RESEARCH ARTICLE

Characterization of a sandwich ELISA for the quantification of all human periostin isoforms

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Background: Periostin (osteoblast-specific factor OSF-2) is a secreted protein occurring in seven known isoforms, and it is involved in a variety of biological processes in osteology, tissue repair, oncology, cardiovascular and respiratory systems or allergic manifestations. To analyze functional aspects of periostin, or the ability of periostin as potential biomarker in physiological and pathological conditions, there is the need for a precise, well-characterized assay that detects periostin in peripheral blood.

Methods: In this study the development of a sandwich ELISA using monoclonal and affinity-purified polyclonal anti-human periostin antibodies was described. Antibodies were characterized by mapping of linear epitopes with microarray technology, and by analyzing cross-reactive binding to human periostin isoforms with western blot. The assay was validated according to ICH/EMA guidelines.

Results: The monoclonal coating antibody binds to a linear epitope conserved between the isoforms. The polyclonal detection antibody recognizes multiple conserved linear epitopes. Therefore, the periostin ELISA detects all known human periostin isoforms. The assay is optimized for human serum and plasma and covers a calibration range between 125 and 4000 pmol/L for isoform 1. Assay characteristics, such as precision (intra-assay: $\leq 3\%$, inter-assay: $\leq 6\%$), spike-recovery (83%-106%), dilution linearity (95%-126%), as well as sample stability meet the standards of acceptance. Periostin levels of apparently healthy individuals are 864 ± 269 pmol/L (serum) and 817 ± 170 pmol/L (plasma) respectively.

Conclusion: This ELISA is a reliable and accurate tool for determination of all currently known periostin isoforms in human healthy and diseased samples.

KEYWORDS

allergy, cardiovascular disease, ELISA, oncology, OSF-2, osteology, periostin, respiratory disease, sandwich ELISA

1 | INTRODUCTION

Periostin, originally identified as osteoblast-specific factor 2 (OSF-2), is secreted as 91 kDa soluble glycoprotein that is encoded by the POSTN gene.¹ It is highly expressed at the periosteal surface but also in other collagen-rich tissues that are subjected to mechanical stress like periodontal ligaments, heart valves, and tendons.² Periostin belongs to the matricellular proteins,³ a group of

extracellular proteins that catalyze cell matrix interactions and cell functions.

The structure of periostin is composed of an N-terminus with a single emilin (EMI) domain and four fasciclin-1 (Fas1) modules. Post-translational modification by vitamin K-dependent γ -carboxylation within these Fas1 modules is still a matter of debate.^{4,5} Sequences within its N-terminal region allow binding to integrins on the cell membrane via the Fas domains (eg $\alpha v \beta 3$, $\alpha v \beta 5$, $\alpha 6 \beta 4$), leading to activation

of signaling pathways such as the Akt/PKB and FAK-mediated pathway.⁶ The C-terminus is prone to alternative splicing which results in seven periostin isoforms with molecular masses from 80 to 93 kDa (Figure 1). It binds to extracellular matrix molecules and regulates cell-matrix organization.⁶

Periostin is associated with a variety of tissues and pathologies.⁷ It is involved in the epithelial-mesenchymal transition in the course of cardiac development⁸ and cancer.⁹ It is re-expressed after

myocardial injury,¹⁰ it participates in myocardial remodeling and it is induced after vascular¹¹ and skeletal muscle injury.¹² In a cohort of patients with ovarian tumors it has been shown that periostin promotes tumor metastatic growth and angiogenesis.¹³ In bone, periostin has a general function in homeostasis¹⁴ and it is upregulated after bone fracture or in response to mechanical stress when bone development and remodeling is required.¹⁵ It acts on bone formation through an increase in osteoblast function as it is able

FIGURE 1 Schematic representation of human periostin, localization of epitopes recognized by the antibodies used in the periostin ELISA, and alignment of all seven currently known periostin isoforms. A, Top: Epitope recognized by the monoclonal coating antibody (m_e1). Bottom: Epitopes recognized by the polyclonal detection antibody (five major epitopes, p_e1 to p_e5) (x-axes: schematic representation of periostin sequence; y-axes: fluorescence units). B, Protein sequence alignment of the C-terminal domain of seven human periostin isoforms. Identical amino acids are indicated by dots and gaps are indicated by dashes. Two major epitopes recognized by the detection antibody (p_e4, p_e5) are localized in the C-terminal domain and are framed in gray

to modulate Wnt-beta-catenin signaling by downregulating sclerostin expression.¹⁶ There is evidence that serum periostin could be a new biological marker of fracture risk.¹⁴ Moreover, a pathological expression of periostin is observed in bone diseases like fibrous dysplasia.¹⁷ In cutaneous wound healing periostin is described to act on keratinocyte proliferation, for example periostin-deficient mice showed delayed wound closure and re-epithelialization.¹⁸ Periostin has further been shown to be a downstream molecule of the T_H2 cytokines IL-4 and IL-13¹⁹ and it is involved in many aspects of T_H2-driven asthma or eosinophilic inflammation.^{20–22} In addition, periostin has been related to atopic dermatitis,²³ allergic rhinitis, and chronic rhinosinusitis.²⁴

In order to evaluate the function of periostin and its potential as a biochemical marker, a precise, well-defined assay to screen peripheral blood is needed. Hence, we developed a sandwich ELISA based on well-characterized monoclonal and affinity-purified polyclonal anti-human periostin antibodies with mapped epitopes and defined cross-reactivities to human periostin isoforms to provide best possible insight into the antibody-analyte reaction.²⁵ We analyzed and validated the assay based on the principles of bioanalytical validation defined by the ICH harmonized tripartite guideline Q2 (R1) and by the EMEA guideline of bioanalytical method validation.

2 | MATERIAL AND METHODS

2.1 | Serum and plasma samples

Venous blood samples were collected from apparently healthy individuals and from patients suffering from asthma or cardiovascular disease. Sample characterization and patients' clinical data from the latter are summarized in Table 1. Blood samples were obtained using blood collection tubes for serum and plasma after informed consent was given. After incubation at room temperature, collection tubes were centrifuged (eg 10 minutes at 2000×g at 4°C) and the obtained serum and plasma was stored at –20°C or lower. Samples were analyzed in an anonymized manner.

2.2 | Epitope mapping of anti-human periostin antibodies used for sandwich ELISA development

Epitope mapping of linear epitopes was performed for a mouse monoclonal anti-human periostin antibody, and a goat polyclonal anti-human periostin antibody, which are used in the sandwich ELISA as coating and detection antibodies respectively. The periostin sequence was covered by 815 synthetic 15mer peptides with an overlap of 14 amino acids. Peptides were printed on a microarray in duplicates (Pepperprint

TABLE 1 Characterization of asthma and cardiovascular disease sample cohorts

Cohort	ID	Matrix	Gender	Age (years)	Diagnosis
Asthma	1	Serum	Male	82	Asthma (moderate), CVD
	2	Serum	Female	70	Asthma (moderate), COPD
	3	Serum	Female	60	Asthma (severe), Allergies, HTN
	4	Serum	Female	52	Asthma (severe)
	5	Serum	Female	54	Asthma (moderate)
	6	Serum	Male	52	Asthma (moderate), AR
	7	Serum	Female	50	Asthma (severe), AR
	8	Serum	Female	45	Asthma (severe)
	9	Serum	Male	40	Allergic Asthma
	10	Serum	Male	33	Asthma
Cardiovascular	1	Serum	Female	81	SCLC, Anemia, HTN, CAD
	2	Serum	Male	81	MI, Abnormal Cardiac Stress Test, By-Pass Surgery, Atherosclerosis, Hypercholesterolemia
	3	Serum	Male	52	Abnormal Cardiac Stress Test, Hypercholesterolemia
	4	Serum	Female	79	Atrial Fibrillation
	5	Serum	Female	52	CVD, HTN
	6	Serum	Female	78	CHF
	7	Serum	Male	64	CAD
	8	Serum	Male	67	HTN, Benign Prostate Hyperplasia
	9	Serum	Male	63	HTN
	10	Serum	Male	64	Stroke, Cavernous Angioma, Allergy (Codine), HTN, Hypercholesterolemia

CVD, Cardiovascular Disease; COPD, Chronic Obstructive Pulmonary Disease; HTN, Hypertension; AR, Allergic Rhinitis; SCLC, Small Cell Lung Cancer; CAD, Coronary Artery Disease; MI, Myocardial Infarction; CHF, Congestive Heart Failure.

GmbH, Heidelberg, Germany). After blocking, the array was incubated overnight with the diluted antibody (1 µg/mL). Bound antibodies were detected with DyLight680 conjugated anti-mouse or anti-goat antibodies, and signals were measured with a LI-COR Odyssey Imaging System. Quantification of spot intensities based on 16-bit gray scale tiff files was done with the PepSlide® Analyzer software. Average intensities were calculated and spot-to-spot variations >40% were zeroed.

2.3 | Sequence alignment of human periostin isoforms

Sequences of the C-terminal regions of human periostin isoforms (UniProt entry: Q15063) were aligned with the multiple sequence alignment program ClustalW²⁶ and edited and visualized with GeneDoc.²⁷

2.4 | Cross-reactivity of the polyclonal goat anti-human periostin detection antibody with different periostin isoforms

Human recombinant periostin isoforms 1-3 were purchased. Isoform 1 was derived from a mouse myeloma cell line, isoforms 2 and 3 were expressed in HEK293 cells. Comparable amounts of recombinant periostin isoforms 1, 2, and 3 were separated by SDS-PAGE under reducing conditions. The proteins were either stained with colloidal coomassie according to manufacturer's protocol or transferred onto nitrocellulose. After blocking with PBS+2% (w/v) BSA+0.1% (v/v) Tween 20, blots were incubated with the goat polyclonal anti-human periostin antibody used as detection antibody, or with buffer for the determination of background reactions. Bound antibodies were detected with a donkey polyclonal anti-goat IgG HRP antiserum (Abcam, Cambridge, UK) and blots were developed with Super Signal West Dura Chemiluminescent Substrate (Thermo Scientific, Waltham, MA, USA).

2.5 | Setup of the periostin sandwich ELISA

Plates were coated with 150 µL of the mouse monoclonal anti-human periostin coating antibody, sealed and incubated for one hour at room temperature (18–26°C). After aspiration of the coating solution, wells were blocked with 300 µL per well of blocking solution overnight at 4°C. Thereafter, each well was aspirated and dried for three hours. The assay was calibrated with mouse myeloma-derived recombinant human periostin isoform 1. Calibrator (0–4000 pmol/L) was spiked into periostin-depleted serum matrix which was produced by immune affinity chromatography. Standards, control sera, and human serum or plasma samples were diluted 1+50 with protein buffered assay buffer. 150 µL of standard, samples, or controls were added to the dried wells and incubated for 2 hours at room temperature. Plates were then washed five times with wash buffer before adding 150 µL of the biotinylated goat polyclonal anti-human periostin antibody and incubated for 2 hours at room temperature. Plates were washed five times as described before. In order to detect bound biotinylated detection antibody, 150 µL of the conjugate solution, consisting of horseradish peroxidase-labeled streptavidin, was added to each well. The mixture was incubated for 1 hour

at room temperature to allow the formation of the biotin-streptavidin complex. After incubation, wells were washed five times, and substrate solution was added. The final incubation step was performed in the dark, and the reaction was stopped after 30 minutes with stop solution. The absorbance was measured with a microplate reader (BioTec, Winooski, VT, USA) at 450 nm with an absorbance correction at 630 nm.

2.6 | Assay evaluation of the human periostin sandwich ELISA and quantification of periostin levels in apparently healthy and diseased subjects

The human periostin ELISA was validated according to ICH and EMEA guidelines. Assay parameters such as the definition of the assay range (including the lowest level of quantification as well as the level of detection), the analysis of precision, the specificity, the accuracy or spike recovery, and the dilution linearity in different sample matrices (serum, EDTA plasma, citrate plasma, heparin plasma) were assessed. The stability of endogenous periostin in human serum and plasma was tested, and the distribution of periostin concentration in different sample matrices (serum, EDTA plasma, citrate plasma, heparin plasma) from apparently healthy subjects was determined. Serum periostin levels of healthy subjects were compared with serum levels of asthmatic patients and of patients suffering from cardiovascular disease.

2.7 | Statistics

Differences of periostin concentrations measured in different sample cohorts were assessed by Mann-Whitney U or Wilcoxon test using GraphPad Prism 6 software. Values of $P < .05$ were considered as significant.

3 | RESULTS

3.1 | Epitope mapping and analysis of cross-reactivity of the coating and detection anti-human periostin antibodies

Linear epitopes of the monoclonal mouse anti-periostin antibody and the polyclonal goat anti-periostin antibody employed in the assay were mapped with microarray technology, where binding intensities (fluorescence units) of the antibodies to linear peptides printed to microarrays were measured. The monoclonal coating antibody recognized a single epitope (m_e1: KGMTSEEK) that is localized in the fourth Fas1 domain (Figure 1A, top) which is conserved in all seven currently known human periostin isoforms. The polyclonal detection antibody recognizes multiple linear epitopes that are distributed across the whole periostin sequence (Figure 1A, bottom), whereas a total of five major epitopes with fluorescence intensities over 10 000 arbitrary units were identified. Of these, two major epitopes are located within the first Fas1 domain (p_e1: TQRYSDASK, p_e2: EIEGKGSF), one epitope lies within the second Fas1 domain (p_e3: EDDLSSF), and two more epitopes are found within the C-terminal region, which shows sequence variations between the isoforms from 71% to 98%

(Figure 1B). Protein sequence alignment reveals that epitope 5 (p_e5: RISTGGGE) is identical between all isoforms, but epitope 4 (isoform 1: p_e4: KTEGPTLT) varies in isoforms 3 and 5, and is even deleted in isoform 2, 4, 6, and 7 (Figure 1B). Therefore, all epitopes that are recognized by the polyclonal detection antibody are conserved between the isoforms except of epitope p_e4.

To assess if the detection antibody is still able to detect all currently known human periostin isoforms, isoforms 1 to 3 covering all epitope variants of the seven described isoforms were selected for further testing. Commercially available recombinant isoforms 1 to 3 were analyzed by SDS-PAGE, and staining revealed varying amounts of periostin and different levels of purities. Matched amounts of isoform 1 to 3 were separated in two concentrations under reducing conditions by SDS-PAGE, and staining with colloidal coomassie revealed a 90 kDa band in all three periostin isoforms (Figure 2A). Additionally, smaller bands representing impurities that have not been removed by the manufacturer's purification procedure were stained. After nitrocellulose transfer and probing with the polyclonal goat detection antibody, the 90 kDa band was identified as human periostin. All three isoforms were

recognized by the detection antibody, and the intensities of reactivities correlated with the applied protein amounts (Figure 2B).

3.2 | Principle of the sandwich ELISA assay, description of the assay range and definition of the assay limits

The setup of the periostin sandwich ELISA consists of a monoclonal mouse coating antibody used for capturing endogenous or recombinant human periostin, and a labeled polyclonal goat antibody used for detection as described before. The assay uses recombinant human periostin isoform 1 as calibrator. The calibrator curve was evaluated with a 4PL algorithm and is shown in Figure 3A. For all seven standards, the coefficient of variation in percent (% CV) for OD values as well as for calculated concentrations was $\leq 2\%$ (data not shown). The dynamic range of the assay is set between 125 pmol/L and 4000 pmol/L. For the calculation of the lower limit of quantification (LLOQ), which is defined as the lowest concentration that can be measured with an acceptable accuracy and precision, ie with a CV $\leq 25\%$, standard 2 was diluted in twofold steps in protein-buffered assay buffer. Five replicas per dilution were measured and the CV (%) was calculated. The LLOQ was assessed to be 62.5 pmol/L (Figure 3B). The limit of detection (LOD), defined as the calculated concentration of the lowest standard (standard 1) plus three times the standard deviation, was measured in five replicas and was calculated to be 20 pmol/L (data not shown).

3.3 | Intra-assay and inter-assay precision

For the determination of the repeatability, intra-assay precision was determined. Seven samples were run in replicates of five by one operator within one kit lot. Inter-assay precision was established by testing seven samples in replicates of ten by three operators in two different kit lots. For each sample in both precision assays, mean concentrations (pmol/L), standard deviation (SD; pmol/L), and the percent CV were determined. A representative sample from the low and high calibration range is shown in Table 2. In the intra-assay precision, the low sample (sample 1) gave an average reading of 249 pmol/L (SD=7.3 pmol/L) and the high sample (sample 2) of 2008 pmol/L (SD=52 pmol/L) respectively. Both samples showed a precision (CV) of 3% (Table 2). Inter-assay precision resulted in an average of 251

FIGURE 2 Binding of the polyclonal goat detection antibody to human periostin isoforms 1-3. A, Colloidal coomassie-stained SDS-PAGE. Protein molecular mass marker (left, kDa) and two concentrations of human periostin isoforms 1-3 are shown. B, Reactivity of the polyclonal goat detection antibody to two concentrations of nitrocellulose-blotted periostin isoforms 1-3 (left: protein molecular mass marker, kDa)

FIGURE 3 A, Definition of the assay calibration range. Concentrations (c [pmol/L], x-axis) are plotted vs measured OD values (y-axis). B, The LLOQ, calculated as the lowest concentration with a % backfit (y-axis, right), defined as ratio of the mean recalculated concentration (y-axis, left [pmol/L]) and the theoretical concentration (x-axis [pmol/L]), that lies between 75 and 125% with a %CV $< 25\%$, is shown as dashed line

TABLE 2 Determination of the intra-assay and the inter-assay precision

	Mean (pmol/L)	SD (pmol/L)	Precision (% CV)
Intra-assay (n=5)			
Sample 1	249	7.3	3
Sample 2	2008	52	3
Inter-assay (n=10)			
Sample 1	251	11.2	4
Sample 2	1996	111.5	6

Mean concentrations (pmol/L), standard deviations (SD; pmol/L) and the precision depicted as CV (%) are shown for two representable samples per determination.

pmol/L with a SD of 11.2 pmol/L for the low sample (sample 1) and of 1996 pmol/L (SD=111.5 pmol/L) for the high sample (sample 2). The determined CV values were slightly higher than the CV values from the intra-assay precision as the low sample (sample 1) had a CV of 4%, and the sample from the high calibration range (sample 2) showed a CV of 6% (Table 2).

3.4 | Characterization of the assay specificity

To exclude potential interference with unspecific matrix components, the coating antibody was subjected to specificity testing. Samples from apparently healthy subjects (n=14) were measured with or without preceding addition of a 10-fold molar excess of coating antibody, and detection of endogenous periostin was performed as described in the material and methods section. Percent competition was calculated and values from 14 patients varied between 93% and 100% (mean value 98%) (Table 3). All competition values were >90% hence demonstrating that the assay is highly specific for human endogenous periostin.

3.5 | Determination of accuracy/spike recovery in different sample matrices

In order to determine the accuracy of the assay, different sample matrices of apparently healthy individuals (serum, n=7; EDTA plasma, n=8; citrate plasma, n=8; heparin plasma, n=7) containing varying amounts of endogenous periostin were spiked with a mid (500 pmol/L) and a high (2000 pmol/L) concentration of recombinant human periostin isoform 1, and recovery was assayed. Mean spike recovery per sample matrix was calculated. Spiking with 500 pmol/L recombinant periostin led to a mean recovery of 106% for serum, 98% for EDTA plasma, 102% for citrate plasma, and 92% for heparin plasma (Figure 4A, top). When a high concentration (2000 pmol/L) of recombinant periostin isoform 1 was spiked in the four different matrices a mean recovery of 95% for serum, 83% for EDTA plasma, 91% for citrate plasma and 85% for heparin plasma was calculated (Figure 4A, bottom). Percent recoveries reside within the accepted range of 80% to 120% indicating high accuracy of the assay.

TABLE 3 Characterization of assay specificity

Serum sample ID	c (pmol/L) w/o competitor	c (pmol/L) with competitor	Competition (%)
1	647	10	98
2	1280	95	93
3	372	0	100
4	953	64	93
5	1440	100	93
6	1466	76	95
7	603	8	99
8	837	33	96
9	456	0	100
10	582	2	100
12	430	0	100
13	265	0	100
14	413	0	100
15	215	0	100
			Mean: 98

Serum samples of 14 healthy subjects (ID 1-15) were tested with and without (w/o) addition of a molar excess of coating antibody acting as competitor. Measured periostin concentrations with and without addition of competitor (pmol/L) and percent competition values are shown.

3.6 | Definition of dilution linearity

Samples (serum, n=12; EDTA plasma, n=4; citrate plasma, n=4; heparin plasma, n=4) with endogenous periostin levels in a mid dynamic range were tested. Samples were pre-diluted 1+50 as described earlier, and then diluted 1+1 and 1+3 in protein-buffered assay buffer. Percent recovery after dilution was calculated. Sample dilution analysis showed linear results across the dynamic assay range (125 pmol/L to 4000 pmol/L). After a 1+1 dilution, the mean recovery was very homogenous and ranged for plasma (EDTA, citrate, heparin) and serum between 95% and 101% respectively (Figure 4B, top). After a 1+3 dilution, the mean recovery ranged between 105% for serum and 117% for citrate plasma. Heparin plasma showed slightly higher mean recovery value of 126%, indicating the influence of a matrix effect in a 1+3 dilution (Figure 4B, bottom).

3.7 | Freeze/thaw cycle stability of endogenous periostin

For the determination of stability of endogenous periostin in different matrices (serum, EDTA plasma, citrate plasma, heparin plasma), specimens were taken from four subjects containing endogenous periostin levels. Samples were stressed by zero (reference), two, three, and four cycles of freezing at -20°C and thawing. Samples were transferred to room temperature prior to testing. Mean values per matrix were calculated and were related to the initial state (reference) as percent recoveries of the sample (zero freeze/thaw; reference value defined

FIGURE 4 A, Spiking of recombinant human periostin into four different matrices. Mean percent recoveries (x-axis) of two concentrations of recombinant human periostin (500 pmol/L, 2000 pmol/L) spiked into serum (n=7), EDTA plasma (n=8), citrate plasma (n=8), and heparin plasma (n=7) of apparently healthy individuals are shown. B, Determination of dilution linearity of endogenous periostin. Mean percent recoveries (x-axis) of serum (n=12), EDTA plasma (n=4), citrate plasma (n=4) and heparin plasma (n=4) samples diluted 1+1 and 1+3 are shown

FIGURE 5 Determination of stability of endogenous periostin. Four sample matrices (serum, EDTA plasma, citrate plasma, heparin plasma) with four samples each were stressed by zero, two, three, and four cycles of freeze/thaw (x-axis). Mean values per sample matrix were related to the initial sample state (reference) and percent recoveries of periostin concentration are shown (y-axis)

as 100%) (Figure 5). Serum shows percent recovery of 100% after two cycles of freeze/thaw, and slight deviation after three (103%) and four (98%) freeze/thaw cycles. Recovery is similar in plasma samples. EDTA plasma shows recovery of 91% after two freeze/thaw cycles and fluctuates to 93% (three cycles) and 90% (four cycles). Citrate plasma measures 93% recovery after two freeze/thaw cycles, and deviates from 91% to 97% after three and four cycles. Heparin plasma shows 96% recovery after two cycles, another 96% after three, and 99% after four cycles of freeze/thaw (Figure 5). These very slight variations after two (plasma) or three (serum) freeze thaw cycles in all four measured matrices allow the conclusion that endogenous periostin levels are stable for at least four cycles of freezing and thawing of samples.

3.8 | Quantification of endogenous periostin in different sample matrices of apparently healthy subjects and diseased individuals

Four different matrices (serum, n=24; citrate plasma, n=24; EDTA plasma, n=20; heparin plasma, n=20) from blood samples of apparently healthy individuals were collected and endogenous periostin concentrations were determined. In serum samples, periostin concentrations ranged from 397 to 1466 pmol/L with a median concentration of 864 ± 269 pmol/L. In citrate plasma, levels ranged from 321 to 1407 pmol/L with a median concentration of 885 ± 252 pmol/L. In EDTA plasma, concentrations reached from 532 to 1109 pmol/L with a median of 817 ± 170 pmol/L. In heparin plasma, levels reached from 569 to 1194 with a median of 891 ± 181 pmol/L (Figure 6A). All measured samples had identical sample histories in terms of freeze and thaw cycles ($\leq$ four cycles).

In addition, periostin levels were measured in a group of serum samples from asthma (n=10) and cardiovascular disease patients (n=10) vs age and gender matched control patients (n=18). Diseased samples were related to the median of control patients as z-scores, and the differences between the diseased sample groups and the control group were tested for statistical significance with Mann-Whitney U or Wilcoxon testing. There was no significant difference between the control and the asthma group ($P > .05$) (Figure 6B). However, when we compared the control group with the group of samples from patients with cardiovascular disease a significant increase in the population with cardiovascular disease was observed ($P < .05$) (Figure 6B).

4 | DISCUSSION

Periostin has been discussed as a promising biomarker to determine bone fracture risk,¹⁴ or for T_H2 -associated asthma and eosinophilic inflammation to select patients who may benefit from IL-13-targeted treatment.^{21,28,29}

FIGURE 6 A, Human periostin levels were determined in serum, citrate plasma, EDTA plasma and heparin plasma samples of apparently healthy individuals. Data are displayed as scatter plot representation whereas lines within the charts represent median values. Periostin concentrations (pmol/L) are shown on the y-axis. B, Human periostin concentrations measured in serum samples of asthma and cardiovascular disease patients related to healthy controls. Z-scores (y-axis) are displayed as scatter plot representation whereas lines within the charts represent median values. P values <.05 are indicated

In this study, we describe the development and performance of a sandwich ELISA assay to quantify endogenous periostin in human serum and plasma. The assay is standardized with periostin isoform 1. However, the characterization of the antibodies employed in the assay shows that all known human periostin isoforms can be detected. In fact, the mouse monoclonal anti-human periostin antibody used for coating detects a linear epitope in the fourth fasciclin-1 (Fas1) domain which is conserved between the seven periostin isoforms. The goat polyclonal anti-human periostin antibody employed for detection recognizes multiple conserved linear epitopes that spread across the complete periostin sequence. Only one of the five major epitopes of the polyclonal detection antibody (epitope p_e4) differs between the isoforms. In detail, epitope p_e4 on isoform 1 has been resolved as sequence "KTEGPTLT", which differs on isoform 3 and 5 that both carry the sequence "TEVIHG". Isoforms 2, 4, 6, and 7 even have a sequence deletion in the region of epitope p_e4. To analyze if the conserved sequences of the remaining epitopes (p_e1, p_e2, p_e3, p_e5) of the polyclonal detection antibody are sufficient to detect all seven currently known isoforms we used commercially available recombinant periostin isoforms 1, 2, and 3 which cover all epitope variants of epitope p_e4 of the seven known isoforms. We could show that the employed detection antibody reacts with the nitrocellulose-blotted proteins in equal intensities, there was no significant difference between the isoforms.

In contrast, other commercial periostin sandwich ELISA assays (USCN, China; Adipogen, Switzerland) do not reveal epitope information. The assay from USCN is set up with a polyclonal antibody raised against the first fasciclin-1 domain (position 97 – 230) and it is supposed that all isoforms can be detected.³⁰ The ELISA assay from Adipogen uses two monoclonal antibodies of unknown epitope specificity.¹⁴ According to the manufacturer they bind to isoform 1 and 2 and they are expected to bind to additional isoforms.

Our assay is a highly specific and precise tool to detect endogenous periostin within a calibration range 125 pmol/L to 4000 pmol/L in small volumes (3 μ L sample per well) of human specimen, with an

LLOQ of 62.5 pmol/L, an intra-assay precision of $\leq 3\%$ and an inter-assay precision of $\leq 6\%$. Assay characteristics have shown to meet the validation criteria of the ICH harmonized tripartite guideline Q2 (R1) and of the EMEA guideline of bioanalytical method validation. For instance, the accuracy, measured as percentage recoveries of spiked recombinant periostin into different sample matrices, ranges from 83% to 106%. The verification of the dilution linearity shows recoveries between 95% and 101% at 1+1 dilution, and between 105% and 117% at 1+3 dilution. The 1+3 dilution of heparin plasma is the only exception with recoveries slightly beyond the validation criteria (126%) which shows the influence of matrix effects.

Currently, two fully automated assays have been described for the detection of human periostin (Elecys[®] Periostin Immunoassay, Roche Diagnostics, Penzberg, Germany;³¹ ARCHITECT[®] Periostin assay, Abbott Diagnostics, Illinois USA³²). Both assays are based on the sandwich principle using monoclonal antibodies able to detect human serum periostin isoforms. These assays are used in companion diagnostics and are also applied in clinical trials employing serum periostin as a biomarker in T_H2-associated airway inflammation and airway eosinophilia.^{20,33} A clinical trial version of the Elecys[®] Periostin Immunoassay³¹ was used to classify patients suffering from moderate-to-severe asthma into periostin-low and periostin-high patients.^{21,28} As threshold for this classification a median periostin value of 50 ng/mL was chosen. This value was defined in a clinical phase II study (MILLY study) analyzing the usefulness of serum periostin as a biomarker to select asthmatic patients who are more likely to benefit from treatment with lebrikizumab, a therapeutic humanized anti-IL-13 antibody.²¹ The ARCHITECT[®] Periostin assay³² was used in a similar setting to screen serum samples of asthmatic patients for periostin concentrations over a predefined threshold who are most likely to benefit from treatment with IL-13 antagonists.²⁹

Fingleton et al. used the Elecys[®] clinical trial version in a cross-sectional study to determine serum periostin levels in adult patients with symptoms of obstructive airway disease.³⁴ They reproduced

cut-off periostin levels of asthmatic patients from the MILLY, LUTE, and VERSE studies^{21,28} by describing a median of 53.7 ng/mL. The same study also described that there was no significant difference between periostin levels of asthmatic and non-asthmatic patients (median non-asthmatics: 54.6 ng/mL),³⁴ a result that was further confirmed by Caswell-Smith et al.³⁵ Serum periostin levels from asthmatic patients measured with our ELISA agree with these data, we also do not show significant differences between the asthmatic and non-asthmatic group.

As serum levels of periostin have further been described to increase in response to cardiovascular injury and hypertension,^{11,36,37} we analyzed a panel of samples from patients with cardiovascular disease (n=10). Consistent with literature we observed significantly increased serum periostin levels in these samples.

We defined periostin concentrations in apparently healthy subjects with median concentrations of 864 pmol/L (78.5 ng/mL) for serum, 885 pmol/L (80.5 ng/mL) for citrate plasma, 817 pmol/L (74.3 ng/mL) for EDTA plasma, and 891 pmol/L (81.0 ng/mL) for heparin plasma. Our serum levels were consistent with serum periostin levels of adult healthy subjects at the age of 30 years or older as measured by Walsh et al. who used the same assay to analyze an age-related role of periostin in cortical modeling.³⁸ Comparison of human periostin serum levels from healthy subjects detected with our assay and the Elecsys® Periostin Immunoassay revealed that samples showed similar assay ranges. For instance, serum levels from healthy subjects obtained with our periostin assay ranged from 397 pmol/L to 1466 pmol/L (36.1 to 133.3 ng/mL), and serum levels obtained with Elecsys® ranged from 15.0 to 164.7 ng/mL and from 28.1 to 136.4 ng/mL as reported by Fingleton et al.³⁴ and Caswell-Smith et al.³⁵ respectively. We further analyzed assay ranges of the periostin sandwich ELISA assays from USCN and Adipogen. They were applied to investigate the correlation of serum or plasma periostin with spine and hip areal bone mineral density in post-menopausal women.^{39,40} In the study by Kim et al. the Adipogen assay measured mean plasma periostin concentrations of 29.2 ng/mL in a group of healthy control subjects without osteoporotic fracture.³⁹ We obtained comparable values in an apparently healthy population with our periostin assay. In contrast, Anastasilakis et al. report mean serum periostin levels of 250 ng/mL from apparently healthy control women with normal bone mass obtained with the USCN assay.⁴⁰ These values are beyond the assay range of our assay and of the Elecsys® assay, which may be explained by the lack of standardization in research and commercial assays.

In conclusion, we developed a reliable, accurate, and robust tool for the quantitative determination of all currently known isoforms of periostin in human healthy and diseased samples. Unlike other commercially available immunoassays, we provide in depth epitope information of the employed antibodies. While the automated immunoassays Elecsys® and ARCHITECT® detect periostin in serum, we are able to quantify periostin concentrations in serum and in various plasma matrices (citrate, EDTA, heparin). Therefore, this assay is a useful tool to further investigate functional aspects of periostin and its potential as a biomarker in various clinical settings.

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