Pro-inflammatory cytokines and leukocyte oxidative burst in chronic kidney disease: culprits or innocent bystanders?

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ABSTRACT

Background. Pro-inflammatory cytokines are elevated in chronic kidney disease (CKD), a condition characterized by microinflammation with oxidative stress as key feature. However, their role in the inflammatory response at uraemic concentrations has not yet been investigated. In this study, the contribution of cytokines on induction of leukocyte oxidative stress was investigated.

Methods. Whole blood from healthy donors was incubated with 20–1400 pg/mL TNFα, 5–102.8 pg/mL IL-6, 20–400 pg/mL IL-1β and 75–1200 pg/mL IL-18 separately or in combination. Oxidative burst was measured, at baseline and after stimulation with fMLP (Phagoburst™). The effect of the TNFα blocker, adalimumab (Ada), was evaluated on TNFα-induced ROS production. Finally, the association between TNFα and the composite end point all-cause mortality or first cardiovascular event was analysed in a CKD population stage 4–5 (n = 121).

Results. While interleukin (IL)-6, IL-1β and IL-18 alone induced no ROS activation of normal leukocytes, irrespective of concentrations, TNFα induced ROS activation at baseline (P < 0.01) and after fMLP stimulation (P < 0.05), but only at uraemic concentrations in the high range (400 and 1400 pg/mL). A similar pattern was observed with all cytokines in combination, but already at intermediate uraemic concentrations (all P < 0.05, except for monocytes after fMLP stimulation: n.s.), suggesting synergism between cytokines. ROS production induced by TNFα (400 pg/mL) and the cytokine combination was blocked with Ada. Uraemia-related oxidative stress in leucocytes of haemodialysis patients was however not blocked by Ada. In patients, TNFα was not associated to adverse events (HR: 1.52, 95% CI 0.81–2.85, P = 0.13).

Conclusion. Among several pro-inflammatory cytokines, TNFα alone was pro-oxidative but only at high-range uraemic concentrations. Adding a TNFα blocker, Ada, blocked this ROS production, but not the oxidative stress in blood samples from haemodialysis patients, suggesting that other uraemic toxins than TNFα are more crucial in this process. However, the lack of association between TNFα and mortality suggests that the role of TNFα-linked oxidative stress is limited.

Keywords: CKD, haemodialysis, mortality, oxidative stress, tumour necrosis factor alpha

INTRODUCTION

The concentration of cytokines gradually increases in chronic kidney disease (CKD) [1], which is thought to be mainly attributed to an increased generation in response to uraemic toxins [2–4] and reduced renal clearance [5, 6]. In clinical studies in CKD, pro-inflammatory cytokines are used as a hallmark of microinflammation [7]. Both in pre-dialysis and dialysed CKD, cytokines, especially interleukin (IL)-6, have been associated to multiple CKD-related adverse outcomes such as malnutrition [8], muscle wasting [9], atherosclerosis [8, 10], anaemia [11] and all-cause or cardiovascular mortality [12–20]. Surprisingly, data on the pathophysiological role of cytokines at concentrations as occurring in CKD as contributors to the leukocyte oxidative burst are to the best of our knowledge not available.

Excessive oxidative stress is one of the key features of CKD-related microinflammation, to which a host of factors such as intravenous iron administration, bio-incompatibility of dialysis membranes, endotoxaemia and uraemic solute retention are contributing [21]. Although increased oxidative stress plays a role in the pathogenesis of atherosclerosis and cardiovascular disease, the causative role for increased oxidative stress in CKD to cardiovascular disease remains to be proven [22, 23].

In vitro, IL-6, tumour necrosis factor alpha (TNFα) and IL-18 induce leukocyte oxidative stress at concentrations in the range or in excess of those observed in extreme clinical conditions, such as sepsis [24–27]. In contrast, whether cytokines at lower levels, as observed in CKD, could induce free radical production has not yet been investigated.
A better understanding of the interaction between cytokines and oxidative stress in CKD could provide more insight into the mechanisms of uraemic leukocyte activation, one of the triggers for CKD-related cardiovascular disease, and on potential therapeutic pathways to prevent these problems.

Therefore, the aim of the present study was to investigate the effects of four pro-inflammatory cytokines, at relevant uraemic concentrations, separately and in a combination, on the induction of leukocyte oxidative burst in whole blood of healthy donors. The effect of a TNFα blocker, adalimumab, on free radical production by TNFα was also evaluated. These findings were then compared with the impact of adalimumab on free radical production in leukocytes of haemodialysis patients. Finally, we analysed an in vivo association between TNFα and adverse outcome in a population with CKD stage 4–5.

**MATERIALS AND METHODS**

**In vitro study**

**Samples.** After informed consent, heparinized whole blood (NH, BD Vacutainer™, Becton Dickinson, Plymouth, UK) and coagulated blood (Venosafe™, Terumo Europe NV, Leuven, Belgium) samples were collected from non-smoking healthy volunteers and from stable, non-smoking haemodialysis patients before dialysis and heparinization. Patients with diabetes, concurrent infection, malignancy or treatment with immunosuppressive drugs were excluded. Patients underwent online haemodialfiltration 3 times 4 h a week. The quality of the dialysis fluid met the ultrapure standards (bacteria <0.1 CFU/mL, endotoxin <0.03 EU/mL) as checked on a regular basis. Blood was allowed to coagulate for 30 min and was then centrifuged (10 min, 3000 r.p.m.) to store the serum in aliquots at −80°C. The protocol of the study was approved by the local ethics committee.

**Reagents.** The human recombinant cytokines such as IL-6, TNFα and IL-1β (R&D Systems®, Abingdon, UK) and coagulated blood (Venosafe™, Terumo Europe NV, Leuven, Belgium) samples were collected from non-smoking healthy volunteers and from stable, non-smoking haemodialysis patients before dialysis and heparinization. Patients with diabetes, concurrent infection, malignancy or treatment with immunosuppressive drugs were excluded. Patients underwent online haemodialfiltration 3 times 4 h a week. The quality of the dialysis fluid met the ultrapure standards (bacteria <0.1 CFU/mL, endotoxin <0.03 EU/mL) as checked on a regular basis. Blood was allowed to coagulate for 30 min and was then centrifuged (10 min, 3000 r.p.m.) to store the serum in aliquots at −80°C. The protocol of the study was approved by the local ethics committee.

**Test solutions and experimental set-up**

**Oxidative burst.**

**Test solutions and experimental set-up**

**Controls.** Saline and DPBS-0.5%alb were used as controls (co) for the experiments with respectively adalimumab and the cytokine solutions, as they were the solutions in which these compounds were diluted.

**Dose–response experiments of cytokines.** The cytokines were tested individually in a dose–response setting at relevant uraemic concentrations, varying from average to high, within a broad range as retrieved from reviews on uraemic toxin concentrations [28–30]. The added test concentrations were as follows: for TNFα: 20, 70, 400 and 1400 pg/mL; for IL-6: 5, 10, 21.5, 95.4 and 102.8 pg/mL; for IL-1β: 20, 80 and 400 pg/mL and for IL-18: 75, 150, 300, 600 and 1200 pg/mL. As they were added to whole blood of healthy donors, it should be noted that the final concentration in the blood was slightly higher than the aimed test concentration since these cytokines are present as such at very low concentrations in normal blood samples. It is highly unlikely that this would have influenced the results of the experiments, since the background concentration of cytokines in donor blood is negligible compared with the added concentrations of the cytokines.

**Effects of a combination of pro-inflammatory cytokines.** Also the combination of the four pro-inflammatory cytokines at a uraemic concentration in the intermediate range was tested in whole blood of healthy donors. The combination contained TNFα at 70 pg/mL, IL-6 at 10 pg/mL, IL-1β at 20 pg/mL and IL-18 at 150 pg/mL.

**Effects of blocking TNFα with adalimumab in healthy controls.** The effect of adalimumab (17.5 mg/L [31]) on burst activity was evaluated as such and in the presence of TNFα (400 pg/mL), which was the lowest TNFα concentration that induced burst activation per se or of the cytokine combination mentioned above.

**Studies of oxidative burst activity in whole blood of haemodialysis patients.** Leukocyte burst activation in heparinized whole blood of haemodialysis patients and healthy volunteers was compared. Furthermore, adalimumab was added to whole blood of haemodialysis patients to evaluate the possible influence of the TNFα blocker on uraemia-related oxidative stress.

**Oxidative Burst test**

Whole blood of healthy donors or haemodialysis patients was incubated for 10 min at 37°C with control solution or the different experimental solutions, after which reactive oxygen species (ROS) production in monocytes and granulocytes was measured. For that purpose, the Burst test (Phagoburst™, Orpégan Pharma, Heidelberg, Germany) was applied according to the manufacturer’s instructions. Burst activity in leukocytes was evaluated at baseline and after stimulation with N-formyl-methionine-leucine-phenylalanine (fMLP), a moderate stimulus. The generation of ROS was measured by assessing the oxidative conversion of the fluorogenic substrate dihydrorhodamine-123 into rhodamine.
The samples were analysed within 30 min by flow cytometry using an FACScan (Becton Dickinson, Erembodegem, Belgium). Monocytes and granulocytes were gated separately and the percentage of rhodamine-positive cells (%) per gate was measured at baseline and after fMLP stimulation.

**In vivo study**

**Study population.** All non-transplanted CKD patients stage 4 and 5 not on dialysis, attending the nephrology outpatient clinic, included in the biobank sample collection of the Nephrology Department of the Ghent University Hospital between January 2011 and July 2012, were eligible for this study (n = 121). Patients were sampled after written informed consent and outcomes were registered prospectively. Plasma samples were processed immediately after collection and stored at −80°C. The study was approved by the local ethical committee.

Baseline clinical parameters (age, gender, blood pressure, pulse, height and weight) were registered. Body mass index (BMI) was calculated as weight/height² (kg/m²), mean arterial pressure (MAP) as the sum of 1/3 of the systolic and 2/3 of the diastolic blood pressure and pulse pressure (PP) as the difference between systolic and diastolic blood pressure. Estimated glomerular filtration rate (eGFR) was calculated based on the creatinine-based CKD-EPI formula [32]. The following comorbidities were recorded: cardiovascular history when at least one of the following was present: arterial cardiovascular disease (coronary, cerebral or peripheral), atrial fibrillation or heart failure (requiring hospitalization); malignancy; diabetes mellitus, defined as a history of diabetes or treatment with insulin or oral antidiabetic drugs; hypertension, defined as current hypertension (>140/90 mmHg) or the use of antihypertensive drugs; hypercholesterolaemia, defined as history of or treatment with lipid-lowering drugs, and smoking status (active versus no/former smoker).

Patients were followed until 12 March 2014 for the occurrence of cardiovascular events (acute coronary syndrome, de novo atrial fibrillation, acute heart failure, coronary artery bypass graft, percutaneous transluminal coronary angioplasty, cerebrovascular accident, percutaneous transluminal angioplasty) or all-cause mortality whichever came first.

**Concentration determination**

The TNFα concentration in the serum of the HD patients used for evaluation of oxidative burst activity as well as that in plasma samples of patients with CKD 4 and 5 in the in vivo study was quantified by ELISA (R&D Systems, Abingdon, UK). Serum creatinine and C-reactive protein (CRP) were measured by routine laboratory tests.

**Statistical analysis**

**In vitro study.** The data of the burst test were non-normally distributed and consequently expressed as medians with interquartile range and analysed with Friedman-ranks test, Wilcoxon signed-rank test or Mann–Whitney test as appropriate.

For the in vivo study, continuous data were expressed as mean with standard deviation or median with interquartile range depending on their distribution, and analysed by Student’s t-test or Mann–Whitney test. Binary categorical data were expressed as frequencies and analysed with χ² test. The association between TNFα concentration as continuous variable and outcome was analysed by univariate Cox proportional hazards model. The primary outcome was the composite end point of mortality or first non-fatal cardiovascular event. The analysis was repeated with all-cause mortality as outcome. In a multivariate analysis, variables with a P-value of <0.1 in univariate analysis together with TNFα were included into the model. The results are reported as hazard ratios with their 95% confidence interval (CI). A P-value of <0.05 was considered as significant. Kaplan–Meier survival curves were made for both outcomes using the median TNFα as cut-off.

Statistical analysis was performed using SPSS statistics 22 (SPSS Inc., Chicago, IL, USA) for Windows (Microsoft Corp., Redmond, WA, USA). Graphs were made with GraphPad Prism 04 (GraphPad Software, La Jolla, CA, USA) or SPSS statistics 22. A P-value of <0.05 was considered as statistically significant.

**RESULTS**

**In vitro study**

**Control solutions (n = 10).** The two control solutions, saline and DPBS-0.5%alb, had no effect on oxidative burst (data not shown).

**Effect of cytokines on leukocyte oxidative burst in healthy donors**

**Dose response of TNFα (n = 8).** TNFα increased ROS production dose dependently in monocytes and granulocytes, at baseline and after stimulation with fMLP (Figure 1). In monocytes, TNFα at 400 and 1400 pg/mL induced free radical production at baseline and after fMLP stimulation compared with control (all P < 0.01) and to 20 pg/mL: (400 pg/mL: P < 0.05; 1400 pg/mL: baseline: P < 0.05; fMLP: P < 0.01) (Figure 1A and C). In granulocytes, TNFα at 400 pg/mL and 1400 pg/mL, induced ROS at baseline compared with control (P < 0.01) (Figure 1B), while after fMLP stimulation, only TNFα at 1400 pg/mL increased free radical production compared with control (P < 0.05) (Figure 1D).

**Dose response of other cytokines (n = 8).** The tested concentrations of IL-6, IL-1β and IL-18 neither induced nor inhibited free radical production in monocytes or granulocytes (data not shown).

**Effect of the combination of the four cytokines (n = 8).** The combined cytokines had a stimulatory effect on leukocyte oxidative burst at baseline in monocytes and granulocytes (both P < 0.05) and after stimulation with fMLP in granulocytes (P < 0.05) (Table 1). The pro-oxidative effects were similar to the ones observed with TNFα alone (400 pg/mL), although it is of note that the concentration of 70 pg/mL TNFα used in the combination solution did not induce ROS when added to normal blood by itself (Figure 1).

**Effects of adalimumab on leukocyte oxidative burst**

**Effects of adalimumab alone (n = 10).** Compared with control, adalimumab had no effect on free radical production at
Effects of adalimumab on oxidative burst induced by TNFα (n = 10). Adalimumab blocked the free radical production induced by TNFα (400 pg/mL) in monocytes (6.9 versus 3.3%, P < 0.05) (Figure 2A) and granulocytes (5.5 versus 2.6%, P < 0.05) (Figure 2B), at baseline. In contrast, adalimumab did not completely inhibit the stimulatory effects of TNFα in monocytes (Figure 2C) or granulocytes (Figure 2D) after fMLP stimulation.

Effect of adalimumab on oxidative burst induced by the cytokine combination (n = 8). The pro-oxidative effects of the cytokine combination could be partially or entirely blocked by adalimumab at baseline in monocytes and granulocytes and after fMLP stimulation in granulocytes (Table 1). Despite the probable synergisms between the cytokines at this intermediate concentration (see above), the blockade with adalimumab suggests that TNFα plays a primordial role in these in vitro data.

Effects of adalimumab on uraemia-related leukocyte oxidative burst in haemodialysis patients (n = 10). Compared with healthy donors, monocytes and granulocytes from haemodialysis patients showed increased burst activity at baseline (P < 0.001) and after fMLP stimulation (P < 0.001). Adalimumab, however, did not blunt this increased uraemia-related oxidative stress present in blood from haemodialysis patients (Figure 3).

**TNFα concentrations in samples of haemodialysis patients used for in vitro assessment.** The serum concentration of TNFα in the samples of our haemodialysis patients was 9.9 ± 3.3 pg/mL which was lower than the concentrations reported in major reviews on uraemic toxin concentrations.

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**Table 1. Oxidative burst in leukocytes of healthy donors in the presence of a mixture of pro-inflammatory cytokines**

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Control</th>
<th>Cytokine mix</th>
<th>Blocking with Ada</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Monocytes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline (%)</td>
<td>2.4 (2.1–2.9)</td>
<td>5.9* (3.7–7.4)</td>
<td>3.1* (2.4–5.8)</td>
</tr>
<tr>
<td>fMLP (%)</td>
<td>7.7 (5.1–11.1)</td>
<td>13.0 (8.0–16.1)</td>
<td>11.6 (10.4–21.0)</td>
</tr>
<tr>
<td><strong>Granulocytes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline (%)</td>
<td>2.7 (2.5–3.0)</td>
<td>5.2* (4.0–9.8)</td>
<td>3.1* (1.2–4.2)</td>
</tr>
<tr>
<td>fMLP (%)</td>
<td>11.4 (8.0–17.9)</td>
<td>17.1* (12.8–20.0)</td>
<td>11.1** (9.7–13.4)</td>
</tr>
</tbody>
</table>

Medians with interquartile range within brackets.

*%: percentage of rhodamine-positive cells; Ada, adalimumab; fMLP, N-formyl-methionine-leucine-phenylalanine.

**P < 0.05 versus control.

*P < 0.05 versus cytokine mixture.

**P = 0.05–0.2 versus cytokine mixture.

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**Figure 1:** Dose response of oxidative burst in monocytes and granulocytes from healthy donors in dose response to TNFα at baseline and after stimulation with fMLP. Y-axis: percentage of rhodamine-positive cells (%); X-axis: concentration of TNFα in pg/mL; the concentrations mentioned on the X-axis are the TNFα concentrations of the test solutions that are added to whole blood of healthy donors. Co, control (DPBS-0.5% alb); *P < 0.05, **P < 0.01 versus Co; ###P < 0.05, **P < 0.01 versus 20 pg/mL.
(Table 2) [28–30], be it close to the average mean reported by Duranton et al. in 2013 (22 ± 25 pg/mL) [30] and within the range reported in clinical studies in haemodialysis patients between 2011 and 2014 (TNFα mean 13.6 ± 13.6 pg/mL, median of 6.4 pg/mL) (Supplementary Table 1). This observation, together with the lack of effect of adalimumab described above, points towards other factors than TNFα being at least as preponderant for causing leukocyte burst activity in dialysis patients treated according to today’s standards. Synergistic effects, however, cannot be excluded.

**In vivo study**

Baseline clinical characteristics of the CKD 4–5 population (n = 121) divided according to the median of TNFα are presented in Table 3. Patients with TNFα concentrations above the median had a higher CRP, higher BMI and pulse pressure. There were no differences in age, gender or eGFR. After a total median [range] follow-up of 33 months [20–38 months], 41 patients reached the composite end point of mortality or non-fatal cardiovascular event (33.9%). Twenty-two patients died (18.2%) and 19 had a non-fatal cardiovascular event (15.7%), of which 6 patients died later on during follow-up, resulting in a total all-cause mortality of n = 28 (23.1%). In univariate analysis, TNFα was neither associated to the composite end point (HR: 1.52, 95% CI 0.81–2.85, P = 0.13) nor to all-cause mortality (HR: 1.01, 95% CI 0.93–1.10, P = 0.85). The Kaplan–Meier curves for TNFα < or ≥ median (4.62 pg/mL) for the composite end point of death or first non-fatal cardiovascular event, as well as for all-cause mortality, show no difference in outcome according to TNFα concentration (Figure 4). In univariate analysis, only higher age (HR: 1.05, 95% CI 1.02–1.08), higher CRP (HR: 1.01, 95% CI 1.01–1.01) or a history of cardiovascular disease (HR: 2.90, 95% CI 1.48–5.67) and diabetes (HR: 2.11, 95% CI 1.14–3.91) were associated to increased risk for the composite end point. The same variables were also significantly associated to all-cause mortality in univariate analysis, except for a history of cardiovascular disease that showed only a trend (P = 0.09) (Supplementary Table 2). In a multivariate model, CRP and age remained significantly associated to the composite end point and CRP, age and gender were significantly associated to all-cause mortality (Table 4).

**DISCUSSION**

The present study evaluated the role of four pro-inflammatory cytokines, IL-6, TNFα, IL-1β and IL-18, in the context of CKD. The main findings of this study generally indicate that the
evaluated cytokines are at best only ancillary contributors to leukocyte oxidative stress, which is one of the pathophysiological mechanisms involved in the inflammatory morbidity and mortality of uraemia, and are as follows: (i) At high uraemic concentrations, only TNFα and not IL-6, IL-18 and IL-1β increased the percentage of ROS producing monocytes and granulocytes at baseline and after fMLP stimulation, in whole blood of healthy controls (Figures 1 and 2). (ii) The free radical production, induced by the combination of the four cytokines, at concentrations at which the individual cytokines induced no free radical production, showed a similar pro-oxidative pattern in monocytes and granulocytes as TNFα alone at higher concentrations (Table 1). (iii) The human monoclonal TNFα antibody, adalimumab, could block partially or entirely the ROS production induced by TNFα alone (Figure 2) and by the combination of cytokines (Table 1). (iv) In contrast, adalimumab could not blunt uraemia-related oxidative stress in blood from haemodialysis patients (Figure 3), suggesting that TNFα is probably not a main contributor to the ROS production as observed nowadays in uraemia. (v) Finally, TNFα was not associated to adverse outcome in a population with CKD stage 4–5 (Figure 4).

**Table 2. Tumour necrosis factor alpha concentrations in uraemia**

<table>
<thead>
<tr>
<th>Uraemic TNFα concentration</th>
<th>Reference Major reviews on uraemic toxin concentrations</th>
<th>Literature search Period 2011–14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum</td>
<td>Vanholder <em>et al.</em> [29]</td>
<td>Meert <em>et al.</em> [28]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Duranton <em>et al.</em> [30]</td>
</tr>
<tr>
<td>Mean</td>
<td>408 pg/mL</td>
<td>1400 pg/mL</td>
</tr>
<tr>
<td>High mean</td>
<td>114 pg/mL</td>
<td>70 pg/mL</td>
</tr>
<tr>
<td>Average mean</td>
<td></td>
<td>58 ± 10 pg/mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22 ± 25 pg/mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14 pg/mL*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>range: 0.4–43 pg/mL</td>
</tr>
</tbody>
</table>

*TNFα, tumour necrosis factor alpha.

The mean uraemic concentration of the literature search is based on concentrations found in haemodialysis patients and is the calculated mean from the mean/median concentrations reported in the individual studies in the period 2011–14 (based on nine studies). For the original mean/median TNFα concentrations, see Supplementary Table 1. The range is the minimum and maximum mean concentration of all individual studies. The time period of the literature search started from the year when the search of Duranton *et al.* stopped until May, 2014.

**Figure 3:** Effect of adalimumab on basal and fMLP-stimulated oxidative burst in uraemic leukocytes. %, percentage of rhodamine-positive cells; co, healthy control; HD, oxidative stress due to uraemia in haemodialysis patient; HD + Ada, adalimumab added to whole blood of haemodialysis patients; fMLP, *N*-formyl-methionine-leucine-phenylalanine. ***P < 0.001 versus co.
By using the leukocyte burst test, which measures NADPH-oxidase activity, this study demonstrated for the first time that TNF-α increased dose dependently free radical production (short-lived superoxide anion) in monocytes and granulocytes, at baseline as well as after fMLP stimulation at concentrations as observed in uraemia, but only significantly at values in the high concentration range of what has been reported in uraemia (400 and 1400 pg/mL) (Figure 1). This is congruent with data.

Table 3. Clinical characteristics of the entire study population and according to a TNF-α concentration < or ≥ median of 4.62 pg/mL

<table>
<thead>
<tr>
<th>Variable</th>
<th>Entire population</th>
<th>TNF-α median</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 121</td>
<td>&lt;4.62 pg/mL</td>
<td>≥4.62 pg/mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n = 58</td>
<td>n = 63</td>
</tr>
<tr>
<td>Age (years)</td>
<td>74 [63–81]</td>
<td>73.5 [67.0–82.0]</td>
<td>74.0 [65.0–81.0]</td>
</tr>
<tr>
<td>Gender (M)</td>
<td>74 (61.2)</td>
<td>35 (60.3)</td>
<td>39 (61.9)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.4 ± 5.8</td>
<td>27.1 ± 4.3</td>
<td>29.6 ± 6.7</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>100 ± 13</td>
<td>100 ± 14</td>
<td>98 ± 11</td>
</tr>
<tr>
<td>PP (mmHg)</td>
<td>62 ± 19</td>
<td>66 ± 21</td>
<td>59 ± 16</td>
</tr>
<tr>
<td>HR (min⁻¹)</td>
<td>69 ± 13</td>
<td>69 ± 13</td>
<td>70 ± 14</td>
</tr>
<tr>
<td>eGFR (mL/min/1.73 m²)</td>
<td>23.0 [16.2–27.0]</td>
<td>21.1 [16.1–27.0]</td>
<td>23.9 [16.6–26.9]</td>
</tr>
<tr>
<td>CVH, n (%)</td>
<td>60 (49.6)</td>
<td>24 (41.4)</td>
<td>36 (57.1)</td>
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<tr>
<td>DM, n (%)</td>
<td>49 (40.5)</td>
<td>25 (43.1)</td>
<td>24 (38.1)</td>
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<tr>
<td>Malignancy, n (%)</td>
<td>31 (25.6)</td>
<td>15 (25.9)</td>
<td>16 (25.4)</td>
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<tr>
<td>Cholesterol, n (%)</td>
<td>83 (68.6)</td>
<td>39 (67.2)</td>
<td>44 (69.8)</td>
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<tr>
<td>AHT, n (%)</td>
<td>101 (83.5)</td>
<td>49 (84.5)</td>
<td>52 (82.5)</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td>10 (8.6)</td>
<td>3 (5.4)</td>
<td>7 (11.7)</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>4.62 [3.68–6.00]</td>
<td>3.60 [2.62–4.03]</td>
<td>5.72 [4.89–7.97]</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>3.0 [1.0–8.0]</td>
<td>2.0 [0.9–5.3]</td>
<td>4.0 [2.0–13.0]</td>
</tr>
<tr>
<td>Composite end point</td>
<td>41 (33.9)</td>
<td>16 (27.6)</td>
<td>25 (39.7)</td>
</tr>
<tr>
<td>All-cause mortality</td>
<td>28 (23.1)</td>
<td>13 (22.4)</td>
<td>15 (23.8)</td>
</tr>
</tbody>
</table>

Continuous data are presented as mean ± standard deviation or median with inter-quartile range within square brackets and categorical data as frequencies with percentages within brackets. TNF-α, tumour necrosis factor alpha; BMI, body mass index; MAP, mean arterial pressure; PP, pulse pressure; HR, heart rate; eGFR, estimated glomerular filtration rate; CVH, history of cardiovascular disease; DM, diabetes mellitus; cholesterol, hypercholesterolaemia; AHT, arterial hypertension; CRP, C-reactive protein; composite end point, death or non-fatal cardiovascular event. Variables in bold: P-value < 0.05.

Figure 4: Kaplan–Meier survival curve for TNF-α < or ≥ median (4.62 pg/mL) with the composite end point death or first non-fatal cardiovascular event in A and all-cause mortality in B. X-axis: follow-up in months, Y-axis: cumulative survival.

Table 4. Multivariate Cox proportional hazards model for the composite end point (Model A) and for all-cause mortality (Model B)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Model A: Composite end point: death or first cardiovascular event</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP (per mg/L)</td>
<td>0.014</td>
<td>1.01 [1.01–1.02]</td>
</tr>
<tr>
<td>Age (per year)</td>
<td>0.052</td>
<td>1.05 [1.02–1.09]</td>
</tr>
<tr>
<td>Model B: All-cause mortality</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP (per mg/L)</td>
<td>0.022</td>
<td>1.02 [1.01–1.03]</td>
</tr>
<tr>
<td>Age (per year)</td>
<td>0.093</td>
<td>0.10 [1.04–1.16]</td>
</tr>
<tr>
<td>Gender (male)</td>
<td>1.187</td>
<td>3.28 [1.27–8.47]</td>
</tr>
</tbody>
</table>

Variables with a P-value in univariate analysis <0.1 (see Supplementary Table 2) and TNF-α were included in the multivariate model. Included variables: Model A: age, CRP, diabetes mellitus, history of cardiovascular disease and TNF-α. Model B: age, gender, CRP, history of cardiovascular disease, malignancy, pulse pressure and TNF-α.

HR, hazard ratio; CI, confidence interval; CRP, C-reactive protein; TNF-α, tumour necrosis factor alpha.

By using the leukocyte burst test, which measures NADPH-oxidase activity, this study demonstrated for the first time that TNF-α increased dose dependently free radical production (short-lived superoxide anion) in monocytes and granulocytes, at baseline as well as after fMLP stimulation at concentrations as observed in uraemia, but only significantly at values in the high concentration range of what has been reported in uraemia (400 and 1400 pg/mL) (Figure 1). This is congruent with data.
obtained in other in vitro studies at concentrations at least 10-fold above those used in the present study [24, 25]. In combination with IL-6, IL-1β and IL-18, TNFα induced already a similar increase in oxidative burst as 400 pg/mL TNFα alone at an intermediate uraemic concentration of 70 pg/mL (Table 1). Since TNFα at 70 pg/mL as well as the three other cytokines at any concentration did not induce ROS by themselves, this points to synergisms between the different cytokines. Synergism between uraemic retention solutes was previously shown for p-cresylsulfate and p-cresylglucuronide on induction of leukocyte oxidative stress [33] and leukocyte rolling and capillary leakage in rat peritoneal vasculature [34].

Our data suggest that it is unlikely that TNFα by itself is one of the main responsible toxins in uraemia-related leukocyte oxidative burst, although synergisms with other pro-oxidative factors in uraemic patients cannot be excluded nor effects of low concentrations of pro-inflammatory cytokines on other cell systems. Even if the test concentrations in this study were within the range that is proposed as uraemic (Table 2) [28–30], the TNFα concentration (400 pg/mL) that provoked ROS production per se, is far above the concentrations that are found in studies in haemodialysis patients over the recent years; those ranged from 0.4 to 43 pg/mL with a mean and median concentration of 14 and 6 pg/mL, respectively (Table 2; Supplementary Table 1). This concentration range is congruent with the mean TNFα concentration of only 9.9 pg/mL measured in our HD samples. Possible explanations for this trend in decreasing cytokine concentrations in HD patients are the use of ultrapure water for preparation of dialysis fluid [35], the better uraemic toxin removal, the use of more biocompatible dialyser membranes and the use of bicarbonate instead of acetate buffers [36]. Moreover, specifically for this in vitro study, in the haemodialysis patients, the presence of comorbidities that could have caused additional inflammation, such as acute or chronic infection, smoking or malignancy, was an exclusion criterion.

Furthermore, the TNFα blocker, adalimumab, did not blunt the increased free radical production present in the whole blood of haemodialysis patients (Figure 3). The relatively low TNFα concentrations in the samples of our haemodialysis patients, together with the fact that other uraemic retention solutes and haemodialysis-related factors are probably more important in the generation of uraemia-related leukocyte ROS, could explain this finding. TNFα blockade is clinically used in the treatment of rheumatoid arthritis, a condition that is also characterized by systemic microinflammation and associated to cardiovascular disease [37, 38]. Also, in studies in rheumatoid arthritis patients, TNFα blockade could not attenuate the increased ROS production present in these patients [39, 40], while, for example chemo-taxis could be decreased [39].

Finally, in our population with advanced CKD, which already have increased TNFα concentrations, but are not yet affected by possible negative effects of dialysis therapy, the concentration of TNFα was not associated to adverse outcome (Figure 4), as also shown earlier in a CKD population stage 2 to 5D [12]. This is in contrast to IL-6 that has repeatedly been shown to be a strong predictor for outcome in CKD/dialysis [12, 15–18, 20] as well as in sepsis [41, 42]. A possible explanation for this finding could be the difference in occurrence pattern of these two cytokines in the inflammatory cascade, with TNFα appearing earlier compared with IL-6. TNFα initiates the inflammatory response, while IL-6 is generated after NF-κB has been activated and is as such the result of the activated inflammatory system [43]. In the same vein, also CRP, which is further downstream the cascade [44], was associated to adverse outcome in this (Table 4) and in other studies in CKD [13, 15].

With all arguments taken together, the results in the present study raise the question whether the reduction of TNFα activity by the administration of targeted monoclonals should be a goal in CKD, even if TNFα is pro-oxidative at high uraemic concentrations and even if causality between TNFα, oxidative stress and cardiovascular outcome is not proved. It is reasonable to accept, based on the present data, that TNFα concentrations, as observed nowadays in most patients with CKD, are insufficiently high to expect a therapeutic benefit of TNFα blockade. It might be more important to prevent the sustained generation of TNFα and other cytokines by targeting first line uraemic toxins such as intestinal-derived protein-bound uraemic toxins and SDMA which are pro-oxidative and pro-inflammatory [2, 3, 33, 45]. Blocking TNFα in the context of uraemia-related oxidative stress may happen too late in the cascade of effects to reduce leukocyte ROS production. In line with this hypothesis, the results of a small randomized controlled trial evaluating the use of anti-TNFα therapy, etanercept, in haemodialysis patients, failed to reduce inflammatory markers after 44 weeks of treatment [46].

In summary, pro-inflammatory cytokines appear not to be the main contributors to uraemia-related leukocyte oxidative stress. Out of the four investigated pro-inflammatory cytokines, only TNFα was pro-oxidative in normal monocytes and granulocytes but only at high uraemic concentrations. This free radical production could be blocked by a TNFα blocker, adalimumab, but the uraemia-related oxidative stress could not be blunted by this TNFα blockade. Furthermore, TNFα concentration was not linked to hard clinical end points in a population with CKD stage 4–5. The present study suggests that preventing the rise in TNFα could be a more useful therapeutic strategy than targeting TNFα itself to reduce oxidative stress in uraemia.

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**CONFLICT OF INTEREST STATEMENT**

None declared.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://ndt.oxfordjournals.org.
REFERENCES


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