

# Circulating levels of osteopontin are closely related to glomerular filtration rate and cardiovascular risk markers in patients with chronic kidney disease

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## ABSTRACT

**Background** The pleiotropic cytokine osteopontin (OPN) is thought to be involved in the pathogenesis of atherosclerosis. However, the relationship between OPN and renal function, a cardiovascular risk factor itself, is not known. Therefore, we assessed the relationship between OPN plasma levels and renal function in patients at different stages of chronic kidney disease (CKD).

**Methods** We studied 49 non-diabetic and non-smoking patients with primary kidney disease at different CKD stages (K/DOQI 1-5). True glomerular filtration rate (GFR) in patients was assessed using the inulin-clearance technique. To examine the role of an abrupt change in GFR on circulating OPN, 15 living related kidney donors were studied before and after unilateral nephrectomy. Twenty matched non-smoking healthy subjects served as controls.

**Results** OPN plasma levels in patients with CKD stage 1 (i.e. GFR above  $90 \text{ mL min}^{-1} 1.73 \text{ m}^{-2}$ ) were comparable with controls. OPN levels increase in a linear fashion with declining GFR ( $r = -0.9$ ,  $P < 0.0001$ ), so that the increase in OPN mirrors the severity of renal impairment. After unilateral nephrectomy, circulating OPN increased significantly in parallel to the decrease in GFR. We found a direct association between OPN and other markers of renal function (serum-creatinine, homocysteine and symmetric dimethylarginine,) as well as with cardiovascular risk factors such as asymmetric dimethylarginine ( $r = 0.36$ ,  $P = 0.0213$ ).

**Conclusion** There is a close inverse association between GFR and circulating OPN in patients with CKD. Furthermore, OPN plasma levels correlate with established cardiovascular risk markers in patients with CKD. Assessment of renal function is important for the interpretation of OPN levels in patients with atherosclerotic disease.

**Keywords** ADMA, atherosclerosis, osteopontin, renal function, SDMA, vascular calcification.

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## Introduction

Chronic renal disease, even with minor impairment of renal function, has only recently been recognized as a cardiovascular risk factor of epidemic proportions as about 17% of the US population suffer from some degree of chronic kidney disease (CKD) [1]. These patients are more likely to die of cardiovascular disease before they develop overt kidney failure. Therefore the American Heart Association views CKD as

a cardiovascular risk factor [2]. Unfortunately, many new cardiovascular risk factors have not been evaluated concerning their potential association with renal function, among them osteopontin (OPN). OPN, also known as bone sialoprotein, is a glycoprotein that has been first described in osteoblasts and is an organic component of bone [3–5]. OPN interacts with a variety of cell surface receptors [6–8] and is involved in the recruitment and retention of macrophages and T cells to sites of inflammation.

<sup>1</sup>These authors contributed equally to the study.

Osteopontin has been implicated as a key factor in the development of atherosclerosis [9–14] and is emerging as an important inducible inhibitor of vascular calcification [15]. Plasma levels of OPN are elevated in essential hypertension, in patients with coronary artery disease, and restenosis [12–14]. Despite its emerging role in cardiovascular disease, it is unclear if renal function *per se* is influencing OPN levels. Therefore, the aim of our study was to investigate OPN levels in relation to glomerular filtration rate (GFR), measured by inulin clearance in patients with CKD. Furthermore, we aimed to investigate the effect of abrupt reduction in GFR, i.e. living kidney donation on circulating OPN. We also aimed to investigate the potential relationship between plasma OPN levels with other cardiovascular risk markers, e.g. asymmetric dimethylarginine (ADMA).

## Patients and methods

### Participants and protocol

The study was approved by the local ethics committee. Informed consent was obtained from all participating individuals. To exclude patients with active vasculitis and diabetes, which have known elevations of OPN, we focused on patients with biopsy-proven IgA glomerulonephritis (IgA GN,  $n = 26$ ), and adult polycystic kidney disease (ADPKD,  $n = 23$ ) confirmed by family history and ultrasound examination. None of the patients showed clinical signs of heart failure. Patients with known diabetes mellitus of any type, liver disease, malignancy, and a history of alcohol abuse were excluded from the study. Patients examined were at different stages of CKD according to the K/DOQI guidelines (stages 1–5), but they had a stable renal function for at least 6 months before the study. Until enrolment none of them had been treated with vitamin B12, folate, antioxidant vitamins, vitamin D, erythropoietin, fish oil or immunosuppressive agents, and none of them had a low-protein diet. Higher grade proteinuria (i.e. more than  $1 \text{ g day}^{-1}$ ) was present in 3 of 15 patients with moderate renal failure (CKD stage 2 and 3) and in 5 of 18 patients with advanced renal failure (CKD stage 4 and 5). Hypertension according to World Health Organization criteria, that is mean arterial blood pressure greater than 105 mmHg, antihypertensive therapy, or both, was present in 7 of 16 patients with normal renal function, in 10 of 15 patients with moderate renal failure and in 14 of 18 patients with advanced renal failure. Antihypertensive drugs, if present, were washed out for time periods depending on their half-life of action. In all patients we assessed true GFR in a supine position by means of the steady-state inulin ( $C_{in}$ ) infusion clearance technique, as described in detail elsewhere [16].

Secondly, we quantified OPN levels in 15 healthy individuals before, 24 and 72 h after kidney donation in whom GFR was estimated by cystatin C clearance. For comparison, 20

normotensive, non-smoking, age-, gender- and body mass index-matched healthy volunteers from the Department of Nephrology, Hanover Medical School, between the ages of 20 and 60 years were included.

Blood samples for measurement of routine chemistry and ADMA, symmetric dimethylarginine (SDMA) and total homocysteine (Hcy) concentrations were taken in the morning after at least 12 h of fasting. In addition, mean arterial blood pressure was measured oscillometrically in supine position and quiet environment with an automated device (Dinamap, Critikon Co., Tampa, FL, USA). The mean of three consecutive measurements 5 min apart after a rest of at least 20 min was taken for analysis.

### Biochemical analyses

For the determination of plasma dimethylarginine levels, we used the high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS-MS) method published recently by our group [17]. The intra-day precision was 4.6%, and the inter-day precision was 3.3%.

Plasma inulin concentration was measured enzymatically by use of inulinase as described by Kuehnle *et al.* [18] and inulin clearance was calculated as described elsewhere [16]. Intact parathyroid hormone was measured with an immunoradiometric assay (normal range,  $1.2\text{--}6.0 \text{ pmol L}^{-1}$ ) and total plasma Hcy with a fluorescence-polarization immunoassay (normal range,  $5.0\text{--}15.0 \text{ } \mu\text{mol L}^{-1}$ ). Osteopontin plasma levels were measured using a commercially available ELISA (R&D Systems, Minneapolis, MN, USA). The detection limit was  $0.011 \text{ ng mL}^{-1}$ , and the intra- and inter-assay coefficients of variance were 2.6 and 5.4% respectively. Measurements were run in duplicate. All other measurements were performed with routine laboratory tests that used certified assay methods.

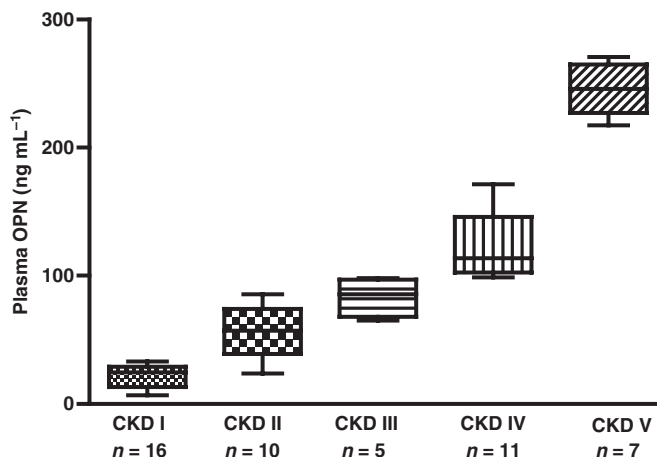
### Statistical analyses

Differences in OPN levels between patients and matched healthy controls were evaluated using Mann–Whitney *U*-test (two sided). Correlations between OPN and parameters of renal function and cardiovascular burden (GFR, ADMA, SDMA and Hcy) were calculated with Spearman's test and linear regression analysis. Correlations between OPN and age, mean arterial BP, serum cholesterol, serum triglyceride, PTH, Calcium, Phosphate levels and calcium-phosphate-product were analysed as well. Friedman's test was used to show that OPN levels in patients at different stages of renal dysfunction were statistically significant. Statistical significance was accepted at  $< 5\%$  probability levels. Data are displayed as mean  $\pm$  SD. Data analysis was performed using SPSS (SPSS Inc., Chicago, IL, USA) and GraphPad Prism software (GraphPad Prism Software Inc., San Diego, CA, USA).

## Results

Clinical data of CKD patients are shown in Table 1. Plasma OPN concentrations in patients with CKD stage 1 were comparable with control subjects (IgA:  $20.4 \pm 9.0$  ng mL<sup>-1</sup>; ADPKD:  $23.8 \pm 8.7$  ng mL<sup>-1</sup>; healthy controls:  $23.8 \pm 3.7$  ng mL<sup>-1</sup>). There was a stepwise increase in circulating OPN in parallel with declining renal function (Fig. 1), and differences in OPN levels in patients with CKD at different stages of renal dysfunction were highly significant ( $P = 0.0007$ ). Consequently, OPN plasma concentrations correlated negatively with GFR ( $r = -0.9$ ,  $P < 0.0001$ ), and positively with Hcy ( $r = 0.76$ ,  $P < 0.0001$ ), SDMA levels ( $r = 0.6$ ,  $P < 0.0001$ ) and serum-creatinine ( $r = 0.9$ ,  $P < 0.0001$ ) (Fig. 2). OPN plasma levels correlated closely with ADMA ( $r = 0.36$ ,  $P = 0.0213$ ) levels. There was almost no overlap in plasma OPN concentrations between different stages of renal dysfunction.

OPN plasma levels correlated closely with calcium-phosphate-product ( $r = 0.66$ ,  $P < 0.0001$ ), serum-phosphate ( $r = 0.7$ ,  $P < 0.0001$ ) and intact parathyroid hormone ( $r = 0.82$ ,  $P < 0.0001$ ). We did not find an association between plasma OPN levels and age ( $r = 0.147$ ,  $P = 0.36$ ), serum cholesterol ( $r = -0.046$ ,  $P = 0.77$ ) and triglyceride levels ( $r = 0.27$ ,  $P = 0.0825$ ), and body mass index ( $r = -0.054$ ,  $P = 0.73$ ).



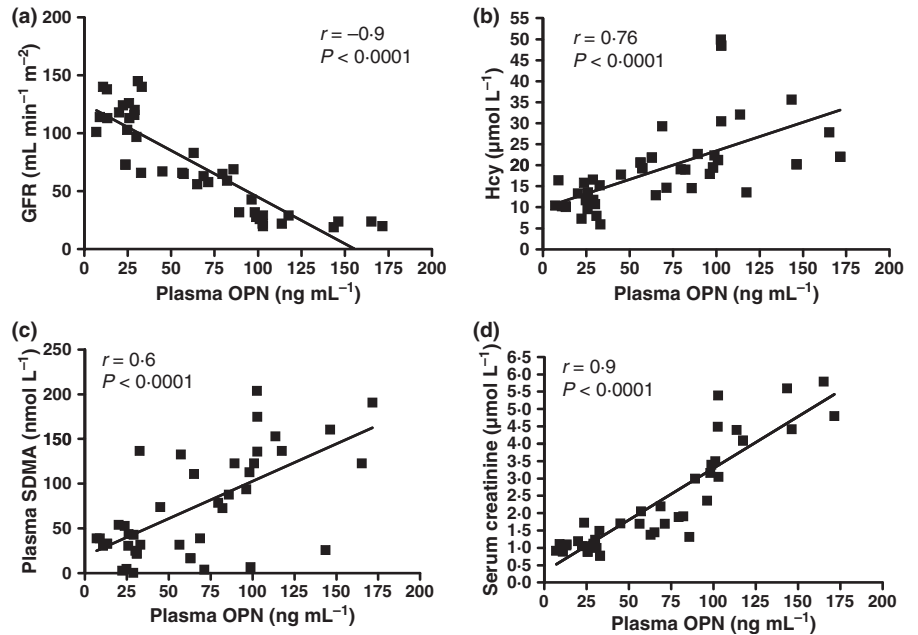
**Figure 1** Osteopontin (OPN) plasma levels increase in a linear fashion with declining renal function in patients with primary chronic kidney disease. Differences are highly significant as assessed by Friedman's test ( $P = 0.0007$ ).

Furthermore, plasma OPN levels were not associated with mean arterial blood pressure ( $r = 0.28$ ,  $P = 0.0773$ ). The separate descriptive analysis of patients with immune origin of renal disease (IgA GN) and patients with non-immune renal disease

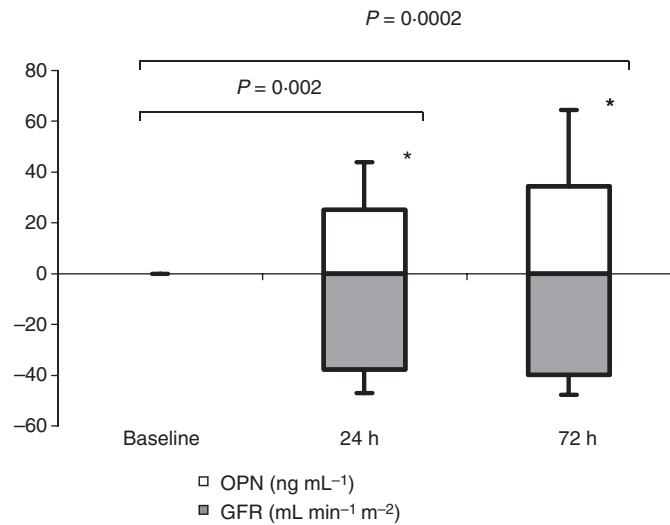
**Table 1** Cardiovascular risk factors in 26 patients with IgA glomerulonephritis (IgA GN), 23 patients with adult polycystic kidney disease (ADPKD)

K/DOQI	I	II	III	IV	V
IgA GN (n)	10	3	3	7	3
Age (years)	44.8 ± 8.7	49 ± 6.1	50 ± 8.7	43.3 ± 8.8	45.4 ± 5.5
BMI (kg m <sup>-2</sup> )	26.2 ± 4.1	26.6 ± 0.8	24.3 ± 2.2	26.4 ± 4.3	25.6 ± 4.4
MAP (mmHg)	104 ± 12	109 ± 10	114 ± 3	107 ± 9	120 ± 6
Hcy (μmol L <sup>-1</sup> )	10.4 ± 2.4	17.7 ± 2.7	17.6 ± 4.3	23.7 ± 7.2	–
Chol. (mg dL <sup>-1</sup> )	210 ± 27	212 ± 25	200 ± 13	204 ± 39	201 ± 10
OPN (ng mL <sup>-1</sup> )	20.4 ± 9.0	53.4 ± 28.1	86.2 ± 13.7	125.9 ± 28.1	242 ± 7.1
ADPKD (n)	6	7	2	4	4
Age (years)	35.3 ± 6.3	46.6 ± 9.2	42.3 ± 6.4	49.3 ± 10.3	45.4 ± 4.6
BMI (kg m <sup>-2</sup> )	26.8 ± 4.1	26.4 ± 2.4	27.2 ± 0.8	26.4 ± 3.8	25.6 ± 5.4
MAP (mmHg)	104 ± 4	108 ± 12	106 ± 8	118 ± 2	120 ± 4
Hcy (μmol L <sup>-1</sup> )	11.9 ± 3.8	19.3 ± 5.3	16.6 ± 3.3	39.6 ± 11.3	–
Chol. (mg dL <sup>-1</sup> )	171 ± 40	202 ± 25	186 ± 36	174 ± 8	180 ± 10
OPN (ng mL <sup>-1</sup> )	23.8 ± 8.7	58.5 ± 18.6	81.1 ± 15.6	121 ± 29.9	250.5 ± 9

BMI, body mass index; MAP, mean arterial blood pressure; Hcy, total plasma homocysteine concentration; Chol., total serum cholesterol concentration; OPN, plasma osteopontin concentration.



**Figure 2** OPN plasma levels correlate significantly with glomerular filtration rate (a), homocysteine (b), symmetric dimethylarginine (SDMA) (c) and serum creatinine (d) (all  $P < 0.0001$ ).



**Figure 3** Delta (difference from baseline in %) of OPN levels in relation to delta in glomerular filtration rate in patients before, 24 and 72 h after living kidney donation.  $*P < 0.05$ .

(ADPKD) showed no differences between both groups with respect to plasma OPN levels (Table 1).

Figure 3 displays differences in OPN levels in relation to GFR in individuals before, 24 and 72 h after kidney donation. OPN levels 24 and 72 h after kidney donation increased by 46% and 56% respectively. OPN levels 24 h ( $P = 0.002$ ) and 72 h ( $P = 0.0002$ ) after kidney donation were significantly different compared with baseline levels. Table 2 shows clinical

**Table 2** Clinical data of individuals before living kidney donation and healthy controls

	Living donors	Healthy controls
<i>n</i>	15	20
Age (years)	53.2 ± 8.9	45.8 ± 4.4
BMI (kg m <sup>-2</sup> )	27.8 ± 3.6	22.4 ± 4.4
MAP (mmHg)	95 ± 8	94 ± 4
Chol. (mg dL <sup>-1</sup> )	208 ± 34	–
OPN (ng mL <sup>-1</sup> )	27.6 ± 7.8	23.8 ± 3.7

BMI, body mass index; MAP, mean arterial pressure; Chol, total serum cholesterol; OPN, plasma osteopontin concentration.

data of individuals before kidney donation and healthy volunteers.

## Discussion

This study is the first clinical evaluation of the association of circulating plasma levels of OPN with renal function. The findings in patients with primary CKD and in healthy individuals after living kidney donation are as follows: (i) OPN plasma concentrations correlate inversely with GFR in patients with CKD (K/DOQI stages 1–5), as well as positively with other indicators of renal function such as serum creatinine, SDMA and homocysteine. (ii) OPN plasma levels correlate closely with calcium-phosphate-product, serum-phosphate and intact parathyroid hormone. (iii) Circulating levels of OPN increase after

abrupt reduction in GFR in individuals after kidney donation. In our study, different parameters of renal function, above all inulin clearance as the gold standard, correlated with OPN levels. Similarly, OPN levels are associated with novel markers of renal function like SDMA [19] and markers that strongly depend on renal function like Hcy [20]. The highly significant correlation between plasma OPN levels and GFR as well as plasma SDMA levels clearly illustrates its dependence upon renal function.

Although the nature of the study does not allow a firm conclusion on the exact pathophysiological relationship between renal function and circulating OPN, the following scenarios seem to be possible. OPN is a 70 kDa protein that is highly negatively charged. It is produced by a variety of tissues including the kidney. In healthy kidneys, OPN is secreted into the urine [21]. It is thus conceivable that increases in circulating OPN are partly resulting from reduced urinary excretion.

OPN is a cell survival factor and may protect cells from entering apoptosis. Despite diminished macrophage infiltration and interstitial fibrosis, the obstructed kidneys of OPN knockout mice exhibit increased levels of tubular cell apoptosis compared with wild-type mice, suggesting that OPN is capable of providing survival signals to tubular epithelial cells *in vivo* [22]. In a rat model, following ischaemia/reperfusion injury, the distal tubular epithelium shows an early and persistent increase in OPN staining in the absence of major morphological injury, whereas OPN staining in proximal tubular epithelial cells is delayed and is mostly associated with morphological regeneration [23]. Thus, in the setting of acute kidney injury OPN might be increased as a result of reparative processes. In CKD, which is characterized by progressive fibrosis of renal tissue, OPN might be a mediator of the fibrotic process [24]. Tubulo-interstitial macrophage accumulation is an important marker of prognosis that correlates closely with declining renal function in a range of human and experimental diseases [25,26]. OPN is closely associated with their trafficking into the tubulo-interstitium of the kidney.

Plasma levels of OPN are elevated in essential hypertension, and in patients with coronary artery disease and restenosis [12–14]. OPN levels are predictive of adverse cardiac events in patients with chronic stable angina [27]. However, in this study renal function was determined using serum-creatinine, which, if used alone, is inadequate to assess renal function. The American Heart Association (AHA) as suggested by Brosius *et al.* [28] recommends the use of the MDRD formula (Modification of Diet in Renal Disease equation) to estimate glomerular filtration rate in patients with or at increased risk of cardiovascular disease. Kato *et al.* who could show that pre-procedural OPN predicts re-stenosis in patients undergoing percutaneous coronary intervention [14] did not assess renal function at all,

which is known to predict major cardiovascular events in this patient population [29]. In other words, future studies on the predictive value of OPN have to correct for a reliable marker of renal function to avoid the mistake the medical community made in the case of Hcy, which could today be viewed as an expensive creatinine [20].

An emerging cardiovascular risk factor in patients with CKD as well as in the general population is the endogenous nitric oxide inhibitor ADMA [30–32], but the association between ADMA and circulating OPN has never been described before. Several experimental and clinical studies have highlighted the role of OPN in cardiovascular disease. OPN has been implicated as a key factor in the development of atherosclerosis [9–13], and its expression was reported to be closely related to arterial smooth muscle cell proliferation both *in-vitro* and *in-vivo* [33,34]. OPN-transgenic mice develop marked atherosclerosis [11]. Thus, the relationship between OPN and ADMA needs further exploration.

OPN levels were also closely associated with serum-phosphate and iPTH, which progressively increase with declining renal function representing hallmarks of secondary hyperparathyroidism in chronic kidney disease. Interestingly, in an *in-vitro* model OPN was required for PTH-induced bone resorption by influencing the increase in the number of osteoclasts through inhibition of receptor activator of NF- $\kappa$ B ligand (RANKL) that directly promotes bone resorption [35]. The association of OPN levels and calcium-phosphate-product underscores its potential significance in the development of extra-osseous calcification, as OPN may function as an inhibitor of calcification. OPN directly inhibits calcification of cultured bovine aortic smooth muscle cells and inhibits aortic valve calcification *in-vivo* [36,37]. OPN may also affect calcification by the stimulation of resorption, as binding of OPN to the  $\alpha_v\beta_3$  integrin-receptor on osteoclasts leads to a decrease in cytosolic calcium, a change associated with osteoclast activation to a resorptive phenotype [38]. Intriguingly, this form of binding also promotes resorption of ectopic calcification by inducing expression of carbonic anhydrase II, which is key in creating the acidic environment required for resorption [36]. Mice deficient in both Matrix-Gla-Protein (MGP) and OPN (MGP $^{-/-}$  OPN $^{-/-}$ ) showed accelerated and enhanced vascular calcification compared with mice deficient in MGP alone (MGP $^{-/-}$  OPN $^{-/-}$ ), consistent with the concept that OPN is an inducible inhibitor of vascular calcification *in vivo* and may play an important role in the adaptive response of the body to injury and disease [15].

We wish to point out important limitations of the study. Firstly, we included a rather small number of CKD patients, yet all patients underwent measurement of true GFR using continuous inulin clearance technique. Secondly, we limited the spectrum of CKD patients to two disease settings, i.e. ADPKD and IgA-nephropathy. Thus our analysis does not include the whole

spectrum of renal diseases. We have chosen such an approach to have both, inflammatory and non-inflammatory 'marker' diseases that were diagnosed state of the art, i.e. by renal biopsy in the case of IgA nephropathy.

In conclusion, circulating OPN is closely and inversely related to GFR. Furthermore, there is a significant association between OPN and cardiovascular risk markers such as ADMA. In future studies pertaining to circulating levels of OPN in human pathology, the influence of renal function has to be taken into account.

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### Disclosures

The authors declare no conflict of interest.

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