Hemoglobin is inversely related to flow-mediated dilatation in chronic kidney disease

Mahmut I. Yilmaz[1,6], Alper Sonmez[2,6], Mutlu Saglam[3], Mahir Gulec[4], Selim Kilic[4], Tayfun Eyileten[1], Kayser Caglar[1], Yusuf Oguz[1], Abdulgaffar Vural[1], Mujdat Yenicesu[1] and Carmine Zoccali[5]

[1]Department of Nephrology, Gülhane School of Medicine, Etlik-Ankara, Turkey; [2]Department of Internal Medicine, Gülhane School of Medicine, Etlik-Ankara, Turkey; [3]Department of Radiology, Gülhane School of Medicine, Etlik-Ankara, Turkey; [4]Department of Epidemiology, Gülhane School of Medicine, Etlik-Ankara, Turkey and [5]Department of Nephrology, Hypertension and Renal Transplantation, CNR-IBIM Clinical Epidemiology of Renal Diseases and Hypertension, Ospedali Riuniti, Reggio Calabria, Italy

The microcirculation is regulated by oxygen gradients and by endothelial release of nitric oxide, which can react with hemoglobin to form S-nitroso derivatives. Here we induced flow-mediated dilatation of the brachial artery in response to ischemia in 141 non-diabetic patients with stage 3–4 chronic kidney disease who had no history of smoking, cardiovascular events or use of erythropoietin-based agents. Patients with hemoglobin concentrations above the cohort median of 11.6 g/dl were found to have significant reductions in flow-mediated dilatation compared to those below the median. This inverse relationship remained significant after adjustment for potential confounders, including insulin sensitivity, glomerular filtration rate, proteinuria, body mass index, serum urate, etiology of underlying renal disease, treatment with anti-hypertensive drugs, and traditional Framingham risk factors. Given that hemoglobin can act as an important nitric oxide carrier and buffer, our studies suggest that the mechanism by which hemoglobin influences the endothelium-dependent microcirculation requires its nitrosylation; however, more direct studies need to be performed.

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Correspondence: *Mahmut I. Yilmaz, Department of Nephrology, Gülhane School of Medicine, Etlik-Ankara 06018, Turkey.*
E-mail: mahmutiyilmaz@yahoo.com

[6]These authors contributed equally to this work.

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Anemia in chronic kidney disease (CKD) is currently considered as a consequence of erythropoietin deficiency and as an adaptive response to illness and inflammation.[1] Although it is plausible that physiological correction of erythropoietin deficiency may be beneficial, studies in patients with CKD and end-stage renal disease[2–4] and a thorough meta-analysis[5] have coherently shown that full pharmacological correction of anemia in this condition may produce harm. The excess risk portended by anemia correction in CKD is currently attributed to a noxious effect of higher doses of erythropoietin given the none of erythropoietin resistance in patients with systemic illness and inflammation.[6] A hitherto still overlooked, additional mechanism may be related with the effects of red blood cell mass and the concentration of the oxygen carrier molecule, hemoglobin (Hb), on the vascular endothelium. Red cells contribute to the regulation of vascular tone by viscosity/shear stress-dependent and -independent mechanisms. Among these mechanisms, the nitric oxide (NO)-carrying and -buffering capacity of Hb appears prominent, because it modulates NO bioavailability.[7,8] This phenomenon depends on a series of biochemical processes, including oxidation of NO and[9] nitrosylation of the Fe molecule and of sulfur amino acids in the globin molecule. The NO-carrying capacity of the Hb molecule is considered as a phenomenon of paramount biological relevance.[8] The potential clinical relevance of Hb in the regulation of NO bioavailability is suggested by the observation that hematocrit exhibits a strong, independent predictive power for cardiovascular disease events beyond blood viscosity[10] and by the finding that in polycythemia vera, a disease characterized by increased red cell mass and high blood viscosity, the endothelial response to vasodilatory stimuli is much decreased.[11] Furthermore, a strong inverse relationship between the forearm blood flow response to acetylcholine and Hb was recently reported in a series of type II diabetics with modest or no anemia.

With this background in mind, we investigated whether endothelial function defined on the basis of the forearm blood flow in ischemia is associated with Hb concentration in a large series of cardiovascular event-free, non-diabetic, non-smoker, vitamin D-naive, and stage 3–4 CKD patients who

had never received angiotensin-converting enzyme inhibitors or angiotensin II blockers and whether such an association is independent of classical and emerging risk factors, including systemic inflammation, insulin sensitivity, serum calcium and phosphate, and uric acid concentration.

RESULTS

The median Hb in the study population was 11.6 g per 100 ml. Demographic, clinical, and biochemical characteristics in the whole study population and as categorized according to the median Hb value are presented in Table 1. Patients with Hb concentrations above the median displayed higher systolic blood pressure as well as higher serum triglyceride and insulin concentration and estimated glomerular filtration rate levels. Flow-mediated dilatation (FMD) was lower in patients with Hb above the median value. These associations were also substantially confirmed by standard regression analysis (Table 1). As shown in Figure 1, both estimated glomerular filtration rate and proteinuria were related with FMD. It is of note that Hb was inversely associated with FMD, and each gram increase in the concentration of this compound reflected a 2.0% decrease in FMD. No significant difference was present between the two groups in terms of age, gender, body mass index, diastolic blood pressure, total, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol, Homeostasis Model Assessment-Insulin resistance (HOMA-IR), high sensitive C Reactive Protein, calcium, phosphate, 24-h proteinuria, endothelium-independent vasodilatation (nitroglycerin-mediated dilatation, NMD), and uric acid levels.

Multivariate analysis

To further analyze the independent contribution of Hb to explain the variance of FMD, we constructed a series of multiple regression models based on traditional and non-traditional risk factors impacting on this variable. In the unadjusted analysis, the Hb and FMD levels were intercorrelated (Figure 1 and Table 2). Adjustment for the full set of Framingham risk factors did not significantly affect the strength of the association. Further adjustment for glomerular filtration rate (GFR), proteinuria, calcium, phosphate, hsCRP, HOMA index, and the uric acid levels did not produce a reduction in the strength of the association between Hb and FMD, which remained relevant and highly significant. In the final model, systolic blood pressure, LDL cholesterol, HOMA index, proteinuria, and GFR resulted to be independent correlates of FMD (Table 2). Forcing etiologies of CKD and antihypertensive drugs into the final model did not materially modify the correlation coefficient of the Hb–FMD relationship ($\beta = -0.28$, $P < 0.001$). There was no interaction between GFR and proteinuria with Hb in explaining the variability in FMD. Indeed, the Hb–FMD link was largely independent of the absolute level of proteinuria or GFR (nonsignificant, NS) and of similar degree in patients with and without proteinuria and in patients with stages 3 and 4 CKD.

DISCUSSION

The main finding of this study is the presence of a negative relationship between Hb and endothelial function in a series of non-smoker, non-diabetic stage 3–4 CKD patients without background cardiovascular complications. This association was moderately strong and independent of established and

Table 1 | Demographic, hemodynamic, and biochemical data in non-diabetic CKD stage 3–4 patients categorized according to median hemoglobin value (11.6 g per 100 ml)

	CKD patients (n = 141)	<11.6 g per 100 ml (n = 79)	⩾11.6 g per 100 ml (n = 62)	P-value
Age (years)	45 ± 13	46 ± 13	45 ± 13	0.85
Male sex, n (%)	70 (49%)	34 (43%)	40 (55%)	0.35
BMI (kg/m^2)	24.8 ± 2.8	24.8 ± 2.8	24.9 ± 2.7	0.85
Systolic blood pressure (mm Hg)	134 ± 9	131 ± 9	136 ± 8	**0.001**
Diastolic blood pressure (mm Hg)	85 ± 4	84 ± 5	85 ± 4	0.55
On antihypertensive treatment	Ca channel blocker: 16, 11% α-blocker: 9, 6% Diuretic: 4, 3%	Ca channel blocker: 9, 11% α-blocker: 6, 8% Diuretic: 4, 5%	Ca channel blocker: 7, 11% α-blocker: 3, 5% Diuretic: –	
Calcium (mg per 100 ml)	8.4 ± 0.5	8.4 ± 0.4	8.4 ± 0.5	0.87
Phosphate (mg per 100 ml)	4.7 ± 1.2	4.7 ± 1.1	4.7 ± 1.1	0.91
LDL cholesterol (mg per 100 ml)	119 ± 16	120 ± 17	118 ± 15	0.40
Glucose (mg per 100 ml)	87 ± 10	90 ± 9	84 ± 10	**0.02**
Insulin (UI/l)	7.2 ± 1.5	6.8 ± 1.3	7.7 ± 1.6	**0.001**
HOMA index	1.5 ± 0.4	1.5 ± 0.3	1.6 ± 0.4	0.25
hsCRP (mg/l)	28 (16–58)	26 (16–58)	36 (17–58)	0.100
GFR (ml/min per 1.73m^2)	33 ± 13	31 ± 13	36 ± 14	**0.035**
Proteinuria (g/24 h)	1.3 ± 0.5	1.3 ± 0.5	1.3 ± 0.5	0.79
Uric acid (g per 100 ml)	4.3 ± 1.1	4.4 ± 1.1	4.9 ± 1.0	0.08
Hb (g per 100 ml)	11.4 ± 1.1	10.7 ± 0.7	12.7 ± 0.8	<**0.001**
NMD (%)	13.0 ± 0.5	13.0 ± 0.5	12.9 ± 0.5	0.27
FMD (%)	7.1 ± 0.6	7.2 ± 0.6	6.9 ± 0.6	**0.04**

BMI, body mass index; CKD, chronic kidney disease; FMD, flow-mediated dilatation; GFR, glomerular filtration rate; Hb, hemoglobin; HOMA, Homeostasis Model Assessment; NMD, nitroglycerin-mediated dilatation.
Data are expressed as mean ± s.d., median (minimum–maximum), or as the percentage of frequency, as appropriate.
Significant values are highlighted in bold.

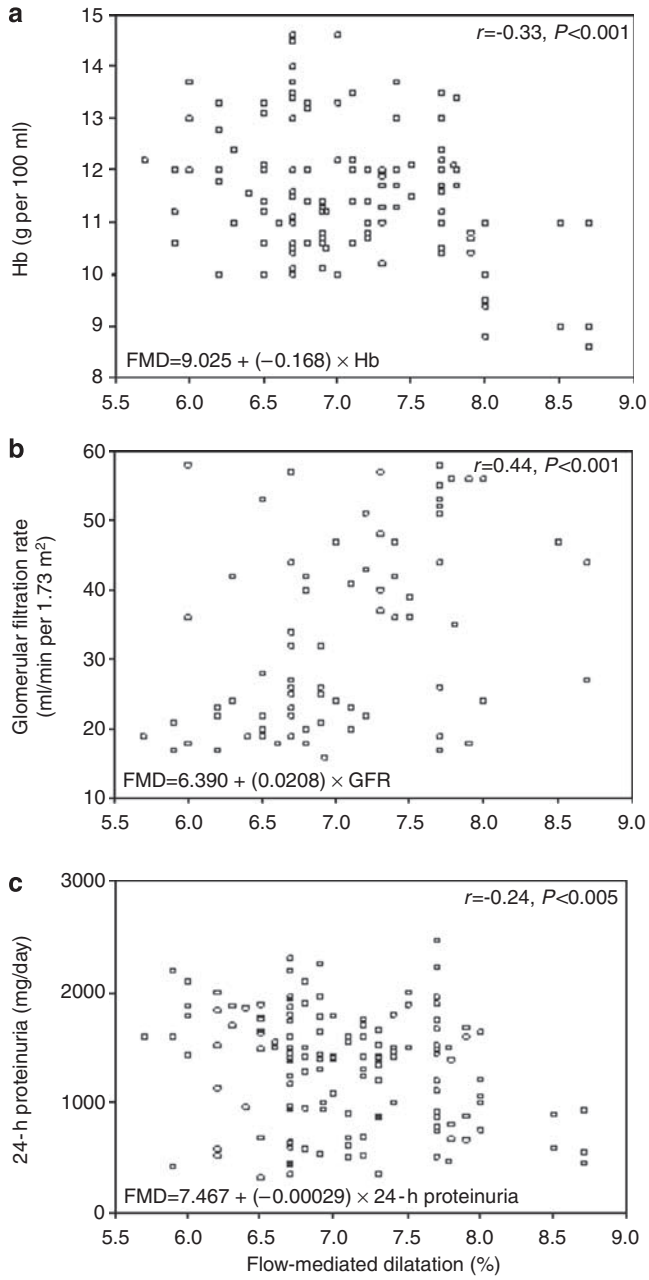

Figure 1 | The association between FMD and Hb, GFR or 24-h proteinuria in CKD 3 and 4 patients. (**a**) FMD and Hb, (**b**) FMD and GFR, (**c**) FMD and 24-h proteinuria.

Table 2 | Multiple regression models of FMD in CKD 3 and 4 patients

	Unadjusted (β, *P*-value)	Model 1 (β, *P*-value)	Model 2 (β, *P*-value)
Hemoglobin (g per 100 ml)	**−0.33 (<0.001)**	**−0.26 (0.002)**	**−0.28 (<0.001)**
Age (years)		0.049 (0.56)	−0.032 (0.77)
Sex		−0.012 (0.89)	0.034 (0.69)
BMI (kg/m^2)		−0.043 (0.61)	−0.043 (0.62)
SBP (mm Hg)		**−0.25 (0.002)**	**−0.37 (<0.001)**
LDL cholesterol (mg per 100 ml)		**−0.23 (0.003)**	**−0.16 (0.01)**
Glucose (mg per 100 ml)		−0.067 (0.44)	−0.07 (0.32)
HOMA index			**0.14 (0.03)**
hsCRP (mg/l)			0.068 (0.43)
GFR (ml/min per 1.73m^2)			**0.54 (<0.001)**
Proteinuria (g/24 h)			−0.16 (0.01)
Ca (mg per 100 ml)			0.051 (0.55)
P (mg per 100 ml)			−0.054 (0.53)
Uric acid (g per 100 ml)			0.128 (0.14)

BMI, body mass index; Ca, calcium; CKD, chronic kidney disease; GFR, glomerular filtration rate; HOMA, Homeostasis Model Assessment; P, phosphate; LDL, low-density lipoprotein; SBP, systolic blood pressure. Significant values highlighted in bold.

novel risk factor for endothelial dysfunction. These observations may have potential relevance for the interpretation of the excess risk caused by Hb normalization in CKD.

The median Hb concentration in this study (11.6 g per 100 ml) was similar to that in patients enrolled in the Cardiovascular risk reduction by early anemia treatment by epoetin beta study,[3] the largest randomized study performed so far in the CKD population with stage 3b–4 CKD. Crude categorical analysis based on this value showed that FMD was reduced in patients with Hb higher than the median value. Further analysis by regression techniques revealed that the best data fit of the Hb–FMD link was linear without any apparent threshold value, indicating that such a link is continuous over a Hb range between 8.5 and 14.5 g per 100 ml. It is of note that this association was unconfounded by age, gender, fasting hyperglycemia or insulin resistance, LDL cholesterol, smoking, arterial pressure, and inflammation. Neither GFR nor proteinuria or the diagnosis of renal disease or antihypertensive treatment affected the Hb–FMD link, which suggest that this phenomenon depends on a peculiar effect of Hb *per se* on endothelial function regulation rather than on confounding by renal disease severity and other risk factors. This hypothesis is in keeping with earlier studies reporting an even stronger Hb–endothelial function association in type II diabetics[12] and in essential hypertensives as well (Perticone F, Maio R, Mastroianni S, Greco L, Tripepi G, Mallamaci F, and Zoccali C. Hemoglobin and endothelium-dependent vasodilation in uncomplicated, untreated subjects with essential hypertension (TH-PO251). *J Am Soc Nephrol* 2008; **19**(Suppl):165A, Abstract). Remarkably, in both diabetic and essential hypertensive patients included in these studies, the inverse relationship between Hb and the vasodilatory response of the endothelium was evident over a range of Hb values narrower than that in this study (11 to 15–16 g per 100 ml). Furthermore, in the study by Natali *et al.*,[12] Hb was inversely related to endothelial function also in an analysis confined to normotensive subjects, which is again in line with our finding that adjustment for arterial pressure did not affect the Hb–FMD link in CKD patients.

Our study in non-diabetic CKD 3–4 patients is the largest performed so far exploring the Hb–endothelial function link in human diseases. The observation that progressively higher

Hb levels signal an increasing risk for endothelial dysfunction may depend on the peculiar role of Hb as a NO buffer/carrier and/or on other factors. Owing to NO oxidation,[13] nitrosylation of the Fe molecule, and linkage to sulfur amino acids in the globin molecule, Hb acts as a transient or permanent NO buffer, thus modulating NO bioavailability.[7,8] It is well demonstrated that NO liberated from the endothelium is taken up and transported by red blood cells as *S*-nitrosohemoglobin and in other chemical forms.[8] The *S*-nitrosohemoglobin binding is fully reversible and NO by *S*-nitrosylated Hb represents an important biological mechanism in the response to tissue hypoxia.[8] However, *S*-nitrosylated Hb releases NO when oxygen is extracted from arterial blood, a phenomenon occurring at the capillary level, that is, posterior to arterioles, which is the main site where vasoregulation is controlled. On the other hand, NO oxidation by Hb and the Fe–NO link are not reversible, thus affecting the vasodilatory potential of the NO molecule. It is of note that it was emphasized that oxidative stress—a pervasive phenomenon in CKD—may limit the release of NO from the Hb molecule.[8] Furthermore, NO bioavailability is compromised in patients with CKD,[14,15] which can make these patients more prone to any negative effect of NO buffering by Hb. Endothelial dysfunction in CKD is typically GFR and proteinuria-dependent, a phenomenon once again confirmed in this study. Even though NO buffering by Hb seems to be a cogent interpretation of our findings, other interpretations are possible. Reduction in Hb level results in the upregulation of eNOS, a phenomenon evident systemically and at renal level in a model of iron deficiency anemia in the rat.[16] Thus, it is possible that, rather than high Hb levels impair the endothelial response to vasodilatory stimuli, low Hb levels enhance the same response. As a third possibility, relatively higher Hb levels in CKD patients may represent a surrogate marker for diffuse endothelial dysfunction, which at the kidney level can stimulate erythropoietin production and hence increase Hb by promoting renal vasoconstriction. Even though this hypothesis cannot be excluded only on the basis of our cross-sectional data, neither the GFR level nor proteinuria appeared to be modifiers of the Hb–FMD relationship, suggesting that the Hb–endothelial function link does not depend on the fact that Hb is a mere marker of renal disease severity. Our findings are apparently in contrast with recent observations by London *et al.*[17] in end-stage renal disease, showing an improvement in endothelium-dependent vasodilatation in patients with moderate-to-severe degree of anemia (9.4 g per 100 ml on average) when Hb was increased to an average level of Hb coinciding with the upper limit of Hb target recommended by the guidelines (12 g per 100 ml). End-stage renal disease patients are a very high-risk population, and severe anemia in these patients is definitely a maladaptative phenomenon.[18] The improvement registered in London's paper conforms with early erythropoietin studies in end-stage renal disease patients showing an improvement in cardiovascular risk profile (as reflected by a marked reduction in left ventricular mass) when severe anemia was partially corrected (to reach an average 10 g per 100 ml value).[19–20] Patients in our study were low-risk CKD patients (non-diabetic, non-smokers, cardiovascular event-free), and the vast majority of them had Hb values in the 10–15 g per 100 ml range.

This study has limitations. On account of the cross-sectional nature, we cannot interpret the Hb—FMD link in causal terms. As alluded to above, endothelial dysfunction at kidney level may cause vasoconstriction and by this mechanism stimulate erythropoietin synthesis and increase the Hb level. Apart from our observations showing that the Hb–FMD link is largely independent of the severity of renal disease, studies in experimental models apparently do not support this interpretation. Indeed, the endothelial NO synthase null mouse, that is, an animal model with severe systemic endothelial dysfunction,[21] does not develop polycythemia, as one would expect if endothelial dysfunction at renal level triggers erythropoietin synthesis through local ischemia and hypoxia. Even though an effect of Hb *per se* on endothelial function *in vivo* in humans is fully compatible, the present cross-sectional data in CKD and with the earlier-mentioned observations in other conditions[12] (Perticone F, Maio R, Mastroianni S, Greco L, Tripepi G, Mallamaci F, and Zoccali C. Hemoglobin and endothelium-dependent vasodilation in uncomplicated, untreated subjects with essential hypertension (TH-PO251). *J Am Soc Nephrol* 2008; **19**(Suppl):165A, Abstract), it should be clearly recognized that the balance between NO synthesized by the endothelium and NO taken-up red blood cell has not been studied in detail in humans. Thus, our data in CKD patients as well as the earlier above-mentioned observations in type II diabetics and in essential hypertensive subjects should be considered as hypothesis generating rather than as hypothesis testing.

In conclusion, the results of this study in a well-selected series of non-diabetic CKD patients show that, independent from classical and emerging risk factors, higher Hb values are associated with reduced endothelium-dependent vasodilatation in this population. Whatever the explanation, the association of endothelial dysfunction with relatively higher Hb levels in patients with CKD is of potential relevance for the interpretation of the increased cardiovascular risk elicited by Hb normalization in CKD. Specifically designed mechanistic studies are warranted to clarify the nature of the endothelial function–Hb link in CKD.

MATERIALS AND METHODS

Patients

Between May 2005 and May 2008, a total number of 350 patients with CKD stage 3–4 were referred to the Nephrology outpatient clinics of Gulhane School of Medicine. By protocol, we aimed at testing endothelial function in non-diabetic, non-smoker patients without background cardiovascular complications (coronary artery disease, congestive heart failure, or peripheral vascular disease). From this group, we selected 201 non-diabetic patients as the candidates for the study. Patients who were being treated with angiotensin-converting enzyme inhibitors ($n = 33$), angiotensin receptor blockers ($n = 21$), statins ($n = 13$), vitamin D ($n = 10$), or supplemental vitamin pills ($n = 7$) at the time of the study were

excluded. No patient was on treatment with erythropoietin-stimulating agents. Finally, 141 non-diabetic patients, classified as Kidney Disease Outcomes Quality Initiative stage 3 ($n=70$) and stage 4 ($n=71$), were enrolled for this study. None had acute infections at the time of the study. Part of the data was published elsewhere.[22]

Patients were studied before starting pharmacological treatment aimed at preventing renal disease progression and complications of CKD. The etiologies of the patients were as follows: glomerulonephritis ($n=33$), hypertensive nephropathy ($n=31$), chronic pyelonephritis ($n=11$), reflux nephropathy ($n=6$), autosomal-polycystic kidney disease ($n=10$), and unknown ($n=50$). Of these patients, 16 were taking calcium channel blockers, 9 were taking α-blockers, 4 were taking diuretics. As alluded to above, owing to the peculiar referral pattern to Gulhane Medical Center preliminary nephrology consultation: is considered before specific drug prescription to patients with suspected renal diseases. Brachial artery endothelium-dependent vasodilatation (FMD) was studied. The Ethical Committee of Gulhane School of Medicine (Etlik-Ankara, Turkey) approved the study, and all patients gave their informed consent.

Laboratory measurements

Arterial blood pressure was measured by a physician three times after a 15-min resting period in the morning, and mean values were calculated for systolic and diastolic pressures for all subjects. All samples were obtained from patients in the morning after 12 h of fasting, for measurement of fasting plasma glucose (FPG), serum albumin, total serum cholesterol, triglycerides, high-density lipoprotein, LDL cholesterol, calcium, and phosphate. Total plasma cholesterol, triglycerides, and high-density lipoprotein cholesterol were measured by the enzymatic colorimetric method with Olympus AU 600 autoanalyzer using reagents from Olympus Diagnostics GmbH (Hamburg, Germany). LDL cholesterol was calculated by Friedewald's formula.[23] A 24-h urine collection was carried out three times, and the average of three 24-h proteinuria measurements was taken as the representative of each participant's 24-h protein excretion rate.

Patients were classified with respect to estimated glomerular filtration rate calculated according to the simplified version of the Modification of Diet in Renal Disease formula as defined by Levey *et al*:[24] [$\mathrm{GFR} = 186 \times \mathrm{Pcr}^{-1.154} \times \mathrm{age}^{-0.203} \times 1.212$ (if black) $\times 0.742$ (if female)].

The serum basal insulin value was determined by the coated tube method (DPC-USA). HOMA-IR was computed by the following formula:[25] HOMA-IR = FPG (mg per 100 ml) × immunoreactive insulin (μIU/ml)/405. Serum total calcium was measured by the Cresolphthalein complex-one method using Menagent Calcium 60 s kits (Menarini Diagnostics, Florence, Italy). Serum phosphorus was measured by the ammonia molybdate complex method using Menagent Phosphofix kits (Menarini Diagnostics). To measure hsCRP, serum samples were diluted at a ratio of 1:101 with the diluents solution. Calibrators, kit controls, and serum samples were all added on each microwell with an incubation period of 30 min. After three washing intervals, 100 μl enzyme conjugate (peroxidase-labeled anti-CRP) was added on each microwell for additional 15 min incubation in room temperature in dark. The reaction was stopped with a stop solution, and photometric measurement was performed at the 450 nm wavelength. The amount of serum samples was calculated as mg/l using a graph plotted by noting the absorbance levels of the calibrators.

Vascular assessment

Endothelium-dependent flow-mediated vasodilatation and endothelium-independent vasodilatation (NMD) of the brachial artery were assessed non-invasively, using high-resolution ultrasound as described by Celermajer *et al.*[26]. The method for the vascular assessment met the criteria that were mentioned by the International Brachial Artery Reactivity Task Force.[27]

Measurements performed made by a single observer using an ATL 5000 ultrasound system (Advanced Technology Laboratories Inc., Bothell, WA, USA) with a 12-MHz prob. All vasoactive medications were withheld for 24 h before the procedure. The subjects remained at rest in the supine position for at least 15 min before the examination started. Subject's arm was comfortably immobilized in the extended position to allow consistent recording of the brachial artery 2–4 cm above the antecubital fossa. Three adjacent measurements of end-diastolic brachial artery diameter were performed from single two-dimensional frames. All ultrasound images were recorded on an S-VHS videotape for subsequent blinded analysis. A pneumatic tourniquet was inflated to 300 mm Hg with obliteration of the radial pulse. After 5 min, the cuff was deflated. Flow measurements were performed 60 s after deflation. After a further 15 min, measurements were repeated, and again 3 min after the administration of sublingual glyceryl trinitrate (400 μg). The maximum FMD and NMD diameters were calculated as the average of the three consecutive maximum diameter measurements after hyperemia and nitroglycerin, respectively. The FMD and NMD were then calculated as the percent change in diameter compared with baseline resting diameters.

Statistical analysis

All the statistical analyses were performed by using SPSS 11.0 (SPSS Inc., Chicago, IL, USA) statistical package. Non-normally distributed variables were expressed as median (range) and normally distributed variables were expressed as mean ± s.d., as appropriate. A P-value <0.05 was considered to be statistically significant. Differences among the groups were analyzed by the Student t-test (continuous variables) and the χ^2 test (categorical variables). Standard correlation analysis was used for testing associations between paired variables. Finally, multiple regression analysis was applied to test the independent link between FMD and potential functional correlates of this variable. To this scope, we computed models of increasing complexity adjusting for traditional (Framingham risk factors) and emerging risk factors, as well as for the diagnosis of renal disease and anti-hypertensive medication type. Multivariate models included at least 10 observations for each covariate in the same models.[28]

DISCLOSURE

All the authors declared no competing interests.

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