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Abstract

Background and objectives Higher serum phosphate is associated with cardiovascular events and all-cause mortality. Explanations of this association have focused on large vessel calcification and stiffness. Studies suggest that a higher serum phosphate induces microvascular dysfunction, but relationships in humans with direct measures of microvascular function are lacking.

Design, setting, participants, & measurements We performed a cross-sectional analysis of 3189 community-living participants that underwent skin capillaroscopy, laser-Doppler flowmetry, and flicker light–induced retinal vessel responses. We used linear regression to assess the association between serum phosphate and each microvascular outcome. The primary outcome was skin capillary recruitment during postocclusive peak reactive hyperemia by capillaroscopy. Secondary outcomes included capillary recruitment during venous congestion, heat-induced skin hyperemic response, flicker light–induced retinal arteriolar, and venular dilation.

Results The mean age of the cohort was 59±8 years, 48% were women, 7% had an eGFR <60 ml/min per 1.73 m^2, and the mean serum phosphate concentration was 3.2±0.5 mg/dl. A 1 mg/dl higher serum phosphate was independently associated with a 5.0% lower postocclusive capillary recruitment (95% CI, −10.0% to −0.1%). Results were similar for capillary recruitment with venous congestion (−4.5%; 95% CI, −9.8% to 0.7%). A 1 mg/dl higher serum phosphate was also independently associated with a 0.23% lower retinal venular dilation in response to flicker light (95% CI, −0.44% to −0.02%). A higher serum phosphate was not associated with change in flicker light–induced retinal arteriolar dilation or heat-induced skin hyperemic response, however a higher serum phosphate was associated with a lower heat-induced skin hyperemic response among men (−149% [95% CI, −260 to −38] per 1 mg/dl higher serum phosphate) but not women (*P* interaction, 0.01).

Conclusions Higher serum phosphate concentrations, even within the normal range, are associated with microvascular dysfunction in community-living individuals.

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Introduction

Higher serum phosphate concentrations are independently associated with cardiovascular events and all-cause mortality. These associations are evident in the general population, in persons with CKD, in persons with overt hyperphosphatemia, and in persons with slightly higher serum phosphate concentrations that remain within the normal laboratory reference range (1–5). Evaluation of potential mechanisms linking hyperphosphatemia with cardiovascular events and mortality has largely focused on large-vessel alterations including arterial calcification and stiffness (6,7). However, recent studies have suggested that higher phosphate exposure may also contribute to microvascular dysfunction (8,9). *In vitro* studies have demonstrated that phosphate inhibits nitric oxide production by reducing nitric oxide synthase expression (9). The few available clinical studies are also supportive, but have largely relied on flow-mediated dilation (FMD) as the marker of endothelial dysfunction (10,11). FMD uses flow through large vessels (typically the brachial or femoral artery) to infer information about bioavailable nitric oxide downstream in the microvasculature, an important determinant of endothelial function (12). To our knowledge, no studies have yet evaluated the relationship between phosphate and microvascular function by direct imaging of the microvasculature or assessment of dynamic changes in dilation of microvasculature in response to stimuli.

Skin capillaroscopy, skin laser-Doppler flowmetry, and flicker light–induced retinal vessel dilation are reproducible noninvasive markers of microvascular function that more directly evaluate the arterioles, venules, and capillaries. Direct skin capillaroscopy is a technique in which an intravital microscope is used in the direct assessment of the dermal capillaries, both at rest and in response to arterial occlusion or venous congestion (13). Laser-Doppler flowmetry measures skin arteriolar and venular flow and changes in response to local heating of the skin, a process partially

mediated by nitric oxide (14). Abnormalities in skin capillaroscopy, laser-Doppler flowmetry, and retinal diameter have all been associated with increased cardiovascular risk in select populations (15–18). Retinal arterioles and venules dilate in response to flicker flight (with the magnitude of dilation thought to be mediated by nitric oxide), which can thus serve as an additional measure of microvascular function (19). These microvascular function measurement techniques have been demonstrated to be altered in persons with hypertension, albuminuria, and (pre)diabetes (13,20,21).

In a population-based cohort, we evaluated the cross-sectional association between serum phosphate concentration and microvascular function using skin capillaroscopy, laser-Doppler flowmetry, and flicker light–induced retinal vessel dilation. We hypothesized that higher serum phosphate concentrations, even within the normal range, would be associated with diminished microvascular function.

Materials and Methods

Study Population

The Maastricht study is an observational, prospective, population-based, cohort study. The rationale and methodology have been described previously (22). In brief, the study focuses on the etiology, pathophysiology, complications, and comorbidities of type 2 diabetes mellitus and is characterized by an extensive phenotyping approach. Eligible participants were aged 40–75 years and living in the southern part of The Netherlands. Participants were recruited through mass media campaigns, from the municipal registries, and the regional diabetes patient registry *via* mailings. Recruitment was stratified according to known type 2 diabetes mellitus status, with an oversampling of individuals with type 2 diabetes mellitus for efficiency. We report a cross-sectional analysis of 3451 participants that underwent phosphate measurement as well as skin capillaroscopy, laser-Doppler flowmetry, or flicker light–induced retinal vessel dilation between November 2010 and September 2013 (20). Any individual that provided a blood sample and underwent at least one of these measurements was included in this analysis. From the initial 3451 participants, 862 underwent capillaroscopy, 1647 underwent laser-Doppler flowmetry, and 2261 underwent retinal vessel analysis. The baseline visit and collection of venous blood samples used for phosphate measurements occurred within 3 months of the microvascular function measurements. The study has been approved by the institutional medical ethical committee (NL31329.068.10) and the Minister of Health, Welfare and Sport of The Netherlands (permit 131088-105234-PG). All participants gave written informed consent. There were 659, 1306, and 1834 participants included in the final capillaroscopy, laser-Doppler flowmetry, and retinal vessel analyses, respectively (Figure 1). The main reasons for the differences in participant numbers within each measurement technique were logistic issues and image quality (23).

Serum Phosphate Measurement

Participants had fasted overnight at the time of blood sampling. Samples were stored at −80°C from collection until testing in 2017–2018. Serum phosphate was measured spectrophotometrically by using an automated analyzer (Cobas 8000, C702 module; Roche). Analytical variation of phosphate in serum, estimated from internal quality controls, was 4%. The detectable range was 0.31–20 mg/dl. Among adults, the normal range for this device is 2.5–4.5 mg/dl.

Microvascular Measurements

For a full description of microvascular measurements, see Supplemental Methods. All participants were asked to refrain from ingesting caffeine or smoking for 3 hours before measurement. A light meal (breakfast or lunch), low in fat content, was allowed at least 90 minutes before the start of the measurements.

Skin Capillaroscopy

Details of the skin capillaroscopy measurements have been described previously (20). Briefly, measurements were performed in a quiet, temperature-controlled room (24°C) with participants in the supine position. Capillaries were visualized in the dorsal skin of the third and fourth finger of the right hand using a digital microscope (Capiscope; KK Technology, Honiton, UK). Capillaries were visualized 4.5 mm proximal to the terminal row of capillaries at the nail fold. All capillary density measurements were made on two fingers, and the average was used for each individual in analysis. Capillary density was first measured at baseline. Next, a finger cuff was inflated to 260 mm Hg to occlude arterial flow for 4 minutes and then released. Capillary density was then measured for 15 seconds. Venous congestion was then applied by inflating the cuff to 60 mm Hg for 2 minutes and all capillaries were counted for 15 seconds in the second minute of occlusion. Operators performing the capillaroscopy were blinded to the measurements and analysis. Considering the known associations between albuminuria (an indicator of microvascular dysfunction) and changes in skin capillaroscopy (20), and to minimize multiple comparisons, we chose to evaluate the percentage change in response to arterial occlusion as our primary outcome *a priori*. Percentage change was calculated as (peak value-baseline value)/(baseline value) ×100. We also evaluated the percentage change from baseline to venous congestion in secondary analyses.

Laser-Doppler Flowmetry

Details of the laser-Doppler flowmetry measurements have been described previously (21). Briefly, skin blood flow was measured using a laser-Doppler flowmetry system (Periflux 5000; Perimed, Järfalla, Sweden) equipped with a thermostatic probe (PF 457; Perimed). The thermostatic probe was placed at the dorsal side of the wrist of the left arm, with care to avoid any visible large blood vessels. Blood flow was measured at baseline for 2 minutes and then skin was heated to 44°C for 23 minutes. The heat-induced skin hyperemic response was expressed as the percentage increase in average perfusion units during the 23-minute heating phase over the average baseline perfusion units. The percentage change was calculated as (peak value−baseline value)/baseline value×100.

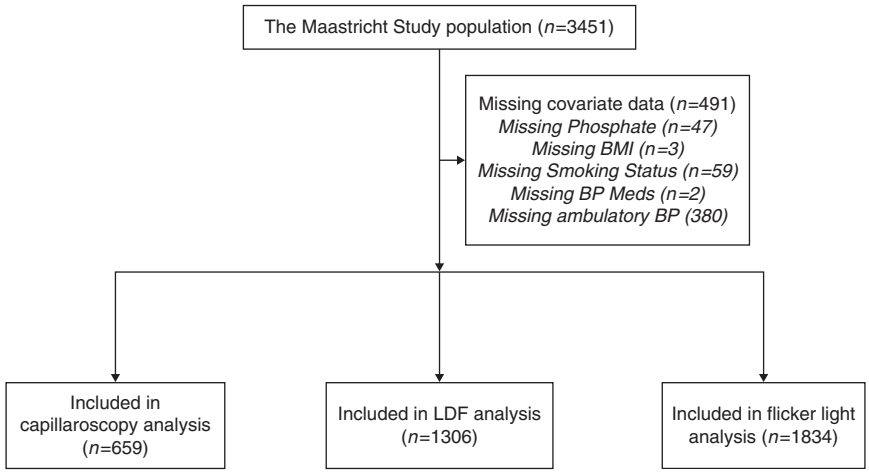

Figure 1. | **Maastricht population for each microvascular end point.** BMI, body mass index; LDF, laser-Doppler flowmetry.

Retinal Vessel Dilation

Details of the retinal vessel dilation response to flicker-light measurements have been described previously (21). Briefly, retinal arteriolar and venular dilation response to flicker light was measured in a dimly lit room using a Dynamic Vessel Analyzer (IMEDOS, Jena, Germany). First, pupils were dilated with 0.5% tropicamide and 2.5% phenylephrine at least 15 minutes before the start of the examination. Participants with an intraocular pressure of >30 mm Hg were excluded. Participants were asked to focus on a needle inside a retinal camera (FF450; Carl Zeiss GmbH, Jena, Germany) and the fundus of the eye was examined under green light. Straight vessel segments of 1.5 mm were examined and the diameter was measured over 50 seconds using the Dynamic Vessel Analyzer. Baseline diameter was calculated as the average diameter between seconds 20 and 50 of the baseline period, and was expressed in measurement units in which 1 U was equal to 1 μm of the Gullstrand eye. Next, flicker-light stimulation was performed for 40 seconds (12.5 Hz, bright-to-dark contrast ratio of 25:1), followed by a 60 second recovery period, and then vessel diameter was remeasured. The percentage dilation above baseline was based on the average dilation as compared with the baseline diameter. Percentage change was calculated as (peak value−baseline value)/baseline value×100.

Covariate Measurements

To assess glucose metabolism status, participants who were not using insulin and had fasting glucose of <200 mg/dl underwent a standardized 2-hour, 75-g oral glucose tolerance test after an overnight fast. Glucose metabolism status was defined according to the World Health Organization 2006 criteria as normal glucose metabolism, impaired fasting glucose, impaired glucose tolerance (combined as prediabetes), and type 2 diabetes mellitus (20).

History of cardiovascular disease, duration of diabetes, menopausal status, physical activity (h/wk), and smoking status (never, former, current) were assessed by questionnaire (22). Use of lipid-modifying, antihypertensive, and glucose-lowering medication as well as postmenopausal hormone replacement therapy was assessed during a medication interview where generic name, dose, and frequency were registered (22). We measured weight, height, body mass index, waist circumference, office and ambulatory 24-hour BP, plasma glucose levels, serum creatinine, spot urine albumin/creatinine ratios (twice), glycated hemoglobin A1c, and plasma lipid profile as described elsewhere (22). eGFR was calculated using the four-variable Modification of Diet in Renal Disease equation (24). The presence of retinopathy was assessed in both eyes by using fundus photographs taken with an Auto Fundus Camera (model AFC-230; Nidek, Gamagori, Japan) (21).

Statistical Methods

Participant characteristics were compared across serum phosphate quartiles. Multiple linear regression was used to assess the association of serum phosphate with each measure of microvascular function. In companion analyses, we evaluated quartiles of phosphate, setting the lowest as the reference category, to assess the functional form of the relationships. We developed a sequence of models. Model 1 adjusted for age and sex. Model 2 additionally adjusted for body mass index, smoking status, 24-hour ambulatory systolic BP, use of antihypertensives, use of lipid-modifying agents, glucose metabolism status, eGFR, and serum calcium. We assessed for sex, diabetes, and CKD interactions by including multiplicative interaction terms in model 2. Lastly, we performed *post-hoc* sensitivity analyses additionally adjusting for office BP instead of ambulatory BP, antihypertensive class, as well as household income and size. Analyses were conducted in Stata SE version 14.1 (College Station, TX). P values <0.05 were considered statistically significant for all analyses including interaction terms.

Results

The mean age of the 3189 participants was 59±8 years, 48% were women and 7% had an eGFR of <60 ml/min per 1.73 m^2. The mean serum phosphate concentration was 3.2±0.5 mg/dl, and 257 (8%) participants had serum phosphate concentrations ≥4.0 mg/dl. Baseline characteristics across quartiles of serum phosphate are shown in

Table 1. Baseline characteristics of participants by serum phosphate quartiles

Characteristics	Phosphate Quartiles (Range)			
	Q1 (1.6–2.8 mg/dl)	Q2 (2.9–3.2 mg/dl)	Q3 (3.2–3.6 mg/dl)	Q4 (3.6–5.2 mg/dl)
	n=809	*n*=855	*n*=781	*n*=744
Age (yr [±SD])	60 (9)	60 (8)	60 (8)	59 (8)
Male (*n* [%])	644 (80)	549 (64)	307(39)	159 (21)
BMI (kg/m^2 [±SD])	28 (4.2)	27 (4.3)	27 (4.5)	26 (5.1)
Smoking (*n* [%])				
Never	283 (35)	297 (36)	260 (34)	226 (31)
Former	433 (54)	429 (51)	394 (51)	375 (52)
Current	82 (10)	110 (13)	118 (15)	123 (17)
Diabetes status (*n* [%])[a]				
Normal	424 (52)	462 (54)	453 (58)	438 (59)
Prediabetes	152 (19)	119 (14)	104 (13)	92 (12)
Type 2 diabetes	233 (29)	274 (32)	224 (29)	214 (29)
HbA1C (% [±SD])	5.9 (0.9)	5.9 (0.9)	5.9 (0.9)	6.0 (1.0)
Retinopathy (*n* [%])	10 (1)	12 (2)	12 (2)	16 (2)
Cardiovascular disease (*n* [%])	117 (15)	145 (17)	131 (17)	118 (17)
BP (mm Hg [±SD])				
Office systolic BP	139 (17)	136 (18)	134 (18)	130 (18)
Office diastolic BP	79 (10)	76 (10)	75 (10)	74 (10)
24-h systolic BP	122 (11)	120 (12)	118 (12)	116 (11)
24-h diastolic BP	76 (7)	74 (7)	73 (7)	72 (7)
BP medication (*n* [%])	331 (41)	380 (45)	304 (39)	270 (36)
Hyperlipidemia medication (*n* [%])	303 (37)	337 (40)	273 (35)	257 (35)
eGFR (ml/min per 1.73 m^2 [±SD])	81 (18)	82 (17)	81 (16)	80 (17)
CKD stage (*n* [%])				
None (eGFR >60 ml/min per 1.73 m^2)	760 (94)	782 (91)	722 (92)	695 (93)
CKD stage 3a	45 (6)	63 (7)	54 (7)	39 (5)
CKD stage 3b	4 (0.5)	10 (1)	5 (1)	8 (1)
CKD stage 4	0 (0)	0 (0)	0 (0)	2 (0.3)
Urine albumin/creatinine ratio (mg/g [±IQR])	4 (2–8)	5 (3–9)	5 (3–10)	5 (2–8)
Calcium (mg/dl [±SD])	9.3 (0.4)	9.3 (0.3)	9.4 (0.3)	9.4 (0.3)

The upper range of Q2 was 3.19, the lower range of Q3 was 3.22, the upper range of Q3 was 3.56, and the lower range of Q4 was 3.60. BMI, body mass index; HbA1C, hemoglobin A1c; IQR, interquartile range.
[a]Glucose metabolism status was assessed by an oral glucose tolerance test and defined according to the World Health Organization 2006 criteria as normal glucose metabolism, impaired fasting glucose, impaired glucose tolerance (combined as prediabetes), and type 2 diabetes mellitus.

Table 1. Baseline characteristics for the subcohorts that underwent each microvascular measurement are reported in Supplemental Table 1. Compared with persons in the lowest quartile, those with higher serum phosphate were more often women, current smokers, had lower BP, were less likely to be taking antihypertensive medications, were more likely to have retinopathy, and had slightly higher serum calcium concentrations.

Among the 659 individuals with available serum phosphate and capillaroscopy measurements, the mean postocclusive capillary recruitment was 45%±29%. In model 1, higher serum phosphate was associated with statistically significantly less capillary recruitment during postocclusive peak reactive hyperemia (−5.2% per 1 mg/dl higher phosphate). In the fully adjusted model, this association remained essentially unchanged (Table 2). When phosphate was evaluated by quartiles, the relationship with postocclusive reactive hyperemia was fairly linear and, compared with persons in the lowest quartile, persons in the highest phosphate quartile recruited 8.4% less capillaries in response to arterial occlusion (Figure 2). This association was similar in either sex, in individuals with and without diabetes, and with and without CKD (Supplemental Table 2).

Next, we evaluated skin capillary recruitment during venous congestion. Among the 659 individuals, the mean percentage recruitment during venous congestion was 46%±31%. Similar to the postocclusive response, in model 1, serum phosphate was inversely associated with capillary recruitment during venous congestion (−4.7% per 1 mg/dl higher in phosphate) but this association did not reach statistical significance (Table 2). In the fully adjusted model the association with capillary recruitment during venous congestion remained similar. There was no significant interaction of sex, diabetes, or CKD status and phosphate for the capillary recruitment endpoint (Supplemental Table 2).

We next evaluated laser-Doppler flow in forearm skin. The mean heat-induced skin hyperemia was 1120%±762% among the 1306 individuals with laser-Doppler flowmetry and serum phosphate data. Serum phosphate was not associated with heat-induced skin hyperemic response

Table 2. Association of phosphate with microvascular measurements

Measurement Used (% [95% CI])	Measurement Results per Phosphate Range Quartile				Per 1 mg/dl Higher Serum Phosphate	P^a
	Q1 (1.6–2.8 mg/dl)	Q2 (2.9–3.2 mg/dl)	Q3 (3.2–3.6 mg/dl)	Q4 (3.6–5.2 mg/dl)		
Capillary recruitment during postocclusive reactive hyperemia (n=659)						
Model 1	Reference	−7.1 (−13.3 to −0.8)	−7.1 (−13.6 to −0.6)	−8.7 (−15.9 to −1.6)	−5.2 (−10.1 to −0.4)	0.04
Model 2	Reference	−6.6 (−12.8 to −0.3)	−6.3 (−12.8 to 0.3)	−8.4 (−15.6 to −1.1)	−5.0(−10.0 to −0.1)	0.04
Capillary recruitment during venous congestion (n=659)						
Model 1	Reference	−5.6 (−12.3 to 1.0)	−6.0 (−12.9 to 0.90)	−8.1 (−15.7 to −0.5)	−4.7 (−9.8 to 0.5)	0.08
Model 2	Reference	−5.2 (−11.7 to 1.6)	−5.0 (−11.9 to 2.0)	−7.9 (−15.6 to −0.1)	−4.5 (−9.8 to 0.7)	0.09
Heat-induced skin hyperemic response (n=1306)						
Model 1	Reference	−132 (−242 to −22)	−112 (−230 to 6)	−120 (−249 to 8)	−53 (−140 to 35)	0.24
Model 2	Reference	−117 (−226 to −7)	−92 (−211 to 27)	−81 (−211 to 415)	−25 (−113 to 63)	0.57
Retinal arteriolar dilation (n=1834)						
Model 1	Reference	−0.18 (−0.53 to 0.18)	−0.12 (−0.50 to 0.25)	−0.21 (−0.61 to 0.20)	−0.19 (−0.47 to 0.07)	0.15
Model 2	Reference	−0.11 (−0.46 to 0.24)	−0.05 (−0.43 to 0.33)	−0.09 (−0.50 to 0.32)	−0.12 (−0.30 to 0.15)	0.39
Retinal venular dilation (n=1834)						
Model 1	Reference	−0.02 (−0.29 to 0.26)	−0.25 (−0.54 to 0.05)	−0.41 (−0.73 to −0.10)	−0.26 (−0.47 to −0.05)	0.01
Model 2	Reference	0.03 (−0.24 to 0.31)	−0.20 (−0.49 to 0.10)	−0.35 (−0.66 to −0.03)	−0.23 (−0.44 to −0.02)	0.03

The upper range of Q2 was 3.19, the lower range of Q3 was 3.22, the upper range of Q3 was 3.56, and the lower range of Q4 was 3.60. Model 1 adjusted for age and sex; model 2 additionally adjusted for body mass index, smoking status, 24-h ambulatory systolic BP, use of antihypertensives, use of lipid-modifying agents, glucose metabolism status, eGFR, and serum calcium.
[a]P values reported for the continuous analysis.

across the series of models. (Table 2). However, we observed that the association differed by sex (P interaction, 0.01). In analyses stratified by sex, we found no association between serum phosphate and heat-induced skin hyperemia among women. However, serum phosphate was strongly associated with heat-induced skin hyperemia among men, such that a 1 mg/dl higher phosphate was associated with a 148% lower heat-induced skin hyperemic response (Table 3). We found no evidence of interaction with diabetes or CKD status.

The mean retinal arteriolar and venular dilation were 3.0%±2.8% and 3.9%±2.2%, respectively, among the 1834 individuals with both retinal imaging and serum phosphate data. Serum phosphate was not associated with flicker light–induced retinal arteriolar dilation in either the demographic or fully adjusted model (P=0.15 and 0.39 for models 1 and 2, respectively) (Table 2). Serum phosphate was, however, inversely associated with flicker light–induced venular dilation in both model 1 and the fully adjusted model, such that a 1 mg/dl higher serum phosphate was associated with a 0.23% lower venular diameter in the fully adjusted model. When evaluating phosphate quartiles, the relationship with flicker light–induced retinal venule dilation appeared fairly linear across increasing quartiles. There were no statistically significant interactions between sex (Supplemental Table 2), diabetes, or CKD status and either retinal imaging outcome.

Considering ambulatory BP data were missing in 380 participants, we performed sensitivity analyses using office BP. Associations using these measurements were essentially unaltered. Similarly, further adjustment for antihypertensive class, household income, and household size did not significantly alter the association of serum phosphate with any of the measured outcomes.

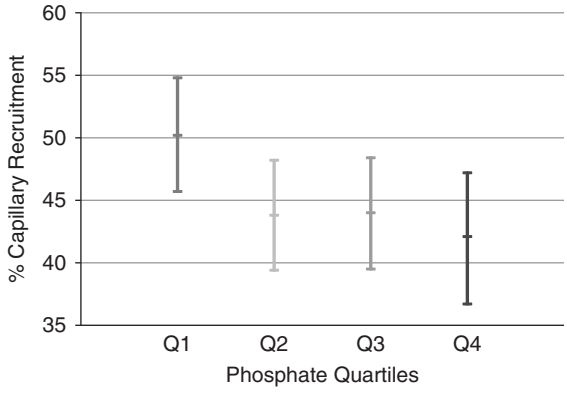

Figure 2. | Percentage capillary recruitment during postocclusive reactive hyperemia is lower at higher phosphate quartiles. Phosphate quartile ranges were (Q1) 1.6–2.8 mg/dl, (Q2) 2.9–3.2 mg/dl, (Q3) 3.2–3.6 mg/dl, and (Q4) 3.6–5.2 mg/dl. Values presented are adjusted for age, sex, smoking status, 24-hour ambulatory systolic BP, use of antihypertensives, use of lipid modifying agents, diabetes status, eGFR, and serum calcium.

Discussion

In a large, well characterized cohort of community-living men and women, we demonstrate that a higher serum phosphate concentration, even within the normal reference range, is associated with lower capillary recruitment. We also found higher serum phosphate concentrations were associated with lower flicker light–induced retinal venular dilation. Lastly, we also found serum phosphate to be associated with a lower heat-induced skin hyperemic response among men. Overall, these findings demonstrate that higher serum phosphate concentrations are independently associated with worse microvascular function in community-living individuals.

Table 3. Association of serum phosphate with heat-induced skin hyperemic response, stratified by sex

Sex	Change per 1 mg/dl Higher Phosphate (% [95% CI])[a]	*P* Value	*P* Interaction
Men	−149 (−260 to −38)	0.01	0.01
Women	88 (−55 to 230)	0.23	

[a]Adjusted for age, sex, body mass index, smoking status, 24-h ambulatory systolic BP, use of antihypertensives, use of lipid-modifying agents, diabetes status, eGFR, and serum calcium.

Putative relationships of phosphate with microvascular disease are only just emerging. Multiple *in vitro* studies have found that endothelial cells exposed to high phosphate concentrations have reduced amounts of endothelial nitric oxide synthase leading to a reduced vasodilation (8,9). These studies suggest phosphate may be directly causative of impaired microvascular function. In a rodent model of CKD and endothelial dysfunction, a low phosphate diet improved vasodilation of the thoracic aorta through induction of increased activation of endothelial nitric oxide synthase (25). Other studies have reported a link between higher serum phosphate and downregulation of Annexin II, a key protein in several biologic processes including endothelial cell adhesion and angiogenesis (26). Endothelial cell apoptosis has also been observed in the setting of hyperphosphatemia (27). Alternatively, α-Klotho deficiency or other emerging pathways may be contributing to both a higher serum phosphate and impaired microvascular function (28). Thus higher phosphate may be associated with impaired microvascular function *via* multiple mechanisms.

Human studies evaluating the relationship between phosphate and microvascular function have had small sample sizes and have used FMD, which reflects nitric oxide production but uses changes in large vessels to infer potential changes in downstream microvasculature (10,11). In a study among 100 persons with severe CKD, individuals were randomized to 8 weeks of an intestinal phosphate binder, which limits dietary phosphate absorption. These patients had significantly elevated serum phosphate levels at baseline. Those randomized to the binder had significant improvement in brachial artery FMD compared with their baseline FMD measurement (11). Similarly, Shuto *et al.* fed 11 healthy volunteers meals containing 400 and 1200 mg of phosphate, using a crossover design, and performed brachial artery FMD measurements 2 hours after each meal. The high-phosphate meal induced significantly higher serum phosphate concentrations, although the average peak phosphate was still around the high end of the normal range (4.6 mg/dl). Compared with the low-phosphate meal, the high-phosphate meal induced significantly less arterial dilation by FMD (10). Thus, these findings suggested that not only does phosphate impair endothelial function, but it can do so acutely, even in healthy persons without CKD. Similarly, in another small, controlled trial of healthy subjects randomized to 11 weeks of a high-phosphate diet versus a low-phosphate diet combined with a phosphate binder, those in the high-phosphate group experienced a 4 mm Hg increase in systolic BP compared with those in the low-phosphate group, possibly due to the effects of phosphate on the microvasculature (29). The persons randomized to the high-phosphate diet still had an average phosphate in the normal range (around 4 mg/dl). In larger community-living populations, Mehta *et al.* reported an association between higher serum phosphate and prevalence of retinopathy, even among persons with a serum phosphate in the normal range in the Multi-Ethnic Study of Atherosclerosis, a finding confirmed in our study (30). In that study, serum phosphate was not associated with central retinal arteriolar or venular diameter; however, these measurements were made without flicker-light stimulation, thus this would only reflect the baseline diameter. Higher serum phosphate has also been associated with cerebral microvascular disease and there have been conflicting studies regarding an association between serum phosphate and albuminuria, a marker of microvascular disease in the kidney (31–33). All of these studies accounted for kidney function, sex, and other key confounders. Our study is consistent with these prior works and we extend these findings by evaluating three distinct techniques that more directly assess microvascular function concurrently, and demonstrate by multiple direct metrics of the microvasculature that higher serum phosphate is associated with impaired microvascular function among healthy individuals.

Older women have higher serum phosphate levels than older men (7,34–36). In rodent models, estrogen induces phosphaturia with a resultant decline in serum phosphate (37). As women transition through menopause and estrogen levels decrease, serum phosphate levels rise, likely due to the loss of estrogen-induced phosphaturia (38). Panwar *et al.* (39) recently reported that sex modified the relationship between fibroblast growth factor 23 (a hormone regulating serum phosphate) and coronary heart disease. Considering the known differences in phosphate handling between older men and women, we evaluated whether the effects of phosphate on the microvasculature were modified by sex. We found a significant interaction with the heat-induced skin hyperemic response: higher phosphate was strongly negatively associated with the heat-induced skin hyperemic response among men but not women. Whether this finding is reflective of differences in estrogen levels and associated phosphate homeostatic control, whether sex differences exist in younger persons, or whether this finding was due to chance, requires further investigation. The very low *P* value observed for the interaction argues against chance as the likely explanation.

The Maastricht study is unique in that there were several distinct microvascular imaging techniques performed on >3000 participants with a range of comorbidities. The concurrent availability of demographics, cardiovascular risk factor status, and kidney function are additional key strengths. This study also has important limitations. First, as the design is cross-sectional, the temporal directions of associations are uncertain and there may be issues of residual confounding for which we could not account. Second, this was a community-living population, which increases generalizability but results in a fairly healthy study population. It is unclear if these findings can be extended to patients with more advanced CKD or overt

hyperphosphatemia. Third, although we found a strong association of serum phosphate with capillary recruitment postarterial occlusion (our prespecified primary outcome), the association between serum phosphate and microvascular function did not reach statistical significance for all end points, despite the direction of point estimates being consistent across all end points irrespective of significant *P* values. Similarly, the magnitude of the association for the retinal vein dilation outcome appears small (0.23% per 1 mg/dl higher serum phosphate,). However, for point of reference, the difference in retinal vessel dilation between persons with normal glucose metabolism and persons with prediabetes was shown to be 0.2%, growing to 0.6% when comparing persons with normal glucose metabolism to those with type 2 diabetes (17). Lastly, there was a significant amount of missing covariate data. However, this was almost entirely due to lack of ambulatory BP measurements. When analyzing the more complete cohort using office BP instead of ambulatory BP, our results were essentially unchanged.

In conclusion, beyond relationships with large-vessel arterial disease, higher serum phosphate is independently associated with impaired microvascular function in community-living individuals predominantly with normal kidney function and with serum phosphate concentrations within the normal laboratory reference range. These findings suggest pathways linking serum phosphate concentrations with cardiovascular disease and mortality may be more complex than phosphate simply promoting arterial calcification and stiffness. The findings also suggest there may be novel avenues to target the effects of phosphate on the vasculature beyond effects in large arteries. Further investigation is needed to evaluate if these associations may be causative. If confirmed, these findings would have important clinical implications regarding phosphate intake in the general population and for the development of novel strategies to improve vascular health by targeting microvascular function.

Disclosures

Dr. Ix reports receiving an Investigator Initiated Research Grant from Baxter International outside of the submitted work. Dr. Webers reports receiving grants from Alcon/Novartis and Santen and personal fees from Alcon/Novartis, Santen, and Thea Pharma, outside of the submitted work. Dr. Berendschot, Dr. Dagnelie, Dr. Ginsberg, Dr. Houben, Dr. Kooman, Dr. Malhotra, and Dr. Stehouwer have nothing to disclose.

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Supplemental Material

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Supplemental Methods.

Supplemental Table 1. Baseline characteristics of participants by subcohort.

Supplemental Table 2. Association of phosphate with microvascular function stratified by sex.

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Supplemental Material Table of Contents

Supplemental Methods

Skin Capillaroscopy

All participants were asked to refrain from smoking and drinking coffee or tea 3 hours before the measurements.[17] A light meal (breakfast and/or lunch) low in fat content was allowed before the start of the measurements. Skin capillaroscopy measurements were performed in a quiet, temperature–controlled room (T=24°C) with participants in the supine position as previously described.

Briefly, capillaries were visualized in the dorsal skin of the distal phalanges of the third and fourth finger of the right hand by use of a digital video microscope (Capiscope; KK Technology, Honiton, United Kingdom) with a system magnification of 3100. Capillaries were visualized 4.5 mm proximal to the terminal row of capillaries in the middle of the nailfold. The investigator selected a region of interest of 1-mm2 skin area. Capillary density (mean of two fields) was measured under three conditions. First, baseline capillary density was measured. Baseline capillary density was defined as the number of continuously erythrocyte–perfused capillaries per 1 mm2 skin and was counted for 15 seconds. Second, capillary recruitment during post-occlusive peak reactive hyperemia was assessed after 4 minutes of arterial occlusion. Arterial occlusion was applied using a miniature cuff at the base of the investigated finger inflated to suprasystolic pressure (260 mmHg) for 4 minutes. Directly after release of the cuff, all (continuously and intermittently) perfused capillaries were counted for 15 seconds. Third, venous congestion was applied, with the cuff inflated to 60 mmHg for 2 minutes, and all (continuously and intermittently) perfused capillaries were counted for 15 seconds. The number of perfused capillaries was counted in the recorded digital raw data with the use of a semiautomatic procedure (CapiAna) by two

investigators who were blinded to participants' clinical status. The intra- and interobserver coefficients of variation for the counting procedure were 2.5% and 5.6%, respectively, as described previously.

Retinal Vessel Dilation Response

The retinal arteriolar dilation response to flicker light, which is thought to be related to nutritive demands of activated retinal neurons, was measured in a dimly lit room by use of the Dynamic Vessel Analyzer (IMEDOS, Jena, Germany).[18] For safety reasons, participants with an intraocular pressure exceeding 30 mm Hg were excluded from retinal measurements. Per participant, we randomly measured the left or right eye.

During the measurement, the participant was instructed and encouraged to focus on the tip of a fixated needle inside the retinal camera (FF450; Carl Zeiss GmbH, Jena, Germany) while the fundus of the eye was examined under green measuring light (530–600 nm, illumination of fundus approximately 6500 lux). A straight arteriolar segment of approximately 1.5 mm in length located 0.5 to 2.0 disc diameters from the margin of the optic disc in the temporal section was examined. When the specific vessel profile was recognized, vessel diameter was automatically and continuously measured for 150 seconds. A baseline recording of 50 seconds was followed by a 40-second flicker light exposure period (flicker frequency 12.5 Hz, bright-to-dark contrast ratio 25:1) followed by a 60-second recovery period. The Dynamic Vessel Analyzer automatically corrected for alterations in luminance caused by, for example, slight eye movements. During blinks and small eye movements, the registration stopped and restarted once the vessel segments were automatically re-identified.

The integrated Dynamic Vessel Analyzer software (version 4.51; Imedos) automatically calculated baseline diameter and percentage dilation. Baseline diameter was calculated as the average diameter size of the 20- to 50-second recording and was expressed in measurement units, where 1 measurement unit is equal to 1 μm of the Gullstrand eye. Percentage dilation over baseline was based on the average dilation achieved at time points 10 and 40 seconds during the flicker stimulation period. Two regression lines were drawn (at intervals of 0–10 seconds and 10–40 seconds during flicker stimulation) and averaged to assess average percentage dilation. The software successfully assessed 2 regression lines in 95.4% of the curves; only 102 dilation curves (4.6%) were based on one regression line. The purpose of taking the average dilation was to account for interindividual variation in the curve shape during dilation.

Retinal venule dilation was performed in an identical fashion as arteriolar dilation, except the venules were used.

Skin Hyperemic Response

Skin blood flow was measured as described previously by means of a laser-Doppler system (Periflux 5000; Perimed, Järfalla, Sweden) equipped with a thermostatic laser-Doppler probe (PF457; Perimed) at the dorsal side of the wrist of the left hand.[18] The laser-Doppler output was recorded for 25 minutes with a sample rate of 32 Hz, which gives semi-quantitative assessment of skin blood flow expressed in arbitrary perfusion units. Skin blood flow was first recorded unheated for 2 minutes to serve as a baseline. After the 2 minutes of baseline, the temperature of the probe was rapidly and locally increased to 44°C and was then kept constant until the end of the registration. The heat-induced skin hyperemic response was expressed as the percentage increase in average

perfusion units during the 23-minute heating phase over the average baseline perfusion units). The response is thought to be related to skin metabolic and thermoregulatory function.

Supplemental Table 1: Baseline Characteristics of Participants by Subcohort

Subcohort	Capillaroscopy	Laser-Doppler	Flicker-Light
Age (years) ± SD	60 (9)	60 (8)	60 (8)
Male, n (%)	421 (54)	818 (52)	1094 (51)
BMI (kg/m^2) ± SD	27 (5)	27 (5)	27 (5)
Smoking, n (%)			
Never	223 (29)	485 (32)	735 (35)
Former	416 (55)	837 (55)	1104 (53)
Current	122 (16)	197 (13)	264 (13)
Diabetes Status*, n (%)			
Normal	413 (53)	823 (53)	1204 (56)
Prediabetes	125 (16)	237 (15)	315 (15)
Type 2 Diabetes	236 (30)	504 (32)	619 (29)
HbA1C (%) ± SD	6.0 (0.8)	6.0 (1.0)	6.0 (1.0)
Retinopathy, n (%)	15 (3)	26 (2)	34 (2)
CVD, n (%)	135 (18)	262 (17)	319 (15)
Office Systolic BP (mm Hg) ± SD	137 (19)	136 (18)	135 (18)
Office Diastolic BP (mm Hg) ± SD	77 (10)	76 (10)	74 (7)
24 hr Systolic BP (mm Hg) ± SD	120 (12)	120 (12)	119 (11)
24 hr Diastolic BP (mm Hg) ± SD	75 (7)	74 (7)	74 (7)
On BP Meds, n (%)	308 (40)	663 (42)	830 (39)
On HLD Med n (%)	292 (38)	616 (40)	765 (36)
eGFR (ml/min/1.73m^2) ± SD	81 (17)	81 (16)	81 (17)

CKD Stage n (%)			
None (eGFR>60ml/min/1.73m^2)	705 (91)	1451 (93)	1989 (93)
CKD 3a	61 (8)	102 (7)	131 (6)
CKD3b	8 (1)	11 (1)	17 (1)
CKD4	0 (0)	0 (0)	1 (0.1)
Urine Albumin/Creatinine Ratio (mg/g) ± IQR	5 [4-9]	5 (3-9)	4 (2-8)
Calcium (mg/dl) ± SD	9.3(0.3)	9.3 (0.3)	9.3 (0.3)

Supplemental Table 2: Association of Phosphate with Microvascular Function Stratified by Sex*

Women		Men		
Per 1 mg/dl increase	P Value	Per 1 mg/dl increase	P Value	P Interaction
%Post-Occlusive Reactive Hyperemia				
-5.3 (-13.5, 2.8)	0.20	-4.9 (-11.2, 1.4)	0.13	0.61
% Capillary Recruitment Increase in Response to Venous Congestion				
-6.0 (-14.6, 2.5)	0.16	-3.9 (-10.7, 2.9)	0.26	0.76
% Retinal Arteriolar Dilation Increase in Response to Flicker Light				
-0.07 (-0.32, 0.46)	0.72	-0.32 (-0.72, 0.07)	0.11	0.22
% Retinal Venule Dilation Increase in Response to Flicker Light				
-0.40 (-0.71, -0.10)	0.01	-0.12 (-0.42, 0.18)	0.42	0.32

* Adjusted for age, sex, BMI, smoking status, 24-hour ambulatory systolic blood pressure, use of anti-hypertensives, use of lipid modifying agents, diabetes status, eGFR and serum calcium.