

# A targeted mass spectrometry immunoassay to quantify osteopontin in fresh-frozen breast tumors and adjacent normal breast tissues



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## ABSTRACT

Osteopontin (OPN) is a multifunctional protein that can activate cell-signaling pathways and lead to cancer development and metastasis. Elevated OPN expression was reported in different cancer types, including breast tumors. Here, we present a new immuno-mass spectrometry method for OPN quantification in fresh-frozen malignant and adjacent normal human breast tissues. For quantification we used two proteotypic peptides: OPN-peptide-1 and OPN-peptide-2. Peptide concentrations were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in multiple reaction monitoring (MRM) mode with stable isotope standards (SIS) and immuno-affinity enrichment for isolation of OPN peptides. Based on the OPN-peptide-1, the average OPN concentration in normal breast tissue was 19.42 µg/g, while the corresponding level in breast tumors was 603.9 µg/g. Based on OPN-peptide-2, the average concentration in normal breast tissue was 19.30 µg/g and in breast tumors 535.0 µg/g. In ER/PR/HER2(–) patients the OPN levels in breast tumors were significantly higher than in corresponding normal breast tissue samples, whereas in the single ER/PR/HER2(+) patient the OPN concentration in tumor samples was lower than in normal breast tissue sample. In conclusion, the current method is considered promising for the quantification of OPN in research and in clinical settings and should be further studied in breast cancer patients.

**Significance:** A new immuno-mass spectrometry method was successfully developed and applied to determine OPN concentrations in malignant tumor and normal breast tissues from six patients, and the method is promising for OPN quantification in both research and clinical settings.

## 1. Introduction

Osteopontin (OPN) is a matricellular protein that modulates cell function and cell-extracellular matrix (ECM) interactions. The protein is of special scientific interest as an aberrant OPN expression disturbs ECM homeostasis [1] and elicits signal transduction cascades that may lead to tumor development and metastasis [2,3]. In breast cancer, one of the most common cancers in women worldwide [4,5], OPN

overexpression contributes to tumor formation, angiogenesis and growth, and mediates metastasis and reduced survival [6–8]. OPN possesses a multitude of functions in health and disease, which are in many cases regulated by alternative mRNA splicing and different post-translational modifications (PTMs) of the protein. Phosphorylations and glycosylations are the most abundant OPN PTMs, but tyrosine sulfations and sialylation have also been observed. Moreover, OPN undergoes extensive proteolytic processing eg. by matrix metalloproteinases and

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thrombin, which also affects its functions. As recently reviewed [9,10], five alternative splice variants as well as secreted and intracellular isoforms of the protein have been identified. In addition, the degree of OPN modifications may vary between species for the same tissue type [11,12], as well as between different types and states of the same cell/tissue [13]. Due to this high number of OPN forms both non-modified OPN and its PTM-dependent cell processes have been intensively studied.

Several studies have thus evaluated OPN as a potential prognostic biomarker of breast cancer, either by analyzing normal tissue/tumor specimens or plasma/serum samples. Until now, OPN protein and gene expression in tissue samples from breast cancer patients has mainly been measured by immunohistochemistry (IHC) [6–8,14–17], western blot analysis [6–8,14] and quantitative polymerase-chain reaction (qPCR) [6,15,16,19]. Using IHC, Rudland et al. [14] showed that positive OPN staining in breast cancer tissue sections correlated with poor prognosis. Elevated OPN expression was also associated with tumor aggressiveness and poor prognosis in lymph node negative [15] and invasive ductal carcinoma [6] breast tumor specimens. Conversely, Wang-Rodriguez et al. [16] found that increased OPN mRNA levels in human breast tissues indeed identified malignant carcinoma, but no difference in transcript levels were found between primary tumors with or without metastases, or between primary and metastatic tumors. Moreover, significantly lower cytoplasmic OPN mRNA was observed in tumor sections from metastatic breast cancer patients when bone metastases were present [17]. In serum and plasma samples, enzyme-linked immunosorbent assays (ELISA) have been typically applied for OPN detection [18,20–22]. Singhal et al. [20] found that elevated OPN concentrations in plasma of breast cancer patients correlated with tumor aggressiveness, disease progression and lower survival rate. Plasma OPN concentrations determined by ELISA also proved to have prognostic value in metastatic breast cancer [21] and locally advanced breast cancer [22]. However, it should be noted that Bramwell et al. [18] did not find elevated OPN plasma or tumor levels in a large cohort of women with hormone responsive early breast cancer treated by surgery followed by adjuvant treatment with tamoxifen +/- octreotide. One exception to this was a subgroup of patients that showed higher OPN plasma concentrations at the time of recurrence [18]. The above examples illustrate that the functional outcomes of differential OPN expression in breast cancer is still poorly understood, particularly regarding concentrations/levels of OPN in tumor tissue vs serum/plasma in patients with different breast cancer subtypes. Hence, further studies on different breast tumor types are necessary to define OPN utility as a prognostic marker of breast cancer.

Currently, ELISA is a standard, routinely applied method for validation and verification of many protein biomarkers, ensuring sensitive detection when specific antibodies are available. However, lack of specific antibodies towards many potential biomarkers, as well as the low capacity of multiplexing in ELISA, has spurred the development of alternative methods. Here, targeted mass spectrometry (MS)-based proteomics in the selected, multiple or parallel reaction monitoring (SRM, MRM or PRM) mode in combination with stable isotope labelled peptide standards (SIS), such as AQUA (absolute quantification) peptides [23], has become increasingly popular [24]. In the SRM/MRM mode, triple quadrupole mass analyzers measure signal intensities of previously selected parent ion/fragment ion transitions characteristic for proteotypic peptides and their isotope labelled counterparts. When employed on Orbitrap or QTOF mass analyzers, this strategy is known as PRM. In either case, endogenous protein concentrations are calculated based on their peptide concentrations, which in turn are determined by comparison to the corresponding labelled standards. The method can easily be multiplexed, allowing parallel quantitative analysis of many proteins. This has proven highly efficient for quantification of high abundance plasma proteins [25]. However, quantification of low-abundance proteins in plasma or serum may require pre-depletion of high abundance proteins, or enrichment of the specific target

protein(s) e.g. by antibodies immobilized on magnetic beads [26]. This technique, commonly abbreviated immuno-SRM/MRM/PRM when coupled to targeted MS analysis, has allowed effective targeted quantitative analysis of several low abundance biomarkers [27]. Moreover, the MS step provides additional analytical specificity and eliminates errors caused by antibody cross-reactivity [28].

In the present work, we aimed at establishing a new, improved method for OPN quantification in normal breast tissues and breast tumors, based on immunoaffinity enrichment and downstream LC-MRM-MS analysis. The OPN quantification method was tested by analyzing breast tumor samples and corresponding adjacent normal breast tissues from six patients.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Glycine, Tris(hydroxymethyl)aminomethane (Tris) and sodium chloride (NaCl) were from Merck KGaA (Darmstadt, Germany). Ammonium bicarbonate (NH<sub>4</sub>HCO<sub>3</sub>), human serum albumin (HSA), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), dithiothreitol (DTT), dimethyl pimelimidate dihydrochloride (DMP), ethanolamine, ethylenediaminetetraacetic acid (EDTA), human recombinant osteopontin expressed in NSO cells (OPN, ≥ 95%, UniprotKB-SwissProt: OSTP\_HUMAN/P10451, the protein lacks the 16 N-terminal residues of the canonical human OPN isoform a), iodoacetamide (IAA), 3-(decyldimethylammonio)-propane-sulfonate inner salt (SB3-10), thiourea, Triton X-100 and urea were from Sigma-Aldrich (St. Louis, MO, USA/ Steinheim, Germany). Acetone was from SDS (Peypine, France) and ampholytes (Bio-Lyte® pH 3–10) from BioRad, (Hercules, CA, USA). Fat-free dry milk was from Fluka (Buchs, Switzerland), phosphate buffered saline (PBS) (Dubecco A tablets) from Oxoid (Basingstoke, Great Britain) and Tween 20 from VWR BDH Prolabo (Briare, France). LC-MS grade acetonitrile (ACN) and formic acid (FA) were from Fisher Scientific (Fair Lawn, New Jersey, USA).

Proteotypic OPN peptides: YPDAVATWLNPDPSQK (OPN-peptide-1, MW<sub>theor.</sub> 1801.97) and AIPVAQDLNAPSDWDSR (OPN-peptide-2, MW<sub>theor.</sub> 1854.99), both of > 70% purity, were synthesized by Thermo Fisher Scientific (Ulm, Germany). The stable isotope labelled standards (SIS) of these peptides: YPDAVATWLNPDPSQK (OPN-heavy peptide-1, MW<sub>theor.</sub> 1809.97 Da), and AIPVAQDLNAPSDWDSR (OPN-heavy peptide-2, MW<sub>theor.</sub> 1864.99 Da), as 5 pmol/μL absolute quantification reagents – HeavyPeptide AQUA QuantPro (> 97%) – were also from Thermo Fisher Scientific (Ulm, Germany). The stable isotope labels (13C, 15N) were incorporated at the lysine or arginine positions, resulting in mass shifts of +8 or +10 Da, respectively. The heavy amino acid was labelled as bold and underlined in the sequences of the peptides. Custom rabbit antisera containing polyclonal antibodies against OPN-peptide-1 (Ab1) and – 2 (Ab2) were obtained from Thermo Fisher Scientific (Ulm, Germany). Protein A-coated magnetic beads (Dynabeads Protein A) were from Invitrogen Dynal AS (Oslo, Norway). Trypsin Gold – Mass Spectrometry Grade, was from Promega (Madison, WI, USA). HRP-conjugated polyclonal swine anti-rabbit antibodies were from Dako Denmark AS (Glostrup, Denmark). In all experiments, Axygen Microtubes (1.5 mL, Maximum Recovery™ Clear MCT-150-L-C, Homo-Polymers, Boil Proof, Axygen Scientific, Union City, CA, USA) were used to minimize binding to test tubes walls. Deionized water was obtained by using the DirectQ – Millipore System (Bedford, MA, USA).

### 2.2. Patient sample collection

Six patients of Caucasian origin, with a newly diagnosed breast cancer and without previous chemotherapy, were admitted to the Department of Surgical Oncology Medical University of Gdansk (MUG). During tumor removal surgery, both cancerous breast tissue and

adjacent normal breast tissue specimens were collected. The breast tumor samples investigated in our study was from its ¼ part. The tissue material denoted as normal was taken during the breast amputation from the most distant site relative to the tumor site. All specimens were snap-frozen in liquid nitrogen in the operating room and stored at  $-80^{\circ}\text{C}$  at the Department of Medical Laboratory Diagnostics – Biobank MUG. The study was approved by the local ethics committee (agreement # NKEBN/781/2005MUG). About three quarters of breast tumors were examined by an experienced pathologist to determine the cancer stage using anatomical pTNM classification (T – tumor, N – nodus, M – metastases) [29] as well as the presence of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). The adjacent normal breast tissue was not checked by the pathologist. There were five breast tissue and tumor sample pairs from patients with ER/PR/HER2 negative and one from a patient with ER/PR/HER2 positive breast cancer. All the investigated breast tumors represented ductal carcinoma type. A detailed description of each sample is available in Supplementary material 1: Table 1S. The clinical sample set is small, as the main focus of this study was on developing the methodology for OPN quantification by mass spectrometry in fresh frozen breast tissue and tumor samples. The clinical samples were used mainly to verify the efficiency of the methodology on the target sample types.

### 2.3. Protein extraction

All tissue handling and extraction steps were performed at  $4^{\circ}\text{C}$  on ice unless specified, to minimize possibility of sample degradation by endogenous proteases. After thawing, blood was removed from the tissues by washing with PBS, which is a routinely used non-toxic, physiological buffer in terms of osmolality and pH, helps preserving the tissues intact and prevents them from rupturing. Tissues were weighted, cut into smaller pieces and manually homogenized in a Dounce tissue grinder. Homogenates were mixed with 400  $\mu\text{L}$  lysis buffer (5 M urea, 2 M thiourea, 1% Triton X-100, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2% (v/v) CHAPS, 0.2% Ampholytes 3–10, 1.5% (v/v) SB3–10, 1 mM EDTA, 1 mM DTT), sonicated  $2 \times 10$  min in a water bath and centrifuged at  $14,000 \times g$  for 20 min. Each supernatant was then mixed with 1.6 mL ice-cold acetone and proteins precipitated for 5 h at  $-20^{\circ}\text{C}$ . After centrifugation at  $14,000 \times g$ , for 20 min, supernatants were discarded. The remaining protein pellets were evaporated to dryness (Vacufuge, Eppendorf, Hamburg, Germany). Finally, protein pellets were dissolved in 100  $\mu\text{L}$  freshly prepared 50 mM  $\text{NH}_4\text{HCO}_3$  pH 7.8 and protein concentrations determined in a NanoDrop ND 1000 (Thermo Fisher Scientific, Wilmington, DE, USA). The protein extracts were either analyzed immediately after extraction or stored at  $-80^{\circ}\text{C}$  until analysis.

### 2.4. Protein digestion

Prior to enzymatic digestion, protein extracts were diluted 1:50 with freshly prepared 50 mM  $\text{NH}_4\text{HCO}_3$  (pH 7.8). Each diluted extract (100  $\mu\text{L}$ ) was then reduced with DTT (10 mM final) at  $37^{\circ}\text{C}$  for 1 h and alkylated with iodoacetamide (20 mM final) in the dark for 30 min at room temperature. Trypsin was added (1:50 E:S ratio) and the samples digested for 12 h at  $37^{\circ}\text{C}$ . The digestion was stopped by reducing the pH to 3 with 5% FA/50% ACN. After vacuum evaporation, the samples were either immunoprecipitated and analyzed immediately or lyophilized and stored at  $-20^{\circ}\text{C}$ .

### 2.5. Peptide immunoaffinity enrichment

Ab1 and Ab2 IgGs were purified from rabbit antisera over HiTrap Protein A HP 1 mL columns (0.7 cm  $\times$  2.5 cm) (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), according to the manufacturer's instructions and purified IgG concentrations measured using the

NanoDrop 1000. IgGs were pre-concentrated by ultrafiltration when needed (Microcon Centrifugal Filters, 10 kDa cutoff, Millipore, Bedford, MA, USA). The purified Ab1 and Ab2 IgGs were covalently cross-linked by DMP to Dynabeads Protein A according to the manufacturer's protocol [30]. The capture efficiencies of the IgG-coated beads against OPN peptide-1 and -2 were monitored. This was done by transferring 20  $\mu\text{L}$  of the magnetic bead-Ab conjugates to new Axygen tubes in a magnetic separator and replacing the supernatant with either 1.25  $\mu\text{g}$  of OPN-peptide-1 or OPN-peptide-2 dissolved in 30  $\mu\text{L}$  extraction buffer, diluted 50 times in  $\text{NH}_4\text{HCO}_3$  solution (50 mmol/L). In parallel experiments, the standard OPN peptide solutions were subjected to detergent removal prior to exposure to the magnetic bead-Ab conjugates, by passing the peptides through 0.5 mL Pierce Detergent Removal Spin Columns (Thermo Scientific Pierce, Rockford, IL, USA) according to the producer's instructions. Subsequently, OPN peptides and the bead-Ab conjugates were incubated for 5 h at  $4^{\circ}\text{C}$ , with constant tilting-rolling prior to magnet-assisted removal of unbound material, then washed twice in 200  $\mu\text{L}$  PBS and finally once in 100  $\mu\text{L}$  of deionized water. To optimize antigen elution conditions compatible with downstream MS analysis, two elution buffers were tested: (1) 0.1% FA and, (2) 50 mM glycine pH 2.8. After adding 30  $\mu\text{L}$  of either elution buffer, samples were incubated for 20 min at room temperature with gentle mixing. After magnetic bead separation, the elution step was repeated for both FA and glycine buffers separately and the eluates from the two subsequent steps were combined and evaporated to dryness in a Vacufuge. To check elution efficiency, the beads were finally stripped by addition of 30  $\mu\text{L}$  of 1% SDS/50 mM glycine pH 2.8 [31]. Peptide pellets were stored at  $-20^{\circ}\text{C}$  until further analysis. The peptide immunoaffinity enrichment of the normal breast tissue and tumor protein extracts was performed as described above except that antigen elution was done with two volumes of 30  $\mu\text{L}$  0.1% aqueous FA only, to ensure complete elution from the beads.

### 2.6. Dot blot analysis

The pellets of immunoprecipitated OPN-peptide-1 and 2, were solubilized in 5  $\mu\text{L}$  of PBS and 2.5  $\mu\text{L}$  of those solutions were spotted on nitrocellulose membranes (Trans-Blot Transfer Medium Pore Nitrocellulose Medium 0.45  $\mu\text{m}$ , BioRad Laboratories, Hercules, CA, USA). The dried membranes were blocked in 5% fat-free dry milk in PBST (0.1% Tween 20 in PBS) for 0.5 h. After overnight incubation with the (purified from crude antisera) IgGs Ab1 and Ab2 (1:2000 in blocking buffer) at  $4^{\circ}\text{C}$ , membranes were washed three times for 10 min in PBST. After 1 h incubation with HRP-conjugated secondary swine anti-rabbit antibodies (1:3000 in blocking buffer), membranes were washed  $3 \times 10$  min in PBST prior to development using Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific Pierce, Rockford, IL, USA) and detection in a Kodak 4000R chemoluminescent scanner.

### 2.7. LC-MS/MS analyses

The LC-MS/MS analyses were performed on a Shimadzu Prominence HPLC System (Shimadzu Scientific Instruments, Columbia, MD, USA) in line with an API5000 Triple Quadrupole LC-MS/MS Mass Spectrometer (AB SCIEX, Foster City, CA, USA) and controlled with Analyst v1.4.2 software. The mass spectrometer was equipped with electrospray TurboV Ion Source and run in either information dependent acquisition (IDA) or multiple reaction monitoring (MRM) mode. The ion source was operated in positive ion mode, applying the following source parameters: ion spray voltage 4.5 kV, curtain gas (CUR) 35, nebulizer gas (GS1) 40, heater gas (GS2) 40. The chromatographic separation of the injected 5  $\mu\text{L}$  peptide mixtures, in both IDA and MRM mode, was carried out on Zorbax SB-Aq column (2.1  $\times$  150 mm, 3.5  $\mu\text{m}$ ) with Eclipse XDB-C8 precolumn (2.1  $\times$  12.5 mm, 5  $\mu\text{m}$ ) placed in Guard Column Hardware Kit (Agilent Technologies, Santa Clara, CA,

USA). The flow rate was set to 200  $\mu\text{L}/\text{min}$  and the gradient was: 0–10 min: 0–5% B, 10–30 min: 5–100% B, 30–35 min: 100% B, 35–40 min: 100–0% B, 40–50 min: 100% A [A: 0.1% aqueous FA, B: and 80% ACN/0.1% aqueous FA].

OPN determination was performed by LC-MRM-MS. The selection of OPN peptides and their parent and fragment ions for further MRM analyses was performed by combining the *in silico* approach and experimental verification. MS-Digest and MS-Fragment tools of the Protein Prospector v 5.14.2 software (available on-line at [32,33]) were first used to obtain theoretical OPN digest and the resulting peptide fragmentation patterns, respectively. Then, the trypsin digested OPN standard (500 ng of protein) was analyzed in LC-MS/MS IDA mode for empirical verification of the peptides and their corresponding fragment ions presence in the MS/MS spectrum. Q1 was scanning the mass range from 500 to 2000  $m/z$  for 51 min, with total cycle time of 1.01 s and unit Q1/Q3 resolution. Then, the two most intense +2 to +4 charged ions were selected for fragmentation in the product ion scan using rolling collision energy. Identification of OPN peptides was based on the obtained MS/MS spectra, by searching MSDB protein sequence database (EBI, Cambridge, Great Britain) with MASCOT v2.2 software (Matrix Science, London, Great Britain). Peptides were defined as correctly identified when the probability of random correct identification,  $p$ , was  $\leq 0.05$  [34]. Two of the peptides identified in the IDA experiment, YPDAVATWLNPDPSQK (OPN-peptide-1) and AIPVAQDLNAPSDWDSR (OPN-peptide-2) (both +2 charge), were selected as signature peptides for OPN quantification. Product ion spectra acquired for the parent ions of  $m/z$  901.5 (OPN-peptide-1  $^{+2}$ ) and 927.9 (OPN-peptide-2  $^{+2}$ ) revealed that the best signals for creation of the MRM transitions for the peptides were:  $m/z$  459.5 corresponding to  $y_4$  ion of OPN-peptide-1 and 262.3 corresponding to the  $y_2$  ion of OPN-peptide-2. The fragment ions monitored for the SIS standards of the investigated OPN peptides were:  $m/z$  467.3 for OPN-heavy peptide-1 ( $y_4$  ion) and 271.9 for OPN-heavy peptide-2 ( $y_2$  ion). Prior to LC-MRM-MS analysis, pellets of light OPN peptides and tissue peptide samples were dissolved in 8  $\mu\text{L}$  of mobile phase A and spiked with 2  $\mu\text{L}$  (100 fmol) of SIS peptides (OPN-heavy peptide-1 and OPN-heavy peptide-2). The analytes were detected in MRM mode at the ion transitions:  $m/z$  901.5  $\rightarrow$  459.5 for OPN-peptide-1 and  $m/z$  927.9  $\rightarrow$  262.3 for OPN-peptide-2. The MRM transitions monitored for the SIS standards of these peptides were:  $m/z$  905.6  $\rightarrow$  467.3 for OPN-heavy peptide-1 and  $m/z$  933.6  $\rightarrow$  271.9 for OPN-heavy peptide-2. The MRM analyses were carried out for 51 min, with total cycle time of 1.01 s, unit Q1/Q3 resolution and 250 ms dwell time per transition. The source parameters of the API5000 were the same as in the IDA experiment. Optimized compound parameters for the analytes were: collision gas (CAD) 6, declustering potential (DP) 40 V, collision energy (CE) 52 V, entrance potential (EP) 10 V and cell exit potential (CXP) 11 V. The chromatograms obtained in the MRM analyses were processed with Analyst v1.4.2 with signal smoothing applied. The area under peaks of the investigated peptides and their SIS standards were used for quantification of OPN in the tissue samples. The OPN concentrations were normalized to the total protein content in the tissue extract during the calculation of OPN concentration basing on OPN-peptide-1 and OPN-peptide-2. (Supplementary material 2: Table 1S and 2S).

## 2.8. Statistical analysis

Pearson correlation analysis was performed with the use of Dell Statistica v. 13 (Dell Inc., 2016) to investigate the similarity between OPN concentrations calculated based on OPN-peptide-1 and OPN-peptide-2 for each of the investigated samples pairs (normal and tumor from the same patient). Descriptive statistics were calculated in Excel, 2016.

## 3. Results and discussion

### 3.1. Development of a targeted LC-MS/MS method for osteopontin quantification

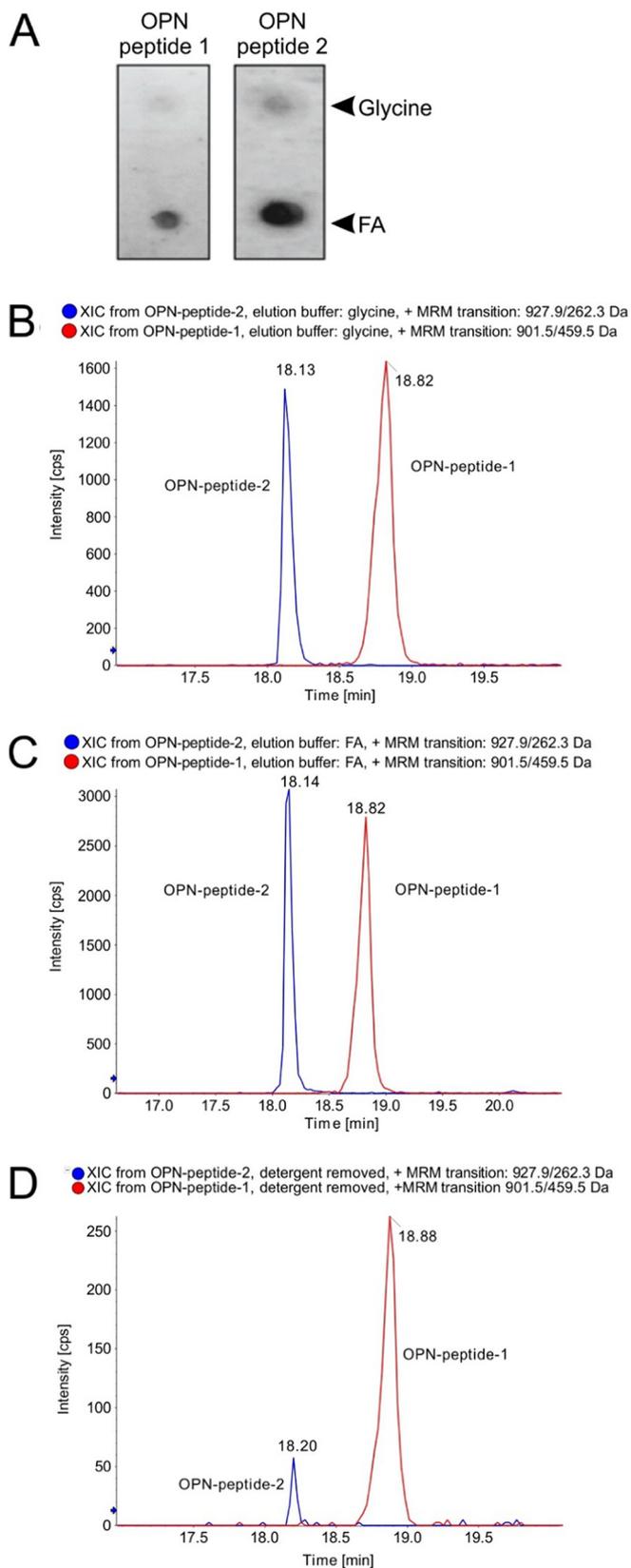
Database search of MS/MS spectra obtained in IDA LC-MS/MS of trypsin-digested OPN standard, in combination with *in silico* fragmentation pattern analysis, suggested that two tryptic peptides, YPDAVATWLNPDPSQK (OPN-peptide-1) and AIPVAQDLNAPSDWDSR (OPN-peptide-2), were the most suitable for quantification purposes. The peptides were present in both the experimental spectrum and the theoretical ions list, and were characterized by the highest ion scores in the Mascot database search (91 and 106 for OPN-peptide-1 and OPN-peptide-2, respectively). The OPN-peptide-1 covers the amino acids 36–51 in the N-terminal part of OPN-a and the OPN-peptide-2 corresponds to amino acids 204–220 located in the C-terminal part of the canonical OPN-a variant. It should be noted that due to the potential phosphorylation on Ser 215 and Ser 219 of OPN-peptide-2 there may be a risk of underestimating the concentration of C-terminal OPN fragment if such phosphorylation would be present in the investigated samples as the current MRM method focuses on detection of non-phosphorylated peptides.

The analysis of the MS/MS spectra obtained during product ion scanning of the OPN peptides parent ions enabled selection of the product ions to be monitored. Based on the signal and quality of the resulting MRM transition the ions of the following  $m/z$  were chosen: 459.5, which is  $y_4$  ion of OPN-peptide-1 and 262.3 being  $y_2$  ion of OPN-peptide-2. Accordingly, the fragment ions monitored for SIS labelled OPN-peptides were:  $m/z$  467.3 for OPN-heavy peptide-1 ( $y_4$  ion) and 271.9 for OPN-heavy peptide-2 ( $y_2$  ion). Four SRM transitions were monitored, which corresponded to the parent ion/fragment ion pairs of the OPN-peptide-1 and OPN-peptide-2 and their stable isotope labelled standards (SIS) – OPN-heavy peptide-1 (+8 Da) and OPN-heavy peptide-2 (+10 Da). Based on the list of potential parent and fragment ions and empirical verification of their signal intensities in mass spectra, the  $m/z$  transitions 901.50  $\rightarrow$  459.5 (OPN-peptide-1), 927.9  $\rightarrow$  262.3 (OPN-peptide-2), 905.6  $\rightarrow$  467.3 (OPN-heavy peptide-1) and 933.6  $\rightarrow$  271.9 (OPN-heavy peptide-2), were chosen for MRM analysis. The absolute peptide concentrations were defined by comparison of their SRM signals with the signals SIS standards of known concentration (Supplementary material 2: Table 1S and 2S).

### 3.2. Development of osteopontin isolation procedure from breast tumor and adjacent normal breast tissue samples

The malignant and adjacent normal breast tissue samples were extracted using buffer containing chaotropes (urea, thiourea) and detergents (Triton X-100, CHAPS, SB3–10) to ensure efficient protein denaturation and solubilization. Additional tissue grinding and sonication during sample preparation ensured efficient protein extraction. After removal of cell debris, proteins were enriched by ice-cold acetone precipitation [35], which mediated no apparent loss of osteopontin peptides (Supplementary Fig. 1S). To reduce potential MS signal suppression, OPN peptides were enriched from the trypsin digest by affinity capture using OPN-peptide specific polyclonal IgGs covalently coupled to Dynabeads Protein A.

We have previously shown that optimal elution conditions from such immune-affinity matrices depend on both the antibody and the antigen, as well as the cross-linker employed for covalent antibody coupling [31]. We thus tested two elution buffers that are compatible with downstream MS analyses. Here, we found that 0.1% FA mediated superior elution compared to 50 mM glycine pH 2.8 (Fig. 1A), which is recommended by the bead manufacturer. 0.1% FA was thus chosen for further quantification of OPN. An additional advantage of employing 0.1% FA is that it constitutes buffer A in the LC step, thus omitting a desalting step prior to LC-MS/MS analysis.



The results from the dot-blot analyses (Fig. 1A) were corroborated by LC-MRM-MS analysis of eluates employing either of the two elution buffers, since 0.1% FA resulted in markedly higher peak intensities (Fig. 1C) compared to 50 mM glycine pH 2.8 (Fig. 1B). The LC-MRM-MS

Fig. 1. Final levels of OPN-peptide-1 and -2 obtained after immunoaffinity capture were dependent on the elution buffer employed as determined by dot-blot (A) and MS analysis (B, C). Very low levels of both peptides were retrieved when employing 50 mM glycine pH 2.8 as elution buffer (A, upper row, B). Considerably increased levels of both peptides were retrieved by employing 0.1% FA as elution buffer (A, lower row, C). A significant signal loss for both analyzed OPN peptides was observed for the sample subjected to detergent removal prior to IP (D) comparing to the immunoprecipitated only samples. Red and blue chromatograms represent OPN-peptide-1 and -2, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

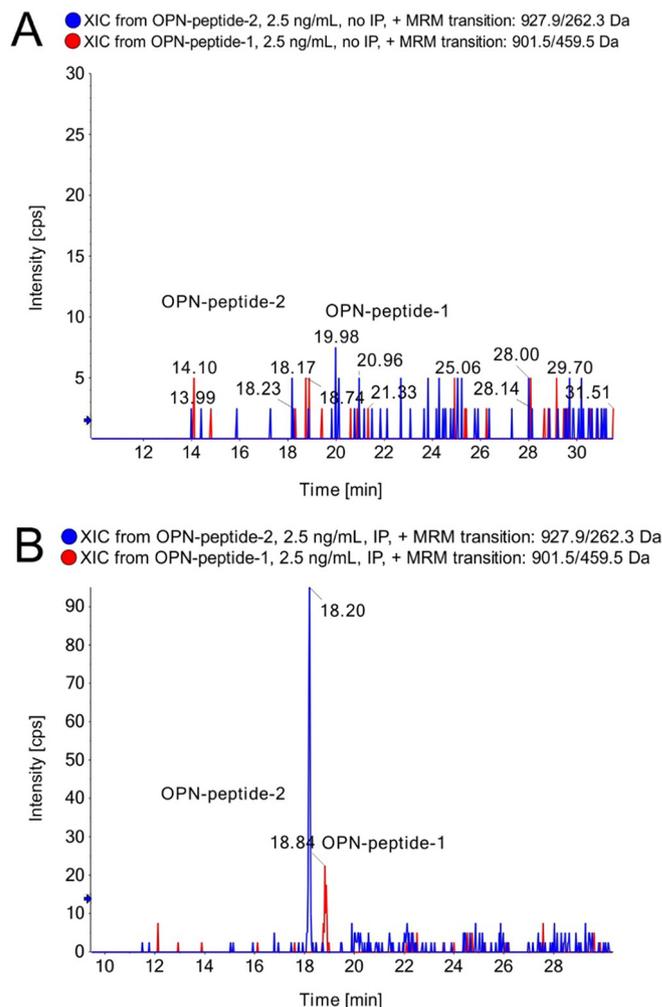


Fig. 2. MRM chromatogram of the 2.5 ng/mL OPN standard before immunoprecipitation (A) and after immunoprecipitation (B).

analysis further demonstrated that detergent removal prior to immunoprecipitation mediated marked reduction in peak intensities (Fig. 1D). This step was thus omitted from further analyses, since the acetone precipitation step would nevertheless ensure removal of detergents prior to MS analysis.

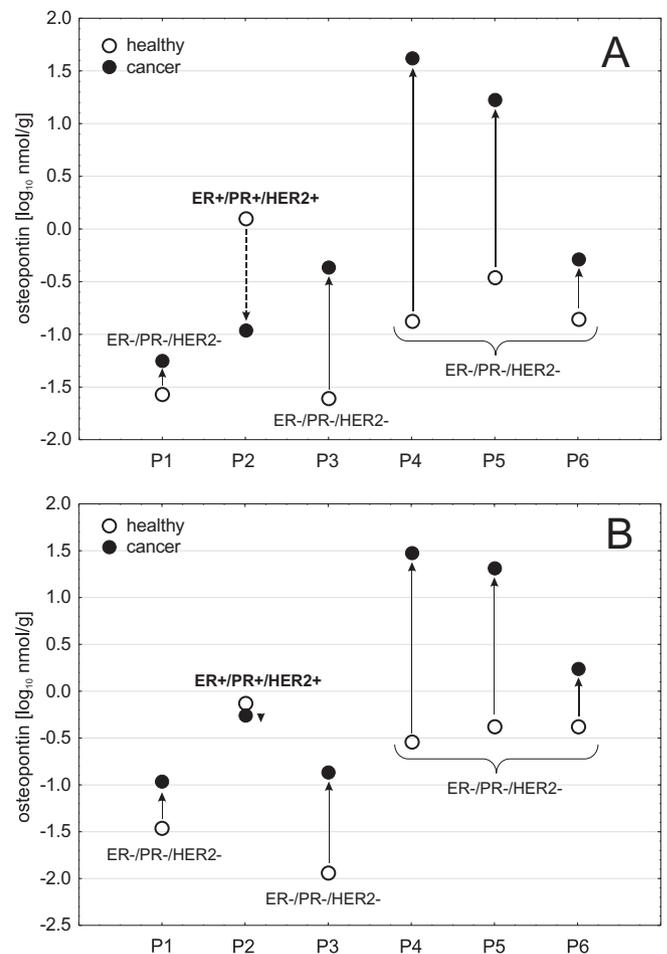
Immunoprecipitation of OPN-peptide-1 and OPN-peptide-2 enabled their robust detection (S/N is 15.3 and 36 for OPN-peptide-1 and OPN-peptide-2, respectively) from the trypsin-digested OPN standard at a concentration of 2.5 ng/mL (Fig. 2). Thus, the procedure enables the OPN quantification between 2.5 ng/mL to 5000 ng/mL. This sensitivity was regarded sufficient to proceed with quantitative analysis in patient breast tissue material. Thus, in the protein extracts from breast tissue and tumor patients' samples, the OPN peptides were enriched by affinity capture, using OPN-peptides specific polyclonal antibodies coupled

to Dynabeads Protein A (magnetic bead-Ab conjugates). This ensured specific analytes separation from biological matrix. The peptides elution from the magnetic bead-Ab conjugates was then carried out using 0.1% FA (used as a mobile phase A). Prepared this way samples were submitted to LC-MRM-MS analysis.

### 3.3. Osteopontin quantitation in breast tumor and adjacent normal breast tissue samples

The finalized workflow was tested by quantifying OPN in breast tumors and adjacent normal breast tissues from six breast cancer patients (Supplementary material 1: Table 1S). In these experiments, the endogenous OPN peptides and their SIS standards co-eluted from the chromatographic column, confirming their structural identity (Supplementary material 1: Fig. 2S–5S). The analyses revealed considerable variation in the OPN concentrations across both the healthy and malignant breast tissue samples. Based on OPN-peptide-1, the values ranged from 1.50  $\mu\text{g/g}$  to 75.80  $\mu\text{g/g}$  in healthy tissue (average 19.42  $\mu\text{g/g}$ , median 8.25  $\mu\text{g/g}$ ), whereas the corresponding values ranged from 0.70  $\mu\text{g/g}$  to 44.47  $\mu\text{g/g}$  (average 19.30  $\mu\text{g/g}$ , median 21.47  $\mu\text{g/g}$ ) based on OPN-peptide 2. OPN levels in the breast tumors ranged from 3.42  $\mu\text{g/g}$  to 2540  $\mu\text{g/g}$  (average 603.9  $\mu\text{g/g}$ , median 28.63  $\mu\text{g/g}$ ), and from 6.56  $\mu\text{g/g}$  to 1799  $\mu\text{g/g}$ , (average 534.98  $\mu\text{g/g}$ , median 69.96  $\mu\text{g/g}$ ) based on OPN-peptide-1 and OPN-peptide-2, respectively (Supplementary material 1: Table 1S and Table 2S). Pearson correlation analysis demonstrated high correlation ( $r = 0.969$ ) between average OPN concentrations determined based on OPN-peptide-1 and OPN-peptide-2 (Supplementary material 1: Fig. 6S). This confirms similarity in the OPN levels when calculation is performed on the basis of both OPN-peptide-1 and OPN-peptide-2.

For both OPN-peptide-1 and -2, the concentrations were higher in the malignant tissues in five out of the six patients (Fig. 3A and B). Those significant differences in the OPN concentrations were identified in the five ER/PR/HER2 negative (triple-negative) patients (paired  $t$ -test, 2-tailed;  $p = .032$ ,  $t = 3.217$ ,  $df = 4$  and  $p = .0161$ ,  $t = 4.000$ ,  $df = 4$  for OPN-peptide-1 and OPN-peptide-2, respectively). These results are consistent with findings reported by Wang et al. [36], who showed by means of immunohistochemistry that OPN expression in triple-negative breast tumors was significantly higher than in non-triple-negative subtypes. In ER/PR/HER2 positive breast cancer subtype expression of estrogen (ER), progesterone (PR) and human epidermal growth factor receptor 2 (HER2) is observed, which makes it sensitive to hormonal and neoadjuvant therapy. The ER/PR/HER2 positive breast cancer is also characterized by low histological grade and is less metastatic. In contrary to that, triple-negative breast tumors do not express ER, PR and HER2 receptors, being not responsive to known hormonal drugs and targeted therapy. Due to this fact, in the treatment of the ER/PR/HER2 negative breast cancer only severe chemotherapy is applied, causing many side effects. Additionally, triple-negative breast cancer has more aggressive histology than other breast cancer subtypes and is associated with poor prognosis and shorter survival. Therefore, it remains a difficult clinical challenge [37]. Several genome and proteome-wide studies have already shown that triple-negative breast cancer is very heterogeneous and not only varies among other breast cancer subtypes but also considering different cancers (reviewed by Miah et al. [38]). Thus, better understanding of its complex nature is crucial for development of more sophisticated diagnostic methods and effective treatment options. The study of Wang et al. [36] and the present study indicate that OPN overexpression is related with ER/PR/HER2 negative breast cancer. In contrary to triple-negative breast cancer patients investigated in our study, in Patient 2, who was an ER/PR/HER2 positive, both OPN-peptide-1 and OPN-peptide-2 concentrations were lower malignant tissue than in normal tissue. Thus, there were no significant differences between the concentrations in the two tissue types when considering all six patients. However, more research on larger number of clinical samples is needed to confirm if there



**Fig. 3.** The log<sub>10</sub> average OPN concentration values calculated based on OPN-peptide-1 (A) and OPN-peptide-2 (B) [nmol/g] for each pair of breast tumor (closed circle) and adjacent healthy breast tissue (open circle) samples obtained for the same person (P1–P6) in the analyzed patients cohort. The arrows indicate the direction of change in OPN concentrations.

are differences in OPN concentrations in breast tumors cells of ER/PR/HER2 positive patients as compared to ER/PR/HER2 negative patients and what is a molecular background of this feature.

Our analyses revealed a high interindividual variability in the OPN concentrations both in the malignant and adjacent healthy breast tissues. This is corroborated by previous studies employing ELISA-based quantification [18,20,21]. In the study of Bramwell et al. [21] plasma OPN levels measured in metastatic breast cancer patients ranged from 1 to 2648 ng/mL. In another study, Bramwell et al. [18] investigated OPN levels in postmenopausal women with hormone responsive early breast cancer treated by surgery followed by adjuvant treatment with tamoxifen +/- octreotide. In that particular study, mean baseline OPN level in plasma was 46 ng/mL (range 22.6 to 290), while mean plasma OPN levels measured in healthy individuals was 28.4 ng/mL (range 11.8 to 109). Surprisingly, Bramwell et al. [18] also found that in patients with recurrence disease, mean OPN plasma levels were higher (60.7 ng/mL) and the range of OPN detected was wider (23.9 to 543) in the recurrence period compared with baseline levels. Singhal et al. [20] reported OPN concentrations in the series of plasma samples obtained from healthy individuals and patients in the initial breast cancer state or with adjuvant therapy did not vary significantly. In their study median OPN concentrations in healthy individuals and patients in the initial breast cancer state or with adjuvant therapy were 60 ng/mL (range 15–117 ng/mL) and 47 ng/mL (range 22–122 ng/mL), respectively [20]. However, in the persons with metastatic breast cancer, the

median plasma OPN levels were higher, 142 ng/mL (range 38–1312 ng/mL), indicating that OPN concentration increase with the number of involved sites and decrease in survival [20]. Together with our study, the above mentioned studies showed that OPN concentrations occurred in wide ranges. Additionally, those ranges varied depending on the characteristics of the studied group of patients, such as breast cancer stage or applied treatment. It can also be noticed that the OPN concentrations determined in breast tissue and tumor samples included in our study were higher than in plasma samples previously reported by other groups [18,20,21]. Among the reasons for such broad range of detected OPN levels in our studies may be extreme heterogeneity of the cancer tissues, where subpopulations of various cancer cells may be located close to each other and resulting in very different phenotype, even though the same TNM stage would be determined for the tissues. Moreover, it is also possible that variability of the OPN estimations based on the two different peptides, both within and between the samples, is a result of post-translational modifications present on the OPN or a cleavage of a C-terminus, eg. in case of the differences in OPN-determinations based on OPN-peptide-1 vs OPN-peptide-2 within the P2, P4 and P6 samples.

To the best of our knowledge, the absolute OPN protein concentrations in human mammary gland tissues have previously not been investigated. Rather, OPN expression levels have been estimated based on mRNA levels or semiquantitatively using immunohistochemical methods with anti-OPN antibodies [16,18,39]. There is also no available literature data on absolute OPN protein levels in breast tumors. Our data thus constitutes the first data on the absolute OPN protein levels in both malignant and healthy human breast tissues.

#### 4. Conclusions

We have developed an immuno-SRM method for targeted OPN quantification. The analytical workflow includes enrichment of peptides by immunoprecipitation with anti-peptide antibodies and LC-MRM-MS analysis with SIS standards. The procedure enables the OPN quantification between 2.5 ng/mL to 5000 ng/mL. The method was applied for OPN quantification in a pilot study encompassing cancerous and adjacent normal human breast tissues from six patients. Our data shows that in patients harboring ER/PR/HER2 negative tumors, the tumor OPN levels were markedly higher than in the corresponding healthy breast tissues. Conversely, in the single ER/PR/HER2 positive patient in our cohort, markedly reduced OPN levels (6.61/33.44 µg/g vs 75.80/44.47 µg/g) were found. Whether this inverse correlation between OPN expression and ER/PR/HER2(+) tumor status represents a general trend will, however, have to await studies in a larger patient cohort. The results of our study as well as those of other previous studies show that OPN concentration ranges overlap in normal and cancerous breast tissues. This indicates that it is difficult to identify with certainty the level of OPN that distinguishes breast tumor tissue from normal breast tissue. As indicated in the present study and previous studies, this is due to that an up- or down-regulation of OPN expression depends on the tumor type and its stage. Another important confounding factor is the complex nature of both breast tumors and OPN itself because of its post-translational modifications, which could introduce bias in the measurements if detection methods are based on the non-phosphorylated variant detection. The developed immuno-SRM method enables selective determination of unmodified OPN peptides in breast tissue and tumor specimens. To what degree this represents the overall level of OPN or specific isoforms, will have to be determined by including additional peptides harboring e.g. phosphorylations, or extending over isoform-specific sequences. Nevertheless, the results from the present study indicate that the differences in OPN expression levels between breast tumor tissue and adjacent normal breast tissue samples may serve as a marker for particular breast cancer types or stages. However, this needs to be verified in studies with higher numbers of clinical samples.

In conclusion, the current method is considered promising for the absolute quantification of OPN in research as well as in clinical settings, but it needs to be validated by additional methods, such as western blot analysis. Immuno-SRM assays have the potential for high sensitivity, ease of automation, ability to be highly multiplexed, and allows high sample throughput. The present work further establishes that immuno-SRM is a flexible alternative to sandwich and ELISA immunoassays, with the potential to triage large lists of candidate proteins or their post-translationally modified variants and thereby help to alleviate the significant bottleneck that exists between unbiased discovery and clinical validation.

#### Declaration of Competing Interest

The authors declare no competing interests.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jprot.2019.103469>.

#### References

- [1] G.S. Wong, A.K. Rustgi, Matricellular proteins, priming the tumour microenvironment for cancer development and metastasis, *Br. J. Cancer* 108 (4) (2013) 755–761.
- [2] L.A. Shevde, S. Das, D.W. Clark, R.S. Samant, Osteopontin: an effector and an effect of tumor metastasis, *Curr. Mol. Med.* 10 (2010) 71–81.
- [3] M.K. El-Tanani, F.C. Campbell, V. Kurisetty, D. Jin, M. McCamm, P.S. Rudland, The regulation and role of osteopontin in malignant transformation and cancer, *Cytokine Growth Factor Rev.* 17 (2006) 463–474.
- [4] L.A. Torre, R.L. Siegel, E.M. Ward, A. Jemal, Global cancer incidence and mortality rates and trends – an update, *Cancer Epidemiol. Biomark. Prev.* 25 (1) (2016) 16–27.
- [5] WHO, Health in 2015: From MDGs, Millennium Development Goals, to SDGs, Sustainable Development Goals, Chapter 6 WHO Press, 2015, pp. 142–143 ISBN 978 92 4 156511 0.
- [6] H. Zhang, M. Guo, J. Chen, Z. Wang, X. Du, P. Liu, W. Li, Osteopontin knockdown inhibits  $\alpha V\beta 3$  integrin-induced cell migration and invasion and promotes apoptosis of breast cancer cells by inducing autophagy and inactivating the PI3/Akt/mTOR pathway, *Cell. Physiol. Biochem.* 33 (2014) 991–1002.
- [7] G. Chakraborty, S. Jain, G.C. Kundu, Osteopontin promotes vascular endothelial growth factor-dependent breast tumor growth and angiogenesis, *Cancer Res.* 68 (1) (2008) 152–161.
- [8] R. Behera, V. Kumar, K. Lohite, S. Karnik, G.C. Kundu, Activation of JAK2/STAT3 signaling by osteopontin promotes tumor growth in human breast cancer cells, *Carcinogenesis* 31 (2) (2010) 192–200.
- [9] K. Viloria, N.J. Hill, Embracing the complexity of matricellular proteins: the functional and clinical significance of splice variation, *BioMol. Concepts* 7 (2) (2016) 117–132.
- [10] J.E. Murphy-Ullrich, E.H. Sage, Revising the matricellular concept, *Matrix Biol.* 37 (2014) 1–14.
- [11] B. Christensen, M.S. Nielsen, K.F. Haselmann, T.E. Petersen, E.S. Sørensen, Post-translationally modified residues of native human osteopontin are located in clusters: identification of 36 phosphorylation and five O-glycosylation sites and their biological implications, *Biochem. J.* 390 (2005) 285–292.
- [12] E.S. Sørensen, P. Højrup, T.E. Petersen, Posttranslational modifications of bovine osteopontin: identification of twenty-eight phosphorylation and three O-glycosylation sites, *Protein Sci.* 4 (1995) 2040–2049.

- [13] B. Christensen, C.C. Kazanekci, T.E. Petersen, S.R. Rittling, D.T. Denhardt, E.S. Sørensen, Cell type-specific post-translational modifications of mouse osteopontin are associated with different adhesive properties, *J. Biol. Chem.* 282 (27) (2007) 19463–19472.
- [14] P.S. Rudland, A. Platt-Higgins, M. El-Tanani, S. de Silva Rudland, R. Barraclough, J.H.R. Winstanley, R. Howitt, C.R. West, Prognostic significance of the metastasis-associated protein osteopontin in human breast cancer, *Cancer Res.* 62 (2002) 3417–3427.
- [15] A.B. Tuck, F.P. O'Malley, H. Singhal, J.F. Harris, K.S. Tonkin, N. Kerkvliet, Z. Saad, G.S. Doig, A.F. Chambers, Osteopontin expression in a group of lymph node negative breast cancer patients, *Int. J. Cancer (Pred. Oncol.)* 79 (1998) 502–508.
- [16] J. Wang-Rodriguez, V. Urquidi, A. Rivard, S. Goodison, Elevated osteopontin and thrombospondin expression identifies malignant human breast carcinoma but is not indicative of metastatic status, *Breast Cancer Res.* 5 (5) (2003) R136–R143.
- [17] P. Winczura, K. Sosińska-Mielcarek, R. Duchnowska, A. Badzio, J. Lakomy, H. Majewska, R. Pęksa, B. Pieczyńska, B. Radecka, S. Dębska-Szmich, K. Adamowicz, W. Biernat, J. Jassem, Immunohistochemical predictors of bone metastases in breast cancer patients, *Pathol. Oncol. Res.* 21 (2015) 1229–1236.
- [18] V.H.C. Bramwell, A.B. Tuck, J.-A.W. Chapman, P.H. Anborgh, C.O. Postenka, W. Al-Katib, L.E. Shepherd, L. Han, C.F. Wilson, K.I. Pritchard, M.N. Pollak, A.F. Chambers, Assessment of osteopontin in early breast cancer: correlative study in a randomised clinical trial, *Breast Cancer Res.* 16 (2014) R8.
- [19] M. Ihnen, R.M. Wirtz, K.T. Kalogeras, K. Milde-Langosh, M. Schmidt, I. Witzel, A.G. Eleftheraki, C. Papadimitriou, F. Jänicke, E. Briassoulis, D. Pectasides, G. Fountzilas, V. Müller, Combination of osteopontin and activated leukocyte cell adhesion molecule as a potent prognostic discriminators in HER2- and ER-negative breast cancer, *Br. J. Cancer* 103 (2010) 1048–1056.
- [20] H. Singhal, D.S. Bautista, K.S. Tonkin, F.P. O'Malley, A.B. Tuck, A.F. Chambers, J.F. Harris, Elevated plasma osteopontin in metastatic breast cancer associated with increased tumor burden and decreased survival, *Clin. Cancer Res.* 3 (1997) 607–611.
- [21] V.H.C. Bramwell, G.S. Doig, A.B. Tuck, S.M. Wilson, K.S. Tonkin, A. Tomiak, F. Perera, T.A. Vandenberg, A.F. Chambers, Serial plasma osteopontin levels have prognostic value in metastatic breast cancer, *Clin. Cancer Res.* 12 (2006) 3337–3343.
- [22] P.H. Anborgh, L.B.R. Caria, A.F. Chambers, A.B. Tuck, L.W. Stitt, M. Brackstone, Role of plasma osteopontin as a biomarker in locally advanced breast cancer, *Am. J. Transl. Res.* 7 (4) (2015) 723–732.
- [23] D.S. Kirkpatrick, S.A. Gerber, S.P. Gygi, The absolute quantification strategy: a general procedure for the quantification of proteins and post-translational modifications, *Methods* 35 (2005) 265–273.
- [24] R. Hüttenhain, J. Malmström, P. Picotti, R. Aebersold, Perspectives of targeted mass spectrometry for protein biomarker verification, *Curr. Opin. Chem. Biol.* 13 (2009) 518–525.
- [25] L. Anderson, C.L. Hunter, Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins, *Mol. Cell. Proteomics* 5 (2006) 573–588.
- [26] L. Callipo, G. Caruso, P. Foglia, R. Gubbiotti, R. Samperi, A. Lagana, Immunoprecipitation on magnetic beads and liquid chromatography-tandem mass spectrometry for carbonic anhydrase II quantification in human serum, *Anal. Biochem.* 400 (2010) 195–202.
- [27] J.R. Whiteaker, L. Zhao, L. Anderson, A.G. Paulovich, An automated and multiplexed method for high throughput peptide immunoaffinity enrichment and multiple reaction monitoring mass spectrometry-based quantification of protein biomarkers, *Mol. Cell. Proteomics* 9 (2009) 184–196.
- [28] J. Whiteaker, A.G. Paulovich, Peptide immunoaffinity enrichment coupled with mass spectrometry for peptide and protein quantification, *Clin. Lab. Med.* 3 (3) (2011) 385–396.
- [29] L.H. Sobin, M. Gospodarowicz, C. Whitekind, International Union Against Cancer. TNM Classification of Malignant Tumors, Wiley-Blackwell-Hoboken, New York, 2009.
- [30] Immunoprecipitation with Dynabeads® Protein A, available at: <https://www.thermofisher.com/pl/en/home/references/protocols/proteins-expression-isolation-and-analysis/protein-purification-protocol/immunoprecipitation-with-dynabeads-protein-a.html>.
- [31] M.M.L. Sousa, K.W. Steen, L. Hagen, G. Slupphaug, Antibody cross-linking and target elution protocols used for immunoprecipitation significantly modulate signal-to-noise ratio in downstream 2D-PAGE analysis, *Proteome Sci.* 9 (2011) 45.
- [32] <http://prospector.ucsf.edu/prospector/mshome.htm>.
- [33] R.J. Chalkley, K.C. Hansen, M.A. Baldwin, Bioinformatic methods to exploit mass spectrometric data for proteomic applications, *Methods Enzymol.* 402 (2005) 289–312.
- [34] <http://www.matrixscience.com>.
- [35] X. Duan, R. Young, R.M. Straubinger, B. Page, J. Cao, H. Wang, H. Yu, J.M. Canty Jr., J. Qu, A straightforward and highly efficient precipitation/on-pellet digestion procedure coupled with a long gradient nano-LC separation and orbitrap mass spectrometry for label-free expression profiling of the swine heart mitochondrial proteome, *J. Proteome Res.* 8 (2009) 2838–2850.
- [36] X. Wang, L. Chao, G. Ma, L. Chen, B. Tian, Y. Zang, J. Sun, Increased expression of osteopontin in patients with triple-negative breast cancer, *Eur. J. Clin. Invest.* 38 (6) (2008) 438–446.
- [37] S.A. O'Toole, J.M. Beith, E.K.A. Millar, R. West, A. McLean, A. Cazet, A. Swarbrick, S.R. Oakes, Therapeutic targets in triple negative breast cancer, *J. Clin. Pathol.* 66 (6) (2013) 530–542.
- [38] S. Miah, C.A.S. Banks, M.K. Adams, L. Florens, K.E. Lukong, M.P. Washburn, Advancement of mass spectrometry-based proteomics technologies to explore triple negative breast cancer, *Mol. Biosyst.* 13 (2017) 42–55.
- [39] N. Patani, F. Jouhra, W. Jiang, K. Mokbel, Osteopontin expression profiles predict pathological and clinical outcome in breast cancer, *Anticancer Res.* 23 (2008) 4105–4110.