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Reduced glomerular filtration rate, inflammation and HDL cholesterol as main determinants of superoxide production in non-dialysis chronic kidney disease patients

MARION MORENA[1,2,3], LAURE PATRIER[1,3,4], ISABELLE JAUSSENT[5], ANNE-SOPHIE BARGNOUX[1,3], ANNE-MARIE DUPUY[1], STÉPHANIE BADIOU[1,3], HÉLÈNE LERAY-MORAGUES[4], KADA KLOUCHE[3,6], BERNARD CANAUD[2,3,4], & JEAN-PAUL CRISTOL[1,3]

[1]*Laboratoire de Biochimie, CHRU Montpellier, F-34000 France; Univ Montpellier 1, Montpellier, F-34000 France* [2]*Institut de Recherche et de Formation en Dialyse, CHRU Montpellier, F-34000 France* [3]*UMR 204, Nutripass, F-34000 France,* [4]*Service de Néphrologie-Hémodialyse et Soins Intensifs, CHRU, Montpellier, F-34000 France; Univ Montpellier 1, Montpellier, F-34000 France,* [5]*INSERM, U1061, Montpellier, F-34093 France; Univ Montpellier 1, Montpellier, F-34000 France, and* [6]*Service de Réanimation Métabolique, CHRU Montpellier, F-34000 France; Univ Montpellier 1, Montpellier, F-34000 France*

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Abstract

Enhanced oxidative stress partly resulting from an over-production of superoxide anion ($O_2^{\bullet-}$) represents a novel and particular risk factor in chronic kidney disease (CKD) patients. This study was therefore designed to evaluate $O_2^{\bullet-}$ determinants in this population. $O_2^{\bullet-}$ production was evaluated using chemiluminescence method in 136 CKD patients (79M/57F, median age: 69.5 [27.4–94.6]). Renal function (evaluated by the glomerular filtration rate using modification of diet in renal disease (MDRD)), inflammation, lipids, nutritional and bone mineral as well as clinical parameters were evaluated. Potential relationships between $O_2^{\bullet-}$ and these clinico-biological parameters were investigated to identify main determinants of such a pathological process. Enhanced $O_2^{\bullet-}$ production has been observed at the pre-dialysis phase: stages 4 and 5 of CKD ($p = 0.0065$). In multivariate analysis, low eGFR (MDRD <30 mL/min/1.73 m^2; $p = 0.046$), high fibrinogen (≥3.7 g/L; $p = 0.044$) and abnormal HDL cholesterol (<1.42 mmol/L and ≥ 1.75 mmol/L; $p = 0.042$) were the main determinants of $O_2^{\bullet-}$ production in CKD patients.

Keywords: *Chronic renal disease, superoxide anion, fibrinogen, lipids.*

Introduction

Oxidative stress results from excessive oxidant production and/or impairment in antioxidant defense mechanisms and induces chemical alterations of biomolecules leading to structural and functional cell and tissue alterations. Existence of an oxidative stress, as attested by the presence of plasma lipid, protein and DNA oxidation markers, has been established in patients with chronic kidney disease (CKD) [1–6]. Initially thought to be related mainly to the dialysis process, an increasing body of evidence attests the multifactorial origin of oxidative stress among which uremia *per se* and inflammation are largely incriminated in its genesis. Uremia-associated metabolic abnormalities include complex alterations of oxidant production [7]. In a CKD patient population, polymorphonuclear neutrophils are in a 'primed' state for superoxide ($O_2^{\bullet-}$) anion production [8,9]. Accumulation of uremic toxins, particularly advanced glycation end products [10] and homocysteine [11], may play a major role in this leukocyte activation. Homocysteine, combined with inflammation, is indeed a major determinant of $O_2^{\bullet-}$ production as we previously reported in a large population of elderly patients

Correspondence: Jean-Paul Cristol, Laboratoire de Biochimie, CHU Lapeyronie, 371 Avenue du Doyen Gaston Giraud, 34295 Montpellier cedex 5, France. Tel: +33 467338314. Fax: +33 467338393. Email: jp-cristol@chu-montpellier.fr

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[12]. The chronic micro-inflammatory process that occured in uremic patients participates also to this oxidant over-production through the up-regulation of NADPH oxidase complex, the major source of $O_2^{\bullet-}$ production [13]. In addition to the effect of inflammation on oxygen radicals, it is noteworthy that oxidants modulate inflammatory mediator release through activation of transcription factors including NFκB, AP-1 and hypoxia inducible factor [14,15], leading to amplification loops between oxidative stress and inflammation. Thus, oxidative stress may exhibit an ambivalent role, as an 'effector' (by oxidant release) but also as a 'modulator' (in regulating transcription factors) of a CKD-dependent chronic micro-inflammatory process [16]. Nevertheless, the implication of oxidative stress as a pathogenesis cofactor in the development of long-term complications including vascular diseases, anaemia, malnutrition and amylosis observed in this population is well recognized, but its determinants remain under scrutiny [17].

We evaluated in this study the relationships between CKD, $O_2^{\bullet-}$ and CKD-related clinical and metabolic disorders (including not only inflammation and hyperhomocysteinemia but also malnutrition, disturbance in bone and mineral metabolism and dyslipidemia). Our purpose was to identify main determinants of an oxidant over-production in a population of non-dialysis CKD patients.

Methods

Patients

One hundred and thirty-six non-dependent dialysis CKD patients (at various stages of kidney disease), issued from the outpatient general nephrology consultation of the Lapeyronie university hospital, entered this cross-sectional study. All were of caucasian origin. Inclusion criteria were age >18 years old and presence of CKD defined by glomerular filtration rate in agreement with the National Kidney Foundation [18]. The study was conducted according to the principles of the Declaration of Helsinki and in compliance with International Conference on Harmonization/Good Clinical Practice regulations. According to the French Law, the study has been registered at 'Ministère de l'Enseignement Supérieur et de la Recherche' after approval by our institution ethical committee with the following number: DC–2008–417.

CKD causes were as follows: glomerulonephritis ($n = 20$), cystic renal disease ($n = 11$), diabetic nephropathy ($n = 24$), angiosclerosis and hypertensive nephropathy ($n = 58$), infectious/obstructive interstitial nephropathy ($n = 4$), renal neoplasia ($n = 1$), necrotizing angiitis ($n = 1$), other cause ($n = 13$) and unknown cause ($n = 4$).

Detailed medical history including age, gender, weight, height, waist-to-hip ratio (defined as the waist girth/hip girth ratio), diabetes mellitus, hypertension, past or current smoking and current medication was recorded.

Existence of hypertension was defined by brachial blood pressure higher or equal to 130/80 mmHg and/or by a current anti-hypertensive treatment. Pulse pressure (PP), as an index of aortic stiffness, was defined by the difference between systolic and diastolic blood pressure [19]. All elements of clinical examination were also recorded.

Fifty healthy normal subjects, free of kidney damage (including absence of abnormalities in the composition of blood or urine or absence of abnormalities in imaging tests) and with a GFR >60 mL/min/1.73 m^2 for $\geq$ 3 months were chosen to serve as a control group.

Laboratory measurements

Blood samples were collected as part of our regular CKD patient follow-up, centrifuged and supernatant was processed for the following parameters: creatinine, urea, high sensitive C reactive protein (CRP), fibrinogen, alpha-1 acid glycoprotein, ceruloplasmin, homocysteine, total cholesterol, LDL and HDL cholesterol, triglycerides, plasma vitamin E, albumin, transthyretin, calcium, phosphate, intact parathormone (PTH), $1.25(OH)_2$ vitamin D, haemoglobin, iron and ferritin. In addition, from these same blood samples, an aliquot of whole blood was immediately prepared after collection in order to perform $O_2^{\bullet-}$ production.

Biological parameters in plasma or serum

Plasma creatinine was measured by enzymatic method (Olympus apparatus, Rungis, France) using reagents from Randox (Mauguio, France) and previously validated with the Roche enzymatic method [20]. Plasma urea was also evaluated by enzymatic method (Olympus apparatus). Plasma CRP was determined by immunoturbidimetry method (Olympus apparatus) using reagents from Randox (Randox). Fibrinogen was measured by Von Clauss method (STA Fibrinogen, Diagnostica Stago, Asnières, France). Serum albumin, transthyretin, alpha-1 acid glycoprotein and ceruloplasmin were measured by nephelometry technique (Immage Beckman Coulter, Villepinte, France). Homocysteine was measured by using a commercial Recipe high performance liquid chromatography (HPLC) analytical kit (Recipe Chemicals & Instruments, Munich, Germany). Triglycerides (TG), total cholesterol (TC) and HDL cholesterol (HDL) levels were measured by enzymatic method (Konelab DPC France, La Garenne Colombes, France). The LDL cholesterol (LDL) rate was calculated by the Friedwald's formula: [LDL] = [TC] - ([TG]/5) - [HDL]. Plasma calcium, phosphate and iron were assessed by

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colourimetric method (Olympus apparatus). Plasma vitamin E concentrations were measured by HPLC using Waters-chromatography (Millipore Waters, Les Ulisses, France). Intact PTH was measured by immunoradiometric assay (Elsa-PTH, Cis Bio International, Gif sur Yvette, France). 1.25$(OH)_2$ vitamin D was measured by radioimmunoassay (Immunodiagnostic Systems, Bolton, UK). Haemoglobin was determined by photometry (ABX Pentra, Montpellier, France). Ferritin was evaluated using immunoenzymometric assay with chemiluminescence detection (UniCel DxI 800, Beckman Coulter, Villepinte, France).

Glomerular filtration rate (GFR) was estimated (eGFR) using the re-expressed 4-variable Modification of Diet in Renal Disease (MDRD) study equation (eGFR $= 175 \times$ standardized $S_{cr}^{-1.154} \times$ age$^{-0.203}$ $\times$ 1.212 [if black] $\times$ 0.742 [if female]) [21].

Determination of $O_2^{\bullet-}$ anion production by whole blood

$O_2^{\bullet-}$ production was determined in 200 µL of fresh whole blood (treated immediately after collection) diluted in 820 µl of DMEM medium and 200 µL of lucigenin (1.5×10^{-4} mol/L) (Sigma Chemical, Saint Quentin Fallavier, France) [12]. After a 20-min incubation at 37°C under gentle agitation, whole blood was stimulated by using phorbol 12-myristate 13-acetate (PMA) (a specific activator of protein kinase C, thus leading to activation of NADPH oxidase) (10^{-7} M) and the luminescence was immediately recorded at 37°C by means of a Victor² Wallac luminometer (Perkin Elmer, Turku, Finland). Luminescence intensity was normalized to leukocyte count. Response of PMA-free whole blood (basal $O_2^{\bullet-}$ production) incubated simultaneously was used as control and considered as equal to 100%.

To rule out auto-production of $O_2^{\bullet-}$ by lucigenin or by plasma compounds, $O_2^{\bullet-}$ production was determined in whole blood, de-leukocyted blood, plasma and culture medium. As expected, these preliminary experiments confirmed an enhanced $O_2^{\bullet-}$ production in whole blood (181 $\pm$ 10%; $p = 0.001$), whereas using de-leukocyted blood, plasma or culture medium did not result in any $O_2^{\bullet-}$ production.

Imprecision studies of $O_2^{\bullet-}$ production measure were as follows: intra-assay CV = 3.5% (basal $O_2^{\bullet-}$ production) and 3.9% (PMA-stimulated $O_2^{\bullet-}$ production); inter-assay CV = 5.0% (basal $O_2^{\bullet-}$ production) and 9.7% (PMA-stimulated $O_2^{\bullet-}$ production).

Finally, in order to evaluate the variability of $O_2^{\bullet-}$ production measure, determination was repeated at 1-year period of time on a sub-group of these CKD patients ($n = 60$). Analysis of sequential samples using Wilcoxon signed-rank test could not evidence any significant difference in $O_2^{\bullet-}$ production ($p = 0.37$). Inflammatory parameters including CRP were not significantly changed as well between the two periods ($p = 0.99$).

Statistical analyses

The sample was described using percentages for categorical variables and median and range for quantitative variables as their distributions were tested with the Shapiro-Wilk statistic and were skewed.

Distribution of some continuous variables were compared according to renal function (MDRD ≥ 60, [60–30] and < 30 mL/min/1.73 m^2) using Kruskall Wallis and Mann-Whitney non-parametric tests with Bonferroni post-hoc test.

To study differences in $O_2^{\bullet-}$ production between healthy control subjects and CKD patients, a multinomial logistic regression (with adjustment for age and sex and further Bonferroni correction) was performed.

In order to evaluate the relationships between clinical characteristics or biological parameters and oxidative stress ($O_2^{\bullet-}$ production level), patients were divided into two groups, those with a low $O_2^{\bullet-}$ production level (<203.358% corresponding to the 1st and 2nd tertiles, as no significant association between variables—except for ceruloplasmin and ferritin—and superoxide production was observed when comparing 1th and 2nd tertiles) and those with a high $O_2^{\bullet-}$ production level ($\geq$203.358% corresponding to the 3rd tertile).

In a first step, a univariate analysis was performed to determine differences in unadjusted clinical characteristics or biological parameters between patients with and without high $O_2^{\bullet-}$ production level. Odds ratios (OR) and 95% confidence intervals (CI) were obtained for each potential characteristic or biological parameter (divided into tertiles or clinical cut-offs). In a second step, multivariate logistic regression analysis was performed with all variables with p-values ≤ 0.1 in the univariate analysis as potential candidates.

Significance of variables was set at $p < 0.05$. All analyses were carried out with SAS software, version 9.1 (SAS Institute, Cary, NC) and STATA software, version 9.2 (Statacorp, 2007).

Results

Characteristics of the population

Clinical and biological characteristics for the 136 CKD patients are summarized in Table I.

Sex ratio of the patients was 79/57 (male/female), median age was 69.5 with a range of 27.4–94.6 years old.

Seventy (51.5%) patients had ever smoked. Diabetes mellitus and hypertension were found in 35 (25.7%) and 124 (91.2%) patients, respectively. One hundred and twenty-two (89.7%) patients received anti-hypertensive drugs including betablockers ($n = 64$; 47.1%), angiotensin receptor blockers and angiotensin converting enzyme inhibitors ($n = 102$; 75.0%). Fifty-five (40.4%)

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Table I. Characteristics of chronic kidney disease patients.

Variable	n	%	Median [min–max]
Gender, male	79	58.1	
Age, years	136		69.5 [27.4–94.6]
BMI, kg/m^2			
< 25	60	44.1	
25–30	52	38.2	
≥ 30	24	17.7	
Smoking habits	70	51.5	
Diabetes mellitus	35	25.7	
Hypertension	124	91.2	
Pulse pressure (mmHg)	136		62.25 [33.00–124.5]
$O_2^{\bullet-}$ production (% activation)	136		181.14 [100.00–586.97]
Homocysteine (µmol/L)	136		20.95 [8.80–75.30]
CRP (mg/L)	136		2.17 [0.20–42.00]
Fibrinogen (g/L)	136		4.00 [2.10–7.80]
alpha-1 acid glycoprotein (g/L)	136		0.95 [0.43–1.89]
Total cholesterol (mmol/L)	136		5.34 [3.13–9.16]
HDL cholesterol (mmol/L)	136		1.54 [0.73–3.37]
LDL cholesterol (mmol/L)	136		2.93 [1.12–6.46]
Triglycerides (mmol/L)	136		1.36 [0.36–4.93]
Albumin (g/L)	136		40.9 [27.4–58.0]
Plasma creatinine (µmol/L)	136		172.0 [60.0–666.0]
MDRD (mL/min/1.73 m^2)	136		32.45 [6.48–91.93]
Plasma iron (µmol/L)	136		14.5 [4.9–35.0]
Ferritin (ng/mL)	136		141.5 [26.0–1229.0]
Haemoglobin (g/dL)	136		13.3 [8.9–16.9]
Ceruloplasmin (g/L)	134		0.34 [0.18–1.04]

Values were described by using proportions for categorical variables and median [minimum–maximum] for quantitative variables.

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patients received statin therapy. Sixteen (11.8%) patients received fibrates. Erythropoiesis stimulating agents were administered to 25 (18.4%) patients. Eighty-seven (64.0%) patients received vitamin D supplementation and finally phosphate binders were administered to 31 (22.8%) patients.

Low eGFR is associated with an enhancement in $O_2^{\bullet-}$ production, fibrinogen and homocysteine

As shown in Figure 1, low eGFR was associated with an increased $O_2^{\bullet-}$ production beginning from stages 4 and 5 of CKD ($p = 0.0065$) (MDRD < 30 mL/min/1.73 m^2 vs MDRD ≥ 30 mL/min/1.73 m^2, $p = 0.0017$; MDRD [30–60] mL/min/1.73 m^2 vs MDRD < 30 mL/min/1.73 m^2, $p = 0.0126$). As shown in Table II, no significant difference ($p = 0.99$) between control subjects and CKD patients with high GFR (MDRD ≥ 30 mL/min/1.73 m^2) was observed while a significant difference ($p = 0.027$) between control subjects and CKD with lower GFR (MDRD < 30 mL/min/1.73 m^2) was reported.

Among biomarkers of inflammation, only fibrinogen significantly changed according to the stage of CKD. An enhancement in fibrinogen activity was observed with higher stages (4 and 5) of CKD ($p = 0.0075$; MDRD < 30 mL/min/1.73 m^2 vs MDRD ≥ 30 mL/min/1.73 m^2, $p = 0.0018$; MDRD [30–60] mL/min/1.73 m^2 vs MDRD < 30 mL/min/1.73 m^2, $p = 0.0105$). In contrast, CRP, as a marker of acute inflammation with earlier kinetics, was not significantly modified according to CKD stages.

A similar pattern was observed with homocysteine level ($p < 0.001$; MDRD ≥ 60 mL/min/1.73 m^2 vs MDRD [30–60] mL/min/1.73 m^2, $p < 0.003$).

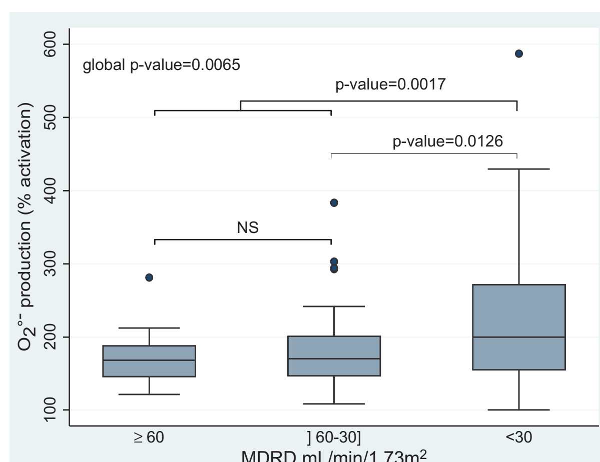

Figure 1. Influence of glomerular filtration rate on whole blood $O_2^{\bullet-}$ production in 136 patients with chronic kidney disease. Patients were divided into tertiles of MDRD (≥ 60, 60–30 and < 30 mL/min/1.73 m^2) and $O_2^{\bullet-}$ production evaluated. Enhanced $O_2^{\bullet-}$ production is already present at the pre-dialysis phase (stage 4 and 5) of CKD ($p = 0.0065$) (MDRD < 30 mL/min/1.73 m^2 vs MDRD ≥ 30 mL/min/1.73 m^2: $p = 0.0017$, MDRD 60–30 mL/min/1.73 m^2 vs MDRD < 30 mL/min/1.73 m^2: $p = 0.0126$) (NS = Not significant).

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Table II. Comparisons of $O_2^{\bullet-}$ production between normal control subjects (n = 50) and CKD patients (n = 136).

	Controls (n = 50)		CKD MDRD ≥30 mL/min/1.73m² (n = 72)		CKD MDRD <30 mL/min/1.73m² (n = 64)			Comparisons between groups		
	n	%	n	%	n	%	global p-value	MDRD ≥30 vs Controls	MDRD <30 vs Controls	MDRD <30 vs MDRD ≥30
MDRD	75.9 [60.0–151.8]		43.5 [30.4–91.8]		20.1 [6.5–29.8]		<0.0001	<0.0003	<0.0003	<0.0003
$O_2^{\bullet-}$ production (%)										
<160	18	36.00	25	34.72	18	28.13	0.0026*	0.9999	0.027	0.0141
160–200	21	42.00	28	38.89	12	18.75				
≥200	11	22.00	19	26.39	34	53.13				

*Adjustment for age and gender
Bonferroni Correction done

Kidney disease, fibrinogen and HDL cholesterol are the major determinants of $O_2^{\bullet-}$ production in CKD patients

On univariate analysis, none of the clinical variables was associated with an increased $O_2^{\bullet-}$ production (see Table III).

Regarding biological variables, as shown in Table IV, kidney dysfunction (plasma creatinine ≥204 µmol/L: OR = 5.05 [1.93–13.20], p = 0.003; and MDRD <30 mL/min/1.73 m²: OR = 5.64 [1.17–27.23], p = 0.0037) was significantly associated with an increased $O_2^{\bullet-}$ production. Among the studied inflammatory parameters, a high fibrinogen (≥3.7 g/L) was the only one to be associated with an increase in $O_2^{\bullet-}$ production (OR = 3.40 [1.23–9.42] and 3.67 [1.38–9.77] in the 2nd and 3rd tertiles, respectively; p = 0.024). An association was also found between high PTH level (≥72 pg/mL) and high $O_2^{\bullet-}$ production (OR = 2.18 [1.04–4.58]; p = 0.039). None of the other calcium phosphate metabolism parameters was associated with $O_2^{\bullet-}$ production. Low (<1.42 mmol/L) and high (≥1.75 mmol/L) HDL cholesterol levels were associated with high $O_2^{\bullet-}$ production (OR = 2.89 [1.10–7.62] vs 3.46 [1.32–9.06] in HDL cholesterol <1.42 and ≥1.75 mmol/L, respectively; p = 0.03), drawing a U-shaped curve. Low haemoglobin level (<12.6 g/dL) was also significantly associated with an increased $O_2^{\bullet-}$ production (OR = 2.77 [1.13–6.81], p = 0.0256). Lastly, no significant association was observed between $O_2^{\bullet-}$ production and all other parameters including homocysteine, nutritional markers or iron status.

Multivariate analysis was performed with all variables with p-values ≤ 0.1 in order not to fail the identification of variables known to be important [22]. The variables retained in the model were renal function, fibrinogen, PTH, HDL cholesterol and haemoglobin. MDRD (instead of creatinine plasma level) has been used as a determinant of renal function. Stages 1, 2 and 3 were pooled vs stages 4 and 5. In the same manner, 1st and 2nd tertiles of fibrinogen were pooled vs 3rd tertile. As shown in Table V, MDRD (p = 0.046), fibrinogen (p = 0.044) and HDL cholesterol (p = 0.042) remained independently associated with $O_2^{\bullet-}$ production. An association between HDL cholesterol and $O_2^{\bullet-}$ production, as a U-shaped curve, was still observed. No independent association between PTH or haemoglobin and $O_2^{\bullet-}$ production was observed.

Discussion

Our study provided new insights and comprehensive elements about the complex relationships between kidney dysfunction, $O_2^{\bullet-}$ production and CKD-related clinical and metabolic disorders. Indeed, our results showed that low GFR (stages 4 and 5 of CKD) was accompanied by an increase in $O_2^{\bullet-}$ production,

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Table III. Associations between clinical parameters and $O_2^{\bullet-}$ production in 136 CKD patients.

	$O_2^{\bullet-}$ production (% activation)					
	< 203.358 (*n* = 90)		≥ 203.358 (*n* = 46)			
Variable	*n*	%	*n*	%	OR [CI 95%]	*p*-value*
Gender						
Male	53	58.89	26	56.52	1	0.79
Female	37	41.11	20	43.48	1.10 [0.54–2.26]	
Median age (min–max), OR for 10 years increased	68.93 [27.39–94.57]		71.31 [36.50–86.45]		1.08 [0.82–1.42]	0.60
Diabetes mellitus						
no	70	77.78	31	67.39	1	0.19
yes	20	22.22	15	32.61	1.69 [0.77–3.74]	
Smoking						
no	44	48.89	22	47.83	1	0.91
yes (past + present)	46	51.11	24	52.17	1.04 [0.51–2.12]	
Hypertension						
no	11	12.22	1	2.17	1	0.08
yes	79	87.78	45	97.83	6.27 [0.78–50.1]	
Pulse pressure (mmHg)						
< 57.5	33	37.08	10	22.22	1	0.22
57.5–69.5	29	32.58	17	37.78	1.93 [0.77–4.89]	
≥ 69.5	27	30.34	18	40.00	2.20 [0.87–5.55]	
Body mass index						
< 25	37	41.11	23	50.00	1	0.41
25–30	38	42.22	14	30.43	0.59 [0.27–1.32]	
≥ 30	15	16.67	9	19.57	0.97 [0.36–2.56]	
Waist-to-hip ratio						
< 1	72	80.00	38	82.61	1	0.71
≥ 1	18	20.00	8	17.39	0.84 [0.34–2.11]	
Anti-hypertensive drugs						
no	13	14.44	1	2.17	1	0.06
yes	77	85.56	45	97.83	7.60 [0.96–60.0]	
Erythropoiesis stimulating agent						
no	74	82.22	37	80.43	1	0.80
yes	16	17.78	9	19.57	1.13 [0.45–2.79]	
Phosphate binders						
no	73	81.11	32	69.57	1	0.13
yes	17	18.89	14	30.43	1.88 [0.83–4.27]	
Vitamin D supplementation						
no	36	40.00	13	28.26	1	0.18
yes	54	60.00	33	71.74	1.69 [0.79–3.65]	
Statins						
no	55	61.11	26	56.52	1	0.61
yes	35	38.89	20	43.48	1.21 [0.59–2.49]	
Fibrates						
no	78	87.64	41	89.13	1	0.80
yes	11	12.36	5	10.87	0.86 [0.28–2.66]	

**p*-value (for variables with more than two categories. the *p*-value of the test for trend is given).

fibrinogen and homocysteine levels. When further attempting to determine possible determinants of this oxidant over-production, our analysis found low MDRD, high fibrinogen and finally low and high HDL cholesterol to be associated with an increased $O_2^{\bullet-}$ production in this sample of 136 patients. PTH and haemoglobin levels were associated with high $O_2^{\bullet-}$ in the univariate analysis only. Finally, no association was observed between medication or iron status and increased $O_2^{\bullet-}$ production.

To our knowledge, no previous study investigated the distribution of $O_2^{\bullet-}$ production according to renal function level. Chen et al. [9] only observed an enhanced $O_2^{\bullet-}$ basal level in haemodialysis vs control subjects, suggesting leukocytes from chronically dialysed patients were in a 'primed' state. We report here that oxygen radical production significantly increases from stages 4 and 5 of CKD. In addition, it was interesting to note that $O_2^{\bullet-}$ levels were not significantly different between lower stages of CKD (representing higher GFR levels) and healthy volunteers. Taken together, these data suggest a late occurrence of oxidative process in the course of kidney disease. Although univariate and multivariate analyses clearly showed that MDRD represents the main determinant of $O_2^{\bullet-}$ enhancement, no significant increase in $O_2^{\bullet-}$

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Table IV. Associations between biological parameters and $O_2^{\bullet-}$ production in 136 CKD patients.

	$O_2^{\bullet-}$ production (% activation)					
	< 203.358 (*n* = 90)		≥ 203.358 (*n* = 46)			
Variable	*n*	%	*n*	%	OR [CI 95%]	*p*-value*
Plasma creatinine (μmol/L)						
< 142	37	41.11	8	17.39	1	0.003
142–204	31	34.44	14	30.43	2.09 [0.78–5.63]	
≥ 204	22	24.44	24	52.17	5.05 [1.93–13.20]	
MDRD (mL/min/1.73 m^2)						
< 30	33	36.67	31	67.39	5.64 [1.17–27.23]	0.0037
30–60	45	50.00	13	28.26	1.73 [0.34–8.75]	
≥ 60	12	13.33	2	4.35	1	
MDRD (mL/min/1.73 m^2)						
< 30	33	36.67	31	67.39	3.57 [1.69–7.56]	0.0009
≥ 30	57	63.33	15	32.61	1	
Plasma urea (mmol/L)						
< 10	34	37.78	11	23.91	1	0.004
10–15.9	35	38.89	11	23.91	0.97 [0.37–2.54]	
≥ 15.9	21	23.33	24	52.17	3.53 [1.44–8.67]	
CRP (mg/L)						
< 1.4	30	33.33	18	39.13	1	0.66
1.4–3.2	30	33.33	12	26.09	0.67 [0.27–1.62]	
≥ 3.2	30	33.33	16	34.78	0.89 [0.38–2.06]	
Fibrinogen (activity) (g/L)						
< 3.7	35	38.89	7	15.22	1	0.024
3.7–4.4	25	27.78	17	36.96	3.40 [1.23–9.42]	
≥ 4.4	30	33.33	22	47.83	3.67 [1.38–9.77]	
alpha–1 acid glycoprotein (g/l)						
< 0.84	34	37.78	13	28.26	1	0.32
0.84–1.06	30	33.33	14	30.43	1.22 [0.50–3.00]	
≥ 1.06	26	28.89	19	41.30	1.91 [0.80–4.57]	
Ceruloplasmin (g/L)						
≤ 0.31	34	38.20	12	26.67	1	0.37
0.31–0.38	27	30.34	18	40.00	1.89 [0.78–4.59]	
≥ 0.38	28	31.46	15	33.33	1.52 [0.61–3.77]	
Calcium (mmol/L)						
< 2.30	36	40.00	12	26.09	1	0.25
2.30–2.42	24	26.67	17	36.96	2.12 [0.86–5.23]	
≥ 2.42	30	33.33	17	36.96	1.70 [0.70–4.11]	
Phosphate (mmol/L)						
< 0.96	35	38.89	11	23.91	1	0.22
0.96–1.15	27	30.00	18	39.13	2.12 [0.86–5.23]	
≥ 1.15	28	31.11	17	36.96	1.93 [0.78–4.78]	
Intact PTH (pg/mL)						
< 72	65	72.22	25	54.35	1	0.039
≥ 72	25	27.78	21	45.65	2.18 [1.04–4.58]	
1.25 $(OH)_2$ vitamin D						
≤ 22	30	33.33	16	34.78	1	0.89
22–37	29	32.22	16	34.78	1.03 [0.44–2.45]	
> 37	31	34.44	14	30.43	0.85 [0.35–2.03]	
Total cholesterol (mmol/L)						
< 4.94	29	32.22	16	34.78	1	0.95
4.94–5.70	31	34.44	15	32.61	0.88 [0.37–2.09]	
≥ 5.70	30	33.33	15	32.61	0.91 [0.38–2.16]	
Triglycerides (mmol/L)						
< 1.09	30	33.33	15	32.61	1	0.50
1.09–1.70	27	30.00	18	39.13	1.33 [0.56–3.15]	
≥ 1.70	33	36.67	13	28.26	0.79 [0.32–1.92]	
Vit E/(Triglycerides + Total cholesterol)						
< 4.68	32	37.21	12	27.27	1.58 [0.66– 3.77]	0.51
4.68–5.5	28	32.56	15	34.09	1	
≥ 5.5	26	30.23	17	38.64	1.03 [0.42–2.52]	
HDL cholesterol (mmol/L)						
< 1.42	28	31.11	18	39.13	2.89 [1.10–7.62]	0.03
1.42–1.75	36	40.00	8	17.39	1	

(Continued)

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Table IV. *(Continued)*

Variable	$O_2^{\bullet-}$ production (% activation) < 203.358 (n = 90) n	%	≥ 203.358 (n = 46) n	%	OR [CI 95%]	p-value*
≥ 1.75	26	28.89	20	43.48	3.46 [1.32–9.06]	
LDL cholesterol (mmol/L)						
< 2.68	30	33.33	15	32.61	1	0.99
2.68–3.24	30	33.33	15	32.61	1.00 [0.42–2.40]	
≥ 3.24	30	33.33	16	34.78	1.07 [0.45–2.54]	
Homocysteine (μmol/L)						
< 17.5	31	34.44	13	28.26	1	0.76
17.5–24.5	29	32.22	16	34.78	1.32 [0.54–3.20]	
≥ 24.5	30	33.33	17	36.96	1.35 [0.56–3.26]	
Albumin (g/L)						
< 39.0	26	28.89	16	34.78	1	0.47
39.0–42.7	35	38.89	13	28.26	0.60 [0.25–1.47]	
≥ 42.7	29	32.22	17	36.96	0.95 [0.40–2.26]	
Transthyretin (g/L)						
< 0.28	30	33.33	11	23.91	1	0.34
0.28–0.36	30	33.33	14	30.43	1.27 [0.50–3.25]	
≥ 0.36	30	33.33	21	45.65	1.91 [0.79–4.64]	
Haemoglobin (g/dL)						
< 12.6	21	23.33	20	43.48	2.77 [1.13–6.81]	0.054
12.6–14.0	35	38.89	12	26.09	1	
≥ 14.0	34	37.78	14	30.43	1.20 [0.49–2.97]	
Ferritin (ng/mL)						
< 108	28	31.11	17	36.96	1	0.78
108–196	31	34.44	14	30.43	0.74 [0.31–1.78]	
≥ 196	31	34.44	15	32.61	0.80 [0.34–1.89]	
Plasma iron (μmol/L)						
< 12.5	27	30.00	18	39.13	1	0.56
12.5–16.6	31	34.44	14	30.43	0.68 [0.28–1.61]	
≥ 16.6	32	35.56	14	30.43	0.66 [0.28–1.56]	

*p-value (for variables with more than two categories. the p-value of the test for trend is given).

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was observed from stage 3. However, due to its heterogeneity, stage 3 could be split into early (3A) and late (3B) components with a cut-off value at 45 mL/min (recommendations from the National Institute for Health and Clinical Excellence. Clinical guideline 73: Chronic kidney disease. London; 2008). Interestingly, the first tertile of MDRD in our population is close to this value (= 40.95 mL/min). If we consider $O_2^{\bullet-}$ production in patients with MDRD ≥ 40.95 mL/min (stages 1, 2 and 3A), it is significantly lower compared with patients of stages 3B, 4 and 5 (MDRD < 40.95 mL/min) (p = 0.007).

This over-production of $O_2^{\bullet-}$ has been previously observed and reported in such settings. In a recent work, Karamouzis et al. [23] reported a significant increase in isoprostanes with the renal function decline without affecting antioxidant defense mechanisms confirming that imbalance between oxidant and antioxidant systems already exists at the pre-dialysis phase. However, increase in oxidative stress concomitantly with progression of kidney disease remains a question of debate. While Terawaki et al. [24] could evidence positive correlation between oxidative stress and plasma creatinine, Oberg et al. [25] failed to do so. The fact that most uremic toxins involved in oxidative stress are protein-bound molecules of high or middle molecular weight not necessarily regulated by glomerular filtration may be one possible explanation. The occurrence of reactive oxygen species overproduction before onset of dialysis is all the more interesting since no significant difference in oxidative stress markers between pre-dialysis stage 5 and haemodialysis patients is hence observed [26] (mainly due to better biocompatibility of dialysis process). This strongly suggests that nowadays uremia *per se* and not dialysis anymore is incriminated in this oxidant over-production.

Regarding inflammatory markers, our study showed that advanced stages of CKD (stages 4 and 5) were accompanied by an increase in fibrinogen level similarly to $O_2^{\bullet-}$ increase. None of the other inflammatory parameters, particularly CRP, was affected by CKD stages. Our observations were also reported by another group [27], whereas Stuveling et al. [28] observed an enhancement in CRP concomitantly with progression of renal failure. This firstly suggests that fibrinogen constitutes a better marker of sub-acute (slow process) inflammation compared to CRP, alpha-1 acid

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Table V. Multivariate analysis to show the association of clinical and biological variables with $O_2^{\bullet-}$ production among 136 CKD patients.

Variable	OR [CI 95%]	*p*-value
Fibrinogen (activity) (g/L)		
< 3.7	1	0.044
≥ 3.7	2.70 [1.03–7.10]	
HDL cholesterol (mmol/L)		
< 1.42	2.39 [0.84–6.74]	0.042
1.42–1.75	1	
≥ 1.75	3.84 [1.34–10.96]	
MDRD (mL/min/1.73 m^2)		
< 30	2.55 [1.02–6.40]	0.046
≥ 30	1	
Intact PTH (pg/mL)		
< 72	1	0.52
≥ 72	1.35 [0.54–3.36]	
Haemoglobin (g/dL)		
< 12.6	1	0.37
≥ 12.6	0.67 [0.28–1.61]	

glycoprotein or ceruloplasmin for prediction of oxidative status. It seems that fibrinogen is a marker with slower kinetics compared to the rapid ones described for CRP. Second, the association observed between fibrinogen and $O_2^{\bullet-}$ in our study is in total agreement with the established amplification loops between inflammatory and oxidative processes previously described.

Interestingly, our analysis revealed that low and high levels of HDL cholesterol were associated with an increase in $O_2^{\bullet-}$. The expected association between low HDL cholesterol levels and $O_2^{\bullet-}$ could be related to antioxidant and anti-inflammatory properties of HDL such as the protective role against LDL oxidation, inhibiting role of chemoattractant molecule MCP-1 expression and adhesion molecules [29]. HDL-associated Apo A1 and ApoE apolipoproteins also confer antioxidant capacity [30]. Presence of enzymes such as LCAT [31], PON1 [32] and PAF-AH [33] which are able to remove oxidized lipids emphasize the antioxidant properties of these particles. In contrast, the link we found between high HDL cholesterol and high $O_2^{\bullet-}$ was more surprising. Several authors including us reported qualitative alterations of HDL from chronic haemodialysis patients, leading to an impairment in their antioxidant capacities [34,35], which could partly explain these results. HDL can also become pro-oxidant and pro-inflammatory molecules consequently to physicochemical modifications [36]. Indeed, oxidants including copper, peroxyl and hydroxyl radicals, aldehydes, peroxidases and lipooxigenase could oxidize HDL cholesterol leading to physico-chemically and biologically modified lipoparticles being able to generate lipid peroxidation and oxygen radicals [37]. Interestingly, in another work, Van Lenten et al. [37] observed that, during acute phase response, a displacement and/or exchange of proteins associated with HDL (ApoA1, PON1 against SAA) resulted in a pro-inflammatory molecule, with a pro-oxidant role in LDL oxidation, monocyte adhesion, pro-inflammatory cytokine release and diminution in PPAR-alpha nuclear receptor expression. CKD being a chronic microinflammatory disease, we could hypothetize such uremia-induced modification of antioxidant HDL into pro-inflammatory lipoparticle.

When considering bone and mineral metabolism disorders, we identified a significant association between PTH (>72 pg/mL) and $O_2^{\bullet-}$ production. This third tertile of PTH (>72 pg/mL) is closed to the upper limit of normal range from our laboratory (=62 pg/mL) recommended by the KDOQI guidelines as a target for PTH level before onset of dialysis. Even though this association did not reach significance in the multivariate analysis, several arguments converge to demonstrate the implication of PTH in oxidative stress. Firstly, Mitnick et al. [38] reported a stimulatory effect of PTH on pro-inflammatory IL-6 secretion with subsequent activation of oxidative stress. PTH can also generate a NADPH oxidase-dependent oxidative stress through intracellular calcium induction [39]. Lastly, a role of PTH in the FGF23/Klotho axis has been evidenced knowing that the Klotho gene would increase resistance to oxidative stress [40].

We observed in this sample of patients an association which tends to significance ($p = 0.054$) between high $O_2^{\bullet-}$ production and low haemoglobin level. These results, which suggest a protective role of haemoglobin against oxidant over-production, may be attributable to the scavenger action of red blood cell superoxide dismutase. However, no independant association remained in the multivariate analysis.

Finally, the lack of association observed between $O_2^{\bullet-}$ production and plasma vitamin E is not surprising and may be explained by the downstream action of vitamin E in the oxidative stress pathway. Moreover, over-production of $O_2^{\bullet-}$ as a signalling molecule in several pathological processes including amplification of inflammation or cell transdifferenciation has been reported [41,42]. These intracellular actions may affect cellular rather than plasma defence mechanisms. Indeed, it has been shown that oxidative stress is not associated with an impairment in plasma (but rather in erythrocyte) vitamin E in CKD patients.

Our study acknowledged some limitations. The relatively small sample size of this cross-sectional study may have prevented some of the detected associations from being statistically significant. This sample of patients being issued from the same area, eliminating possible regional variations in oxidative stress (as we previously reported with LDL oxidizability [43]) may have influenced the results.

To conclude, CKD patients present an increased cardiovascular mortality that cannot be explained entirely by conventional cardiovascular risk factors. Enhanced $O_2^{\bullet-}$ is a novel non-traditional risk factor

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in CKD patients that occurs at stages 4–5 and clearly prior onset of dialysis. We identified inflammation (fibrinogen) and abnormal HDL levels as the main determinants of this oxidant over-production suggesting that these clinico-biological disorders should be targeted for more efficient preventive intervention.

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