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# Bone matrix vesicle-bound alkaline phosphatase for the assessment of peripheral blood admixture to human bone marrow aspirates

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ABSTRACT

*Purpose:* Peripheral blood (PB) admixture should be minimized during numerical and functional, as well as cytokinetic analysis of bone marrow (BM) aspirates for research purposes. Therefore, purity assessment of the BM aspirate should be performed in advance. We investigated whether bone matrix vesicle (BMV)-bound bone alkaline phosphatase (ALP) could serve as a marker for the purity of BM aspirates.
*Results:* Total ALP activity was significantly higher in BM serum (97 (176–124) U/L, median (range)) compared to PB serum (63 (52–73) U/L, $p < 0.001$). Agarose gel electrophoresis showed a unique bone ALP fraction in BM, which was absent in PB. Native polyacrylamide gel electrophoresis revealed the high molecular weight of this fraction, corresponding with membrane-bound ALP from bone matrix vesicles (BMV), as evidenced by electron microscopy. A serial PB admixture experiment of bone cylinder supernatant samples, rich in BMV-bound ALP, confirmed the sensitivity of this proposed quality assessment method. Furthermore, a BMV ALP fraction of ≥15% is suggested as cut-off value for minimal BM quality. Moreover, the BM purity declines rapidly with larger aspirated BM volumes.
*Conclusion:* The exclusive presence of BMV-bound ALP in BM could serve as a novel marker to assess purity of BM aspirates.


## 1. Introduction

Relatively large amounts of bone marrow (BM) are aspirated for cellular analysis and cytokinetic profiling of BM in both diagnostic and research settings. However, inevitable peripheral blood (PB) contamination during BM aspiration could lead to misinterpretation of the obtained results. In the setting of autologous stem cell therapy for cardiovascular diseases for example, numerical and functional differences between BM could explain the magnitude of response to stem cell therapy [1], but ideally should take PB contamination into account. Therefore, quality assessment of the BM aspirate prior to BM analysis in research settings is essential and should be reinstated into the field [2].

The current method in daily clinical practice to assess purity of a BM aspirate is a qualitative method based on the observation of spicules in the BM smear. Quantitative assessment on the other hand is challenging and is often neglected, and to date, no consensus is reached on an acceptable gold standard.

A first quantitative approach dating from the 80s was based on the difference of nucleated cells in S-phase index between BM biopsies (i.e. bone cylinders) and BM aspirates, which was considered as

*Abbreviations:* ALP, alkaline phosphatase; BC, bone cylinder; BM, bone marrow; BMV, bone matrix vesicle; Hb, hemoglobin; MW, molecular weight; NA, neuraminidase; PAGE, polyacrylamide gel electrophoresis; PB, peripheral blood; PI-PLC, phosphatidyl-inositol-phospholipase C; SN, supernatant; TEM, transmission electron microscopy.

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a consequence of PB cell contamination exclusively [3]. Secondly, flow cytometric quantification of the percentage CD45+ gated lymphocytes and monocytes in the BM aspirate was introduced to evaluate BM quality, since this percentage is inversely related to the percentage of cells in the S-phase for the total BM cell population [4]. A total lymphocyte–monocyte count of >30% was used as cut-off value to exclude BM aspirates with an unacceptable degree of PB contamination [4]. Nowadays, the most common technique is the Holdrinet method [5,6]. By means of $Cr^{15}$ labeled autologous erythrocytes, Holdrinet et al. showed that 97% of the hemoglobin (Hb) content in BM is derived from PB. Based on the assumption that a proportional number of PB cells is present, the fraction of PB admixture could be calculated by determining the Hb levels in blood and bone marrow.

However, the aforementioned methods for the quantification of PB admixture to BM aspirates are based on *cellular* assessments and should not be used in conditions that possibly influence cellular function of BM such as heart failure [7–9]. Therefore, we searched for an alternative stable marker, which is exclusively present in BM and is independent of BM cellular characteristics.

To this purpose we investigated bone alkaline phosphatase (ALP), an important bone turnover marker produced by bone forming osteoblasts from BM [10]. The enzyme ALP consists of different isoenzymes and, in humans, four ALP genes are identified: tissue-nonspecific, intestinal, placental and germ-cell ALP [11,12]. After posttranslational modifications of the tissue-nonspecific ALP gene product, bone and liver ALP isoforms are formed [11].

Whereas the tertiary structure of ALP in PB promotes dimerization, tetrameric ALP and high molecular weight (MW) isoforms have also been described for all the ALP isoenzymes/isoforms [11]. The high MW isoforms consist of ALP anchored to the plasma membranes of shedded microvesicles and incubation of these microvesicles with a non-ionic detergent results in the release of anchor-bearing ALP from the membrane which, in the presence of detergents, has a MW that is consistent with tetrameric ALP [11]. Phospholipases, such as phosphatidyl-inositol-specific phospholipases, are responsible for the conversion of this tetrameric ALP into the soluble dimeric form of ALP [11,13–15].

In PB serum from healthy subjects, liver and bone ALP mainly circulate as soluble dimers, and tetrameric liver and bone ALP are absent. In pathological conditions, the finding of membrane-bound bone ALP in PB is extremely rare, in contrast to membrane-bound liver ALP [11].

We hypothesized that a high activity of membrane-bound bone ALP, originating from bone matrix vesicles (BMV) formed by budding of osteoblasts that reside in the BM [16], is present in BM aspirates but absent in PB. As such, the activity of BMV-bound ALP could serve as a novel marker to assess BM purity.

## 2. Materials and methods

### 2.1. Patients and collection of BM and PB samples

After approval of the Antwerp University Hospital Ethics Committee and written informed consent, patients undergoing elective cardiac surgery were included in the study. Exclusion criteria were chronic inflammatory or malignant disease, chronic kidney disease (Creatinine clearance <30 ml/min), severe liver failure and disorders with abnormal bone turnover. Table 1 shows the patient characteristics and indicates in which cohort the experiments were performed.

Prior to sternotomy and under general anesthesia, BM samples were aspirated by sternal puncture (15G Jamshidi BM aspiration needle) as described previously [17] and arterial PB was sampled. Both BM and PB were collected in Vacutainer® serum tubes (Becton Dickinson (BD) Benelux, Erembodegem, Belgium). Serum of BM and PB was obtained by 15 minute centrifugation at 1500 *g*. For the quantification of PB admixture by the Holdrinet method, PB and BM samples were collected in Vacutainer® EDTA tubes (BD Benelux) to determine Hb levels.

**Table 1**
Patient characteristics and indication in which cohort the different experiments were performed. The results are expressed as mean ± SD or as percentage (%) of patients.

| Characteristic | Cohort 1 N = 55 | Cohort 2 N = 20 | Cohort 3 N = 9 | Cohort 4 N = 4 |
|---|---|---|---|---|
| Age (yrs) | 63 ± 10 | 64 ± 11 | 70 ± 11 | 42 ± 15 |
| Male (%) | 84 | 90 | 89 | 50 |
| BMI (kg/m$^2$) | 27 ± 4 | 27 ± 4 | 28 ± 3 | N.A. |
| Creatinine clearance (ml/min) | 77 ± 18 | 74 ± 22 | 74 ± 20 | N.A. |
| Left ventricular ejection fraction (%) | 53 ± 19 | 52 ± 18 | 44 ± 12 | N.A. |
| Framingham risk score (%) | 14 ± 8 | 15 ± 8 | 19 ± 10 | N.A. |
| CRP (mg/l) | 5.7 ± 7.8 | 15.1 ± 33.3 | 27.7 ± 46.3 | N.A. |
| Diabetes (%) | 5 | 5 | 22 | N.A. |
| Pulmonary disease (%) | 9 | 3 | 22 | N.A. |
| Surgery | | | | |
| - CABG (%) | 71 | 68 | 67 | N.A. |
| - Aortic valve surgery (%) | 7 | 11 | 33 | N.A. |
| - Mitral valve surgery (%) | 24 | 11 | 0 | N.A. |
| - VAD implantation (%) | 7 | 5 | 0 | N.A. |
| - Heart transplantation (%) | 2 | 5 | 0 | N.A. |
| Medication | | | | |
| - Statins (%) | 66 | 63 | 44 | N.A. |
| - B-blockers (%) | 64 | 68 | 89 | N.A. |
| - ACE-inhibitors (%) | 31 | 21 | 33 | N.A. |
| - Diuretics (%) | 35 | 37 | 33 | N.A. |
| Total aspirated BM volume (ml) | 10 | 10 | 20 | N.A. |
| Sampled arterial PB volume (ml) | 10 | 10 | 10 | N.A. |
| Experiment | | | | |
| - Total ALP activity quantification | X | | | |
| - ALP isoenzymes/isoforms differentiation | X | | | |
| - MW ALP isoforms determination | X | | | |
| - In vitro PB admixture experiment of BC SN | | | | X |
| - BMV ALP versus Holdrinet comparison | | X | | |
| - Influence of aspirated BM volume on BMV ALP | | | X | |

Abbreviations: ALP, alkaline phosphatase; ACE-inhibitors, angiotensin-converting-enzyme inhibitors; BC SN, bone cylinder supernatant; BM, bone marrow; BMI, body mass index; BMV, bone matrix vesicle; CABG, coronary artery bypass grafting; CRP, C-reactive protein; VAD, ventricular assist device.

### 2.2. Quantification of total ALP activity

Total ALP activity (U/L) of BM and PB serum was measured spectrophotometrically on the Dimension Vista® System (Siemens), using the ALP Flex® reagent cartridge (Siemens). This colorimetric assay uses p-nitrophenylphosphate (p-NNP) as substrate, which is catalyzed into p-nitrophenol (p-NP) by all ALP isoenzymes/isoforms. For this IFCC calibrated method, the reference interval for healthy individuals in PB serum is 42–98 U/L in women from 15 to 59 years old, 53–141 U/L for women older than 59 years, 53–128 U/L in men from 20 to 59 years old and 56–119 U/L for men older than 59 years [18,19]. Coefficients of variation for total ALP activity quantification were 4.5% for a mean ALP activity of 27 U/L (n = 86 PB serum samples) and 2.7% for a mean activity of 137 U/L (n = 85 PB serum samples).

### 2.3. ALP isoenzyme/isoform differentiation

To separate the different ALP isoenzymes and isoforms present in PB and BM serum, agarose gel electrophoresis was performed with the Isopal Plus® kit (Beckman Europe, Analis S.A, Suarlée, Belgium) as described previously [20]. In addition, samples were subjected to a set of treatments for ALP isoenzyme differentiation. Treatment with neuraminidase (2 U/ml, purified from Clostridium perfringens, Analis S.A.)

enhances the separation of bone and liver ALP on the agarose gel, due to a different degree of sialylation [21,22]. By incubating the samples at 56 °C for 10 min, bone ALP will be inhibited by heat inactivation, while liver ALP is less sensitive to heat treatment. PI-PLC (≥200 U/mg, purified from *Bacillus cereus*, Sigma-Aldrich) treatment is responsible for the in vitro dissociation of tetrameric ALP into dimeric ALP [13]. For the detection of intestinal and placental ALP, a single DAKOCytomation polyclonal placental ALP antibody that binds both isoenzymes was used (3 g/l, a purified immunoglobulin fraction of rabbit antiserum in 0.1 mol/l NaCl and 15 mmol/l $NaN_3$, Analis S.A.). A positive control for BMV ALP consisted of supernatant (SN) of a bone cylinder (BC) from the vertebra, collected from 1 organ donor at the time of organ prelevation (see Supplementary methods).

### 2.4. Quantification of ALP isoenzyme/isoform activity

To quantify the activity of the different ALP isoenzymes and isoforms, gels were scanned at 600 nm in a densitometer (Appraise, Beckman Instruments Inc.). The ALP isoenzyme activity was expressed in U/L and as a percentage of total ALP activity.

### 2.5. Estimation of MW for different ALP isoforms

Native polyacrylamide gel electrophoresis (PAGE) was performed to separate the different MW isoforms of ALP (see Supplementary methods). For the MW estimation of the ALP isoforms, a protein standard was loaded onto the gel. After electrophoresis, this protein standard was cut off and stained with Coomassie blue, followed by overnight destaining. The other part of the gel was incubated for 30 min at 45 °C with the ALP-substrate 5-bromo-4-chloro-3-indolyl phosphate (Analis S.A.) in order to visualize the ALP activity. BM and PB serum samples were also treated with the non-ionic detergent n-butanol (pH 8–8.5) [13].

### 2.6. Visualization of bone matrix vesicles (BMV)

Transmission electron microscopy (TEM) was used for direct visualization of BMV in BM and PB serum. After purification of high-MW ALP on the polyacryamide gel, high-MW bands of untreated PB and BM serum were cut out of the gel. The bands were fixed in 0.1 M sodium cacodylate-buffered (pH 7.4) 2.5% glutaraldehyde solution for 2 h at room temperature, and then washed three times in 0.1 M sodium cacodylate-buffered (pH 7.4) 7.5% saccharose solution. Post-fixation was performed by incubating cells for 2 h with 1% $OsO_4$ solution. After dehydration, the samples were embedded in EM-bed812. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined in a Tecnai G2 Spirit Bio TWIN microscope (Fei, Europe BV, Zaventem, Belgium) at 120 kV.

### 2.7. In vitro admixture of BC SN with PB serum

Bone cylinders (BC) from the vertebra were collected from 4 deceased heart-beating organ donors at the time of organ prelevation, which was approved by the Antwerp University Hospital Ethics Committee. Spongiosa BC were softly shaken overnight in 1 ml saline in order to isolate the BMV. For each donor, 5 of the obtained BC supernatant (BC SN) samples, which were rich of BMV, were pooled and filtered prior to further analysis. Total ALP activity and the electrophoretic isoenzyme pattern of the BC SN were evaluated as described in Sections 2.2.–2.4. These BC SN samples were used for the setup of an in vitro addition experiment of BC SN with PB serum in different degrees (0–20–40–60–80–100%). For each percentage admixture, total ALP activity as well as the percentage of BMV ALP was determined. This addition experiment was performed on the pooled BC SN sample of each donor.

### 2.8. Correlation between BMV ALP% in BM and PB admixture% in BM quantified by Holdrinet

In order to ascertain if there is a correlation between the measured BMV ALP percentage in BM and the PB admixture degree to the BM aspirate quantified according to the previously proposed Holdrinet method [5], the Hb content and number of nucleated cells in BM and PB, collected in EDTA tubes, were measured on the Sysmex Automated Hematology Analyzer XS-1000i (Sysmex). The PB admixture of each BM aspirate according to Holdrinet et al. was calculated using the following formula: percent nucleated PB cells = (Hb BM asp / Hb PB) × (nucleated cells PB / nucleated cells BM asp) × 100.

### 2.9. BMV ALP measurement in different aspirated BM volumes

To investigate whether the PB admixture increases with larger aspirated BM volumina, BM was aspirated in a fractionated way. In each patient 2, 3, 5, 5 and 5 ml BM was aspirated successively in different syringes without replacing the Jamshidi BM aspiration needle. Total ALP activity and different ALP isoenzyme/isoform at each stage were compared to the first aspirated 2 ml BM.

### 2.10. Statistical analysis

Results are expressed as median (interquartile range—IQR). IBM SPSS Statistics version 22.0 was used for statistical analysis. A p-value $< 0.05$ was considered statistically significant. The comparison between total ALP activity in PB and BM serum was made by the Wilcoxon signed rank test. Differences in BMV ALP fraction between the different aspirated BM volumes were calculated using the Friedman test, followed by a pairwise comparison by the Wilcoxon signed rank test. The correlation between the measured BMV ALP fraction and the PB admixture to the BM aspirate quantified according to the Holdrinet method was evaluated by the Spearman test. Multivariate regression analysis was performed for the determination of independent predictors of BMV-ALP percentage.

## 3. Results

### 3.1. Quantification of total ALP activity

Total ALP activity was measured in BM and PB serum samples in 55 patients undergoing cardiac surgery (Table 1, cohort 1). Total ALP activity was significantly higher in BM serum (97 (176–124) U/L) compared to PB serum (63 (52–73) U/L, $p < 0.001$).

### 3.2. ALP isoenzyme/isoform separation and quantification in PB and BM samples

When comparing the electrophoretic pattern of untreated BM serum with the pattern of untreated PB serum, a distinct fraction with a more anodal migration was detected exclusively in BM serum (Fig. 1). This fraction was identified as bone type ALP based on neuraminidase treatment (a clear separation between bone and liver ALP occurred) and incubation of BM serum at 56 °C during 10 min (the bone ALP fraction disappeared). In other words, BM serum contains a unique bone ALP fraction, which is absent in PB serum. One sample of bone cylinder supernatant (BC SN) with a total ALP activity of 217 U/L of 1 organ donor was used twice as positive control for bone samples without PB admixture. The samples mainly consisted of the same unique bone ALP fraction that was encountered in BM aspirates, without any detection of liver ALP. Based on these results we hypothesized that this unique bone ALP fraction was high-MW bone ALP.

Quantification of the activity of the ALP isoenzymes/isoforms was performed in both BM and PB serum. A representative example of a densitometer scan is presented in Supplementary Fig. 1. Table 2 shows

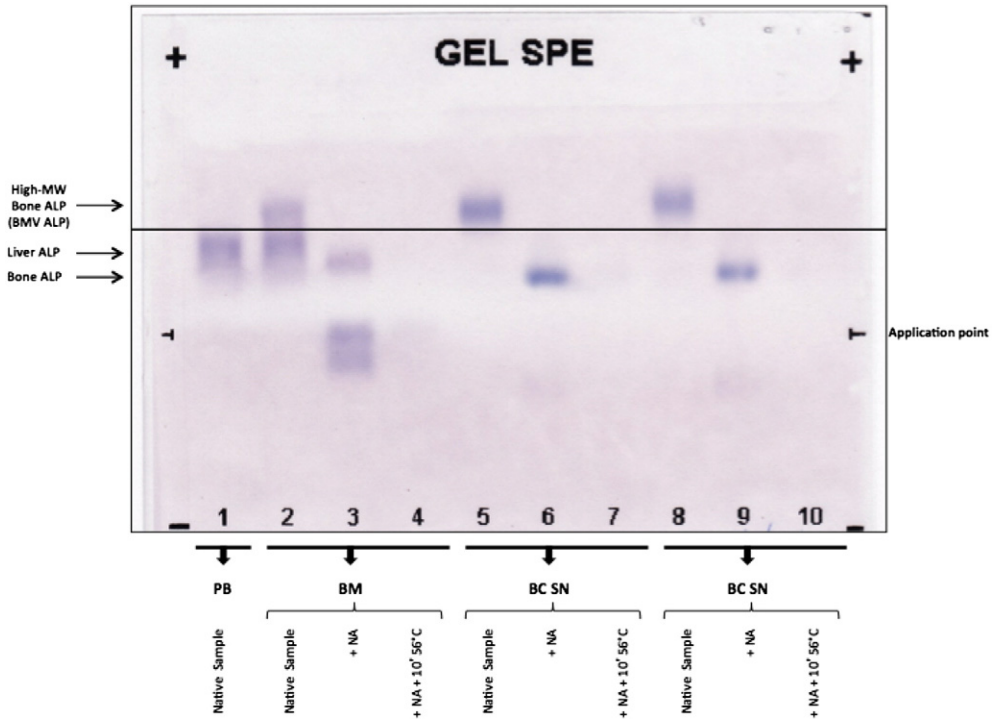


**Fig. 1.** Agarose gel electrophoresis of alkaline phosphatase (ALP) in peripheral blood (PB) serum, bone marrow (BM) serum and bone cylinder supernatant (BC SN). Untreated PB serum (total ALP of 71 U/L, lane 1); untreated BM serum (total ALP of 113 U/L, lane 2); BM serum after treatment with neuraminidase (NA; lane 3), BM serum after treatment with NA and heat inactivation by incubation at 56 °C during 10 min (lane 4); untreated BC SN (total ALP of 217 U/L, positive control, lane 5 and 8); BC SN after treatment NA (lane 6 and 9); and BC SN after treatment with NA and heat inactivation (lane 7 and 10). BM serum contains a unique high-molecular weight (MW) bone ALP fraction, which is absent in PB serum.

the percentages of the different ALP isoenzymes/isoforms in BM and PB serum of the first cohort of 55 patients. The high-MW bone ALP fraction was detected in BM of all patients and represented 25% (15–43%) of total ALP. In contrast, this fraction was completely absent in all PB serum samples. Next to high-MW bone, bone and liver ALP, also a very small high-MW liver ALP fraction was detected in BM in only 6 out of 55 patients. Intestinal ALP was never detected in BM, but 8 out of 55 patients contained a very small intestinal ALP fraction in PB. The intra-assay coefficient of variation for the quantification of high-MW bone ALP with mean ALP activity of 56.7 U/L in a BM serum sample was 7.12% (10 measurements). The inter-assay coefficient of variation was 15.3% for a mean bone ALP activity of 19 U/L (n = 14 PB serum samples) and 9.5% for a mean liver ALP activity of 50 U/L (n = 14 PB serum samples).

The unique high-MW bone ALP fraction showed a rather wide range in our cohort (IQR 15–43%, minimum 5%, maximum 83%). Therefore, we performed multivariate regression analysis for the detection of independent determinants for this high-MW bone ALP fraction. Clinical variables, such as left ventricular ejection fraction, Framingham risk score (i.e. 10-year cardiovascular disease risk), CRP, renal function as well as age and medication were included in the analysis, however, no significant determinants were detected.

**Table 2**
Percentages of the ALP isoenzymes/isoforms present in BM aspirates and PB serum of 55 patients. The presence of a unique high-MW bone ALP fraction was confirmed in all 55 patients and expressed as a percentage of the total ALP activity measured in BM or PB serum. Results are expressed as median (IQR).

| ALP isoenzyme/isoform % of total ALP activity | BM | PB |
|---|---|---|
| Bone ALP | 23.1 (13.8–31.7) | 29.2 (22.8–37.3) |
| High-MW bone ALP (BMV ALP) | 25.0 (15.2–42.8) | 0 (0–0) |
| Liver ALP | 46.9 (31.5–58.5) | 64.1 (58.3–73.6) |
| High-MW liver ALP | 0 (0–0) | 2.8 (0–4.6) |
| Intestinal ALP | 0 (0–0) | 0 (0–0) |

Abbreviations: ALP, alkaline phosphatase; BM, bone marrow, BMV, bone matrix vesicles; MW, molecular weight; PB, peripheral blood.

### 3.3. Identification of different MW ALP isoforms

The native PAGE experiment (Fig. 2) shows that the high-MW ALP isoforms (>1236 kDa) were predominantly present in the untreated BM serum as compared to the untreated PB serum. Heat-inactivation resulted in a significant reduction in ALP activity of the high-MW isoforms in BM serum, but not in PB serum. This observation confirmed that the high-MW ALP isoforms in BM serum correspond with bone type ALP and those in PB serum with liver type ALP.

ALP in PB serum is mainly present in its dimeric form (estimated MW 242 kDa), while ALP in BM serum is mainly present in the high-MW form. N-butanol treatment of BM serum resulted in the release from the high-MW fraction of ALP with an estimated MW of 480 kDa, hence this fraction is called 'tetrameric' ALP [11,13]. This tetrameric ALP was also heat-sensitive, proving its bone origin. Since no 480 kDa protein band is visible in PB serum, we confirmed previous findings that in normal conditions no tetrameric ALP is found in PB serum from healthy adults [11].

Treatment with PI-PLC of PB serum and BM serum showed a reduction of the ALP activity at the level of high-MW bone type isoforms and BM serum also showed a smear between the molecular weights of tetrameric and dimeric ALP, which is in line with the finding of Hawrylak et al. [13] that PI-PLC is responsible for the dissociation of tetrameric ALP into dimeric ALP. In conclusion, native PAGE revealed that the unique ALP fraction found in BM serum is indeed a high-MW bone type ALP.

### 3.4. Visualization of BMV in high-MW PAGE bands of BM

To determine whether the high-MW bone ALP fraction in BM corresponds with BMV-bound ALP, TEM of the high-MW PAGE bands of BM was performed. Indeed, TEM images showed the presence of BMV (Fig. 3), which were identified based on their properties as described previously by Golub et al. [16]. The vesicles had a size of 20–200 nm

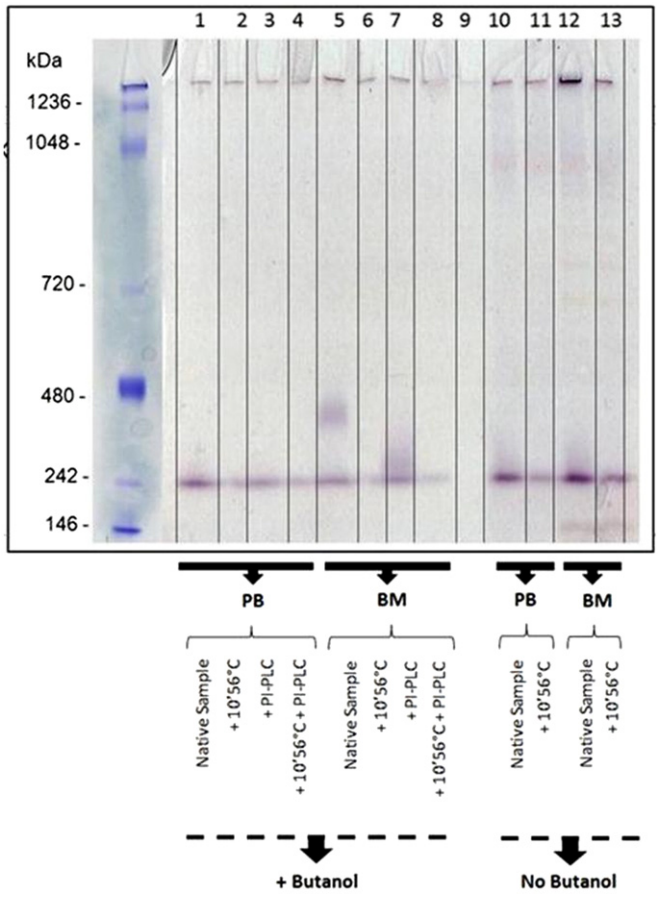


**Fig. 2.** Separation of the different molecular weight (MW) isoforms of bone and liver ALP in PB and BM serum. With n-butanol treatment: PB serum (1); PB serum after heat-inactivation (2); PB serum after PI-PLC (phosphatidyl-inositol-phospholipase C) treatment (3); PB serum after heat-inactivation and PI-PLC treatment (4); BM serum (5); BM serum after heat-inactivation (6); BM serum after PI-PLC treatment (7); BM serum after heat-inactivation and PI-PLC treatment (8). Without n-butanol treatment: PB serum (10); PB serum after heat-inactivation (11); BM serum (12); BM serum after heat-inactivation (13).

with a phospholipid bilayer membrane. Henceforth, the unique high-MW bone ALP fraction present in BM serum will be called bone matrix vesicle-bound ALP (BMV-ALP).

### 3.5. In vitro admixture of BC supernatant with PB serum

Total ALP activity of the bone cylinder supernatant (BC SN) samples of 4 different organ donors was 394 (252–765) U/L. The BMV ALP fraction accounted for 87 (81–98) % of the total ALP activity in these samples, the remaining ALP activity was due to the presence of dimeric bone ALP.

Serial dilution of the pooled BC SN samples with PB serum of a healthy subject (total ALP activity of 71 U/L) was performed. The results of the 4 admixture experiments are summarized in Supplementary Fig. 2. This PB admixture experiment to BC SN samples demonstrates that the measured BMV ALP fraction (%) diminishes with increasing PB admixture (%) and confirms that the established BMV ALP technique is sensitive for the detection of PB admixture to BM aspirates.

### 3.6. Correlation between BMV ALP and the Holdrinet method for quality assessment of BM aspirates.

In a second cohort of 20 patients (Table 1), the BMV ALP fraction was similar to the first cohort with a median fraction of 30% (14–64%). Furthermore, the degree of PB admixture of these BM aspirates quantified by the Holdrinet method was 23 (14–43) %. A significant, although weak correlation was found between BMV ALP % and PB admixture % quantified by the Holdrinet method ($r = -0.496$; $p = 0.026$).

### 3.7. BMV ALP measurement in ascending aspirated BM volumes

Not only total ALP activity, but also the fraction of BMV ALP decreases significantly with increasing aspirated BM volume, as shown in Figs. 4a and b respectively. For every BM aspirate, the first 2 ml contains the highest total ALP activity (146 (107–213) U/L) and highest BMV ALP percentage (37 (14–54) %). The decrease in BMV ALP % is significant when aspirating 10 ml BM or more compared to the first aspirated 2 ml BM. These results show that the purity of BM aspirates decreases with increasing aspirated BM volume.

Fig. 4c shows BMV ALP and liver ALP in BM aspirates. The presence of liver ALP is considered to be a result of PB admixture. As expected, there is an inverse correlation between liver ALP % and BMV ALP % in BM ($r = -0.608$, $p < 0.001$). In other words, the presence of liver ALP increases with decreasing purity of the BM aspirate and thus, also with increasing aspirated BM volume. When more than 3 ml of BM is aspirated, liver ALP becomes more abundantly present than BMV ALP.

### 3.8. Determination of minimal BM quality

A gold standard for 100% pure BM is needed in order to calculate the percentage of PB admixture to the aspirate by means of BMV ALP %. BC SN is very rich in BMV ALP, but as it is directly derived from bone it cannot be used as gold standard for BM. Neither can the first 2 ml BM of an aspirate be used as, although this is the most pure BM that we can obtain, even this small quantity of aspirate still shows a large variation in BMV ALP % between patients.

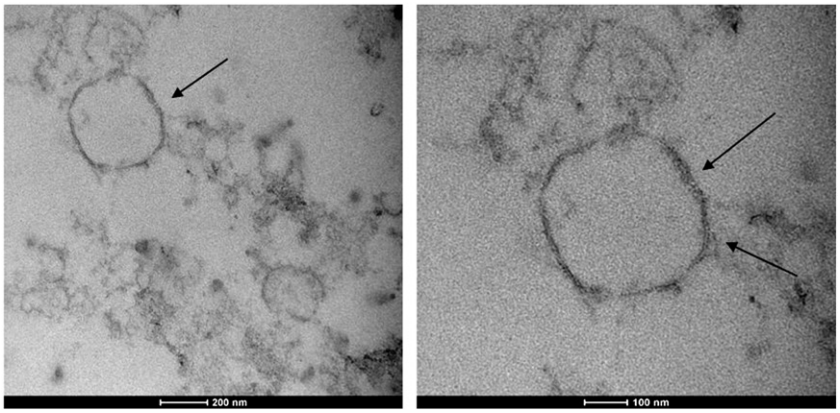


**Fig. 3.** Bone matrix vesicles (BMV) visualized in BM by TEM after purification on PAGE. The bone matrix vesicles have a size of 20–200 nm with a phospholipid bilayer membrane (black arrow).

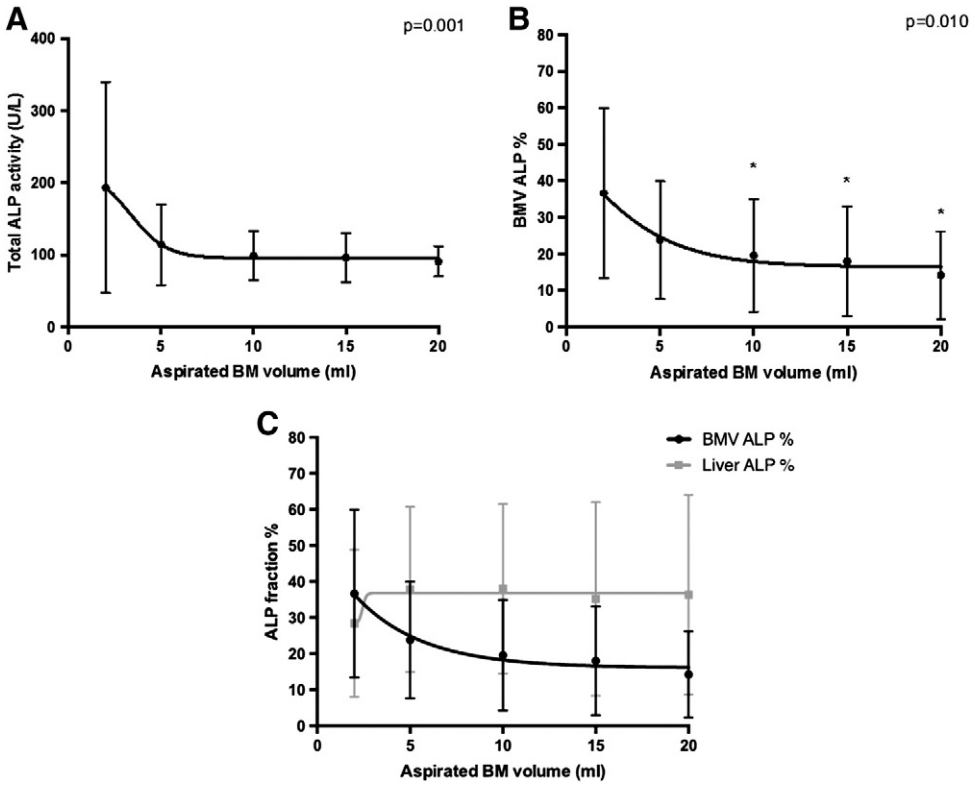


**Fig. 4.** A–C: Analysis of total ALP activity, BMV ALP and liver ALP in ascending aspirated BM volumes. **A.** Total ALP activity (U/L) and **B.** BMV ALP (%) were quantified in the first 2, 5, 10, 15 and 20 ml BM of the total aspirated BM volume (20 ml) of 9 patients. Both total ALP activity and BMV ALP decrease significantly (Friedman; $p = 0.001$ and $p = 0.010$ respectively) with increasing aspirated BM volume. The decrease in BMV ALP fraction is significant ($p < 0.05$) when aspirating 10 ml BM or more compared to the first 2 ml aspirated BM. **C.** In contrast, the percentage of liver ALP in BM increases with increasing aspirated BM volume.

Therefore, we suggest a minimum purity for a BM aspirate, based on the BMV ALP %. As minimum BMV ALP % we suggest the first quartile value of the measured BMV ALP % that was determined in a cohort of 55 patients (Table 1, cohort 1), i.e. 15%. As such, we suggest rejecting BM aspirates with a BMV ALP % ≤ 15% for further analysis.

## 4. Discussion

In the present study, we investigated whether bone matrix vesicle (BMV)-bound bone alkaline phosphatase (ALP) could serve as a marker for the purity of bone marrow aspirates. Several findings emerged from this study. Firstly, we demonstrated by electrophoresis that bone marrow (BM) contains a unique bone ALP fraction, with a high molecular weight, which is absent in peripheral blood (PB). Electron microscopy showed ample presence of BMV, which strongly suggests that the unique high-HW bone ALP fraction corresponds to membrane-bound bone ALP originating from BMV. Due to its exclusivity to the bone marrow, BMV-bound ALP could serve as a novel marker to assess the purity of BM aspirates. Furthermore, serial dilution with PB of bone cylinder supernatant, containing high levels of BMV ALP, demonstrated well how the BMV ALP fraction decreases with increasing PB admixture, proving the sensitivity of this proposed quality assessment method. In addition, purity of BM aspirates declines rapidly when larger volumes are aspirated.

### *4.1. Comparison between BMV ALP and the Holdrinet method for assessing the degree of PB admixture in BM aspirates*

As a consequence of inevitable PB admixture to BM aspirates [23], quality assessment of the BM aspirate is essential in order to prevent unreliable results during numerical, functional and cytokinetic analyses of BM aspirates for research and diagnostic purposes. Although a gold standard for quantification of PB admixture to BM aspirates is still lacking, a few methods have been proposed previously, such as the flow cytometric quantification of lymphocytes and monocytes in BM [4] and the method by Holdrinet et al., [5] which is based on the ratio of the hemoglobin (Hb) content in BM compared to PB. These methods are definitely of value in some conditions. However, since the number of nucleated PB cells in BM can be influenced by pathophysiological conditions associated with cellular BM dysfunction, the application of these 2 methods is hampered in these conditions [7].

Therefore, identification of a stable marker, that is exclusively present in BM and is independent of BM cellular characteristics is mandatory.

Comparison between the proposed BMV ALP fraction and the Holdrinet method revealed a significant, although weak correlation. However, all patients in this study had overt cardiovascular disease, which could have been associated to a potential BM dysfunction and hence, incorrect interpretation when using the Holdrinet method [9].

### *4.2. Variation in BMV ALP between patients*

The measured BMV ALP fraction in BM from 55 cardiovascular patients without underlying bone disorder, showed a wide range of BMV-ALP (15–43%, IQR). However, comprehensive multivariate analysis with several clinical and patient characteristics could not identify important determinants for the level of BMV ALP. In addition, we could not detect a significant influence of the intake of cardiovascular medication on BMV ALP levels. This is in agreement with previous studies on beta-blockers [24] and ACE-inhibitors [25], in which no effect was seen of this medication on bone type ALP activity. However, concerning statin use, Stein et al. observed that in vitro treatment of serum with statins decreases the bone ALP levels in serum samples [26]. In contrast, the ALP activity is significantly enhanced by statin treatment in bone tissue [27,28]. Yet, we entered statin use, which was taken by 66% of the

patients, as variable in the statistical analysis, but no influence of statins on BMV ALP levels could be detected.

### 4.3. Molecular weight determination of the different ALP isoenzymes/isoforms

As mentioned, MWs were estimated by native gradient PAGE. Our results are in agreement with previous reports, wherein TNALP released by a nonionic detergent from membrane-bound bone ALP in cultured human osteosarcoma cells (SAOS-2 cells) [29], or membrane-bound liver ALP from human liver cells [13], had a MW of 440 and 445 kDa respectively. In these studies, the estimated MW of dimeric bone ALP or dimeric liver ALP released by PI-PLC was 200 and 214 kDa respectively. There are, however, discrepancies between these MW estimates and those obtained by native gradient gels in other publications [30–33]. This holds true especially for the dimers, with estimated MWs in these reports ranging from 130 to 150 kDa, which is more in agreement with the theoretical MW (108 kDa) calculated from the known amino acid sequence for tissue-nonspecific ALP. Differences in hydrophobicity and/or oligosaccharide content between bone ALP produced by cultured SAOS-2 osteosarcoma cells, and bone ALP produced by native (non-cultured) non-malignant osteoblasts might account for the observed discrepancies.

Although the main circulating ALP fractions are dimeric and hydrophilic, we have demonstrated previously by means of gel electrophoresis, gel chromatography and 2D electrophoresis that hydrophobic, heterogeneous forms of bone, liver, intestinal and placental ALP can circulate in human serum [11,34,35]. Because of the hydrophobic nature of these fractions, we concluded that they retained at least part of their GPI anchor. In the presence of detergents, their MW could be estimated to be approximately 400 kDa, hence they were called 'tetrameric' ALP. We hypothesize that the two dimers are held together by interactions between the hydrophobic tails, as the enzyme takes on a dimeric structure when the GPI-anchor is lost, and as no obvious surface domain that could be involved in tetramer formation could be demonstrated in the 3D-structure of human ALP [36].

### 4.4. Aspirated BM volume and minimal quality cut-off value for BM purity

Quality assessment by means of BMV ALP determination with ascending aspirated BM volume confirmed the hypothesis that the BM purity decreases by increasing aspirated BM volume, which implicates that only the minimum needed BM volume should be aspirated and that the complete aspirated BM volume should be well mixed prior to BM analysis experiments. Moreover, when aspirating more than 3 ml BM, liver ALP becomes more abundantly present in BM compared to BMV ALP. Therefore, we suggest that a maximum of 3 ml BM should be aspirated when high quality BM is required for further analysis.

Additionally, since a gold standard for 100% pure BM in order to calculate the percentage of PB admixture by means of BMV ALP % is still lacking, we suggest a minimum purity for a BM aspirate, based on the BMV ALP %. The first quartile value of the measured BMV ALP fractions in a cohort of 55 patients, which is currently the largest cohort that is available in literature, was suggested as cut-off value, i.e. $\geq 15\%$. Obviously, this percentage can be re-considered, as more measurements will be performed in the future by different research groups and using different techniques.

Furthermore, we recommend the use of the proposed BMV ALP based quality assessment of BM aspirates for research purposes rather than for daily practice. Due to its labor intensity, the method is less applicable as a routine method for the conventional cytogenetic analysis of BM for diagnosis and prognosis of hematological disease, where also only a minimal volume of BM is aspirated, in contrast to the higher BM volumes (20 ml) needed for research purposes.

### 4.5. Technical aspects taken into account during ALP quantification

We previously showed that ALP activity in healthy adult non-pregnant subjects is quite constant [11]. However, since lower total ALP levels have been measured in patients after cardiac surgery, it is important to sample blood prior to sternotomy [37]. Furthermore, we used BM and PB serum instead of EDTA or heparin plasma during the ALP experiments, since EDTA inhibits ALP activity [18] and heparin causes ruptures in the agarose gel. In contrast, for the measurements necessary for the PB admixture quantification in BM aspirates using the Holdrinet method, the collection of BM in EDTA tubes was required.

In addition, several liver diseases are responsible for elevated ALP levels in PB [11]. Since the presence of liver ALP in BM is completely due to PB admixture, high liver ALP activity in PB could lead to a lower percentage of BMV-bound ALP in BM, resulting in a false elevated estimation of PB admixture. In these cases the use of the suggested method is hampered. Elevated bone ALP activity on the other hand, will not influence the relative percentage of BMV-bound ALP in BM, since bone ALP activity in PB reflects the bone ALP activity in BM.

## 5. Conclusion

The developed technique for quality assessment of BM aspirates is based on the presence of a unique BMV-bound bone ALP fraction in BM. To our knowledge, this is the first report of the presence of BMV-bound ALP in human BM aspirates. Since total ALP activity and the ALP isoenzyme pattern are significantly different in BM as compared to PB, these markers can serve to assess PB admixture to BM samples, independently of hematological indices. Furthermore, a minimal BMV ALP fraction of 15% of total ALP activity in BM aspirates is suggested as cut-off value. Lastly, the purity of BM aspirates is inversely correlated with the aspirated BM volume.

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## Disclosures

None declared.

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