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Review Article

# Alkaline Phosphatase-Biochemistry, Biological Functions Measurement and Clinical Relevance

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**Abstract**

A class of isoenzymes known as alkaline phosphatases (ALPs) is mainly found on the cell membrane's outer layer. Their main function is to catalyze the hydrolysis of organic phosphate esters present in the extracellular space. Zinc and magnesium serve as important co-factors for this enzyme. ALPs have distinct physiochemical features and are real isoenzymes, catalyzing similar processes. ALP is cytosolic in the liver and can be found in hepatocyte canalicular membranes. ALPs are also found in other organs (placenta, ileal mucosa, kidney, and bone). The majority of ALP concentration in serum (>80%) is derived from the liver and bone, with lower quantities from the intestines. While ALPs play a crucial role in processes such as phosphate metabolism and bone mineralization, the exact physiological function of ALPs remains largely unknown. Further research is necessary to fully understand their functions and regulatory mechanisms across different tissues.

**Keywords:** Bone ALP, Hypophosphatasia, Paget disease, Tissue-nonspecific ALP, Total ALP

## Introduction

Alkaline Phosphatases (ALP) are enzymes with specificity for a wide range of substrates, particularly evident in vitro. They derive their name from their optimal activity at pH levels above neutral, typically within the range of pH 8–11[1]. The versatile properties of ALPs render them valuable for diverse biotechnological uses, such as the dephosphorylation of DNA fragments and phosphoproteins, as well as serving as end-point detectors in immunoassays and as in vivo reporter molecules[2].

ALPs are ubiquitous in nature, found across bacteria and all animals, including mammals. Their activity is prevalent in diverse tissues and organs, primarily located ectoplasmically and tethered to the cytoplasmic membrane through glycosylphosphatidylinositol (GPI). ALPs found in mamals show wide substrate selectivity in vitro, but for some of the AP isozymes in vivo, natural substrates are just a small fraction of substances that have been verified. These include the dephosphorylation of proteins like osteopontin and nucleotides like ATP as well as pyrophosphate (PPi) and pyridoxal-5α-phosphate (PLP)[3].

Mammalian ALPs are zinc-containing metalloenzymes that are expressed in different organs and encoded by several genes. They are dimeric molecules. Three necessary metal ions are needed for enzymatic activity; these are two $Zn^{2+}$ and one $Mg^{2+}$ in the active site. These metal ions indirectly control subunit-subunit interactions and provide a substantial contribution to the ALP monomer's structure[4].

## Structure and expression of ALP

In humans, there are 4 separate genes which are encoding unique ALP isoforms . Three of these genes (named *ALPI, ALPP*, and *ALPPL2*) are encoding the tissue-specific ALPs (intestinal, placental, and germ-cell ALPs, respectively), while the 4$^{th}$ gene (*ALPL*, also known as *TNSALP*) encodes the tissue non-specific ALP (TNSALP), which is expressed mainly

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in the skeleton, the liver, the kidney, and the developing teeth. Intestinal, placental, germ cell, and tissue non-specific ALPs are characterized as true isoenzymes because their amino acid sequences differ. TNSALP undergoes posttranslational modifications, resulting in bone and liver ALP formation. Although these two products strictly share the same amino acid sequence, they are not true isoenzymes but rather isoforms of TNSALP, differing only in carbohydrate composition. TNSALP is highly expressed in the bones, liver, and the kidneys (alternatively named L/B/K ALP). TNSALP is also expressed, at lower levels, in several other tissues (i.e., cerebral cortex, developing spinal cord)[5]. The tissue specific isoenzymes share a high degree of similarity (90-98%), and the genes that encode them are located on the long arm of chromosome 2 (bands q34-q37.1). They are closely related, and their structures are nearly similar. This structural similarity between these three genes indicates that they probably evolved from a common ancestor. TNSALP is 50% identical to the other three ALPs, however is at least 5 times larger, mostly due to changes in intron size. The *ALPL* gene is situated on the short arm of chromosome 1 (bands p34–36.1). Moreover, the ALPL gene exists in a single copy and contains 12 exons which are distributed over more than 50 kb. The first exon is part of the 5'-untranslated region (5'-UTR) of the ALPL mRNA and consists of either exon 1A or exon 1B by alternative transcription initiation, giving rise to two different kinds of mRNA, deriving from a single gene[6,7]. Exon 1A is mainly initiated in osteoblasts whereas exon 1B is more often initiated in the liver and the kidneys. The remaining 11 exons encode the 524 amino acid monomer which contains one active site generated by evolutionarily conserved nucleotide sequences.

Until recently, the only information we had about the molecular structure of ALP was from Escherichia coli. However, now the three-dimensional structure of human placental ALP has been resolved, providing valuable information about the structure of human ALPs[4,8]. All ALP isoenzymes are homodimers and are linked to the cell membrane via a GPI anchor. Soluble ALP is released into the blood with the action of an enzyme a GPI-specific phospholipase D[9].

After their synthesis each monomer has a MW of 66 kDa and the native protein is transported to the endoplasmic reticulum, where glycosylation takes place as carbohydrate chains are added as O- and N-linked sugar chains. These posttranslationaly modified proteins are subsequently processed through the Golgi apparatus before being localized to the outer cell membrane via the GPI anchor. The two tissue-specific isforms of ALP (bone and liver) have distinct sugar chains, mostly having different O-linked glycosylations[4,10]. Further research utilizing high-performance liquid chromatography (HPLC) found that bone alkaline phosphatase (BALP) has four distinbct isoforms, while liver alkaline phosphatase (LALP) has three isoforms[11,12].

In order TNSALP to express its enzymatic activity, two $Zn^{2+}$ one $Mg^{2+}$ and one $Ca^{2+}$ ions are necessary as cofactors. Moreover, TNSALP is optimally active in alkaline pH environments. No ALP has been shown to be enzymatically active in a metal-free state. The amino acid sequence of human TNSALP is 57% similar and 74% homologous to the human placental ALP (PLALP) protein[13].

## ALP function in bone mineralization

The critical role of ALP bone formation was identified initialy by Dr Robinson in 1923. He was the first to propose that ALP was involved in skeletal mineralization by providing inorganic phosphate (Pi) for the creation of hydroxyapatite (HA)[14].

The first theory relating ALP to hard tissue formation was formed relating ALP to the raise of the local concentration of Pi, also known as the 'booster hypothesis'. ALP's role as an osteogenic activity marker has been confirmed as our understanding of hard tissue biology and mineral metabolism has grown. However, the precise method by which ALP operates in hard tissues is still debated, despite the fact that its importance in bone metabolism is widely acknowledged. This became clear when we discovered mutations in the gene which encodes the TNSALP. These mutations can to hereditary disorders known as hypophosphatasia[15].

TNSALP plays a crucial role in the physiological mineralization of hard tissues by hydrolyzing pyrophosphate to produce the inorganic phosphate required for mineralization[1,16]. Bone mineralization is a meticulously regulated process involving the deposition of minerals onto an organic matrix (bone matrix) essential for bone development. This process depends on a complex interaction between hormones (such as parathormone (PTH) and fibroblast growth factor 23 (FGF23), inorganic ions ($Ca^{2+}$, Pi, and PPi), and enzymes, primarily TNSALP.

Hydroxyapatite (HA) is the mineral that has been deposited in certain areas of the extracellular matrix (ECM) in humans, as well as in most vertebrates. Hard tissues including bone, growth-plate cartilage, and teeth go through a number of physicochemical and metabolic processes that lead to physiological mineralization. This mineralization is caused by different cells that are particular to certain tissues[16,17].

The hypertrophic zone in growth-plate cartilage is where mineralization takes place, and it is here that chondrocytes—both early and late hypertrophic—form HA[18,19]. Osteoblasts and matrix viens (MV) are the main components of mineralization in bone.

Osteoblasts, which line the osteoid, are responsible for HA formation, while odontoblasts perform this function in teeth. The role of the MVs is crucial, as they are thought to initiate mineralization[17,18]. Hydroxyapatite crystals are formed inside these MVs, which are extracellular vessels and their size is between 50 and200 nm. MVs are formed from the polarized budding of the surface membrane of

osteoblasts, chondrocytes or odontoblasts. The process of the MV-mediated skeletal mineralization is described below.

Inorganic phosphate (Pi) is transported throughout MVs via both sodium ($Na^+$)-dependent and independent routes. The sodium-dependent process is most likely mediated by type III $Na^+$/Pi co-transporters PiT-1 and PiT-2, in a manner similar to Pi absorption by the MVs' originating cells[19].

Calcium ($Ca^{2+}$) and Pi ions absorbed by MVs create HA crystals that can proliferate on collagen fibrils in the extracellular matrix. TNSALP, which is found on the outer surface of MVs, hydrolyzes its substrates, including pyrophosphate (PPi), adenosine triphosphate (ATP), and the protein-bound form of phosphate, to create Pi[18,20]. TNSALP stimulates skeletal mineralization by decomposing PPi and generating Pi (since PPi inhibits HA formation).

According to recent animal investigations, another phosphatase, PHOSPHO1, has been implicated in the initial stage of mineralization by generating Pi within the MVs from phosphocholine and phosphoethanolamine. However, its relevance in humans has to be determined[21,22].

TNSALP on the other is responsible for the calcification of soft tissues in cases of pathological mineraliztion. This type of pathological or uncontrolled mineralization can occur in any soft tissue, although the kidneys and the cardiovascular tissue are particularly prone to this disorder[23]. A more detailed review on the role of ALP in mineralization can be found elsewhere[24-26].

## Tissue Non-Specific ALP in disease

BALP activity (measured in human serum) can be found elevated during physiological and pathological events linked to elevated osteoblastic activity[27]. In healthy individuals, increased serum BALP activity can be seen during fracture healing and bone growth[28-30]. Several diseases like osteoporosis, rickets, osteomalacia, Paget's disease, neoplastic disorders such as osteosarcoma, and metastatic bone disease are all associated with elevated BALP activity as well as primary and secondary hyperparathyroidism, chronic kidney disease (CKD), and vascular calcification.

Conversely, decreased total ALP and BALP activity is rarely observed in humans but can occur in conditions such as hypophosphatasia (a rare metabolic bone disease), multiple myeloma with osteolytic lesions, growth hormone deficiency, and hypoparathyroidism.

Clinical interest in ALP continues to grow, as recent studies have demonstrated that ALP levels can be associated with increased morbidity and mortality in various clinical populations[3,12,31,32]. Elevated BALP levels have also been linked to increased mortality in patients with advanced CKD[33-36].

### *Rickets and Osteomalacia*

Rickets, a condition that affects developing children, is characterized by changes in chondrocyte differentiation and decreased bone matrix mineralization. The primary aetiology of rickets is vitamin D deficiency, however primary phosphate homeostasis and kidney tubular diseases can also be the cause of rickets in children[37,38].

| Age group | Total ALP activity in IU/L | |
|---|---|---|
| | Male | Female |
| 0 - 15 days old old | 90 -273 | |
| 15 - 30 days old | 134 -518 | |
| 1 - 10 years old | 156 - 369 | |
| 10 - 13 years old | 141 - 460 | |
| 13 -15 years old | 127 - 517 | 62 -280 |
| 15 -17 years old | 89 - 365 | 54 - 128 |
| 17 - 19 years old | 59 -165 | 48 - 95 |
| Adults | 43 - 115 | 33 - 98 |

**Table 1.** Age and sex specific reference intervals for total ALP (adapted from: Adeli K, Higgins V, Trajcevski K, White-Al Habeeb N. The Canadian laboratory initiative on pediatric reference intervals: A CALIPER white paper. Crit Rev Clin Lab Sci. 2017;54(6):358-413.[44] published under the terms of the Creative Commons Licence BY-NC-ND 4.0 (https://creativecommons.org/licenses/by-nc-nd/4.0/)

Vitamin D deficiency can lead to rickets through several mechanisms, including inadequate exposure to UV light, insufficient dietary intake, intestinal malabsorption, impaired renal function resulting in inadequate production of $1,25(OH)_2$-vitamin D, vitamin D receptor abnormalities, and inherited deficiencies of the enzyme 1α-hydroxylase, which is essential for synthesizing $1,25(OH)_2$-vitamin D[39]. In children with vitamin D deficiency-related rickets, total ALP and BALP levels are typically moderately to highly elevated. These levels may rise further shortly after the initiation of treatment, reflecting the onset of healing, and then gradually decrease if the therapy is effective. ALP serves as a surrogate marker for osteoblast activity in this context[40,41].

Total ALP and BALP activity are also elevated in X-Linked hypophosphatemia (XLH), an inherited disease caused by mutations in the PHEX gene (phosphate-regulating endopeptidase X-linked), and in autosomal dominant hypophosphatemic rickets (ADHR), another inherited disease resulting from mutations in the gene encoding FGF23. The main characteristic of these conditions is progressive and severe skeletal deformities and dwarfism. Rickets can also occur in various disorders that impair proximal tubular function, leading to increased renal clearance of Pi and subsequent hypophosphatemia. In these cases, the glomerular filtration rate (GFR) may be normal or nearly normal. Hypophosphatemia results in defective mineralization of the bones since serum concentrations of Pi and calcium (Ca) are crucial for the formation of HA crystals and bone mineralization.

Serum total ALP activity levels can be used as a biomarker

| Clinical form | Inheritance | Bone symptoms | Dental symptoms | Diagnosis |
|---|---|---|---|---|
| Lethal prenatal | Autosomal recessive | Hypomneralization, osteohondral spurs | NA | Radiographs, ultrasonography |
| Benign perinatal | Autosomal dominant | Bowing and shortening of long bones | NA | Ultrasonography, clinical examination |
| Infantile | Autosomal recessive | Craniosynostosis, Hypomineralization, Rachitic ribs, hypercalcemia, hyperphosphatemia, hypecalciuria, low PTH | Premature loss of deciduous teeth | Clinical examination. Low serum ALP activity, increased PEA and PLP). |
| Childhood | Autosomal recessive (frequent) or dominant (rare) | Short stature, skeletal deformity, bone pain and fractures | Premature loss of deciduous teeth | Clinical examination. Low serum ALP activity, increased PEA and PLP). |
| Adult | Autosomal recessive or dominant | Stress fractures (metatarsal), osteoarthritis | +/- | Clinical examination. Low serum ALP activity, increased PEA and PLP). |
| Odonto | Autosomal recessive or dominant | Loss of alveolar bone | Reduced thicknes of dentin, enlarged pulp chambers of teeth | Clinical examination. Low serum ALP activity, increased PEA and PLP). |

*NA = not applicable, PLP = pyridoxal 5'-phosphate, PEA = urinary phosphoethanolamine*

**Table 2.** The six clinical forms of Hypophosphatasia. Reproduced from: Mornet E. Hypophosphatasia. Orphanet J Rare Dis. 2007;2:40.[175] published under the terms of the Creative Commons Attribution 4.0 International License (CC BY 4.0) (https://creativecommons.org/licenses/by/4.0/).

reflecting osteoblast activity and can be found elevated in all forms of rickets[38,42]. In children, 80–90% of total ALP originates from bone. Therefore, the measurement of total ALP may be used as an alternative to BALP in pediatric patients provided liver disease has been ruled out through liver enzyme evaluation[43]. ALP levels peak during infancy and puberty, and reach troughs during mid-childhood and post-puberty[44]. Total ALP levels must be interpreted in relation to age- and sex-specific normative values. See Table 1 for reference intervals derived from caliper study performed in Canada[44]. Pediatric reference values for bone-specific ALP are also available[45]. However, reference ranges can vary significantly depending on the population and the laboratory assay used. consequently, laboratories are advised to create their own local reference intervals based on their local population.

High ALP serum levels can be used to confirm the diagnosis of rickets in patients who show clinical and radiographic symptoms. ALP levels can be normal or lowered in several other diseases like metaphyseal dysplasia, Blount's disease, and also in hypophosphatasia, which may mimic rickets[39].

In untreated patients with calcipenic rickets, ALP levels are also elevated, with values potentially exceeding the upper normal limit (UNL) by tenfold or even more. In contrast, in patients with phosphopenic rickets ALP levels, are moderately elevated (usually 1 to 3 times the UNL)[39].

Osteomalacia, the adult equivalent of rickets in children, is defined by a deficiency in the mineralization of the osteoid matrix produced by osteoblasts. Adult growth plates, unlike those in children, are closed, hence epiphyseal cartilage problems do not exist, and consequently growth abnormalities are not noticed. The principal effect is the formation of unmineralized bone, which can be identified by X-rays or other imaging techniques. Vitamin D metabolism disorders are the primary cause of osteomalacia, and BALP levels are typically elevated in affected patients[40].

### *Hypophosphatasia*

The most direct link between TNSALP and human disease is hypophosphatasia (OMIM: 171760). Hypophosphatasia (HPP) is an inherited systemic bone disease caused by mutations in the *TNSALP* gene leading to reduced enzyme activity in specific organs. This reduced TNSALP production results in under-mineralization of hard tissues, primarily affectings bone and teeth mineralization)[46-48].

The severity of HPP can vary widely, ranging from the severe, often lethal, infantile form to the milder adult form. HPP can be classified into six types based on disease severity and age of onset (Table 2)[48,49]. TNSALP has also been implicated in non-HPP related medical conditions[50,51].

### *Inheritance and epidemiology of HPP*

As of today over 400 mutations in the TNSALP gene have been described (see for more details https://alplmutationdatabase.jku.at.). HPP is predominately inherited as an autosomal recessive trait, although some milder cases autosomal dominant inheritance have been reported (Table 2). The most severe cases of the disease are uncommon. Its prevalence has been estimated to 1:100,000 in North America and to 1:300,000 in Europe. In contrast, the frequency of the milder adult forms have been estimated between 1:3,100 and 1:508 in Europe[52-54].

### *Pathophysiology of HPP*

TNSALP deficiency leads to both poor enzymatic and mineralizing activities. The primary characteristic of HPP patients is bone and tooth hypomineralization. These patients have fewer extracellular HA crystals and poor mineralization. However, in HPP, the first phase of mineralization proceeds normally within the MVs since the cytosolic phosphatase PHOSPHO1 is expressed normally in these patients[13,22,55]. This has been verified in a study using double knock-out mice for TNSALP and PHOSPHO1[22]. Patients with HPP usually show an increase in osteoid tissue that contains non mineralized bone extracellular matrix without HA crystals, which leads to rickets and osteomalacia[56]. Additionally, deficient mineralization of acellular cementum results in premature loss of deciduous teeth.

In severe HPP cases, decreased mineral incorporation in the skeleton leads to increased Ca and Pi levels. This causes lower PTH levels and hypercalciuria. In less severe cases, mineral homeostasis is normal; nevertheless, a modest rise in Pi levels and hypercalciuria are common.

The observed hyperphosphataemia is mostly due to an enhanced tubular renal reabsorption rate of Pi. Possible explanations include TNSALP's direct function in renal Pi reabsorption, competition with excess PPi for the same transport route, and abnormally normal or lowered levels of phosphatonines (e.g., FGF23).

Another physiological activity of TNSALP is to synthesize neurotransmitters in the central nervous system (CNS). One of the physiological substrates of TNSALP is pyridoxal 5´-phosphate (PLP). PLP is one of the main metabolites (vitamers) of vitamin B6, and is necessary for the biosynthesis of γ-aminobutiric acid-transaminase (GABA). GABA is a cofactor of glutamic acid carboxylase in neuronal cells and is known to act as repressive neurotransmitter.

### *Role of ALP in Vascular Calcification*

Vascular Calcification (VC), is a pathological process characterized by the deposition of calcium-phosphate crystals in the form of hydroxy-apatite mineral in the arteria wall (aortic media and/or intima), is an important risk factor for morbidity and mortality and increases with age[57,58]. It is linked to an increased risk of heart disease, stroke and atherosclerotic plaque rupture[59]. Several mechanisms have been linked and can lead to vascular calcification, which suggest the presence of multiple etiologies rather than only one mechanism.

VC is linked to physiological aging, genetic disorders, and a variety of pathological illnesses, including chronic kidney disease (CKD) and diabetes[60]. VC lowers aortic and artery compliance and elasticity.

Intimal calcification is linked to arterial obstruction atherosclerotic plaque rupture whereas medial calcification leads to vessel stiffness systolic hypertension leading to increased diastolic dysfunction and heart failure[61].

Tissue mineralization can occur at normal calcium and phosphate concentrations, thus the body has evolved many regulatory systems to confine the process to bone and cartilage. Several endogenous VC inhibitors have been found, with pyrophosphate (PPi) being the most important. PPi inhibits the creation of calcium-phosphate crystals as well as their development.

Inorganic PPi is produced by the hydrolysis of the phosphodiester link in nucleotide triphosphates like ATP or UTP. As a result, it is a metabolic consequence of many intracellular biochemical activities as well as external signaling cascades[62]. It is critical to distinguish between the functions of intracellular and extracellular pyrophosphate. Intracellular pyrophosphate is created as a byproduct of over 200 different enzyme activities, and enormous amounts of pyrophosphate are formed within cells every day during the creation of numerous macromolecules such as proteins, nucleic acids, lipids, and carbohydrates from their precursors. This intracellular PPi remains within cells, where it undergoes hydrolysis by intracellular enzymes[62].

The major source of is extracellular ATP. Extracellular PPi antagonizes the ability of calcium to crystallize with phosphate to form hydroxyapatite. PPi also binds strongly to the surface of HA crystals and prevents further crystal growth. Extracellular PPi therefore has a dual role: first it acts to prevent harmful soft tissue calcification and second to regulate bone mineralization[62-64]. TNSALP has been found to play a central role in VC as it acts upon PPi degrading it to phosphate (Pi) and thus promoting VC.

Calcification of advanced lesions resembles endochondral ossification of long bones and appears to stabilize plaques. This procedure involves the transdifferentiation of vascular smooth muscle cells (VSMCs) into chondrocyte-like cells. In contrast, the poorly known microcalcification of early plaques is thought to be hazardous. Tissue Non-Specific Alkaline Phosphatase (TNSALP) and collagen are two proteins required for physiological mineralization.

Under pathological conditions, TNSALP is activated by pro-inflammatory cytokines in VSMCs (such as TNFα and interleukin-6) and reactive oxygen species (ROS). Collagen production is constant. Activation of TNSALP appears to induce calcification by enhancing mineralization and promoting osteogenic differentiation.

Calcifying vascular specimens contain osteoclasts,

osteoblasts, and chondrocytes, all of which are produced from stem cells or VSMC transdifferentiation. Local TNSALP levels are elevated along with bone-related proteins like osteocalcin and osteopontin. This activation triggers significant VC in arteries due to its overexpression in VSMCs and endothelial cells. Elevated circulating TNSALP is also associated with increased mortality in patients with chronic kidney disease (CKD)[65].

### *Paget's disease*

Paget's disease (PD) of the bone is a nonmalignant skeletal disease defined by specific abnormalities in bone remodeling at one (monostotic) or many (polyostotic) skeletal locations[66]. Any bone of the body can be impacted, although the pelvis, spine, femur, tibia, and skull are most susceptible. At the cellular level, Paget's disease (PD) of the bone is distinguished by an increase in the number (and activity) of osteoclasts, as well as an increase in osteoblastic activity. Bone growth is enhanced yet chaotic, resulting in braided bone that is mechanically weak and susceptible to deformation and fracture. vascularity of bone.

The pathophysiology of PD is poorly known, but hereditary factors appear to play an important role. Inheritance may play a role, as many of the affected people have family histories. Several cases may have an autosomal dominant inheritance pattern with incomplete penetrance[67].

Age and sex may play a role, as the disease typically affects older adults, with men being at a higher risk than women. The most common symptom is pain, and the presentation of the disease may vary depending on which bones are affected, the extent of involvement, and the presence of complications. The disease may also be asymptomatic, and we often it from incidental findings of elevated serum total ALP levels or abnormalities on imaging tests performed for an unrelated cause[68].

Bone turnover markers (BTMs) are not required to diagnose Paget's disease; however, they are useful in evaluating disease activity and monitoring response to bisphosphonate therapy. Total ALP offers comparable value to the newer BTMs and may serve as a cost-effective replacement. However, it iscrucial to confirm that the patient does not suffer from concomitant hepatobiliary disease.

### *Clinical use of total ALP and BALP in bone metastases and multiple myeloma*

In solid tumors, the bone is the third most common location of metastasis, behind the lungs and liver. Bone metastasis disease arises due to various interactions between malignant cells and bone cells, causing changes in normal bone metabolism[69].

Bone metastases are typically associated with significant bone pain, the causes of which are unknown but appear to be related to osteolysis. Osteolysis is also followed by increased bone fragility and a higher risk of fractures. Pathological fractures frequently occur in load-bearing bones due to bone metastases and pose significant treatment challenges when they are present in the neck or shaft of the femur or in the pelvis.

Other side effects of bone metastasis include leukoerythroblastic anemia, bone deformities, hypercalcemia, and nerve-compression disorders such as spinal cord compression. Bone metastases are classified as osteolytic or osteoblastic, with each type having a distinct cause.

Osteolytic metastases are thought to be generated by activated osteoclasts. Osteoclast-activating factors, particularly parathyroid hormone-related peptide (PTHrP), which are released by tumor cells in the bone microenvironment are important for osteoclast activation. On the other side, osteoblastic metastases are caused by cancer cells producing substances that promote osteoblast proliferation, differentiation, and bone creation. However, it is now recognized that osteoblastic and osteolytic lesions are two extremes, and studies have shown that bone metastases, in the majority of patients, contain both osteoblastic and osteolytic features.

Prostate and breast cancers are the two most common causes of bone metastases. However, the outcome of metastasis from each of these tumors is typically fairly varied. Bone metastases in breast cancer are primarily osteolytic, resulting from osteoclast activation rather than cancer cells' direct effects on bone. The major lesion is lytic and destructive, but there is also a local bone formation response, which probably represents an effort at bone repair. This increase in bone formation is reflected by increased levels of serum activity of ALP. However, the predominant effect is osteolysis, despite this secondary increase in local bone formation.

Prostate cancer bone metastases are often osteoblastic. ALP and osteocalcin levels in these metastases indicate that osteoblasts close to metastatic tumor cells are being stimulated locally. However, up to 25% of patients with bone metastases from breast cancers show osteoblastic lesions that are comparable to those found in metastatic prostate cancer. In contrast, some prostate cancer patients develop osteolytic lesions similar to those found in metastatic breast cancer patients. As a result, the idea of two discrete types of bone metastases is probably overly simplified[70].

ALP, specifically the BALP isoform, appears to be a sensitive and consistent indicator of osteoblastic activity[71]. High ALP levels of activity have been reported in a variety of cancers and appear to be associated with an increased risk of unfvourable clinical outcomes[72]. However, due to a low specificity and conflicting evidence in the literature, this test is not used routinely clinical practice. Its potential as a diagnostic or prognostic marker for bone metastases remains intriguing, and it is being studied for numerous solid malignancies[73].

Several studies have shown that ALP is a valuable predictive marker of bone metastasis from solid tumors.

BALP exhibits a good sensitivity and specificity for the prediction of bone metastases and progression of disease in prostate cancer. The majority of patients with prostatic cancer who also have metastases to the bone have elevated ALP values. Furthermore, several studies have found that higher ALP levels before to treatment beginning are related with a poorer response and prognosis. These findings imply that ALP could be a biomarker to be employed in the evaluation of patients with metastatic prostate cancer not only to predict bone metastases but also to monitor the response to treatment[74-79]. The role of ALP as a predictive biomarker of bone metastases for other solid tumors (renal lung or gastric) has been studied in several studies with mixed results[80-83].

Multiple myeloma (MM) is a neoplasm of bone marrow with an increasing prevalence. The clonal development of malignant plasma cells in the bone marrow causes hypogammaglobulinemia, cytopenia, osteolytic bone disease, hypercalcemia, and, ultimately, kidnel failure[85]. Osteolytic bone disease is caused by increased osteoclastic activity and decreased osteoblast function, both of which are associated with myeloma. The bone loss observed in MM is multifactorial, with the majority of patients developing severe osteolytic bone disease during the course of the illness. However, it is not clear why bone destruction is a result of this disease[84,85].

Pain and pathological bone fractures in various areas of the body are common clinical features in many individuals. Some typical examples include vertebral osteolysis, which can cause pathological fractures, as well as spinal collapse with medullary compression and a variety of neurological symptoms. Among all of the clinical repercussions of MM, bone damage has the greatest influence on patients' quality of life[86,87]. Studying bone markers is an essential step in tackling MM. It is critical to identify serum biomarkers that can be used to quantify and predict future severe bone lesions.

Plain radiographs are commonly used to assess bone disease in MM. Although radiographs are valuable for identifying osteolytic lesions, they do not reveal any information about continuing bone remodeling. Therefore, bone turnover markers have been utilized as an alternative to measure the rate of bone turnover in individuals with MM[84,88].

Biomarkers of bone formation have been used in several studies but the results were inconsistent. Some studies showed elevated BALP and osteocalcin in myeloma patients compared with controls whereas in others were either within normal range or even reduced[89-91].

In a study involving 440 patients with and 461 patients with solid tumors with metastases, the levels of ALP were generally found to be normal or slightly elevated, regardless of the stage and the number of osteolytic lesions and were consistently lower than those observed in solid tumors. The distinctions between MM and solid tumors, particularly in comparison to prostate cancer, are significant. Multiple myeloma has a different mechanism of bone involvement than solid malignancies because of decreased bone turnover and/or osteoblastic activity, which coincides with lower levels of plasma ALP expression[73].

## The measurement of ALPs

### *Measuring total-ALP activity*

The assessment the activity of total-ALP in serum or plasma is one of the most commonly performed tests in clinical chemistry laboratories. ALPs hydrolyze a wide range of organic monophosphate esters, resulting in the production of an alcohol or phenol and phosphate ion. Over the years several analytical methods have been proposed, each one of them based on a distinct substrate and distinct assay parameters (such as pH, buffers, and reaction temperature[92]. The choice of buffer and reaction temperature are the most important. Currently, most tests use conventional photometric method which involves the following reactions in two steps: In the first step, ALP catalyzes the hydrolysis of p-nitrophenylphosphate (p-NPP), which is colorless, to phosphate and free p-nitrophenol (p-NP). In the second step, and under alkaline pH conditions, p-NP is converted to 4-nitrophenoxide , which has an intense yellow color and absorbing at 405nm. This is the method that is used today on most automated clinical chemistry analyzers and was initially standardized by IFCC in 1983[93-95]. The reaction temperature was set at 30°C. As the market was populated with more methods, it became clear that these different methods produced different results. The need for assay standardization became urgent. IFCC started a working group with aim to "standardize the measurement of the catalytic activity of enzymes establishing reference systems in clinical enzymology". The major scope of this effort was to achieve inter laboratory agreement of enzyme activity measurements. ALP was one of the target enzymes in this project. The current IFCC primary reference measurement process for ALP is based on prior IFCC work, with the only alteration being an increase in the measurement temperature from 30°C to 37°C[96].

IFCC's recommendation is to use a colorless substrate (p-nitrophenylphosphate) in an alkaline buffer (2-amino-2-methyl-1-propanol) at 37°C[96,97].

Unfortunately, not all clinical laboratories employ the IFCC reference measurement approach. A recent investigation conducted 5 years after the publication of the standard measurement protocol found "substantially poor comparability" among clinical laboratories[98]. However, the existence of an internationally agreed reference system for the measurement of ALP activity does not necessarily imply that all manufacturers will instantly apply traceability to it[98]. It is also critical to emphasize the importance of well-designed external quality assessment schemes (EQAS) in which ALP target values are assigned using the IFCC RMP rather than consensus values (such as peer-group means

or other indicators of central tendency). With such EQAS schemes in place quality specifications indicators are deriving from objective models[97,99,100].

These tests measure the enzymatic activity of total ALP in the serum, tthey cannot distinguish between the many isoenzymes and isoforms, limiting the utility of total ALP activity as a bone formation marker, particularly in patients with hepatic diseases[101]. As a result, immunoassays capable of quantifying the bone isoform of ALP (BALP) have been developed and are now are frequently utilized in biochemical laboratories[102,103].

### *Separation and Quantification of ALP isoenzymes*

The challenge in BALP quantification is to differentiate the bone isoform of ALP from its liver isoform (LALP). In human serum, these are the two main isoforms, under non-pathological conditions. What makes the measurement of the bone isoforms challenging is the fact that both liver and bone isoforms share the same amino acid sequence and that their differences are limited only to the rate of post-translational glycosylations.

Over the years researchers developed various techniques that exploit these carbohydrate differences to measure specifically the bone isoform. Various separation techniques (including electrophoresis and chromatography) were used to separate the two major isoforms however these techniques lacked specificity, reproducibility and were extremely laborious.

Advancements in antibody production and assay technology during the decades resulted in the development of both polyclonal and monoclonal antibodies with high specificity for bone ALP, although in some cases the discrimination between the liver and the bone isoforms has not been adequate to satisfy the clinical needs[104-107]. These antibodies have been used to develop various immunoassays, that allowed more widespread use in clinical chemistry laboratories. Initially two methods have been developed using these antibodies.

Hybritech invented the first immunoradiometric test (IRMA), known commercially as Tandem-R-Ostase[108,109]. This was a "sandwich" immunoassay which used two unique murine monoclonal antibodies directed at separate antigenic regions (epitopes) on the bone-ALP molecule. The assay was calibrated with calibrators made of purified bone-ALP isolated from osteosarcoma cells (SAOS-2 cells). The assay determined the mass of bone-ALP and the results were expressed the ng/mL[110,111]. This type of assay was difficult to perform as it was fully manual required overnight incubations and required radioisotope labeled reagents. According to evaluation tests, there was significant cross reactivity with the liver isoform which ranged between 14.3 and 18.3%[108,109,112,113]. However, this assay demonstrated potential as a tool for monitoring and diagnosing bone disorders characterized by enhanced osteoblastic activity[108,114-116].

The next assay that was developed was an immunocapture enzymatic immunoassay created by Gomez et al. in 1995[102]. This assay utilized only one monoclonal antibody produced on mice, vaccinated with ALP isolated from SAOS-2 cells, and purified[119]. This assay in fact was measuring the activity of BALP that was captured on the solid phase and the results were expressed in U/L where 1U represents the activity to the enzyme which hydrolyses 1µmol of p-NPP per min at 25°C. A calibration curve was also used to quantify the unknown samples. The calibrators were also made of ALP produced from SAOS-2. This assay was commercialised by Metra Biosystems as Alkphase-B[117]. The comparison these two first assays gave inconsistent results[113,118,119].

The Tandem-MP-Ostase, a novel commercial enzyme immunoassay released in 1998, claimed to assess the ALP bone isoform. This was an immunocapture ELISA with a single monoclonal antibody. This assay, like the previous immunoassay, measured the activity of bone isoforms[103]. However the calibration of this assay was designed to match the calibration of the IRMA Tandem-R-Ostase. Calibrators were also produced using purified ALP extracted from SAOS-2 cells, however their values were given in µg/L and therefore using the calibration curve the activity values that were extracted from the test were transformed into mass values to match the immunometric assay results[103].

Today the immunoradiometric (IRMA) assay is still in production and it is distributed by Beckman-Coulter. It is actually the same assay only the new version uses coated tubes with the first antibody whereas the Hybritech version used coated plastic beads.

## Conclusion

ALP is one of the most extensively studied enzymes, however its metabolism is not even today entirely understood. The diagnostic utility of measuring blood total ALP activity is well established, and it is one of the most commonly used laboratory tests for the detection of cholestasis and bone diseases. ALP is an essential enzyme that regulates the mineralization of skeletal tissue. It has important physiological activities (skeletal and dental tissue mineralization), is critical for the dephosphorylation of numerous physiological substrates and also has some extra-skeletal functions (implicated in ectopic pathological calcification of soft tissues, particularly in the vasculature).

Total ALP activity assays provide important information about the total ALP level in human serum, but they do not distinguish between ALP isozymes and isoforms. Although the IFCC has standardized these assays, their clinical relevance and reliability in assessing bone development in patients with liver diseases is limited. Nonetheless, these assays remain critical for identifying hypophosphatasia.

Immunoassays claiming to measure the bone-specific isoform of ALP also have limitations, including significant inter-method variability. These discrepancies likely stem from a lack of standardization due to the absence of a an

**international standard and a reference method for BALP. Standardizing these assays is a primary objective of the IFCC.**

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