Searching for biomarker patterns characterizing carotid atherosclerotic burden in patients with reduced renal function

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Abstract

Background. Evidence linking chronic kidney disease (CKD) with the increased risk of cardiovascular (CV) complications is firm. It is becoming clear that testing functions of the vascular endothelium in conjunction with various biomarkers may better the definition of CV risk profile in CKD patients.

Methods. Using Multiplexed analysis of 16 markers reflecting pro-inflammatory, anti-inflammatory, angiogenic, metabolic and anti-fibrinolytic factors in a series of 75 patients with CKD, who underwent echo-coloured Doppler study of carotid arteries, we analysed the contribution of each parameter to the characterization of atherosclerotic burden. As control group we enrolled 33 healthy individuals.

Results. On univariate analysis, intima-media thickness, number of atherosclerotic plaques and internal diameter of carotid arteries were strongly interrelated. Starting with these indicators of carotid atherosclerosis, we extracted by principal component analysis a single composite atherosclerosis severity score that accounted for 68% of the total variance of original variables. Biomarkers significantly related to severity of carotid atherosclerosis were MMP9, t-PAI-1, IL-6, NT-proBNP, IL-8 and VEGF. In multiple linear regression models adjusting for clinical variables the gain in prediction power was achieved when we added MMP9 and t-PAI-1 to the basic model. IL-6 produced the highest increase in the multiple R² of the basic model. The combination of MMP9, t-PAI-1, IL-6, NT-proBNP, IL-8 and VEGF added to the basic model achieved gain in prediction power of +7.5%. In this expanded model, IL-6 was the only biomarker that significantly predicted the severity of atherosclerosis.

Conclusion. Multiplexed screening revealed that IL-6, MMP-9, tPAI-1 and VEGF showed a tight correlation with carotid atherosclerotic burden in patients with CKD.

Keywords: atherosclerosis; chronic kidney disease; cytokines; IL-6; MMP-9; multiplexed analysis

Introduction

There is unequivocal evidence linking chronic kidney disease (CKD) with the increased risk of cardiovascular (CV) complications [1–6]. Large numbers of studies have been performed in the recent years that established correlations between CV risk and individual parameters characterizing endothelial dysfunction [7–12]. Taking into consideration the range and diversity of endothelial functions, it is becoming increasingly clear that testing various functions of the vascular endothelium and jointly measuring biomarkers of inflammation and oxidative stress may better the definition of CV risk profile in CKD patients. Several examples of the potential value of such a multifaceted testing including various markers of inflammation, oxidative stress and endothelial function have recently been presented [13–16].

Recent progress in high-throughput technological platforms makes it now possible to simultaneously detect multiple parameters in a minute amount of blood sample. One of such platforms, Luminex multiplex antibody-coated beads, meets this specification [17,18]. The existing CV panel allows exploring the individual risk profile by considering 16 markers reflecting pro-inflammatory, anti-inflammatory, angiogenic, metabolic and anti-fibrinolytic factors that are considered to be of critical importance in the pathogenesis of CV complications. In this survey we aimed at defining the association between biomarkers included in the Luminex platform with carotid...
atherosclerosis in a series of 75 patients with CKD. Herein we report the individual associations of Luminex biomarkers with carotid atherosclerosis as well as an exploratory analysis testing the association of these biomarkers considered in a combined way with the same outcome measure.

**Subjects and methods**

The study protocol was in conformity with the ethical guidelines of our institution, and informed consent was obtained from each participant.

**Patients and controls**

We studied 75 incident patients referred to our outpatient clinic because of CKD. Their mean age was 52 ± 16 years (43 M and 32 F) and their Glomerular Filtration Rate (GFR) (Modification of Diet in Renal Disease (MDRD) formula) ranged from 5 to 71 ml/min/1.73 m² [GFR (mean ± SD): 29 ± 13 ml/min/1.73 m²]. Thirteen patients had had major CV events (myocardial infarction and stroke) before the start of the study. The main demographic, somatometric, clinical and biochemical characteristics of patients included in the study are detailed in Table 1. The prevalence of diabetes mellitus in this group was 17% (i.e. 13 patients out of 75). Thirty-six patients were habitual smokers and 69 patients (92%) were on anti-hypertensive treatment. As control subjects we enrolled 33 healthy individuals (age: 50 ± 9 years; 18 M and 15 F) accurately matched to patients as for age and sex.

**Laboratory measurements**

Blood sampling was performed after 20-30 min of quiet resting in a semi-recumbent position. After an overnight fasting blood sample for serum lipids, creatinine, albumin, calcium and phosphate, haemoglobin was obtained from all patients. Plasma was stored at −80°C until analysis. GFR was estimated using the MDRD formula.

The multiplex human CV diseases biomarkers panels 1 and 3 were used (HCVD1-67AK, HCVD3-67CK, Linco Inc, St Louis, MO) for the simultaneous quantification of the following analytes: soluble E-Selectin (sE-Selectin), soluble ICAM (sICAM-1), Matrix Metalloproteinase-9 (MMP-9), Myeloperoxidase (MPO) and total Plasminogen Activator Inhibitor-1 (tPAI-1), Interleukin-8 (IL-8), Interleukin-10 (IL-10), gamma interferon cytokine (IFN-γ), Monocyte chemotactic protein-1 (MCP-1), tumour necrosis factor alpha (TNF-α), N-terminal prohormone brain natriuretic peptide (NT-proBNP), adiponectin (ADPN) and vascular endothelial growth factor (VEGF).

All examined analytes were tested individually and in combination to ensure that there were no cross-reactions. Briefly, the multibiomarkers and cytokines standards were resuspended in assay buffer and then differently serially diluted. Twenty-five microlitre of standard, Quality Controls or sample were added to each well of a 96-well plate with 25 μl of the bead solution. The plate was sealed, covered with aluminium foil and incubated overnight (16–18 h) with agitation on a plate shaker at 4°C. Then the plate was washed twice with 200 μl/well of wash buffer, removing buffer by vacuum filtration between each wash. This was followed by addition of 25 μl of a detection antibody cocktail into each well and incubation at room temperature for 1.5 h. Twenty-five μl of a streptavidin–phycoerythrin solution was added to each well and incubated at room temperature for 30 min. The plate was then analysed on the Lumine × IS100 analyzer (Luminex Inc, Austin TX). The data were saved and evaluated as Median Fluorescence Intensity (MFI) using appropriate curve-fitting software (Lumine × 100IS software version 2.3). A 5-parameter logistic method with weighting was used. All measurements were performed in duplicate.

**Eco-colour Doppler study**

In all patients ultrasonographic studies on common carotid arteries were performed bilaterally by a single observer that was blinded to the clinical and biochemical data. All studies were performed with a GE-VINGMED System five using a 10 MHz high resolution probe. Intima media thickness (IMT) was defined as a low level echo grey band which does not project into the arterial lumen and was measured during end-diastole as the distance from the leading edge of the second echogenic line of the far walls of the distal segment of the common carotid artery, the carotid bifurcation and the initial tract of internal carotid artery on both sides. Measurements were performed 0.5, 1 and 2 cm below and above the bifurcation (six measurements on each side) and the average measurement was taken as IMT. The internal diameter of the common carotid artery (DCCA) was measured bilaterally 2 cm below the bifurcation during end diastole and the average measurement was taken as DCCA. IMT and DCCA measurements were always performed in plaque-free arterial segments. The number of atherosclerotic plaques [either as faint grey echoes (soft plaques) or bright...
white echoes (calcified plaque) protruding into the lumen detected in the bulbar area (from 2 cm below to 2 cm above the bifurcation) of the carotid arteries was recorded on both sides and summed up.

Statistical analysis

Data are expressed as mean ± SD, median and inter-quartile range or as percent frequency and comparisons between two groups were made by t-test, Mann-Whitney U-test or chi-squared test, as appropriate. Variables that showed a positively skewed distribution were log transformed (lg10) before the correlation study. The association between the number of atherosclerotic plaques (an ordinal variable) with all risk factors listed in Table 1 and in Table 2 was analysed by Spearman rank correlation coefficient (\( r \)).

To investigate the association between carotid atherosclerosis and the series of risk factors listed in Tables 1 and 2 we calculated a composite score of atherosclerosis severity by using principal components analysis (PCA). PCA is a multivariate method for transforming a number of interrelated variables (in this instance: IMT, internal diameter of carotid arteries and total number of plaques) into a smaller number of uncorrelated variables (principal components) that retain, in all or in part, the same information of original variables. The variances of these components are called eigenvalues. By rescaling each of the three original indicators of carotid atherosclerosis in order to have zero mean and variance 1, the maximal possible eigenvalue in our study is 3.

Multiple regression analyses

We first considered univariate associations between traditional (age, sex, smoking, diabetes, previous myocardial infarction or stroke, cholesterol, triglycerides and arterial pressure) and factors peculiar to CKD (creatinine, GFR, haemoglobin, albumin and calcium \(^{2+}\) phosphate) with carotid atherosclerosis. A multiple regression model was then built on the number of atherosclerotic plaques to the basic model and calculated association of Linco biomarkers with the outcome measure (with all risk factors listed in Table 1 and in Table 2 was analysed by Spearman rank correlation coefficient (\( r \)).

Results

Echo colour Doppler study of the carotid arteries

In the whole study population, IMT (0.65 ± 0.14 mm) was above the upper limit of the normal range in our laboratory (cut-off: 0.8 mm) in 11 out of 75 CKD

| Table 2. Circulating levels of Linco biomarkers in patients and in controls |
|-----------------------------|-------------------|-----------------|---|
|                            | CKD patients \( n = 75 \) | Healthy subjects \( n = 33 \) | \( P \) |
| IL-10 (pg/ml)               | 1.3 (0.6–3.4)      | 0.6 (0.6–2.4)   | NS |
| NT-proBNP (pg/ml)           | 12.1 (11.5–40.0)   | 9.4 (3.2–19.8)  | 0.004 |
| VCAM (ng/ml)                | 1507 ± 303         | 780 ± 176       | <0.001 |
| ICAM (ng/ml)                | 223 (184–258)      | 120 (87–144)    | <0.001 |
| MMP9 (ng/ml)                | 117 (82–192)       | 71 (50–108)     | <0.001 |
| MPO (pg/ml)                 | 49 (25–90)         | 44 (27–64)      | NS |
| ADNP (ng/ml)                | 18.6 (14.3–27.9)   | 22.4 (17.7–25.0) | NS |
| t-PAI-1 (pg/ml)             | 29.3 ± 13.6        | 19.9 ± 9.0      | <0.001 |
| IL-1β (pg/ml)               | 0.6 (0.6–0.6)      | 0.6 (0.6–0.6)   | NS |
| IL-6 (pg/ml)                | 0.7 (0.7–2.6)      | 0.6 (0.6–0.6)   | <0.001 |
| IL-8 (pg/ml)                | 7.4 (3.1–11.5)     | 0.6 (0.6–1.4)   | <0.001 |
| IFN-γ (pg/ml)               | 0.6 (0.6–0.6)      | 0.6 (0.6–0.6)   | NS |
| TNF-α (pg/ml)               | 11.4 ± 5.3         | 1.1 ± 0.8       | <0.001 |
| MCP1 (pg/ml)                | 168.7 ± 84.9       | 129.8 ± 103.6   | <0.001 |
| VEGF (pg/ml)                | 65.5 (46.2–105.4)  | 30.1 (8.0–83.8) | 0.005 |
| E-Selectin (ng/ml)          | 33.4 ± 10.7        | 20.2 ± 6.5      | <0.001 |

Data are expressed as mean±SD or as median and inter-quartile range, as appropriate.

patients (i.e. 15%). Thirty-nine patients had no plaques while 36 had at least one plaque (1–2 plaques in 20 cases, 3–4 plaques in 8 cases and 5 plaques in the remaining 8 cases). The internal diameter of carotid arteries was on average 6.6 ± 0.8 mm. On univariate analysis, IMT, the number of atherosclerotic plaques and the internal diameter of carotid arteries were strongly interrelated (\( r \) ranging from 0.54 to 0.68, \( P < 0.001 \)). Starting with these three indicators of carotid atherosclerosis, we extracted by PCA a single composite atherosclerosis severity score (eigenvalue: 2.05) that accounted for 68% of the total variance of original variables considered in a three-dimensional space (i.e. 2.05/3.00 = 0.68 or 68%). The relative contribution of IMT to the principal component of atherosclerosis severity (45%) was higher than that of internal DCCA (30%) and total number of plaques (25%).

Lincoplex biomarkers in patients in and controls

All Lincoplex biomarkers, except IL-10, MPO, ADNP, IL-1β and IFN-γ, were significantly higher in CKD patients than in healthy subjects (Table 2).

Lincoplex biomarkers and indicators of carotid atherosclerosis (Table 3): univariate and multiple regression analysis

As reported in Table 3, the atherosclerosis score was strongly and directly related to NT-proBNP (\( r = 0.41, P < 0.001 \)), IL-8 (\( r = 0.40, P < 0.001 \)), MMP9 (\( r = 0.37, P < 0.001 \)), t-PAI-1 (\( r = 0.35, P = 0.002 \)), VEGF (\( r = 0.34, P = 0.003 \)) and IL-6 (\( r = 0.33, P = 0.004 \)). Associations of similar strength were found between these biomarkers and each of the three indicators of carotid atherosclerosis considered individually (Table 3).
Among factors listed in Table 1, only age \((r = 0.76, P < 0.001)\), male sex \((r = 0.46, P < 0.001)\), systolic blood pressure \((r = 0.38, P < 0.001)\), smoking \((r = 0.37, P = 0.002)\), serum glucose \((r = 0.29, P = 0.01)\) and previous myocardial infarction or stroke \((r = 0.27, P = 0.02)\) were significantly associated with the severity of carotid atherosclerosis at univariate analysis. When these risk factors were jointly included into the same multiple regression model (basic model) they explained 63.6\% of the total variance in the atherosclerosis severity score.

The independent association of NT-proBNP, MMP9, t-PAI-1, IL-6, IL-8 and VEGF (i.e. the Linco biomarkers significantly related to carotid atherosclerosis at univariate analysis) with the severity of carotid atherosclerosis was tested in separate multiple linear regression models (i.e. one model for each bio-marker) adjusting for age, sex, smoking, previous myocardial infarction or stroke, systolic pressure and serum glucose (the basic model variables). In this analysis the additional predictive power for atherosclerosis of Linco biomarkers was estimated by calculating the percent gain in the multiple \(R^2\) of the basic model. As shown in Figure 1 the addition of NT-proBNP, IL-8 and VEGF produced a small and not significant increase in the prediction power of the basic model (+0.6\%, +1.3\% and +1.9\%, respectively; \(P = \text{NS}\)). The gain in prediction power was more marked when we added MMP9 and t-PAI-1 to the basic model, these two variables adding an independent predictive power +2.5\% and +2.6\%, respectively \((P = 0.03)\). Of note, IL-6 resulted to be the biomarker that produced the highest increase in the multiple \(R^2\) of the basic model (+5.3\%, \(P = 0.001\)), a figure from 1.5 to 2.0 times higher than those of remaining biomarkers (Figure 1). When NT-proBNP, MMP9, t-PAI-1, IL-6, IL-8 and VEGF were jointly added to the basic model the achieved gain in prediction power was +7.5\%. In this expanded model, IL-6 was the only biomarker that significantly predicted the severity of atherosclerosis \((\beta = 0.20, P = 0.01)\).

**Discussion**

Among different parameters characterizing arterial wall changes and atherosclerotic plaque burden in carotid arteries of CKD patients, IL-6 and MMP-9 featured prominently as they not only showed correlation with ultrasonography-derived parameters of coronary arteries, but also contributed to the independent predictive power of the multivariate model of atherosclerosis severity. IL-6, a major mediator of inflammation, was the only biomarker that significantly predicted the severity of atherosclerosis in the expanded model. This finding is strikingly similar to the observation that IL-6 showed the highest consistency and independent predictive value, at 5 and 12 years of follow-up, in a population cohort (Edinburgh...
Artery Study) as a predictor of atherosclerosis [13]. Similar conclusion was reached in the Augsburg case-cohort study, 1984–2002 [19]. Similarly, IL-6 was found to be an independent predictor of severity of carotid atherosclerosis in 392 volunteers during a 12-year follow-up [20] in the Rancho Bernardo Study. In dialysis cohorts IL-6 appears to be the strongest inflammation marker predicting death and CV complications [12,21]. This apparently crucial role of IL-6 may be related to its ability to up-regulate expression of other pro-inflammatory mediators (e.g. E-selectin, ICAM-1, VCAM-1, CRP) [22]. Thus, our data obtained in patients with CKD are in agreement with results obtained in the general population and in patients with end-stage renal disease.

Our results related to the role of MMP-9 are equally cohesive with the existing experimental and clinical data. Experimental studies in apolipoprotein E-deficient mice show that inflammation and atherosclerosis may be prevented by knocking-out the MMP9 gene [23]. In humans, MMP9 is a well-recognized marker of plaque vulnerability [24] and of atherosclerosis burden [25] and is seen as the strongest matrix metalloproteinase that predicts rapid coronary artery narrowing, incidence of ischaemic heart disease and worse outcome in patients with stroke and CV death [26], a hypothesis in keeping without data in CKD patients.

Elevated levels of plasminogen activator inhibitor-1 signals an increased risk for ischaemic and thrombotic CV events and underlie progression of vascular disease [27]. The finding that VEGF is linked to atherogenesis in our cohort of CKD patients is a novel finding suggesting that this growth factor may play a role in atherosclerosis in these patients. In ApoE knockout mice, VEGF was found to induce plaque expansion [28]. VEGF levels are elevated in hypercholesterolaemic rabbits and in humans with hypercholesterolaemia [29,30]. Oxidative stress is a fundamental pathway in the pathogenesis of atherosclerosis. However, in our study MPO did not correlate with the measured parameters of atherosclerotic burden. Thus markers of oxidative stress other than MPO need to be considered in clinical studies investigating atherosclerosis in CKD.

While several parameters of the Luminex CV panel showed strong correlations with individual parameters of ultrasonography of carotid artery and atherosclerotic burden, as discussed above, others displayed a surprising lack of correlations. The latter was observed for IL-1β, IL-10, IFN-γ, TNF-α, sVCAM, sE-selectin, sICAM-1, adiponectin and MCP-1, all shown to be of diagnostic value in the general population. It is obvious, therefore, that the CV Luminex panel exhibits redundancy and will require further refined selection for the purposes of CKD. Furthermore, the lack of significant differences in circulating levels of IL-10, MPO, ADPN, IL-1β and IFN-γ between patients and controls (Table 2) in our study may depend on the relatively low number of controls and for this reason these differences still remain to be tested in a specifically designed, well-powered, case-control study.

In conclusion, Multiplexed screening of pro-inflammatory, anti-inflammatory, angiogenic, metabolic and anti-fibrinolytic factors revealed that IL-6, MMP-9, tPAI-1 and VEGF showed tight correlation with carotid atherosclerotic burden in patients with CKD. Whether the longitudinal profile of these parameters in this same cohort provides additional information on the predictive power of these biomarkers for the progression of atherosclerosis remains a clue question to be tested in future studies.

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Conflict of interest statement. None declared.

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