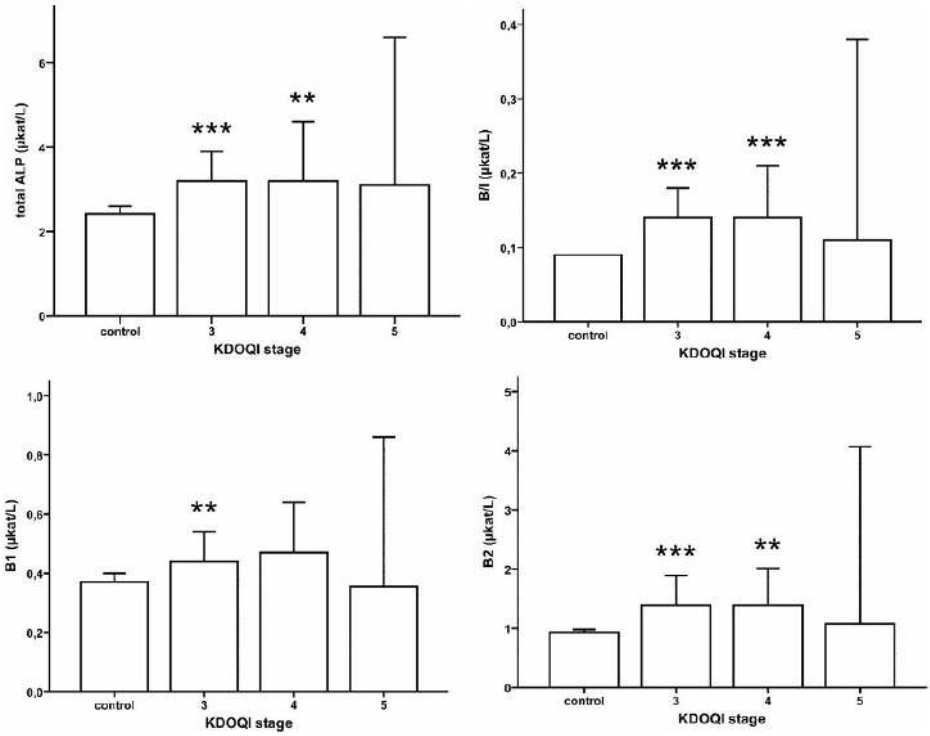

Figure 9. BALP isoforms in different stages of CKD. The box plots show the median serum total ALP and BALP isoform activities in patients with CKD stages 3–5, not on dialysis (n = 46) compared with healthy individuals (n = 153). Error bars demonstrate the 95% confidence intervals. Statistics were calculated using the Mann–Whitney test.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

DXA measurements revealed that none of the patients in the current study had osteoporosis at the total hip, a location with mostly trabecular bone, but 17 patients had osteopenia according to the WHO definition (224). Four patients had osteoporotic and 7 patients osteopenic BMD at the distal 1/3 radius, where cortical bone predominates. Multiple regression analyses using biochemical bone markers and variables with clinical relevance for BMD revealed that total ALP, all three BALP isoforms (B1x was excluded from the analysis due to low patient number), PINP and TRACP5b were significant predictors of total hip BMD (Table 1). None of the biochemical bone markers could predict BMD at the distal 1/3 radius. These site-specific differences of associations of biochemical bone markers with BMD are consistent with previous findings *in vivo* of a greater abundance of BALP in trabecular bone in comparison with cortical bone (44, 50). Several studies have assessed the association of ALP,

BALP, and other biochemical bone markers with BMD at different sites in CKD with variable results (225-229) These inconsistencies can be attributed to a number of confounders, among them differences in CKD stages, localization for and method of BMD measurement, corrections for different co-factors, and different methods for the determination of ALP or BALP. Monoclonal antibodies, widely used in immunologic methods for the quantification of BALP, have been demonstrated to have some cross-reactivity with liver ALP and, furthermore, do not distinguish the different isoforms of BALP (1). The HPLC method, used in the current study for the detection of BALP isoforms, is highly specific and is the only method that can separate and quantify the 4 different BALP isoforms (1),

Table 1. Multiple regression analysis of predictors of total hip BMD

	Model 1 Beta	Model 2 Beta	Model 3 Beta	Model 4 Beta	Model 5 Beta	Model 6 Beta	Model 7 Beta
Gender	0.01	0.15	0.13	0.00	0.09	0.09	0.11
Age	−0.19	−0.04	−0.14	−0.19	−0.23	−0.25	−0.21
Diabetes	0.13	0.16	0.25	0.08	0.04	0.19	0.11
BMI	0.34*	0.23	0.20	0.38*	0.35*	0.24	0.29
GFR	0.16	0.24	0.26	0.14	0.03	0.22	0.16
Intact PTH	−0.22	−0.10	−0.13	−0.25	−0.12	−0.08	−0.16
Total ALP	−0.33*						
B/I		−0.44**					
B1			−0.46**				
B2				−0.31			
CTX					−0.31		
TRACP5b						−0.38*	
PINP							−0.37*

BMD, bone mineral density; BMI, body mass index; GFR, glomerular filtration rate; PTH, parathyroid hormone; ALP, alkaline phosphatase; B/I, B1x, B1, B2, isoforms of bone alkaline phosphatase; CTX, type I collagen carboxy-terminal telopeptide; TRACP5b, tartrate-resistant acid phosphatase isoform 5b; PINP, type I procollagen intact amino-terminal propeptide.
∗P < 0.05; ∗∗P < 0.01.

This study demonstrated, for the first time, the presence of the novel BALP isoform B1x in CKD patients not on dialysis treatment. B1x was detected in 9 out of 46 patients (20%), most of these patients were in CKD stages 4-5. We have previously described the presence of B1x in serum from 60% of patients on chronic dialysis treatment (51), which corresponds well with the finding of B1x in serum from 63% of patients with CKD stage 5 in the study I. B1x has never been observed in serum from healthy individuals or from any other patient

population (43, 54-56, 230). In spite of the more frequent occurrence of B1x in patients with more advanced CKD, no correlation with GFR was observed. The appearance of B1x in serum cannot be explained by reduced renal excretion, since none of the ALP isozymes and isoforms are cleared from the circulation by the kidneys.

In addition to lower GFR, patients with B1x had higher serum phosphate and serum calcium × phosphate product. No differences were observed for intact and biointact PTH, calcium, 25(OH) vitamin D, other markers of bone metabolism, age, and BMD at all examined sites (Table 2). These findings were in contrast with our earlier findings of an association of B1x in serum from adults on maintenance dialysis treatment with lower PTH and biochemical markers of bone turnover (51). Possible explanations for this discrepancy are the wide range of GFR in the current study, the greater variability of PTH, and the relatively low number of patients with B1x in the current study.

Table 2. Differences between patients with and without B1x

	B1x present (n = 9)		B1x missing (n = 37)		P-value
	Median	Min–Max	Median	Min–Max	
Age (years)	66	56–80	71	28–86	NS
GFR (ml/min/1.73 m^2)	14	11–35	33	13–59	0.001
Alphacalcidol treatment	4 patients (44%)		14 patients (38%)		
Calcium ionized (mmol/l)	1.24	1.05–1.53	1.23	1.12–1.37	NS
Phosphate (mmol/l)	1.4	0.9–2.1	1.1	0.7–1.7	<0.05
Total calcium × phosphate	3.5	2.0–5.2	2.5	1.4–3.6	<0.05
Intact PTH (ng/l)	120	0–410	81	6–410	NS
Biointact PTH (ng/l)	75	5–271	62	8–227	NS
25(OH) vitamin D (nmol/l)	26.7	9.0–45.0	32.0	6.0–147.2	NS
CTX (µg/l)	1.36	0.39–1.6	0.65	0.25–2.78	NS
TRACP5b (U/l)	2.6	1.5–5.5	2.7	1.2–5.0	NS
PINP (µg/l)	57	23–100	41	13–115	NS
BMD total hip (g/cm^2)	1.04	0.82–1.1	0.99	0.79–1.34	NS
BMD distal 1/3 radius (g/cm^2)	0.70	0.59–0.9	0.76	0.54–0.92	NS

GFR, glomerular filtration rate; PTH, parathyroid hormone; CTX, type I collagen carboxy-terminal telopeptide; TRACP5b, tartrate-resistant acid phosphatase isoform 5b; PINP, type I procollagen intact amino-terminal propeptide; BMD, bone mineral density.

The associations of B1x in serum with higher serum phosphate and calcium × phosphate product lead us to hypothesize that B1x could originate from vascular calcification since strong evidence was accumulating in support of an association of phosphate and calcium × phosphate product with vascular calcification and mortality (231, 232). Furthermore ALP had been identified as an independent predictor of mortality in CKD and in the general population (171, 174). Experimental studies demonstrated an association of ALP or BALP with vascular calcification (119, 161, 163) and the suppression of VSMC calcification through inhibition of ALP (233). A possible connection between this new putative origin of B1x in serum and our earlier hypothesis of an association with low bone turnover was provided by London *et al.* (234), who described an association of low bone turnover with vascular calcification in CKD patients. Studies II and IV were designed to test this novel hypothesis. Study III addressed our earlier hypothesis of an association of B1x with low bone turnover.

4.2 Study II

Vascular calcification is a common complication of CKD and is associated with increased CV morbidity and mortality (235, 236). Both medial and intimal calcification are increased in patients with CKD (237). In addition to classical risk factors, hyperphosphatemia and hypercalcemia have evolved as well-established risk factors for vascular calcification and mortality in CKD (238). Vascular smooth muscle cells play a central role in the process of vascular calcification by transdifferentiation to an osteoblast-like phenotype (239). A profound increase of ALP activity has been demonstrated in calcifying VSMCs (240). Further characterization of the ALP activity in calcifying VSMCs by immunoassay indicated that these cells express BALP (163). Our aim with study II was to confirm the finding of Shioi *et al.* (163) and to identify the ALP isoforms expressed in calcifying human VSMCs with our HPLC based method, which, in contrast to immunological methods, is free from cross-reactivity with other ALP isoforms (1). Based on our findings in study I of an association of elevated serum phosphate and calcium × phosphate product with the appearance of B1x in serum, we hypothesized that B1x is associated with the calcification of VSMCs (241). To test this hypothesis we cultured HASMCs in calcification medium containing β-glycerophosphate at concentrations of 5 or 10 mmol/L and calcium at a concentration of 2 mmol/l, with or without the specific ALP inhibitor tetramisole (161, 242). We chose β-glycerophosphate as a phosphate donor, since it has been shown to consistently induce ALP when used as a

component of calcification medium for VSMCs (148, 149, 161, 163, 242, 243). Other phosphate donors have had stimulatory (244, 245), absent (164), or even inhibitory (246) effects on ALP activity in calcifying VSMCs. In addition to differences in phosphate donors, these divergent findings could be due to differences in species, cell types, number of passages, length of induction period, and co-stimulation with other procalcific stimulators.

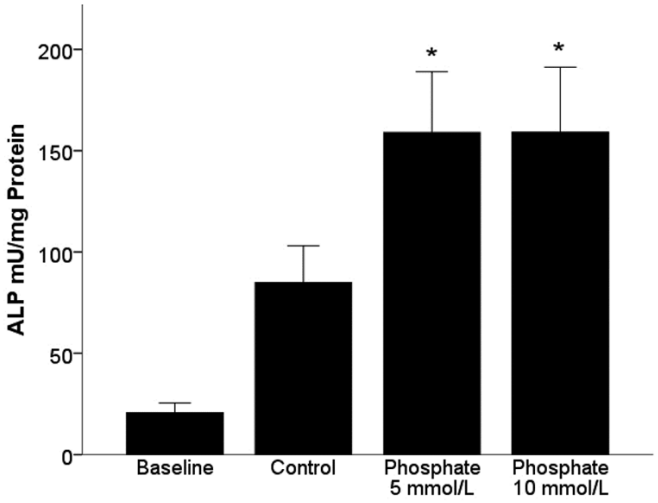

Figure 10. ALP activity in HASMCs. Cells were cultured in growth medium until confluence (baseline) and then cultured for 30 days in growth medium (control) or calcification medium with β-glycerophosphate at indicated concentrations. Data represent the results of three independent experiments, each performed in triplicate.
* $p < 0.01$ compared with control.

In study II, mineralization of HASMCs cultured in calcification medium was paralleled by a two-fold increase of ALP activity in the cell layer (**Figure 10**). The total ALP activity was 21.1 ± 3.9 mU/mg protein (mean ± SD) at baseline. All previously known BALP isoforms were detected in quiescent cells and after stimulation of calcification, including the novel B1x isoform (**Figure 11**). The B2 isoform had the largest relative activity (44%) among the BALP isoforms, whereas the other isoforms demonstrated lower activities: B/I 6%, B1x 21% and B1 29%. No other ALP isozymes or TNALP isoforms were found. The induction of ALP in calcifying VSMCs *in vitro* has been demonstrated repeatedly (161, 242, 247), but the reported

activities differ depending on culture conditions, type of VSMC and type of species. To our knowledge, until now only one group has investigated the activity of BALP in calcifying VSMCs (163). However, they did not further characterize the BALP isoforms, nor did they analyze other isoforms of TNALP or relate the BALP activity to total ALP. Our results demonstrate for the first time, that the increased ALP activity in calcifying HASMCs consists exclusively of BALP isoforms.

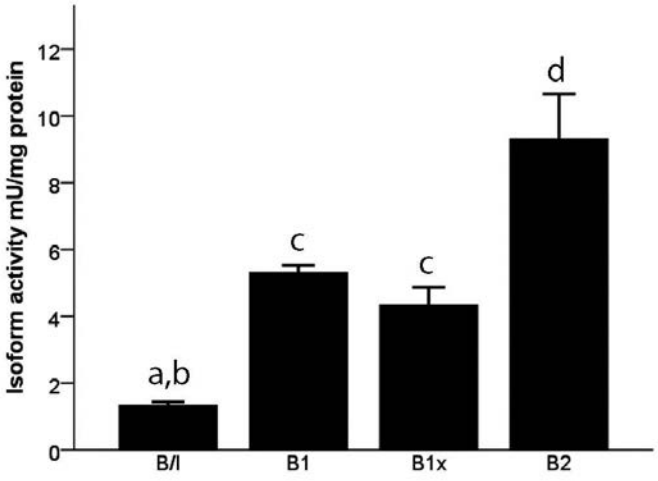

Figure11. BALP isoform activities in the cell layer of quiescent HASMCs. Cells were cultured in growth medium until confluence. Data represents the results of three independent experiments. ANOVA with Fisher's least significant difference test as post hoc analysis revealed significant differences between groups as indicated. a = $p < 0.01$ compared with B1; b = $p < 0.05$ compared with B1x; c = $p < 0.01$ compared with B2; d = $p < 0.001$ compared with B/I.

In our model, β-glycerophosphate stimulated the accumulation of calcium in the cell layer (**Figure 12**), which was paralleled by higher activities of the BALP isoforms B/I, B1x and B2 (**Figure. 13**). The stimulatory effect of β-glycerophosphate on the cell layer B/I and B1x activities followed a dose-dependent pattern, whereas the increase of B2 activity lacked this tendency (**Figure 13**). Tetramisole inhibited the mineralization of HASMCs (**Figure 12**). It also inhibited the β-glycerophosphate-induced activity of the B1x and B/I isoforms in the cell layer, but had no effect on B1 and B2 activities (**Figure 13**).

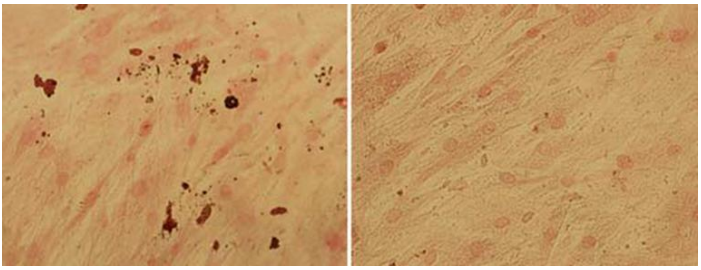

Figure 12. Von Kossa staining of HASMCs after 30 days in calcification medium containing β-glycerophosphate 5 mmol/l: without tetramisole (left) and with 0.1 mmol/l tetramisole (right).

Our results suggest different biological functions among the BALP isoforms in calcifying HASMCs. The BALP isoforms differ due to post-translational glycosylation and 4 of the 5 highly branched N-linked oligosaccharide structures are located within close proximity to the active site (45). Tetramisole (synonymous with levamisole) inhibits TNALP specifically by interaction with amino acids His434 and Tyr371, which are situated in the vicinity of the active site (248). It can therefore be speculated, that tetramisole can interfere with some of these glycosylation properties. Another possible explanation is that the BALP isoforms are not expressed all at the same time, but follow a stepwise activation pattern, where one isoform is expressed before the other. By inhibiting the initial BALP isoform(s), tetramisole could therefore indirectly interfere with these stepwise reaction mechanisms, affecting the feedback regulation. Still, since this is the first study of the effect of tetramisole on the activity of BALP isoforms, our interpretation of the current findings can only be suggestive.

The present study supports our hypothesis that B1x is associated with vascular calcification. The inhibition of vascular ALP has been proposed as a possible treatment strategy for vascular calcification (233, 249). Our finding, that BALP isoforms B/I, B1x and B2 are pivotal for calcification of HASMCs, identifies these BALP isoforms as potential therapeutic targets for the pathological process of vascular calcification. Furthermore, the differential inhibitory effect of the BALP isoforms in HASMC calcification is suggestive of different biological functions. However, these findings are not conclusive and the rise of the BALP isoforms in vascular calcification needs to be elucidated further in experimental and clinical studies.

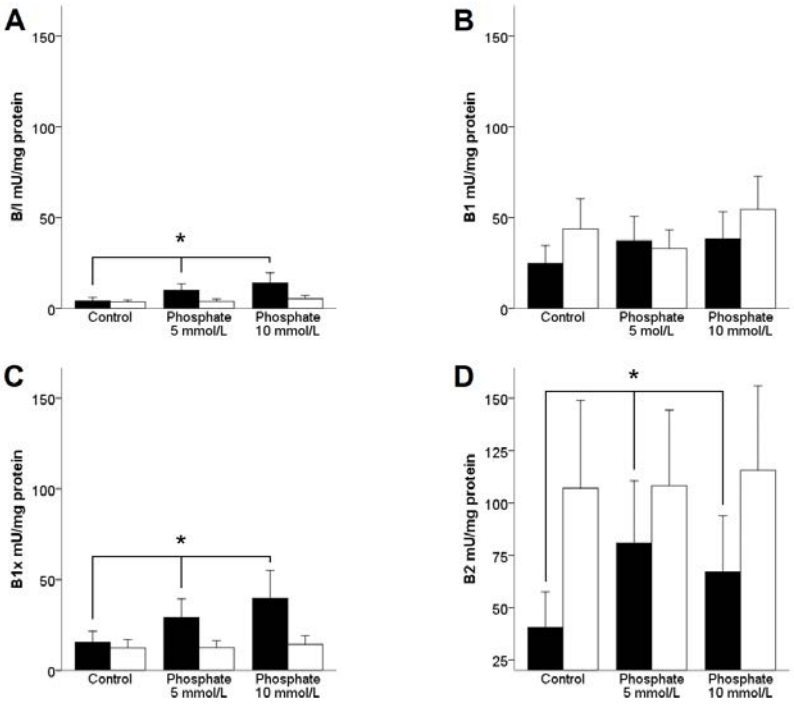

Figure 13. BALP isoform activities in HASMCs cultured for 30 days in growth medium (control) or calcification medium with β-glycerophosphate at indicated concentrations without (black bars) or with 0.1 mmol/l tetramisole (white bars). Data represent the results of three independent experiments. Friedman's two-way analysis of variance by ranks revealed significant differences between groups (* $p < 0.05$) only for the BALP isoforms B/I (a), B1x (c) and B2 (d) in cells not treated with tetramisole. Paired comparisons were not performed due to low sample number.

4.3 Study III

Bone disorders in CKD-MBD are common and associated with a high incidence of fractures and CV complications (250, 251). Different types of ROD can be identified based on bone histologic abnormalities, which consist of changes in bone turnover, mineralization and volume (104, 252). Adequate bone turnover, mineralization and volume are maintained through the interaction of osteoblasts and osteoclasts. A reliable diagnosis of different types of ROD is necessary to adequately direct treatment options. Several biochemical bone markers have been suggested as substitutes for the histomorphometric analysis of bone biopsies, which is the gold standard for the diagnosis of ROD. Parathyroid hormone, ALP,

and BALP, the most widely used biochemical markers in clinical practice, are helpful for screening for high osteoblastic activity, but sensitivity and specificity for the identification of low osteoblastic function are insufficient (107, 253). In study III we investigated the associations of the BALP isoforms with different types of ROD and tested the hypothesis that the B1x activity in serum is reflective of osteoblastic dysfunction.

The main finding of study III was the association of the BALP isoform B1x in serum with low osteoblast activity. B1x was the only biochemical parameter that inversely correlated with histomorphometric parameters of osteoblastic number and activity, while PTH and BALP demonstrated significant positive correlations with several parameters of bone turnover and mineralization. The BALP isoforms B/I, B1, and B2 demonstrated a weaker relationship with these histomorphometric parameters (Table 3). B1x identified low MAR (cut off 0.36 µm/day), a parameter of osteoblastic insufficiency. AUC for B1x was 0.83 compared to 0.11 for BALP and 0.15 for PTH (**Figure 14**).

Table 3. Correlation coefficients between serum markers and histomorphometric parameters of bone formation in patients on chronic hemodialysis.

	N.Ob/B.Pm	Ob.Vg	BFR/BS	MAR	Mlt	MS/BS	B1x
B/I	0.18	0.12	0.08	0.03	-0.10	0.09	-0.19
B1x	-0.30 [a]	-0.26 [a]	-0.26 [a]	-0.29 [a]	0.28 [a]	-0.25 [a]	-
B1	0.19	0.16	0.16	0.14	-0.15	0.17	-0.25[a]
B2	0.28 [a]	0.19	0.20	0.16	-0.18	0.20	-0.23[a]
BALP	0.43 [a]	0.38 [a]	0.39 [a]	0.36 [a]	-0.37 [a]	0.38 [a]	-0.35[a]
PTH	0.40 [a]	0.39 [a]	0.37 [a]	0.33 [a]	-0.35 [a]	0.37 [a]	-0.32[a]

Note: Kendall's tau-b rank correlation coefficients.
Abbreviations: BALP, bone-specific alkaline phosphatase; PTH, parathyroid hormone; N.Ob/B.Pm, number of osteoblast per bone perimeter; Ob.Vg, osteoblast vigor; BFR/BS, bone formation rate per bone surface; MAR, mineral apposition rate; Mlt, mineralization lag time; MS/BS, mineralizing surface per bone surface.
[a]Significant association.

Twenty-one patients (53%) had detectable activities of B1x in serum. This finding was comparable to our previous report on the frequency of B1x in serum from dialysis patients (51) and patients with CKD stage 5, not on dialysis treatment (241). All patients demonstrated serum activities of the BALP isoforms B/I, B1 and B2. Patients with detectable B1x showed significantly lower MAR, mineralization lag time, mineralizing surface, activation frequency,

bone formation rate/bone surface, number of osteoblasts, and osteoblastic activity at the cell level (osteoblast vigor) (Table 4).

Some patients with low bone turnover had no detectable B1x activities in serum. This finding may be explained by a relatively lower activity of B1x compared to B/I and B1 peaks, which may conceal its detection (44). On the other hand, B1x was detected in serum from 8 patients with non-low bone turnover. These patients could be in a transient phase, moving from high to low bone turnover. In these cases B1x could be indicative of an emerging or incipient osteoblastic defect, which will eventually lead to manifest low bone turnover.

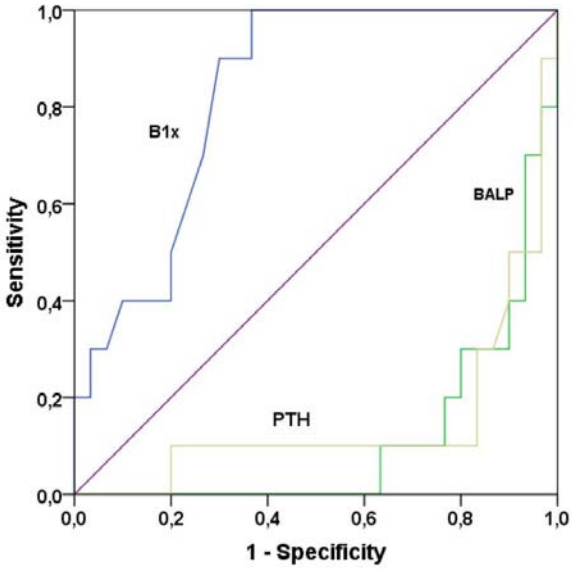

Figure 14. ROC curves of B1x (blue line), BALP (green line) and PTH (grey line) for a diagnosis of low osteoblast activity (mineral apposition rate below <0.36 µm/day).

Table 4. Bone histomorphometric parameters in patients on chronic hemodialysis with absence or presence of the circulating BALP isoform B1x.

	B1x absent (n = 19)	B1x present (n = 21)	P Value
Parameters of bone structure			
Bone volume/Tissue volume (%)	20.1 (16.5-23.1)	19.8 (15.8-24.7)	0.85
Trabecular thickness (µm)	109 (74.7-120)	93.9 (79.7-110)	0.59
Trabecular separation (µm)	342 (295-546)	375 (305-475)	0.90
Static parameters of bone formation			
Osteoid volume/Bone volume (%)	8.25 (5.20-20.8)	6.44 (3.95-13.0)	0.29
Osteoid surface/Bone surface (%)	43.8 (24.6-54.6)	35.2 (15.7-46.5)	0.28
Osteoid thickness (µm)	13.1 (9.79-15.1)	10.2 (8.28-14.6)	0.19
Number of osteoblast/bone perimeter (#/100 mm)	178 (61.5-349)	33.5 (10.5-136)	<0.001
Static parameters of bone resorption			
Erosion surface/Bone surface (%)	2.44 (1.56-6.15)	0.98 (0.55-3.01)	0.06
Erosion depth (µm)	13.0 (11.8-16.4)	11.0 (9.04 -13.8)	0.21
Number of osteoclast/Bone perimeter (#/100 mm)	23.8 (14.3-80.7)	12.9 (10.8-30.7)	0.07
Dynamic parameters of bone formation and turnover			
Mineral apposition rate (µm/d)	0.83 (0.75-0.93)	0.52 (0.10-0.88)	0.02
Mineralizing surface/Bone surface (%)	6.52 (4.32-12.6)	3.17 (0.80-8.7)	0.02
Bone formation rate/Bone surface ($mm^3/cm^2/yr$)	2.43 (1.57-3.86)	0.55 (0.03-3.04)	0.02
Activation frequency (yr^{-1})	0.57 (0.33-0.65)	0.16 (0.01-0.65)	0.02
Mineralization lag time (days)	70.1 (46.1-136)	225 (90.1-972)	0.01
Bone formation rate/osteoblast ($mm^3/cell/yr*10^3$)	15.3 (5.07-38.5)	9.97 (2.59-21.6)	0.38
Osteoblast vigor (%/day)	0.49 (0.23-0.62)	0.14 (0.02-0.37)	0.02

Note: Data are expressed as median (lower quartile to higher quartile).
Abbreviations: BALP, bone-specific alkaline phosphatase.

The correlations between BALP, PTH and high bone turnover have been established before in patients with CKD-5D (109, 254). The identification of B1x as a marker for impaired osteoblastic activity is novel and is unmatched in its diagnostic superiority to PTH and BALP by any other serum parameter. Circulating sclerostin is, like B1x, negatively associated with PTH and BALP (227, 255). Recently, an association of serum sclerostin with low bone turnover and low osteoblast number was demonstrated, but its diagnostic accuracy for the detection of low bone turnover was inferior to PTH and sclerostin serum levels were not associated with parameters of bone mineralization (255). Hypothetically, these discrepant

findings could be explained by the functional differences between BALP and sclerostin. BALP is expressed in active osteoblasts and directly involved in the propagation of matrix mineralization (16). Sclerostin, on the other hand, is secreted by osteocytes and down-regulates bone turnover via inhibition of the Wnt pathway in osteoblasts (256).

In conclusion, study III verified our hypothesis that the release of B1x into serum from bone is a sign of perturbed osteoblast activity. B1x was identified as a novel diagnostic parameter for osteoblastic insufficiency. Due to the pre-selection of patients in study III the ability of the BALP isoforms to diagnose other forms of ROD could not be determined. Further studies, including larger numbers of unselected patients are needed to address this question.

4.4 Study IV

Chronic kidney disease is associated with vascular stiffening and accelerated vascular calcification, both of which are associated with increased mortality (122). Alkaline phosphatase is a strong predictor of all-cause and CV mortality in different CKD populations (175, 176, 183, 187), as well as in the general population (171). Drechsler *et al.* (75) demonstrated recently the superiority of BALP over ALP as predictor of short term all-cause mortality as well as short- and long-term CV mortality in dialysis patients.

In CKD stage 4 to 5D, progressive arterial calcification is associated with increased serum ALP activities (257). Vascular calcification involves transdifferentiation of VSMCs to an osteoblast-like phenotype, which demonstrates increased expression of BALP (163). In study II we found that BALP isoforms B/I, B1x and B2 are involved in the calcification of VSMCs in vitro (258). Furthermore, in study I we found an association of B1x with hyperphosphatemia and increased calcium×phosphate product in CKD stages 3-5, both of which are associated with CV mortality in CKD (241). Based on these earlier findings, our hypothesis for study IV was that serum B1x is associated with vascular calcification in CKD stage 5D. We therefore aimed to investigate the associations of the BALP isoforms with vascular stiffness and aortic calcification in a cohort of Swedish dialysis patients.

Sixty-eight patients from four Swedish dialysis centers, participating in the CORD (Calcification Outcome in Renal Disease) study (122, 201) were included.

Baseline serum activities for total ALP and the BALP isoforms are shown in **Figure 15**. As in previous studies all patients had detectable activities of the three isoforms B/I, B1, and B2 in serum, and the relative activities of the three isoforms were comparable to earlier findings in predialysis (241) and dialysis patients (51).

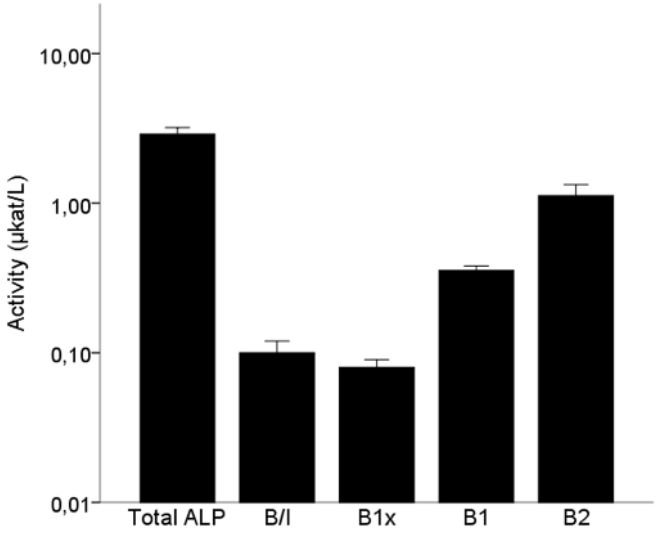

Figure 15. Total ALP and BALP isoforms at baseline. Data is shown as median with error bars indicating the 95% confidence interval.
Abbreviations: ALP, alkaline phosphatase; B/I, B1x, B1, B2, isoforms of bone alkaline phosphatase.

In study IV, 53 patients (78%) had detectable B1x activities in serum. This was the highest prevalence of B1x in serum observed in any population until now. We have previously described an increasing prevalence of detectable serum B1x activities with decreasing kidney function; B1x was not detected in healthy patients or patients with CKD 3, whereas 63% of patients with CKD 5 not on dialysis (241) and 60% of patients on maintenance dialysis treatment (51) had B1x in serum. In spite of this association with higher stages of CKD, it is unlikely that the appearance of B1x in serum is due to differences in renal clearance, since serum ALP is cleared by the liver. Furthermore, in predialysis patients with detectable B1x, serum activities were not correlated with GFR (241). An explanation for the higher prevalence of B1x in study IV compared to earlier reports could be changes in treatment

strategies for CKD – MBD, since PTH concentrations in patients with B1x were lower in study IV than in in our previous study involving dialysis patients (131 vs. 183 ng/L) (51). In both studies patients with B1x had lower PTH levels than patients without B1x. The publication of the KDOQI guidelines for bone metabolism and disease in 2003 (200) and the approval of cinacalcet by the Swedish Medical Products Agency in 2004 could have caused this trend towards a more pronounced PTH suppression. Possible further explanations for the higher proportion of patients with detectable serum B1x in study IV are differences in patient recruitment and study design and changes in the use of phosphate binders and vitamin D.

Aortic calcification score and PWV were determined in all patients at baseline. The correlation between AAC score and PWV at baseline (Kendall's tau =0.329, P< 0.001), found in the whole cohort, could not be reproduced in the highly calcified patients who experienced a combined CV event during follow-up (Kendall's tau = 0.21, NS). Multiple regression analyses identified B1x (β = 0.242, P = 0.03) and AAC score (β = 0.244, P = 0.047) as independent determinants of PWV at baseline, and albumin (β = -0.232, P = 0.046), PWV (β = 0.279, P = 0.021), and PTH (β = -0.330, P = 0.016) as independent determinants of AAC score at baseline. After a follow-up period of 2 years, AAC score was determined in 46 patients and PWV in 49 patients. While AAC scores increased in the majority of patients (30 patients or 65% of baseline), more patients demonstrated a regression than a progression of PWV (26 vs. 22 or 53% vs, 45% of baseline). A regression of AAC score was noted in only two patients (4% of baseline). Mixed model analyses demonstrated that the variation of B1x over time was independently associated with the variation of PWV over time (Table 5), but no association with variation of AAC score over time was found.

Table 5. Mixed model for the prediction of PWV variation over time

	Estimate	SE	*P*
Baseline	0.4975	0.6891	0.47
Year 1	-0.5135	0.6830	0.45
Year 2	0		
BMI	0.03777	0.1114	0.74
PTH	0.000019	0.001102	0.99
No tobacco use	-3.5403	1.9172	0.07
Tobacco use	0	.	.
ALP	0.08274	0.2104	0.70
B1x	14.1427	6.4643	0.03

Abbreviations: ALP, alkaline phosphatase; B1x, isoform of bone alkaline phosphatase; BMI, body mass index; PTH, parathyroid hormone; SE, standard error

During the observational period of 2 years, 21 patients (31%) experienced a combined event of all-cause mortality or a first CV event and ten patients received a functional kidney transplant during the follow-up period. In our multivariate Cox regression model B1x, PTH, PWV, and age, were independent predictors of the combined outcome (Table 6). Total ALP was associated with the highest HR in the whole cohort and after exclusion of patients who were transplanted during the 2-year follow-up, but the effect did not quite reach significance.

Table 6. Cox regression analyses of predictors of all-cause mortality or a first CV event

	All-cause mortality or first CV event					
	All patients (N=68)			Patients censored for kidney transplantation (N=58)		
	HR	95% CI	*P*	HR	95% CI	*P*
Age (years)	1.058	1.003 - 1.116	0.04	1.059	1.002 - 1.121	0.04
ALP (μkat/L)	1.347	0.973 - 1.864	0.07	1.388	0.997 - 1.930	0.05
B1x (nkat/L)	0.987	0.977 - 0.997	0.01	0.987	0.977 - 0.997	0.02
PTH (ng/L)	0.996	0.993 - 1.000	0.04	0.997	0.993 - 1.000	0.05
AAC score	1.041	0.967 - 1.121	0.29	1.042	0.964 - 1.126	0.3
PWV (m/s)	1.067	1.008 - 1.129	0.03	1.070	1.009 - 1.135	0.02

Abbreviations: AAC, abdominal aortic calcification; ALP, alkaline phosphatase; B1x, isoform of bone alkaline phosphatase; CI, confidence interval; CV, cardiovascular; HR, hazard ratio; PTH, parathyroid hormone; PWV, pulse wave velocity.

Study IV is the first report of the association of the four known BALP isoforms, including the novel B1x, with aortic calcification and aortic stiffness in patients with CKD. B1x in serum was associated with baseline PWV and with the variation of PWV over time. These findings are supportive of our hypothesis of a possible role of B1x in vascular pathology. In agreement with our previous findings and with our additional hypothesis of an association of B1x with lower bone turnover, patients with detectable B1x had lower levels of PTH, ALP and all BALP isoforms at baseline.

Verbeke *et al*. (122) described a weaker association of baseline PWV with the combined outcome of all-cause mortality or a first CV event in heavily calcified patients in the whole CORD cohort, which is in agreement with our findings of a loss of association between PWV and AAC score in patients who experienced a combined outcome. They suggested that this could be the effect of an underestimation of PWV due to difficulties in path length calculation in patients with highly calcified vessels. Another possible explanation could be that both

techniques identify different aspects of vascular pathology in CKD. The AAC score is determined at a site with predominantly intimal calcification with a high frequency of coexistence with medial calcification (112). The propensity for intimal calcification is not the same throughout the aorta, since autotransplants of thoracic aorta into abdominal aorta do not develop intimal calcification under atherogenic conditions and vice versa (113). In contrast to intimal calcification, which is strongly associated with inflammation, macrophage accumulation and lipid deposits, BALP producing VSMCs probably play a central role in medial calcification, which is more often found in arteries of the extremities and thoracic aorta (139, 141). Leroux-Berger *et al.* (116) demonstrated a higher propensity of VSMCs from the aortic arch to calcify in response to phosphate in comparison with VSMCs from the abdominal aorta. This was associated with an earlier increase of ALP activity in the neural crest derived VSMCs from thoracic aortas compared to the mesodermal derived VSMCs from abdominal aortas. Our findings of an association of B1x with PWV, but not with AAC score, led us to hypothesize that B1x in serum could be an indicator of predominantly medial calcification. Since patients with extensive intimal calcification seem to have a higher risk of mortality or CV morbidity (147), this could also explain the slight risk reduction for the combined outcome of death or a first CV event associated with B1x in study IV.

5. METHODOLOGICAL CONSIDERATIONS

A general limitation of the studies in this thesis is the requirement of the HPLC method for measurement of BALP isoforms. This method is time consuming, thereby limiting the possible number of analyses or participants for each study. Development of easier methods for measurements of B1x is in progress. The HPLC method has, however, several important advantages. Thus far, it is the only existing method that can separate different BALP isoforms. Furthermore, an advantage compared with immunological and other methods for the determination of BALP is the low cross-reactivity with LALP.

In addition to the low patient number, the cross-sectional design of study I and study III are limitations. Prospective studies are needed to investigate the changes of BALP isoforms during the progression of CKD and to determine the association of the BALP isoforms with changes in BMD. Furthermore, prospective studies for the comparison of detectable B1x with bone changes indicating alterations in bone turnover should be performed. One of the strengths of study III is the use of bone biopsies for the determination of parameters of ROD. Histomorphometric analyses are superior to non-invasive diagnostic markers for the diagnosis of ROD.

A strength of study II is the use of human cells, since our group recently demonstrated species differences for the BALP isoforms (259). The relatively low BALP activity in the calcifying HASMCs is a limitation of study II. Others have observed higher activities, due to the addition of further stimulators of VSMC calcification to the culture medium (163). A further limitation is the use of only one time period for the experiments. Since VSMCs undergo changes during very long time in culture this could have influenced our results. Therefore, experiments to determine the time course of BALP isoform expression in calcifying VSMCs are needed.

A strength of study IV is its design as a prospective multicenter study of prevalent dialysis patients. It is also the largest study of BALP isoforms in CKD patients ever performed; however, also in this study the relatively small number of patients is a limitation. Further limitations are the predominantly Caucasian origin of the participants, the observational character of the study, the possibility of a selection bias of the recruiting physician in spite of the randomization process, which in fact would imply an underestimation of the actual risk for the combined outcome in dialysis patients, and the large loss of patients during follow up,

mainly due to the high combined event rate, which on the other hand compensates somewhat for the short follow-up period of 2 years.

6. CONCLUSIONS

The BALP isoform B1x can be detected in serum from patients with advanced predialysis CKD. It has previously been detected in serum from patients on maintenance dialysis treatment and in some children with CKD. In predialysis CKD we found an association with hyperphosphatemia and increased calcium × phosphate product, two risk factors for cardiovascular complications and mortality in CKD.

The BALP isoforms B/I, B1, and B2, as well as TRACP5b and PINP can predict BMD at the predominantly trabecular hip in patients with CKD stages 3-5, not on dialysis treatment.

Quiescent HASMCs express all known BALP isoforms. No other ALP isoforms were detected.

Calcification of HASMCs is associated with an increased activity of the BALP isoforms B/I, B1x and B2, indicating functional differences between the isoforms in HASMC calcification.

B1x is a novel diagnostic parameter for osteoblastic insufficiency. Its release into serum from bone is a sign of perturbed osteoblast activity. We confirm prior reports that PTH and BALP are useful for diagnosis of high bone turnover associated with increased number of osteoblasts.

The BALP isoform B1x is associated with baseline and time varying aortic stiffness in prevalent maintenance dialysis patients. These findings imply that B1x might play an important role in the vascular pathology of CKD.

B1x was associated with a lower hazard ratio for all-cause mortality or a first CV event. Further experimental and clinical studies are warranted to elucidate the exact function of B1x in the vascular pathology of CKD and its possible role as a future therapeutic target for the treatment and prevention of CV complications in patients with CKD.

7. FUTURE PERSPECTIVES

7.1 Use of B1x as marker of disturbed osteoblastic function

The identification of the novel BALP isoform B1x as an indicator of perturbed osteoblastic function in study III opens for further research to explore B1x as a diagnostic tool in the evaluation of ROD and possibly also in non-CKD populations. The long-term use of bisphosphonates in the treatment of osteoporosis has been associated with atypical fractures (260). An over-suppression of bone turnover has been suggested as a possible rationale for these atypical fractures (261). Further research could investigate the putative role of B1x as indicator for osteoblastic dysfunction in the identification of bisphosphonate-treated patients at risk for atypical fractures.

7.2 BALP isoforms in the development of inhibitors of vascular calcification

Alkaline phosphatase and BALP have emerged as predictors of mortality. The role of BALP as a key player in the mineralization process of bone and soft tissue renders it a possible treatment target for the prevention of CV disease, and investigations to identify possible therapeutic TNAP inhibitors are ongoing (249, 262). We have previously demonstrated catalytic differences between the BALP isoforms with respect to the inactivation of the calcification inhibitor PPi. Our findings in study II of possible differences between the BALP isoforms in the process of VSMC calcification could have an impact on the development of more specific inhibitors of vascular calcification. Therefore, further research should clarify the role of the BALP isoforms in vascular calcification.

7.3 Development of new methods for quantification of BALP isoforms

Our findings in study IV of differences in the association of B1x with aortic stiffness and AAC score are interesting, but need to be verified in larger studies. To this end, the

development of an easier method for the quantification of B1x in serum is mandatory. Our group has recently identified the BALP isoforms in mouse serum and tissues (259). All known BALP isoforms, including isoform B1x, have been detected in mouse serum. These results pave the way for the development of new assays for the quantification of BALP isoforms.

8. ACKNOWLEDGEMENTS

I would like to express my deepest thanks to Linköping University, the Division of Clinical Chemistry at the Department of Clinical and Experimental Medicine, and to everybody who has made the writing of this thesis possible. I would particularly like to thank:

My supervisor **Per Magnusson**: You have been a true inspiration for me with your compassionate dedication to science, your great knowledge and curiosity, your generosity, both with immaterial and material resources, your analytical acuity, honesty and friendship. I look forward to hopefully many years of continued collaboration.

My assistant supervisor and former chief **Anders Fernström**: You have been an inspiring leader and colleague, a safe haven to rely upon in the stormy seas of science and medical uncertainness. Thank you also for unforgettable ice skating expeditions on the Kinda channel and for teaching me the very basics of golf!

My second assistant supervisor **Bengt Lindholm**: You believed in me when I needed it most! Thank you for your cheerful optimism, the great generosity with which you share your immense wisdom and experience, and all in all for your wonderful way of being a true gentleman!

My former assistant supervisor and chief **Martin Magnusson**: You not only set me out on my way into the scientific realm of CKD-MBD and alkaline phosphatase, you also hooked me up with nephrology. When you went on to follow your call you left an empty space behind that could not be filled.

My sister in science and grand master of the bench **Cecilia Halling Linder**: For all the laughs and the fun we had on our travels and for the immense work you did in the lab – without you I wouldn't be here. Thank you and good luck with your own research!

My collaborator overseas, **Hartmut Malluche**, and his team: You most generously welcomed me as a visitor to your department and to your home, and with the same generosity you shared your vast knowledge and experience in the field of CKD-MBD and especially renal osteodystrophy. Thank you for believing in my ideas, I hope we will continue our fruitful collaboration.

My collaborator in Linköping, **Hans Arnqvist**: You were the one who taught me the very first steps in science, long before any PhD studies were thought of. Thank you for that inspiration and for opening your lab once more for me – it finally led to a joint publication!

Anna-Kristina Granath, **Micael Gylling**, **Ingrid Göransson**, **Cecilia Halling Linder** (can't mention you often enough!), **Sonja Mattson**, **Abdul Rashid Qureshi**: For the outstanding work all of you contributed to make this thesis possible. You are a part of it and whoever might find it praiseworthy gives praise also to you.
Tony, you are a magician. Thank you for invaluable help with statistics!

Patrick D'Haese and **Geert Behets:** For supplying great photographs of bone biopsies for this thesis and for our fruitful clinical collaboration concerning bone biopsies.

My colleagues and staff at the **Departments of Nephrology** at **Karolinska University Hospital** and **Linköping University Hospital**: Thank you for backing me up during my work with this thesis. I needed your support. Hope I can make it up to you.

My good friend **Andreas Münch**: Our friendship started with a big bang and has expanded ever since. It feels good to know you are out there somewhere, never farther away than a phone call, to share the deepest thoughts and feelings as the true friend you are and will remain.

My cousin **Malin Nyström**: You cheer me up with your witty humor, you comfort me in difficult times, you inspire me with your courage. Thank you for being such a good friend!

All friends and family, who have in one way or another supported me during the writing of this thesis, among them **Tobias Kühme**, my long-time friend and sailing buddy, my brothers **Heinz** and **Oliver Haarhaus** in far-away Germany, "körgänget" **Kroko Karin Lundqvist, Ellen Wennersten, Heléne Cimander, Fredrik Lundholm** and **Anders Lindman** with their partners, and all newfound friends in Stockholm.

My parents in law **Hans Boberg** and **Madeleine Loberg** and all members of the **Boberg** and **Loberg clans**: For opening your family havens for me and letting med take part of the loving warmth that exists there. Madeleine and Hans, thank you especially for all help with child care, homework assistance and other unforeseeable needs.

My parents **Erika** and **Friedrich Haarhaus**: For all your love and inspiration, for the seed that you planted some time ago and nourished during many years and that now has grown to become a bearing tree. God bless you!

My wife and companion **Malena Loberg Haarhaus**: For your love and understanding, that fills my heart with joy every day. For being the beautiful person you are, for your believe in me and your own dreams. For letting me stand shoulder by shoulder with you to fight the wonderful struggle of life. I love you!

Finally my wonderful children **Joseph, William, Leopold, and Ludwig**: Seeing you gives my life meaning, more than anything else on earth.

As a personal statement, I would like to reproduce the complete sentence by John Ruskin, the first part of which I chose as a motto for this thesis:

"All true science begins in the love, not the dissection of your fellow creatures; and it ends in the love, not the analysis of God." John Ruskin, 1819 - 1900

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