Alkaline phosphatase and cardiovascular disease

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Abstract: Alkaline phosphatase (AP) is a plasma membrane-anchored enzyme that is widely distributed in nature from bacteria to humans. AP has a broad specificity and catalyzes the hydrolysis of phosphate monoesters from a wide range of substrates. The best known physiological function of AP is participation in the bone mineralization by increasing the availability of phosphate that is used for hydroxyapatite synthesis. Measurement of AP activity in plasma is widely used for the diagnosis of bone and liver diseases. Population-based longitudinal studies strongly suggest an association between elevated AP and increased risk of incident cardiovascular disease (CVD) or all-cause mortality. Elevated AP in subjects with pre-existing coronary heart disease (CHD) is associated with the increased risk of mortality with no evidence for a stronger association between AP and mortality in these subjects compared to subjects free of CHD. AP is associated with cardiometabolic risk factors including conventional CVD risk factors, metabolic syndrome, systemic inflammation, vitamin D deficiency and various morbid conditions that may signify a poor CVD risk profile or impact unfavorably on prognosis. Experimental studies strongly suggest an involvement of AP in the vascular calcification and, through this mechanism, a direct role of the enzyme in the pathophysiology of CVD. However, criteria for a causal relationship between AP and CVD or mortality remain unfulfilled. Further explorations of the molecular mechanisms of the involvement of AP in the pathophysiology of CVD and the use of the enzyme as a therapeutic target to reduce CVD risk remain to be elucidated in future studies.

Keywords: Alkaline phosphatase; cardiovascular disease (CVD); coronary heart disease (CHD); prognosis; vascular calcification

Historical perspective

Alkaline phosphatases [AP; orthophosphoric monoester phosphohydrolase (alkaline optimum); Enzyme Commission number (EC) 3.1.3.1.] are plasma membrane-anchored enzymes that are widely distributed in nature from bacteria to humans. The enzymes catalyze the hydrolysis of phosphate monoesters from a wide range of substrates as well as a transphosphorylation reaction in the presence of abundant concentrations of phosphate acceptors (1). AP was discovered by Suzuki *et al.* (2) who named the enzyme "phytase" (because the enzyme released phosphate by hydrolyzing phytic acid). A series of studies by Robison and colleagues in 1920s made a great contribution to biochemical characterization of AP including postulation of

its physiological function. In 1923 Robison (3) found that bone extracts from rabbits and rats but not extracts from non-ossifying cartilage hydrolyzed hexosephosphoric ester to liberate phosphate. Subsequently, the enzyme which was present in bone was named "monophosphoric esterase" by Robison and Soames (4). In 1924 Kay and Robison (5) measured the enzyme activity in human blood and called it "bone enzyme". Subsequently, terms "phosphatase" or "bone phosphatase" were used by Martland and Robison (6). Notably, Robison (3) hypothesized that AP may participate in bone physiology by increasing phosphate availability needed for bone mineralization—a hypothesis that has stood the test of time. Historical aspects of AP discovery, characterization and evolution of laboratory assays used for its measurement have recently been reviewed by Siller and

Whyte (7). AP has been considered the most frequently assayed enzyme in medicine (7).

Structure and function

Human AP consists of four different types: the tissue non-specific AP (TNAP) and three tissue-specific AP (intestinal, placental and germ-cell types). TNAP is expressed in many tissues throughout the body but its highest activity is found in bone, liver and kidney (also known as bone/liver/kidney AP type). The tissue specific APs are 90% to 98% homologous whereas TNAP is 50% homologous to the other three tissue specific APs (8). TNAP is a homodimeric glycoprotein with each monomer consisting of 524 amino acid residues (57.2 kDa) (9,10). TNAP has several domains including active site, several mammalian specific domains like long-terminal α-helix domain that is responsible for catalysis, a non-catalytic Ca^{2+}-binding domain and a crown domain that is responsible for uncompetitive inhibition, allosteric properties, heat stability and interaction with extracellular matrix components including collagen (11). The active site has two binding sites for two Zn^{2+} ions, a binding site for a Mg^{2+} ion and serine 102 to which phosphate binds (12). TNAP undergoes multiple post-translational changes including extensive N-glycosylation in at least 5 sites (asparagine residues 140, 230, 271, 303 and 430) and at least 1 undetermined O-glycosylation (13,14). The glucan content is highly variable but it contributes at least 20% of the dry weight of most preparations (15). All mammalian AP have five cysteine residues per unit that form two disulfide bonds that contribute to enzyme conformational stability (1). The enzyme is anchored to the plasma membrane via its carboxyl terminal end by a phosphatidylinositol-glycan moiety (16). All four AP are coded by four different genes. The TNAP gene is located at the tip of chromosome 1 (1p36.1-p34) whereas the genes for the other three tissue specific AP are located at the end of the long arm of chromosome 2 (2q34-q37). The complete sequence of TNAP gene is known. TNAP gene is comprised of at least 50 kb of DNA and consists of 12 exons, of which the first and part of the second are noncoding (17,18). There are also two leader exons (exon 1B and 1L) with regulatory (promoter) function (10). Regulation of TNAP gene expression is complex and far from being fully understood. However, transcription factors Sp1 and Sp3, retinoic acid (activated form of vitamin A), progestin, granulocyte colony stimulating factor and 1.25-dihydroxyvitamin D have been reported to regulate TNAP gene expression either by increasing transcriptional activity or by increasing TNAP mRNA stability (19,20). Mutations of TNAP gene cause hypophosphatasia, a rare inherited systemic bone disease that is characterized by bone hypomineralization (12,21). As of end of 2013, 275 mutations in the TNAP gene have been reported to cause hypophosphatasia (11).

Physiological functions of AP in tissues other than bone remain largely unknown. The participation of bone isoenzyme of AP in the skeletal mineralization—a hypothesis first proposed by Robison (3)—is already confirmed and is the best known AP physiological action. The role of AP in bone mineralization has been verified, particularly after recognition of an inborn error of metabolism involving TNAP which was associated with defective bone mineralization (22). Although, TNAP has a broad specificity and may cleave phosphate from multiple substrates, natural substrates are pyrophosphate, pyridoxal 5'-phosphate (PLP) and perhaps, phosphoethanolamine (23,24). Hydrolysis of pyrophosphate to produce two inorganic phosphates by the enzyme is important for bone mineralization for at least two reasons: first it increases the availability of phosphate which afterwards is incorporated in the structure of hydroxyapatite together with Ca^{2+}; and second it decreases the concentration of pyrophosphate, a potent inhibitor of mineralization process (22). Pyridoxal-5'-phosphate (activated vitamin B6)—a co-enzyme that is required for >150 enzymatic reactions (25)—is also a physiological substrate for TNAP in leukocytes (26). Although, physiological consequences of TNAP action on pyridoxal-5'-phosphate remain largely unknown, in adults, common variants of TNAP gene influence plasma concentrations of this co-enzyme (27). Abnormal metabolism of pyridoxal-5'-phosphate may also explain epileptic seizures that experience some patients with hypophosphatasia (1). Other hypothetical but less known physiological AP actions have been reviewed (1,12,21,28). Lipopolysaccharide—a constituent of the cell wall of gram-negative bacteria—is also a substrate of intestinal AP. It is suggested that dephosphorilation of lipopolysaccharide by intestinal AP reduces its load and contributes to microbiome homeostasis in the intestinal tract (29).

Isoelectric focusing technique has detected more than 17 different AP isoforms in circulation (30). AP activity in serum originates mostly from liver and bone in similar proportions. Intestinal AP contributes to approximately 10% of overall AP activity (31). Circulating (soluble) AP is physiologically inactive (21). Placental type is

expressed only during the last semester of pregnancy (32). After fatty meal, AP of intestinal origin is elevated in some individuals but it makes a small contribution (less than 20%) (33). Mechanism of AP release from tissues in circulation is unknown, but C or D phosphatidases, detergents, cell turnover, proteolysis, lipolysis or membrane fragmentation have been proposed (21). The AP activity in plasma ranges between 40 and 130 U/L in healthy men and between 35 and 105 U/L in healthy women (measured by a standardized, colorimetric enzyme-assay according to the International Federation of Clinical Chemistry and Laboratory Medicine method) (31). Measurement of circulating AP activity is extensively used for the diagnosis of bone and liver disease. Both elevation (more common) and decrease of circulating AP activity have clinical utility (28,34).

Even though, measurement of AP is not used for the diagnosis of cardiovascular disease (CVD), emerging evidence suggests that elevated AP activity indicates an increased risk for CVD and total or CVD-related mortality. This review aimed to summarize the existing knowledge linking AP with CVD.

AP and CVD and mortality

Epidemiological evidence

Epidemiological evidence gathered from population-based, longitudinal studies suggests an association between elevated AP levels and CVD or mortality. Tonelli *et al.* (35) analyzed the association between AP and cardiovascular outcomes in survivors of an acute myocardial infarction [the Cholesterol And Recurrent Events (CARE) study; n=4,115] and in a general population sample [the Third National Health and Nutrition Examination Survey (NHANES III); n=14,716 adults collected between 1988–1994 and followed up for a mean of 12 years]. The CARE study findings are discussed later in this review. In the NHANES III sample there was a graded association between AP and all-cause mortality [mortality rates, 4.7%, 8.2% and 13.0% in the 1st, 2nd and 3rd AP tertiles; adjusted hazard ratio (HR) =1.27, 95% confidence interval (CI): 1.06 to 1.52, P=0.01 for 3rd *vs.* 1st AP tertile]. With respect to cardiovascular mortality (mortality rates, 1.9%, 3.5% and 5.9% in the 1st, 2nd and 3rd AP tertiles), the association was significant in partially (age, sex and race-adjusted) adjusted analysis [HR =1.61 (1.24–2.09) for 3rd *vs.* 1st AP tertile] but it was attenuated after full adjustment [HR =1.27 (0.98–1.65), P=0.07]. In the NHANES III analysis, the strength of association did

not differ according to presence of chronic kidney disease, proteinuria, smoking status, body mass index (dichotomized at 30 kg/m^2), diabetes or C-reactive protein (CRP; dichotomized at 3 mg/L). The findings remained consistent in analyses restricted to participants without pre-existing coronary heart disease (CHD) or when participants were categorized according to AP quintiles. Of note, cubic spline regression showed a linear relationship between higher AP levels and the risk of all-cause mortality. The association between AP and mortality was stronger in the presence of elevated phosphate levels (35). Wannamethee *et al.* (36) assessed the association of AP with CVD outcomes and mortality in a sample of 3381 men, 60 to 79 years of age recruited in the British Regional Heart Study, all of whom were without pre-existing diagnosis of myocardial infarction or stroke. During a mean follow-up of 11 years, there were 317 CHD events [adjusted HR =1.15 (1.03–1.28)], 230 stroke events [adjusted HR =1.09 (0.96–1.25)], 345 CVD deaths [adjusted HR =1.07 (0.96–1.18)] and 605 CVD events [fatal CHD, nonfatal myocardial infarction, stroke or CVD death; adjusted HR =1.09 (1.01–1.18)], with all risk estimates calculated per standard deviation (SD) increase in log-AP. Overall there were 984 deaths [adjusted HR =1.09 (1.03–1.16) per each SD increase in log-AP]. Notably, all associations remained consistent after exclusion of subjects with chronic kidney disease. The study showed an association between AP and the risk of CHD and CVD events that was partially explainable by AP association with CVD risk factors and inflammation in elderly men free of myocardial infarction or stroke (at entry). The association between AP and total mortality was independent of inflammation or chronic kidney disease.

Other studies offer additional evidence for an association between elevated AP and increased risk of CVD or related outcomes. Abramowitz *et al.* (37) tested the association of AP (or phosphate) with the risk of mortality or hospitalization in a retrospective cohort study of 10,743 outpatients with preserved renal function (estimated glomerular filtration rate of >60 mL/min/1.73 m^2) recruited between 2000 and 2002 and followed up to a median of 6.8 years. The mean age was 51 years and women comprised 64% of the cohort. Overall 949 patients died during the follow-up. There was a progressive increase in the risk of mortality from AP quartiles 1 (AP ≤66 U/L) to 4 (AP ≥104 U/L; P for trend <0.001). In the risk assessment analysis, the unadjusted and fully adjusted HR for the association of AP with mortality were 2.05 (1.70–2.48) and 1.65 (1.36–2.01), respectively (for AP quartile 4 *vs.* quartile 1). AP was also

associated with the risk of all-cause [adjusted HR =1.14 (1.03–1.25)], CVD [adjusted HR =1.28 (1.10–1.48)], infection [adjusted HR=1.53 (1.28–1.84)] or fracture-related [adjusted HR =1.63 (1.03–2.58)] hospitalization (with all risk estimates calculated for 4th vs. 1st AP quartile). Filipowicz et al. (38) assessed the association of skeletal and non-skeletal AP with CRP or mortality in a sample of 10,707 adults from the 1999–2004 NHANES participants. The total serum AP showed a moderate correlation with skeletal AP (Pearson r=0.74, P<0.001) assuming that 55% of variation in serum AP could be due to variation in skeletal AP. There were 715 deaths over a follow-up of 54,089 person-years. Total AP was associated with the risk of elevated CRP [adjusted odds ratio (OR) =1.96 (1.74–2.22)] and mortality [HR =1.28 (1.08–1.52)]. However, there were differences with respect to the association of skeletal and non-skeletal AP with CRP or mortality. Skeletal AP was not associated with CRP or mortality in subjects with or without chronic kidney disease. Conversely, non-skeletal AP was associated with elevated CRP in subjects without chronic kidney disease [OR =4.51 (3.80–5.35)] and those with chronic kidney disease [OR =5.98 (3.40–10.51)]. Of note, non-skeletal fraction was associated with the risk of mortality in subjects without chronic kidney disease [HR =1.96 (1.37–2.80)] but not in those with chronic kidney disease [HR =0.92 (0.51–1.67)] with all risk estimates calculated per doubling of serum skeletal AP (P for interaction =0.03). The study showed that non-skeletal sources of AP elevation, but not bone disease, may explain the association between total AP and increased risk of mortality or inflammation. Koehler et al. (39) assessed the association of liver enzymes with all-cause and cause-specific mortality in 5,186 participants (61.6% women) aged 55 years or older, embedded in the Rotterdam Study. During a follow-up of up to 19.5 years (median: 14 years), 2,997 participants (57.8%) died. Of all the deaths, 672 were of CVD origin and 703 were due to cancer. AP was associated with all-cause [adjusted HR =1.51 (1.28–1.77)], CVD [adjusted HR =1.43 (1.01–2.04)] and cancer-related [adjusted HR =1.57 (1.12–2.20)] mortality, with all risk estimates calculated for AP>95th percentile (124 U/L in women and 119 U/L in men) vs. <25th percentile (61 U/L in men and 64 U/L in women). Findings remained consistent when hemoglobin and phosphate values were added into the multivariable models. The study showed that an elevated AP level is associated with the risk of mortality from cancer and CVD in subjects >55 years of age. Fulks et al. (40) investigated the relationship between liver enzymes (including AP) and all-cause mortality in life insurance

applicants (n=1,905,664). There were 50,174 deaths over a median follow-up of 12 years. Using the risk of the middle 50% of the population (25th to 74th percentiles) as reference, the relative risk (RR) of mortality increased significantly and was linear from very low to relatively high values of AP. Recently, Kunutsor et al. (41) assessed the association of AP with CVD risk in 6,974 participants of the Prevention of Renal and Vascular End-stage Disease (PREVEND) study—an observational, general population-based cohort study. AP correlated with several CVD risk factors, notably age and CRP. Seven hundred and thirty seven participants developed CVD over a median follow-up of 10.5 years. AP showed a "J-shaped" relationship with the risk for developing CVD. After adjustment for conventional CVD risk factors, the HR for an association between AP and CVD was 1.34 (1.14–1.56) for 5th vs. pooled 1st–4th AP quintiles. The association persisted after adjustment for additional confounders but was attenuated after adjustment for CRP [HR =1.24 (1.05–1.45)]. Of note, addition of AP in multivariable risk prediction models containing established CVD risk factors, did not improve the risk prediction for incident CVD by these models, as judged by the degree of change of C statistic or net reclassification improvement. In a sample of 1,054 participants aged 65 years or over (49% women) from the British National Diet and Nutrition Survey, the association between bone-related markers (including AP) and subsequent mortality was analyzed. Over a 13 to 14 years of follow-up, AP was associated with mortality in women [age-adjusted HR =1.08 (1.01–1.16)] but not in men [age-adjusted HR =1.06 (0.89–1.26)]. However, after full adjustment, the association was attenuated to below the significance level (42).

AP has been shown to predict mortality in patients with chronic kidney disease and dialysis. A study by Regidor et al. (43) that included a 3-year cohort of 73,960 hemodialysis patients showed that an AP level ≥120 U/L was associated with a 25% increase in the adjusted risk for all-cause mortality [HR =1.25 (1.21–1.29)]. A rise in AP by 10 U/L over the first 6 months was incrementally associated with higher risk of death in the subsequent 2.5 years. Three-year cardiovascular mortality was also increased with the increase of AP level. There was a somewhat linear relationship between AP and mortality. Recently, bone AP has been proposed as a potential target of drug therapy to improve cardiovascular outcomes in patients with chronic kidney disease (44).

Three recent meta-analyses have summarized epidemiological evidence on the association between AP

and the risk of CVD or mortality (45-47). A meta-analysis by Kunutsor *et al.* (45) that included four prospective cohort studies with 33,727 participants and 2,097 cases reported a 8% increase [RR =1.08 (1.03–1.14)] in the risk for CVD for each SD increase in AP level. Another meta-analysis of 4 prospective studies reported a 38% increase in the risk for all-cause mortality [RR =1.38 (1.17–1.63)] in individuals in the top third *vs.* those in the bottom third of AP level (45). Finally, a recent meta-analysis by Li *et al.* (47) that included only studies of subjects with preserved renal function reported a 2% increase in the risk for CVD death [RR =1.02 (1.01–1.04)] and a risk of 57% [RR=1.57 (1.27–1.95)] for all-cause mortality for high *vs.* low AP group. The AP-mortality relationship was non-linear.

In conclusion, epidemiological evidence is strong for an association between elevated AP activity and the risk of all-cause mortality in general population. Although several studies have reported an increased risk for incident CVD in subjects with elevated AP level, evidence is weaker for an association between elevated AP and the risk for incident CVD or CVD-related mortality. Evidence remains inconclusive with respect to gender-related disparities in the association between AP and the risk for mortality or CVD. The association between elevated AP and the risk of atherosclerosis, CHD or acute coronary events such as acute myocardial infarction remains partially investigated. An elevated AP level appears to be associated with reduced survival in patients with chronic kidney disease.

AP and stroke

Epidemiological evidence suggests an association between elevated AP and the risk of stroke. In the British Regional Heart Study, elevated AP was associated with the increased risk of stroke over a mean follow-up of 12 years in men without myocardial infarction or stroke at baseline. In subjects with AP in the 1st, 2nd, 3rd and 4th AP quartiles, the stroke rates per 1,000 person-years were 5.5, 6.9, 6.5 and 9.5, respectively. The association was significant in age-adjusted analysis [HR =1.15 (1.03–1.29)] but it was attenuated after full adjustment [HR =1.09 (0.96–1.25)], with both HRs calculated per SD increase in log-AP (36). Shimizu *et al.* (48) assessed the association of AP with the risk of stroke in a cohort of 10 754 Japanese subjects (4,098 men and 6,656 women; aged 40–69 years) recruited in the Circulatory Risk in Communities Study (CIRCS) study. During a 16-year follow-up, there were 264 strokes (164 ischemic; 69 hemorrhagic) in men and 225 strokes

(118 ischemic; 89 hemorrhagic) in women.

There was a U-shaped relationship between AP level and incidence of stroke in men and women which for the most part was confined to non-drinkers. The fully adjusted HR for association of AP with total strokes was 1.79 (1.20–2.68) in men and 1.29 (0.85–1.96) in women. According to drinking status the fully adjusted HRs in non-drinkers were 5.90 (2.01–17.33) in men and 1.43 (0.92–2.22) in women; in drinkers, the fully adjusted HRs were 1.32 (0.83–2.11) and 0.31 (0.06–1.74), in men and women, respectively, with all HR calculated for AP 5th quintile *vs.* 3rd quintile (used as reference in the setting of U-shaped relationship). In non-drinkers, AP was associated with the risk of ischemic stroke in men and hemorrhagic stroke in women. Lower AP levels were associated with higher risk of hemorrhagic stroke in both genders. Although, alcohol consumption is known to be associated with the risk of stroke (both ischemic and hemorrhagic) (49), the CIRCS study showed an association between AP and stroke only in non-drinkers (48). In a cohort of 3,916 stroke-free participants ≥55 years of age from the Rotterdam Study, AP was associated with the risk of hemorrhagic stroke [55 hemorrhagic strokes over 50,375 person years of follow-up: adjusted HR=1.30 (1.02–1.68) per SD increment of AP level]. The association was stronger in excessive alcohol drinkers than in non-excessive alcohol drinkers (50).

Several studies have shown that increased AP is associated with poor outcomes after stroke (51,52). A prospective study of 2,029 stroke patients, followed up for a mean of 923 days showed a significant association between baseline AP and all-cause death with adjusted HR for 3rd, 4th and 5th AP quintiles of 1.67 (1.12–2.49), 1.79 (1.20–2.67) and 2.83 (1.95–4.10) compared with 1st AP quintile. The associations were significant for ischemic and hemorrhagic strokes. The risk for vascular death was also higher in patients with elevated AP level with HR for 4th and 5th AP quintiles (*vs.* the 1st quintile) of 1.81 (1.14–2.86) and 2.78 (1.87–4.15), respectively (51). In another more recent study of 1,034 patients with first-ever acute cerebral infarction, AP levels were associated with a poor functional outcome [OR =1.25 (1.04–1.50) for each SD increment of AP] but not with cerebral atherosclerosis (52). In other studies, AP has been associated with cerebral small vessel disease—a marker of silent brain (lacunar) infarction (53), multi-cerebral microbleeds in patients with acute ischemic stroke (54) or transformation of ischemic to hemorrhagic stroke in patients with atrial fibrillation (55). In another recent study of 1,082 neurologically healthy

subjects undergoing brain magnetic resonance imaging, elevated AP was independently associated with white matter hyperintensities and cerebral infarct but not with microbleeds (56). Although such conditions help to explain an elevated risk of stroke in subjects with elevated AP and may impact unfavourably on prognosis of patients after stroke, causality remains unproven.

In aggregate, there is limited (particularly compared to evidence linking AP with the risk of all-cause mortality) but strongly suggestive epidemiological evidence for an association between AP and increased risk of stroke. Evidence available also suggests that subjects with elevated AP may be at higher risk of a poor functional outcome or mortality following a stroke event. Gender-related disparities in the association between AP and the risk of stroke and confounding effect of drinking on the AP-stroke association require further study.

AP and mortality in patients with CHD

Patients with known CHD are at higher risk of mortality primarily due to CVD causes and biomarkers are valuable tools for risk stratification in these patients. A number of studies have investigated the prognostic value of AP in various subsets of patients with CHD. The association between AP and subsequent outcomes was assessed in 4,115 subjects with a previous myocardial infarction in the setting of CARE study (35). A graded, independent association was noted between AP tertiles and the risk for all-cause mortality over a median follow-up of 58.9 months [mortality rates 7%, 9% and 11% in the 1st, 2nd, and 3rd AP tertiles: fully adjusted HR =1.43 (1.09-1.89)]. However, there was no independent association between AP and CHD death [adjusted HR =1.13 (0.78–1.63)] or nonfatal myocardial infarction [adjusted HR=1.23 (0.94–1.62)]. For the association between AP and the composite end point of fatal CHD, nonfatal myocardial infarction, symptomatic heart failure or stroke, the significance was borderline [adjusted HR =1.21 (0.98–1.46). P=0.05] with all risk estimates calculated for highest vs. lowest AP tertile. Thus the CARE study showed an independent association between AP and all-cause mortality but not an association with CHD-related death or acute coronary events (35). Park et al. (57) assessed the association of AP with prognosis of patients with CHD who underwent percutaneous coronary intervention with drug-eluting stent implantation in 1,636 patients without known liver disease or cancer (at baseline) over a median follow-up of 762 days. The rates of all-cause and cardiac

mortality in the 1st, 2nd and 3rd AP tertiles were 3.1%, 5.5% and 13.2% and 1.8%, 2.5% and 6.6% respectively: adjusted HR =4.21 (2.03–8.71) for all-cause mortality and adjusted HR =3.92 (1.37–11.20) for cardiac mortality. AP also was associated with the risk of nonfatal myocardial infarction [adjusted HR =1.98 (0.91–4.29)], and stent thrombosis [adjusted HR =2.73 (1.33–5.61)], with all HR calculated for highest vs. lowest AP tertile. Of note, AP was significantly associated with the presence of angiographic coronary calcium (P=0.046). Our group assessed the association between AP and 3-year outcome in 5,540 patients with angiography-proven coronary artery disease treated with percutaneous coronary intervention (58). The 3-year mortality rates according to AP tertiles were 7.2%, 8.1% and 11.8% in the 1st, 2nd and 3rd AP tertiles with HR =1.33 (1.19–1.50) calculated per each tertile increment. After adjustment, the association between AP and all-cause mortality remained significant [adjusted HR =1.33 (1.18–1.51), calculated per unit increment in log-AP]. Adding AP in multivariable models alongside cardiovascular risk factors and CRP improved the risk prediction for all-cause mortality as assessed by C-statistic changes [C-statistic without AP, 0.820 (0.797–0.843) vs. C-statistic 0.825 (0.804–0.849) after AP inclusion; P<0.001]. The association with cardiac mortality was also significant [adjusted HR =1.40 (1.20–1.62) per unit log-AP]. The study did not find an association between AP and coronary calcium or the risk of acute coronary events (nonfatal myocardial infarction or stent thrombosis) or stroke. Moreover, the association between AP and all-cause mortality was stronger in subjects of younger age (<65 years). Of note, chronic statin therapy was associated with reduced levels of circulating AP (58).

Few studies have investigated the association between AP and prognosis in patients with acute coronary syndromes and ST-segment elevation myocardial infarction (STEMI). In a recent study by our group, the 3-year prognostic value of AP was tested in 2,134 patients with acute coronary syndromes after percutaneous coronary intervention (59). Using a cut-off of 98 U/L (defined by receiver operating characteristic curve analysis), mortality rates were 19.5% and 9.3%, in patients with AP >98 and ≤98 U/L, respectively [adjusted HR =1.37 (1.10–1.70) for each unit increment in log-AP]. AP was also associated with the risk of cardiac mortality [mortality rates 14.3% and 6.0% in patients with AP >98 and ≤98 U/L, respectively (adjusted HR =1.32 (1.02–1.70), for each unit increment in log-AP]. The C-statistic was improved by AP inclusion in multivariable models of all-cause mortality [0.836 (0.807–0.866)

before *vs.* 0.842 (0.814–0.874) after AP inclusion; P=0.045] but not in the models of cardiac mortality [0.854 (0.823–0.884) before and 0.857 (0.824–0.889) after AP inclusion; P=0.385]. The study found a stronger association between AP and all-cause mortality in patients of younger age (<65 years) and those with preserved renal function (P for interaction=0.006 and 0.038, respectively). With respect to cardiac mortality the association was stronger in patients of younger age (<65 years; P for interaction =0.028) (59). A recent study that assessed the prognostic value of liver enzymes in 847 patients in acute phase of STEMI showed that AP was the strongest correlate of in-hospital major adverse cardiovascular events [OR =1.02 (1.01–1.03)] (60). Another study of diabetic patients presenting with acute myocardial infarction showed that elevated AP was associated with reduced subsequent 5-year survival (61). A recent study in 1178 Korean STEMI patients assessed the association of AP measured at presentation and major adverse cardiovascular events—a composite of all-cause death, non-fatal myocardial infarction, non-fatal stroke, and ischemia-driven revascularization over a median follow-up of 25 months. The AP level was below the upper limit of normal in about 95% of patients. There was a progressive increase in the incidence of adverse events from 8.7% to 11.7% and 15.7% across AP tertiles (<64, 65–82 and >83 U/L, respectively) with adjusted HR =1.69 (1.01–2.81) for 2nd *vs.* 1st AP tertile and HR =2.46 (1.48–4.09) for 3rd *vs.* 1st AP tertile (62).

In summary, available evidence suggests that elevated AP is associated with the increased risk of death in patients with stable CHD or acute coronary syndromes. However, several aspects of this association remain unclear. In the absence of comparative studies in patients with proven CHD and subjects without CHD, the question whether the presence of CHD strengthens the association with mortality remains unanswered. Although patients with CHD are expected to die mostly from cardiac causes, the association of AP with cardiac mortality seems to be weaker than the association of AP with all-cause mortality and the reasons for this remain unknown. The association between AP, coronary calcium and acute coronary events remains controversial. Finally, the impact of percutaneous coronary intervention or current cardiovascular drugs (particularly statins) on the association between AP and outcome in patients with CHD is plausible but needs further study.

AP and congestive heart failure

Abnormal liver tests, including abnormal AP values are common in patients with congestive heart failure (63-65) and caused by liver congestion and injury in the setting of this syndrome (66). Very high AP levels in patients with congestive heart failure have been described (67,68). In the CARE study, a progressive increase in the frequency of symptomatic congestive heart failure with the increase in AP activity was observed [adjusted HR=1.38 (1.01–1.88), for highest *vs.* lowest AP tertile] (35). Several studies have suggested that elevated AP is associated with poor prognosis in patients with congestive heart failure. In an analysis from the SURVIVE (Survival of Patients With Acute Heart Failure in Need of Intravenous Inotropic Support) trial that included 1,134 patients, 11% of patients had abnormal AP values (64). Of note, patients with abnormal AP had marked signs of congestion, elevated right-sided filling pressures and increased 6-month mortality (64). Poelzl *et al.* (69) assessed the association of liver function tests in 1,032 patients with stable congestive heart failure. In this study cholestatic enzymes, but not amino transferases were associated with severity of disease. Over a median follow-up of 36 months, death or heart transplantation (primary end point) was recorded in 339 patients. Total bilirubin, AP and gamma-glutamyl transferease were associated with the outcome in bivariate analysis. In multivariable analysis, AP [adjusted HR =1.52 (1.09–2.12)] and gamma-glutamyl transferase [adjusted HR =1.22 (1.06–1.41) with both HR calculated per log units of the biomarkers] remained independently associated with the risk of primary endpoint (69). In other studies, however, the association between elevated AP and prognosis was attenuated after adjustment in multivariable analysis (65,70). A recent retrospective analysis of 972 patients showed that elevated AP was associated with the risk of worsening of renal function in the course of acute decompensated heart failure [adjusted OR =1.69 (1.02–2.79) for 2nd *vs.* 1st AP tertile and adjusted OR=1.95 (1.20–3.21) for 3rd *vs.* 1st AP tertile] (71). These studies almost unanimously showed that liver function tests including AP are frequently abnormal in patients with congestive heart failure and they signify a poor prognosis in these patients.

AP and cardio-metabolic risk factors

Many studies have shown an association between AP and several cardio-metabolic risk factors. In the British Regional Heart Study, AP was significantly associated with older age, smoking, physical inactivity, elevated systolic blood

pressure, low-density lipoprotein cholesterol, CRP, reduced renal function, von Willebrand factor and reduced lung function (36). In the CARE study that included subjects with definite diagnosis of previous infarction, AP was strongly associated with older age, sex (higher proportion of women in top AP tertile), alcohol consumption, smoking, proteinuria, systolic blood pressure, lower high-density lipoprotein, fasting glucose and diabetes (35). In the NHANES III data set, AP was strongly associated with older age, male sex, smoking, alcohol consumption, body mass index, diabetes, albuminuria, lower high-density lipoprotein level, higher systolic blood pressure, lower 25(OH)-vitamin D and higher CRP-level (35). In the Rotterdam Study, higher AP levels were associated with older age, female sex, diabetes, current smoking and arterial hypertension as well as lower alcohol intake and elevated cholesterol (39). A particularly strong correlation between AP and elevated CRP has been reported (41,72-74). It has been suggested that AP represents an acute phase reactant and a marker of inflammation of hepatic origin (72) or that the association between AP and increased CVD risk may be mediated by inflammation (41). A retrospective analysis of 15,234 adult participants in the NHANES III study showed a progressive increase in the frequency of metabolic syndrome with the increase in AP level (frequency 14% among subjects in lowest AP quartile and 41% among subjects in highest AP quartile). In subjects with AP in 2nd, 3rd and 4th AP quartiles, the adjusted ORs for the risk of metabolic syndrome were 1.41, 1.66 and 1.75 compared with AP 1st quartile (reference). However, the association of AP with mortality remained significant after adjusting for demographic factors [HR =1.52 (1.35–1.72)] or demographic factors plus metabolic syndrome [HR =1.37 (1.21–1.56)]. The association was stronger in a subgroup of subjects without any component of metabolic syndrome [HR =1.83 (1.36–2.46)] (75). A large Chinese case-control study that included 6,268 subjects with metabolic syndrome and 6,330 matched healthy controls showed that the risk of metabolic syndrome increased progressively with the increase in AP activity with OR=1.43 (1.28–169); OR=1.87 (1.68–2.08) and OR=2.80 (2.52–3.10) for the 2nd, 3rd and 4th AP quartiles vs. the 1st quartile. The risk of metabolic syndrome was increased by 9% [adjusted OR=1.09 (1.08–1.10)] for each 5 units increase in the AP activity (76). Based on these studies, the evidence is clear that elevated AP levels signify a worse cardio-metabolic risk profile and the close association between AP and CVD risk factors may account at least in part for the association between AP and the risk of CVD or mortality.

Putative mechanisms of the association between AP and CVD risk and mortality

Outside clinically overt bone or liver disease (both conditions may affect CVD risk and survival), mechanisms for the association of AP with CVD risk or mortality remain incompletely investigated. Nevertheless, a number of potential mechanisms may be mentioned.

First, as stated above, elevated AP level is associated with a plethora of cardio-metabolic risk factors. This association has at least two implications: firstly, the association implies that a part of the risk associated with elevated AP levels is mediated by cardio-metabolic risk factors; secondly, the association raises concerns with respect to whether AP has a direct role in the pathophysiology of CVD and the risk of mortality or it is simply an epiphenomenon of CVD risk factors which tend to cluster in subjects with elevated AP level.

Second, AP is strongly associated with markers of systemic inflammation, in particular CRP. According to current paradigm, inflammation plays a crucial role in all stages of atherosclerosis including atherosclerotic plaque initiation, progression and transition from stable to unstable plaques with subsequent clinical events (77).

Third, elevated AP has been reported to reflect low levels of vitamin D in population-based studies (35) and patients with chronic kidney disease on dialysis (78). A prospective study by Giovannucci et al. (79) showed that vitamin D deficiency was associated with the risk of myocardial infarction in a graded manner and independent of CVD risk factors in men. Other studies have shown that up to 90% of patients presenting with ST-segment elevation myocardial infarction had low levels of vitamin D (80) or that vitamin D deficiency was associated with 1-year mortality in patients presenting with acute coronary syndromes (81). In fact, vitamin D deficiency is an emerging CVD risk factor and potentially relevant mechanisms explaining the association between vitamin D deficiency and cardiovascular risk and mortality may include the association of low vitamin D levels with smooth muscle cell proliferation, endothelial dysfunction, vascular inflammation, vascular calcification, increased activity of renin-angiotensin system and atherosclerosis (82-84). Despite this evidence a recent randomized, double-blinded, placebo-controlled study found no effect of vitamin D supplementation for 8 weeks on bone turnover markers (including bone AP) in 197 hypertensive patients (85).

Fourth, elevated AP may be associated with liver disease, primarily non-alcoholic fatty liver disease—a highly prevalent morbid condition and a common cause of elevated liver enzymes (86). Multiple lines of evidence suggest that non-alcoholic fatty liver disease is closely associated and shares many features with metabolic syndrome and other factors predisposing for coronary atherosclerosis and CVD (87). CVD remains a common cause of death in patients with non-alcoholic fatty liver disease and patients with this condition may benefit from CVD risk assessment (88). However, a recent analysis from the PREVEND study involving 6,340 participants aged 28–75 years without pre-existing CVD found no association between non-alcoholic fatty liver disease and the risk of CVD, once the association was adjusted for established CVD risk factors and other potential confounders (89).

Although important for understanding the association between AP and the risk of CVD or mortality, none of the above-cited mechanisms points out to a direct role of AP in the pathophysiology of CVD or CHD. Demonstration of the involvement of AP in vascular calcification (90) has led to the hypothesis that AP mediates elevated CVD risk and a deleterious prognostic impact through vascular calcification. Mechanisms of vascular calcification have been recently reviewed (91). Several lines of evidence seem to support such a role. AP activity is crucial for formation of hydroxyapatite—a crucial molecule in the endochondral calcification or vascular calcification when AP is expressed in osteoblast-type cells originating from phenotypic transformation of smooth muscle cells (92). AP stimulates vascular smooth muscle cell trans-differentiation into chondrocyte-like cells (93), a pivotal step in vascular calcification. Up-regulation of AP expression in vessels with medial calcification (94) or advanced calcified atherosclerotic lesions (95), hydrolysis of pyrophosphate—an inhibitor of calcification—by up-regulated AP (96) and inhibition of vascular calcification by AP inhibitors (97) appear to offer evidence in favor of AP involvement in vascular calcification. An experimental mouse model involving overexpression of human TNAP in vascular smooth muscle cells demonstrated extensive medial vascular calcification early in life, in the absence of systemic changes in calcium, phosphate, or renal function (98). Medial calcification leads to vascular stiffness and consequently to increased pulse pressure (99) and left ventricular hypertrophy (100)—factors associated with the risk of mortality in patients with CHD. Although, the association of vascular calcification with atherosclerosis remains poorly understood (92), medial

artery calcification is associated with CHD and increased risk of mortality in patients with chronic kidney disease (101) and diabetes (102). Coronary calcium also predicts mortality in community-living individuals (103). Whether, atherosclerotic plaque calcification contributes to plaque instability remains controversial (92). Some authors have suggested that presence of calcium destabilizes the plaque either because of a physical stress exerted by calcified nodules (104) or propensity of plaques to rupture at areas of interface between high and low density tissue (105). Spotty calcification has been suggested to predispose for atherosclerotic plaque rupture, as well (106). Putative mechanisms of AP association with CVD are shown in *Figure 1*.

A number of studies have suggested an association of AP with several conditions that may increase CVD risk or mortality. Thus AP has been shown to be associated with the extent of coronary artery disease (107), lack of collateral circulation (108), contrast-induced nephropathy in patients with acute coronary syndromes (109), arterial stiffness after renal transplantation (110), left ventricular hypertrophy in mice (111), endothelium-dependent vasodilation and endothelial dysfunction in hypertensive patients (112), adipogenesis (113), atherogenic diet (33), phosphate metabolism (36), low adiponectin (114), high leptin (115), fibroblast growth factor-23 (116) or environmental pollutants (117). Although these conditions may increase the risk of CVD or have a negative impact on prognosis, causality relationship between them and AP remains unproven.

Conclusions and perspective

Population-based longitudinal studies suggest an association between AP and increased risk of incident CVD or all-cause mortality. Clinical studies involving patients with pre-existing CHD also suggest an association between AP and mortality in these patients. The association between AP and increased risk of mortality seems to be stronger for all-cause mortality than for cardiac mortality. Experimental studies strongly suggest an involvement of AP in vascular calcification and a direct role of the enzyme in the pathophysiology of CVD. There is no consistent evidence with respect to association of AP with extent of CHD, angiographic coronary calcification or acute coronary events such as acute myocardial infarction. The association between AP and other CVD such as arterial hypertension or cardiac arrhythmias, primarily atrial fibrillation remains unexplored. AP is associated with cardiometabolic risk
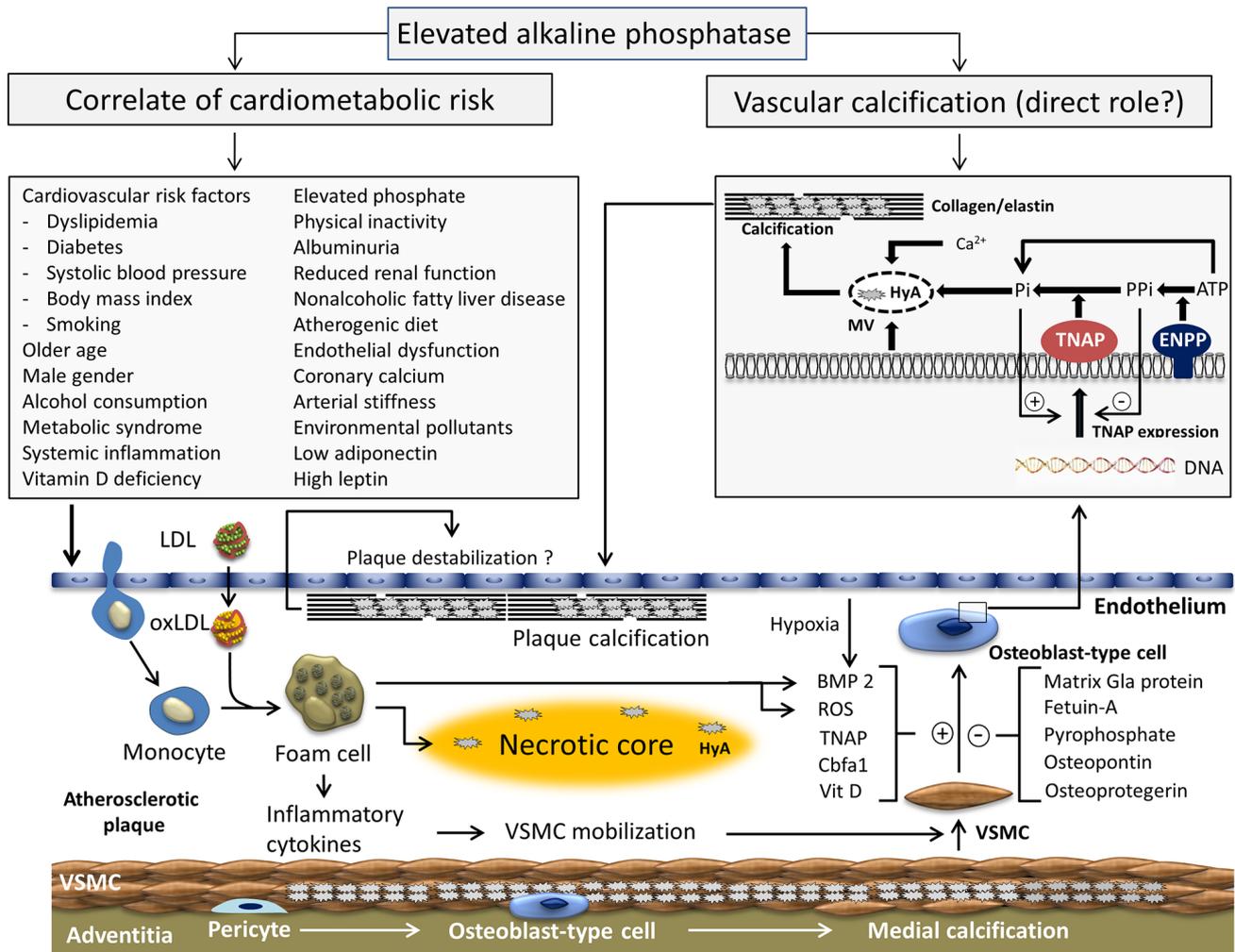
Figure 1 Putative mechanisms of the association between alkaline phosphatase and cardiovascular disease. ATP, adenosine triphosphate; BMP, bone morphogenetic proteins; Ca^{2+}, calcium ion; DNA, deoxyribonucleic acid; Cbfα1, transcription factor core binding factor-α1; ENPP, ectonucleotide pyrophosphatase/phosphodiesterase-1; HyA, hydroxyapatite; LDL, low-density lipoprotein; oxLDL, oxidized low-density lipoprotein; MV, microvesicles; Pi, phosphate; PPi, pyrophosphate; ROS, reactive oxygen species; TNAP, tissue-nonspecific alkaline phosphatase; Vit D, vitamin D; VSMC, vascular smooth muscle cell. Plus sign shows a stimulatory effect; minus sign shows an inhibitory effect.

factors including conventional CVD risk factors, metabolic syndrome, systemic inflammation, vitamin D deficiency and several morbid conditions that may signify a poor CVD risk profile and increased risk for mortality. The intricate relationship between AP and cardio-metabolic risk factors and failure to improve risk prediction for incident CHD (41) or cardiac mortality in patients with acute coronary syndromes (59) by the enzyme seem to weaken arguments for a causal relationship between AP and CVD. Further explorations of the molecular mechanisms of the involvement of AP in the pathophysiology of CVD and the

use of the enzyme as a therapeutic target to reduce CVD risk remain to be elucidated in future studies.

Footnote

Conflicts of Interest: The author has completed the ICMJE uniform disclosure form (available at http://dx.doi.

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Ethical Statement: The author is accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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