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A Toll-like receptor-4/NLRP3 inflammasome pathway promotes inflammation in skeletal muscle of chronic kidney disease patients

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Abstract

Background An emerging hypothesis is that the activation of innate immunity in the muscle of patients with chronic kidney disease (CKD) is implicated in the development and progression of wasting and cachexia. We previously observed that Toll-like receptor-4 (TLR4) and its downward NF-κB-dependent pro-inflammatory pathways are activated in CKD muscle. It is however unknown if TLR4 can activate the TLR4/NOD, LRR, and pyrin domain-containing protein 3 (NLRP3) inflammasome pathway, which is implicated in cardiovascular disease and frailty, clinical settings that are commonly observed in CKD patients.

Methods In a case–control cohort study, we hypothesized that a TLR4/NLRP3 inflammasome pathway is activated in skeletal muscle in uraemia. First, we studied the regulation TLR4/NLRP3/caspase-1 in skeletal muscle biopsies (20M/11F) obtained from 31 non-diabetic CKD5 patients (eGFR 8 ± 1 mL/min 1.73 m^2) scheduled for peritoneal dialysis catheter insertion and 15 controls (10M/5F, eGFR 99 ± 6 mL/min 1.73 m^2). Second, the effects of uraemic serum on the TLR4/NLRP3 inflammasome pathway were studied in C2C12 cells.

Results In the muscle of CKD subjects, NLRP3 mRNA as well as its protein were overexpressed (by ~16-fold, respectively, $P < 0.05$ both vs. controls). Both IL-1β and IL-18 mRNA expressions were also up-regulated (~11.8–3.2-fold, respectively, $P < 0.05$). Also, cleaved caspase-1 was overexpressed in CKD muscle samples ($P < 0.001$ vs. controls). Both muscle NLRP3 mRNA ($n = 22$, $r = -0.606$, $P < 0.01$) and logIL-1 β protein ($n = 26$, $r = -0.460$, $P < 0.02$) were inversely associated with residual renal function, which suggests that the inflammasome is progressively activated in skeletal muscle of CKD patients as the residual renal function deteriorates. In addition, we observed that in C2C12 myotubes, uraemic serum up-regulates NLRP3 mRNA (~11-fold increase, $P < 0.05$), cleaved caspase-1 (by ~5-fold, $P < 0.05$), Il-1β mRNA (~3-fold increase, $P < 0.05$) and oxidative stress markers respect to normal serum. These effects were prevented by TAK-242, a selective TLR4 inhibitor.

Conclusions Overall, our data demonstrate the activation of TLR4/NLRP3/caspase-1 inflammasome and its downward inflammatory cascade in the muscle of subjects with advanced-stage CKD and suggest targeting TLR4/NLRP3 inflammasome as a new therapeutic strategy to blunt muscle inflammation in CKD.

Keywords Cachexia; CKD; Inflammasome; Oxidative stress

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Introduction

One of the most emerging hypotheses concerning the origin of two major consequences of chronic kidney disease (CKD), that is, accelerated atherosclerotic cardiovascular disease and cachexia, is that CKD results in unregulated activation of innate immunity and inflammation in circulating cells and tissues,[1,2] mainly through the NOD, LRR, and pyrin domain-containing protein 3 (NLRP3) inflammasome and interleukin-6 (IL-6) signalling pathways. In patients with CKD, the increase in the blood of inflammatory and oxidative stress markers predicts cardiovascular complications,[2,3] and, in analogy to cancer, cachexia is associated with lower physical performance and wasting.[4,5] In addition, very recent evidence has shown that inflammation activates specific catabolic pathways in uraemic muscle[6,7] and that whole body and muscle net protein catabolism is increased in inflamed maintenance haemodialysis patients.[8,9] Despite these major steps in our understanding of uraemic pathophysiology, mechanisms and mediators inducing tissue inflammation in CKD are still partially understood.

Skeletal muscle possesses both the afferent and efferent limbs of the innate immune system, including Toll-like receptors (TLRs).[10] TLRs are a family of pattern recognition receptors (PRRs) that mediate signal transduction pathways through the activation of transcription factors that regulate the expression of pro-inflammatory cytokines in several cell types and tissues, including skeletal muscle.[10,11] In addition to endotoxins and microbial products, TLRs can be activated by several damage-associated molecular pattern (DAMPS) molecules, including debris from apoptotic and necrotic cells, oligosaccharides, heat shock proteins, and nucleic acid fragments.[11] In a previous study,[12] we observed the up-regulation of TLR4 and its downward NF-κB-dependent production of TNF-α and pro-inflammatory cytokines in the skeletal muscle of patients with stage 5 CKD. Of note, DAMPs can activate several PRRs types, including not only TLRs but also nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs).[11] Intracellular NLRs constitute the inflammasomes, which activate and release caspase-1, IL-1β, and IL-18, which are critically involved in inflammatory responses.[11]

NLRP3 inflammasome signalling is contained and active in myocytes.[10] Deletion of NLRP3 protects mice from multiple aspects of age-related dysfunction,[13] and its overexpression has been suggested to contribute to 'sterile' age-related inflammation and wasting in models of ageing[13,14] and Duchenne-associated muscle atrophy[15] and sepsis.[16] Aberrant NLRP3 inflammasome activation is also pathogenic in atherosclerosis,[17] frailty, and cognitive decline,[13,18] all conditions that are commonly observed in patients with CKD.

The possible role of NLRs in mounting an inflammatory response in muscle has never been explored in patients with CKD. In this study, we hypothesized that the TLR4/NRLP3/caspase-1 pathway is up-regulated in the skeletal muscle of patients with advanced-stage CKD. We tested this postulate with different measures in a case–control cohort study and in vitro studies in muscle cells. First, we studied the activation of NLRP3 inflammasome and selected inflammasome genes in muscle biopsies of patients with advanced-stage CKD. As a second step, we studied the NLRP3 inflammasome response to the uraemic serum (US) of C2C12 myotubes; we observed that US up-regulates the inflammasome, an effect that can be prevented by TLR4 inhibition. Overall, our data demonstrate the activation of TLR4/NLRP3 inflammasome and its downward inflammatory cascade in the muscle of patients with advanced-stage CKD, suggesting that TLR4 may be an upstream target for dietary or pharmacological approaches for treating CKD-associated muscle wasting.

Patients and methods

Patients

Thirty-one non-diabetic CKD patients (20M/11F) scheduled for peritoneal dialysis catheter insertion were eligible for enrolment in this protocol at the Nephrology Divisions, Department of Internal Medicine, University of Genoa, and Ospedale Santa Chiara, Trento. Subjects were enrolled in the study on a consecutive basis if they did not meet the following exclusion criteria: New York Heart Association Class III–IV congestive heart failure, a recent (<12 months) myocardial infarction, liver cirrhosis, infection, or diabetic nephropathy. Patients younger than 18 and older than 85 years were excluded from the study. Patients were taking drugs, including antihypertensive drugs, sodium bicarbonate, and calcium carbonate, which were prescribed as appropriate for each individual. The clinical and biochemical characteristics of the subjects are shown in *Supplementary Table 1*. All subjects had a sedentary lifestyle. Their mean age was 61 ± 3 years (range 28–79). All subjects, except for two Hispanic White patients, were Europeans. Their estimated (CKD-EPI) GFR was 8 ± 1 mL/min 1.73 m^2 (range 5–12 mL/min). Causes of CKD were hypertensive nephrosclerosis (n = 10), chronic glomerulonephritis (n = 9), tubulointerstitial nephritis (n = 4), ADPKD (n = 6), and obstructive uropathy (n = 2). Their mean estimated protein and calorie intakes were 0.90 g/kg and 27 kcal/kg, respectively. Seventeen subjects had a 7-point subjective global assessment (SGA) index <6. Albumin levels were low (<3.8 g/100 mL) in eight subjects, whereas BMI was low (<23 kg/m^2) in six subjects. The evidence of an inflammatory response [C-reactive protein (CRP) > 5 mg/L] was shown in 12 subjects. Control biopsies were taken in 15 otherwise healthy subjects, during elective surgery for abdominal wall hernias. Controls were selected based on having no chronic

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illnesses or acute inflammatory processes. All subjects had a sedentary lifestyle and were Europeans. In these subjects, baseline physical examination and eGFR (99 ± 6, range 78–120 mL/min) and screening biochemical tests of kidney, liver, haematological, and metabolic function were unremarkable. All groups were closely matched for age and gender.

Muscle biopsies and tissue analysis

mRNA analysis

Muscle biopsies were obtained from the rectus abdominis muscle, at the beginning of surgery. Tissue (∼90–100 mg) RNA was isolated using the QIAzol Lysis reagent (Qiagen Sciences, Maryland, USA), and complementary DNA (cDNA) was obtained using iScript™ cDNA Synthesis Kit (Bio-Rad, Segrate, Italy). PCR amplification was carried out in a total volume of 10 μL, containing 1 μL cDNA solution, 5 μL SYBR solution Precision 2xqPCR MasterMix (Primerdesign, Southampton, United Kingdom), 0.5 μL each primer, and 3.5 μL of nuclease-free water. Primers were obtained from Tibmolbiol (Genoa, Italy), and sequences are reported in *Supplementary Table 2*. Relative mRNA levels were calculated from cycle threshold (Ct) values using β-actin as a housekeeping gene.

Immunohistochemistry and image analysis

Paraffin sections (5 μm) of 2% paraformaldehyde-fixed muscle were de-paraffined, hydrated, and treated with 3% H2O2 in methanol. Each sample was analysed for the detection of NLRP3, IL1-β, NOX 4, NRF-2, and nitrotyrosine. Antibodies characteristics are reported in *Supplementary Table 3*. Staining was performed by immunohistochemistry after microwave oven treatment in 0.1 M sodium citrate. Immunostainings were completed with a streptavidin-peroxidase method, performed as previously described.[19] Digital images of immunohistochemical sections were obtained using a Leica microscope (Leica Microsystems GmbH Wetzlar, Germany) equipped with a digital camera controlled by Q500MC Software-Qwin (Leica).[12] Image analysis was quantified by two independent evaluators, who were blind to the experimental conditions according to a previously reported protocol.[20]

Muscle fibre cross-sectional (CSA) was determined in 5-μm paraffin sections by image analysis. For each sample, at least 100 fibres were measured. Of note, whereas NLRP3 inflammasome expression and CSA were studied in all the enrolled patients, other determinations were performed in a different number of samples (indicated in the figure legends), according to the availability of biological material.

Western blot

Tissues were lysed in cold buffer (20 mM HEPES, 150 mM NaCl, 10% [v/v] glycerol, 0.5% [v/v] NP-40, 1 mM EDTA, 2.5 mM DTT, 10 μg/L aprotinin, leupeptin, pepstatin A, 1 mM PMSF, and Na3VO4). Protein concentration was determined by using the Bicinchoninic Protein assay kit (Euroclone S.p.A.), and 10–100 μg was resolved on SDS-polyacrylamide gels and electro-transferred to a PVDF membrane (Serva, Euroclone, Pero, Italy). Blots were incubated in anti-NLRP3 (AdipoGen Life Sciences, Vinci-Biochem) and anti-caspase 1 (cleaved Asp210) (Thermo Fisher Scientific) (o.n. at 4°C) and re-probed with β-actin (Santa Cruz Biotechnology) and then in horseradish peroxidase secondary antibodies (Cell Signalling Technology, Euroclone) for 1 h. Immunoblots were developed with Immobilon Western chemiluminescent HRP substrate (Merck Group, Milan, Italy). Band intensities were determined using the Alliance imaging system (Uvitec, Cambridge, UK).

Cell cultures and treatments

The mouse skeletal muscle cell line C2C12 was propagated as myoblasts in DMEM (Euroclone, Milan, Italy) containing 2 mmol L-glutamine and 100 U/mL penicillin–streptomycin (Euroclone, Milan, Italy), with 5% FBS and incubated at 37°C. For differentiation into myotubes, the myoblasts at 90% confluence were differentiated in myotubes with DMEM plus 2% horse serum (Merck Life Science, Milan, Italy). After 7 days, C2C12 myotubes were incubated in the presence of US or normal serum (NS) (10% *v*/v) for 2–48 h to assess NLRP3, IL-1β, caspase-1, Nrf2, and Nox 4 expressions that were studied by western blot and/or real-time PCR performed as previously described.[12] We performed three independent experiments, and, in each experiment, we had two repetitions for every treatment. In selected experiments, TLR4 engagement was blocked by adding TAK-242 (Cayman Chemical, Vinci-Biochem, Vinci, Italy) (1 μM) to the cells 1 h before US stimulation. Finally, in a series of experiments, C2C12 myotubes were also incubated (250 μmol) for 48 h in the presence of indoxyl sulfate (IS) (potassium 3-indoxyl sulfate, Merck Life Science Milan, Italy), a recognized uraemic toxin.

US

US was collected from patients with end-stage renal disease on maintenance haemodialysis recruited randomly from a pool of 150 patients at the Nephrology Division at Genoa University.[12] Informed consent was obtained, and 3 mL of blood was collected before the next subsequent haemodialysis. Healthy age-matched donors were used as controls. Blood urea nitrogen, creatinine, and glucose were assayed in all patients, and control sera were excluded if creatinine was >1.0 mg/dL. Exclusion criteria were the presence of inflammatory disease, acute or chronic infection, autoimmune or liver diseases, diabetes, and malignancy. Neither patients nor controls were smokers. Serum samples were frozen at −80°C until analysis. In preliminary experiments, 10% was found to be the highest non-toxic concentration of US. Inflammatory markers' composition of US has been previously described.[12]

Statistical analysis

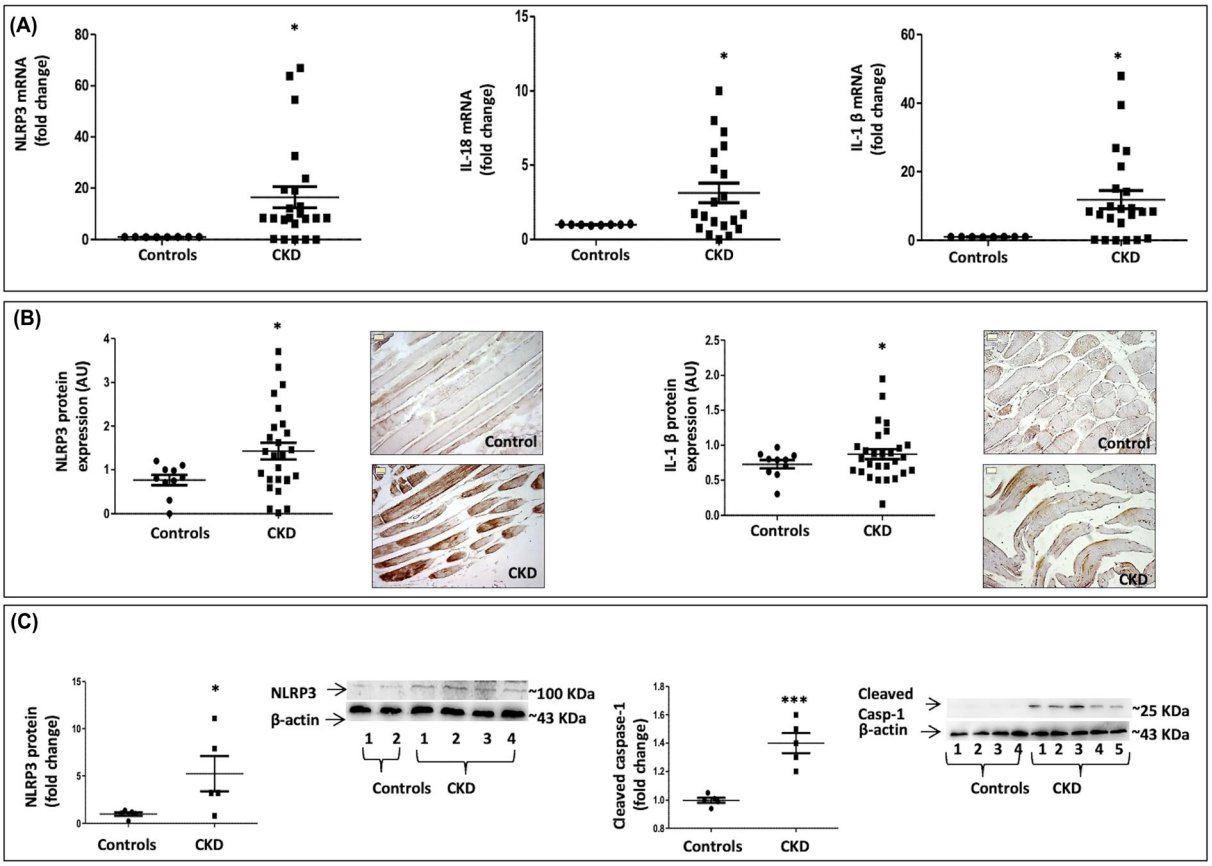
All data are presented as mean ± standard error of mean or median (IQR). Specific mRNAs were normalized for the internal control gene (β-actin) and are expressed as transcript/housekeeping gene ratios. The control treated group mean was given a value of 1, and individual values are expressed relative to this value. Comparisons between groups were performed by one-way ANOVA with a post hoc Bonferroni correction or by a Kruskal–Wallis non-parametric test when appropriate. Relationships between parameters were analysed using simple regression analysis; variables that did not have a Gaussian distribution were logarithmically transformed. Statistical analysis was performed by using the SPSS statistical package (version 16; SPSS, Chicago, IL). Statistical significance occurred if a computed two-tailed $P < 0.05$.

Results

CKD up-regulates NLRP3 inflammasome in skeletal muscle

Muscle fibre CSA was lower in patients with CKD as compared with age-matched controls (median = 881.5 μm^2, range 241–1618; controls, median = 1438 μm^2, range 1100–1974) ($P < 0.05$), indicating muscle atrophy. We previously observed that TLR4 is overexpressed in uraemic muscle.[12] Here, we observed that muscle from CKD patients shows a strong up-regulation of the NLRP3 inflammasome (*Figure 1*). NLRP3 mRNA as well as its protein was overexpressed (by ∼16-fold, respectively, $P < 0.05$ both vs. controls) (*Figure 1A–C*). NLRP3 immunostaining was very faintly expressed in control muscle, although it was clearly detectable as diffuse staining of

Figure 1 NLRP3 inflammasome in muscle of CKD patients. mRNA levels of NLRP-3, IL-1β, and IL-18 were determined by rt-PCR in control and CKD muscle (A). NLRP3 and IL1- β protein expression were evaluated by immunohistochemistry and image analysis (B). NLRP3 mRNA (A) as well as its protein (B,C), were overexpressed in CKD muscle (n = 31) in comparison with control subjects (n = 15). NLRP3 immunostaining was faintly expressed in control muscle, but it was detectable as a diffuse staining of muscle fibres in CKD patients (B). Both IL-1β and IL-18 mRNAs were up-regulated in CKD muscle (A). In CKD, the increased IL-1β expression, detected by immunohistochemistry and image analysis, was observed marginally along the fibres (magnification 400×) (B). Western blot analysis of cleaved caspase-1 (D); cleaved caspase-1 was found overexpressed in CKD muscle (CKD n = 6, controls N = 6) (magnification 400×) (D). For rt-PCR and western blot, values are expressed as fold change ± SEM to the controls, whereas for immunohistochemistry and image analysis, data are expressed as AU ± SEM (*$P < 0.05$, ***$P < 0.001$ vs. controls). AU, arbitrary units; CKD, chronic kidney disease; IL-18, interleukin-18; IL-1β, interleukin-1β; NLRP3, NOD, LRR, and pyrin domain-containing protein 3; rt-PCR, real-time PCR. Bar = 10 μm.



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muscle fibres in CKD patients. Moreover, while we already observed that IL-1β protein is up-regulated in CKD patients,[8] here we found that also IL-1β mRNA expression, such as IL-18 expression, were both up-regulated (by ~11.8–3.2-fold, respectively, $P < 0.05$) (*Figure 1A*). The IL-1β immunosignal was 20% higher in CKD subjects ($P < 0.05$) and was detected marginally along the fibres (*Figure 1B*). Finally, cleaved caspase-1 was overexpressed in CKD muscle samples ($P < 0.001$ vs. controls; *Figure 1D*). Together, these findings show that the components of TLR4/NLRP3/caspase-1 inflammasome cascade are up-regulated in the skeletal muscle of patients with CKD stage 5.

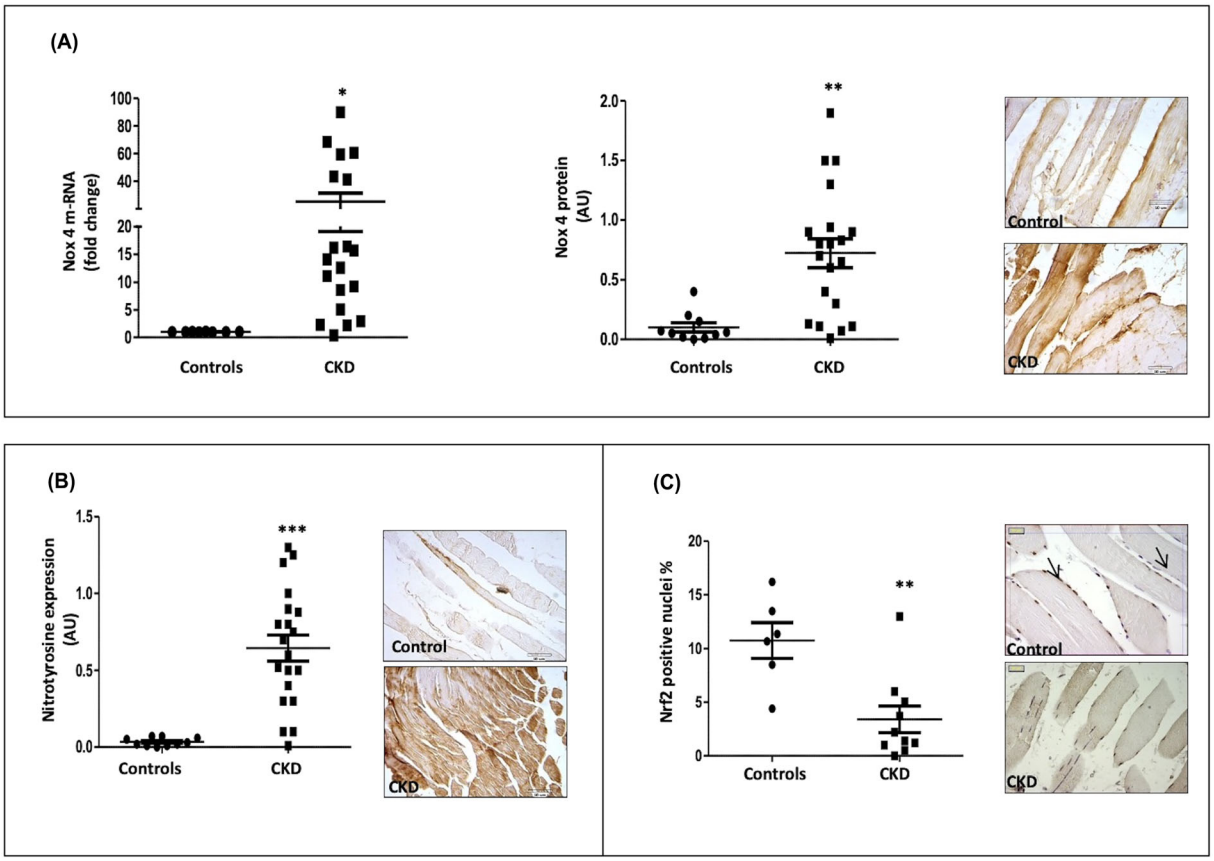
NADPH oxidase 4 (Nox 4) is up-regulated in CKD muscle

As a next step, we studied Nox 4, which regulates the level of oxidative stress, nitrotyrosine, a marker for in vivo tyrosine nitration, and nuclear factor erythroid 2-related factor 2 (Nrf2), a master regulator of redox homoeostasis. Nox 4 mRNA and its protein were markedly overexpressed in CKD muscle (by ~25-fold and ~7-fold, respectively, $P < 0.05$–0.01) (*Figure 2A*). Also, nitrotyrosine was strongly overexpressed in CKD muscle ($P < 0.001$) (*Figure 2B*). As already observed,[21,22] the Nrf2 immunosignal was markedly down-regulated in CKD muscle as compared with control subjects (*Figure 2C*).

Clinical determinants of NLRP3 inflammasome and oxidative stress in muscle

Table 1 shows the clinical determinants of inflammasome activation and oxidative nitrative stress in skeletal muscle. We previously observed that a low eGFR contributed individually and significantly to the prediction of muscle

Figure 2 Oxidative stress in CKD muscle. Nox-4 expression was evaluated by rt-PCR, immunohistochemistry, and image analysis in control and CKD muscle (A). Nox-4 mRNA was markedly overexpressed in CKD muscle (by ~25-fold) ($n = 21$) vs. controls ($n = 15$) (A). Nox-4 as well as nitrotyrosine (B) immunosignals were strongly present along muscle fibres in CKD ($n = 21$) vs. controls ($n = 15$); contrariwise, Nrf2, expressed as percentage of positive nuclei, was severely down-regulated (CKD, $n = 10$, controls, $n = 6$) (C). Arrows point out positive nuclei. mRNA expression was reported as fold change ± SEM to the controls, while protein expression as AU ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. controls. A,B = magnification 400×, bar = 10 μm; C = magnification 630×, bar = 50 μm. AU, arbitrary units; CKD, chronic kidney disease; Nox-4, NADPH oxidase 4; Nrf-2, nuclear factor erythroid 2-related factor 2; rt-PCR, real-time PCR.



TLR4 expression.[12] Here, we observed that also both NLRP3 mRNA (n = 22, r = −0.606, $P < 0.01$) and logIL-1 β protein (n = 26, r = −0.460, $P < 0.02$) were inversely associated with residual renal function (*Table 1* and *Figure 3A* and *3B*), which suggests that in skeletal muscle of advanced-stage CKD patients, the inflammasome is progressively activated as the eGFR reaches very low (∼5 mL/min) levels. Muscle LogIL-1β protein was also associated with a higher CRP plasma level (n = 23, r = 0.437, $P < 0.03$). Surprisingly, plasma CRP was inversely associated with muscle nitrotyrosine expression (n = 22, r = −0.496, $P < 0.05$). A lower SGA score (an index of wasting) was associated with higher muscle NLRP3 expression (n = 28, r = −0.473, $P < 0.05$) (*Table 1*). A low CSA, an expression of muscle atrophy, was related to both up-regulated Nox 4 mRNA (n = 17, r = −0.460, $P < 0.035$) and nitrotyrosine (n = 19, r = −0.494, $P < 0.03$), suggesting that nitrative/oxidative stress is a major determinant of muscle protein content (*Table 1* and *Figure 4*).

US up-regulates NLRP3 and IL-1β in C2C12 myotubes

As a further step, we studied if the uraemic milieu up-regulates inflammasome in C2C12 myotubes. NLRP3 mRNA underwent an ∼11-fold increase ($P< 0.05$) after a 2-h exposure of cells to 10% US, an effect that was boosted after 5 h (+71%, $P < 0.01$) (*Figure 5A*). NLRP3 protein was similarly up-regulated after a 5-h US exposure ($P < 0.001$) (*Figure 5A*) as shown by western blot and immunofluorescence analysis. NLRP3 binds caspase-1, causing its cleavage and activation. In muscle cells, US increased the fraction of activated (cleaved) caspase-1 by ∼5-fold with respect to NS ($P < 0.05$) (*Figure 5B*). Caspase-1 activation is responsible for the maturation and secretion of the pro-inflammatory Il-1β. Indeed, in the presence of US, there was a time-dependent increase in Il-1β mRNA: After 2 h, Il-1β mRNA rose by ∼3-fold ($P < 0.05$), and at 5 h, its expression was even greater (by ∼12-fold, $P < 0.01$) (*Figure 5C*).

Nrf2 and Nox 4 are inversely regulated by the US in C2C12 cells

Then, we evaluated the Nrf2 and Nox 4 responses in US-treated myotubes. After 5 h, Nrf2 mRNA was initially increased after 5 h ($P < 0.05$), but it was down-regulated at 48 h from US exposure (−30%, $P < 0.05$) (*Figure 6A*). As shown in *Figure 6B*, both Nox 4 mRNA and its protein expression were enhanced by the US.

Table 1 Univariate analysis of the correlation between inflammasome components and clinical characteristics in patients with CKD

	Age	p	Gender	p	Weight	p	BMI	p	CSA	p	SGA	p	eGFR	p	BUN	p	Albumin	p	CRP	p
logNLRP3 mRNA	−0.251	NS	−0.126	NS	0.213	NS	0.233	NS	−0.22	NS	−0.331	NS	−0.488	0.037	0.356	0.06	−0.449	NS	0.126	NS
logNLRP3 protein	0.235	NS	0.12	NS	0.167	NS	0.168	NS	0.150	NS	−0,473	0.02	−0.166	NS	−0.04	NS	0.164	NS	0.144	NS
logIL-18 mRNA	0.257	NS	−0.264	NS	0.217	NS	0.027	NS	0.310	NS	0.331	NS	−0.366	NS	−0.301	NS	0.146	NS	0.105	NS
logIL-1β mRNA	0.299	NS	−0.180	NS	0.01	NS	0.01	NS	−0.13	NS	0.02	NS	−0.200	NS	0.10	NS	−0.490	NS	0.110	NS
logIL-1β protein	0.149	NS	−0.255	NS	0.176	NS	0.165	NS	0.225	NS	0.103	NS	−0.392	0.039	−0.33	NS	0.32	NS	0.434	0.03
logNox4 mRNA	0.191	NS	0.042	NS	0.126	NS	0.32	NS	−0.46	0.035	0.264	NS	−0.214	NS	0.065	NS	0.258	NS	0.117	NS
logNox4 protein	0.31	NS	0.19	NS	−0.166	NS	−0.23	NS	−0.361	NS	−0.301	NS	0.184	NS	−0.243	NS	0.239	NS	0.283	NS
logNitrotyrosine	0.025	NS	0.21	NS	−0.099	NS	−0.24	NS	−0.608	0.02	−0.305	NS	0.285	NS	−0.251	NS	0.099	NS	0.170	NS

BMI, body mass index (kg/m^2); CRP, C-reactive protein (mg/L); CSA, cross-sectional area (μm^2); eGFR, estimated glomerular filtration rate (mL/min.1.73 m^2); NS, not statistically significant; SGA, subjective global assessment.

Figure 3 **(A,B)** Relationships between NLRP3 mRNA (A, *n* = 22), logIL-1 β protein (B, *n* = 26), and estimated glomerular filtration rate (eGFR) in patients with chronic kidney disease. IL-1β, interleukin-1β; NLRP3, NOD, LRR, and pyrin domain-containing protein 3.

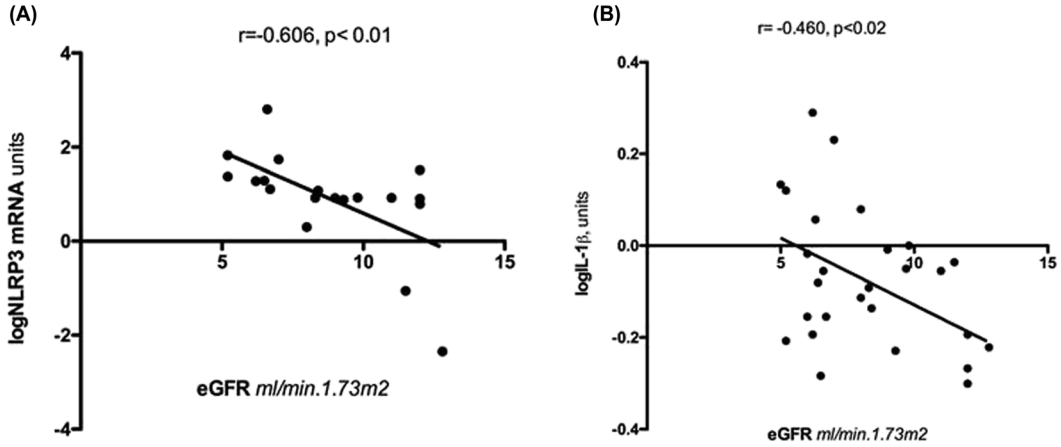
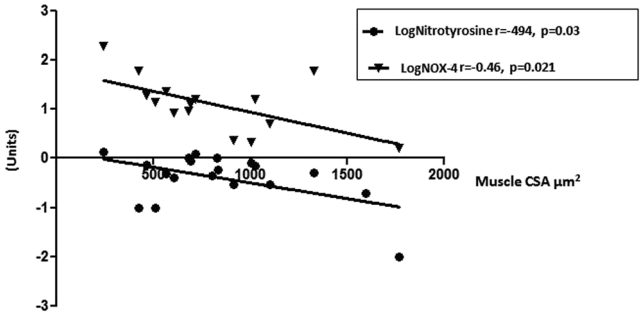


Figure 4 Relationships between muscle cross-sectional area (CSA), an expression of muscle atrophy, Nox-4 mRNA (*n* = 17), and nitrotyrosine (*n* = 19) in patients with chronic kidney disease. CKD, chronic kidney disease; CSA, cross-sectional area; Nox-4, NADPH oxidase 4.



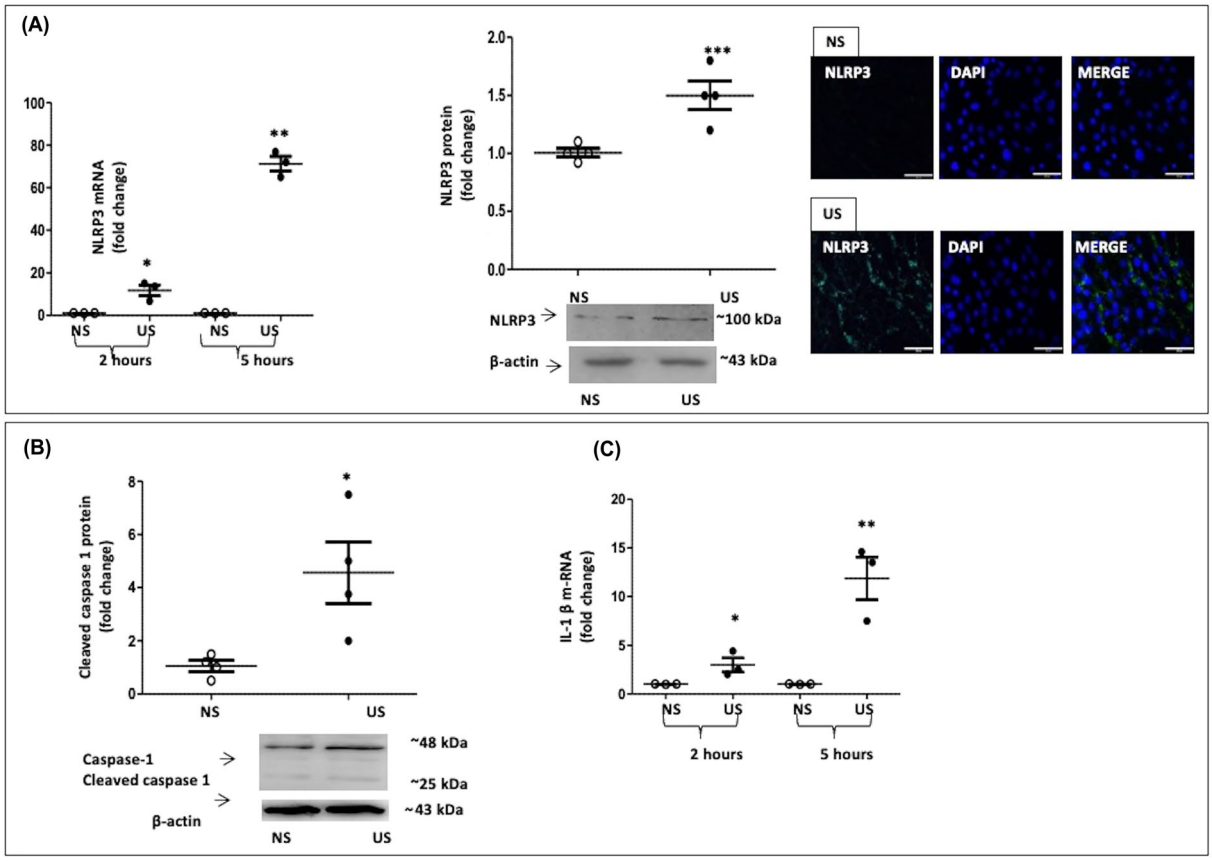
IS activates TLR4/NLRP3 inflammasome signalling in C2C12 cells

As a next step, we studied if IS, a recognized uraemic toxin, can promote TLR4/NLRP3 expression in myotubes. As shown in *Figure 7*, IS treatment was associated with an overexpression of TLR4 (both mRNA and protein), NLRP3, and Il-1β mRNA ($P < 0.05$–0.01) as well as of atrogin-1 mRNA (∼2.5-fold increase, $P < 0.05$).

Effects of the TLR4 antagonist TAK-242 on US-stimulated C2C12 myotubes

We studied whether the inhibition of TLR4 signalling reverses NLRP3 inflammasome activation in C2C12 myotubes. To this end, we pre-treated (for 60 min) C2C12 myotubes with TAK-242 (1 μM), a small molecule that binds selectively to TLR4 and interferes with the interaction between TLR4 and its adaptor molecules.[23] TLR4 blocking had an inhibitory action on US-induced up-regulation of NLRP3, Il-1β, and caspase-1 expressions (*Supplementary Figure 1A*). TAK-242 pre-treatment decreased NLRP3 and Il-1β mRNAs by 82 and 62%, respectively ($P < 0.001$–0.05), with respect to the US only. In addition, NLRP3 protein and cleaved caspase-1 were down-regulated by TAK-242 (by ∼60–50%, $P < 0.01$). This effect was also observed for atrogin-1 (−40%, $P < 0.05$) (*Supplementary Figure 1A*), an E3 ubiquitin ligase, which targets both structural and contractile proteins for UPS-dependent degradation. Similarly, TAK-242 treatment blunted NLRP3, IL1β, and atrogin-1 expression in myotubes exposed to IS (by −20–40%, $P < 0.05$) (*Supplementary Figure 1B*). Finally, we evaluated the genic expression of NLRP-3 and IL-1 β by rt-PCR in C2C12 myotubes exposed to NS + TAK 242, and we found no differences with respect to only NS treatment. Furthermore, NLRP-3 expression, detected by immunofluorescence, was similar in NS and TAK-242 + NS-treated cells (*Supplementary Figure 2*).

Figure 5 Effects of US on inflammasome expression in C2C12 myotubes. (A) 2–5-h US exposition increased mRNA and protein NLRP-3 levels, as well as cleaved caspase-1 (B) and IL-1β (C). mRNAs were evaluated by rt-PCR and proteins by western blot and immunofluorescence (only NLRP-3). Blots were stripped and reprobed with anti-β-actin antibody. All results represent means ± SEM obtained from three/four independent experiments and are expressed as fold change to NS. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Magnification 400×; bar = 35 μm. IL-1β, interleukin-1β; NLRP3, NOD, LRR, and pyrin domain-containing protein 3; NS, normal serum; rt-PCR, real-time PCR; US, uraemic serum.



Discussion

NLRP3 plays a unique role among NLRs because it can sense a large array of endogenous metabolic 'danger signals' to induce sterile inflammation and acceleration of atherosclerosis.[17,24,25] In addition, the NLRP3 inflammasome is implicated in the cognitive decline and functional measures of frailty,[13] conditions that also are commonly observed in CKD patients. This study aimed to learn more about the role of the TLR4/NLRP3 pathway in up-regulating the muscle inflammatory response in uraemic patients. Using muscle biopsies samples, our study shows that a TLR4/NLRP3/caspase-1-driven inflammatory response occurs in the skeletal muscle of non-dialysed patients with advanced-stage CKD. This response is associated with up-regulated Nox 4 and nitrotyrosine, which are both related to enhanced oxidative/nitrative stress. In addition, using well-established mouse culture models, our study shows that following exposure to US, several components of NLRP3 inflammasome are strongly and rapidly activated in muscle, leading to the up-regulation of IL-1β gene expression. In addition, the US-induced NLRP3 inflammasome response is prevented by TAK-242, a small-molecule-specific inhibitor of TLR4 signalling. Overall, our data suggest that the uraemic muscle senses some still unrecognized circulating DAMPS in uraemia and responds by the activation of a TLR4/NRLP3/caspase-1 inflammasome pathway.

Our findings also suggest that maintaining residual kidney function may be protective against inflammasome activation. Both muscle NLRP3mRNA and IL-1β, the final product of the inflammasome, were inversely associated with eGFR (*Figure 4*), implying that inflammasome may be progressively activated in skeletal muscle of CKD5 patients as their residual renal function deteriorates. In addition, muscle LogIL-1β protein was also associated with a higher CRP plasma level, an index of systemic inflammatory response.

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Figure 6 (A,B) Opposite effects of US on Nrf-2 and Nox-4 in C2C12 myotubes. A 5-h US exposition rose up Nrf-2 mRNA levels, which fell down at 48 h, as well as its protein (A). Differently, Nox-4 expression was already increased at 5 h (B). mRNAs were evaluated by rt-PCR and protein by western blot. Blots were stripped and reprobed with anti-β-actin antibody. All results represent means ± SEM obtained from three/four independent experiments and are expressed as fold change to NS. *$P < 0.05$. Nox-4, NADPH oxidase 4; Nrf-2, nuclear factor erythroid 2-related factor 2; NS, normal serum; rt-PCR, real-time PCR; US, uraemic serum.

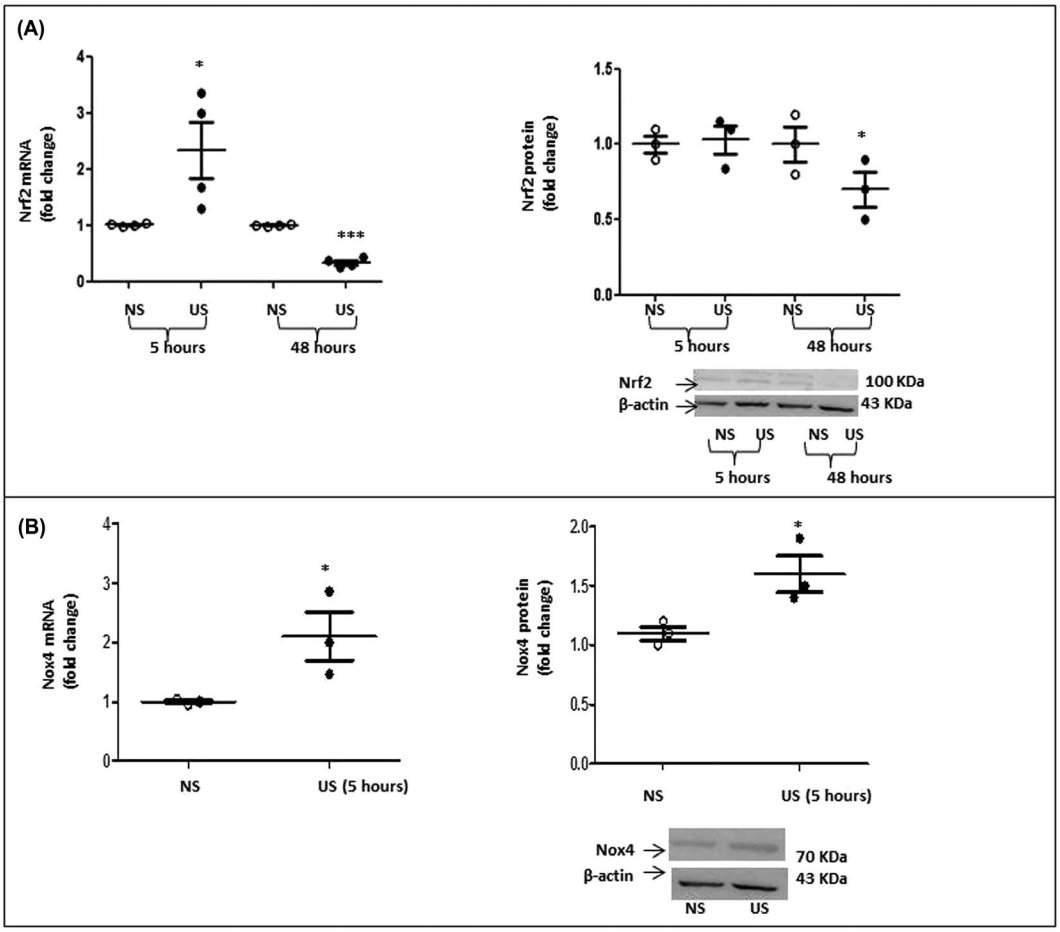
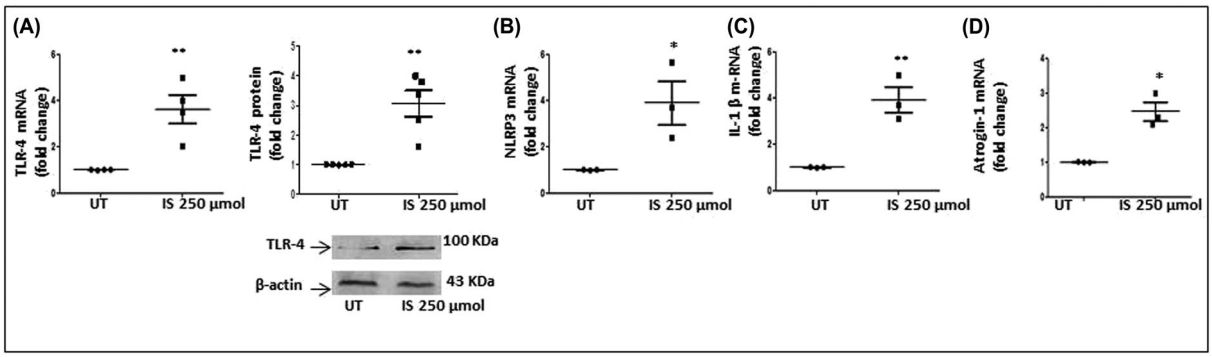


Figure 7 (A–D) Effects of IS on TLR-4 and inflammasome expression in C2C12 myotubes. A 48-h IS exposition increased mRNA and protein TLR-4 levels (A), as well as NLRP-3 (B) and IL1-β mRNAs (C). IS also up-regulated atrogin-1 mRNA expression (D). mRNAs were evaluated by rt-PCR and protein by western blot. Blots were stripped and reprobed with anti-β-actin antibody.All results represent means ± SEM obtained from three/four independent experiments and are expressed as fold change to UT.*$P < 0.05$, **$P < 0.01$. IL-1β, interleukin-1β; IS, indoxyl sulfate; NLRP3, NOD, LRR, and pyrin domain-containing protein 3; TLR-4, Toll-like receptor-4; UT, untreated cells.



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The individual DAMP(s) that may elicit the NLRP3 inflammasome response in skeletal muscle when uraemia develops is not known. Many DAMPs are intracellular and are released to the extracellular space when cells are exposed to stress. Besides endotoxins,[11] the NLRP3 inflammasome can also be activated by DAMPs of non-microbial origin, such as nuclear and mitochondrial DNA, RNA, nucleotides and nucleosides, DNA-binding molecules, temperature-shock proteins, ceramides, free cholesterol, uric acid, ATP, and ROS.[11,13,17,26] Several of the already recognized DAMPs are known to accumulate in the blood in uraemia[27–29] and might elicit a tissue inflammatory response. In our study, IS, a putative uraemic toxin implicated in cardiovascular disease and muscle wasting,[18] was able to activate the inflammasome in myotubes, suggesting that it may act as one of the circulating DAMPs in uraemia.

As a new finding, our study shows that Nox 4 is up-regulated in the muscle of CKD patients. Studies in rodent models of CKD offer discordant views on oxidative stress regulation in uraemic muscle.[28,29] In our study, CKD patients showed an up-regulation of oxidative/nitrative stress markers, an effect that was also observed in vitro when muscle cells were incubated with US or IS. The most commonly accepted activating stimuli for NLRP3 include the relocalization of NLRP3 to the mitochondria and the sensation of mitochondrial factors released into the cytosol with mitochondrial ROS release.[29] In addition, ROS can be produced in the TLR4/NOX 4/NF-κB pathway.[30,31] However, both in the heart and skeletal muscle, the transmembrane NOXs play a major role in cytoplasmic and mitochondrial ROS production.[32]

Nitrotyrosine may derive from the interaction of oxidants derived from peroxynitrite (ONOO−) with tyrosine residues contained in proteins or through alternative pathways, including one mediated by myeloperoxidase (MPO). In several cachexia models, oxidative, as well as nutritional, mechanical, and energy stresses, can modulate signalling pathways that ultimately converge on protein turnover.[33] In our study, both inflammasome and overexpressed oxidative stress proteins accounted for indexes of nutritional status. On the one hand, the 7-scale SGA, an index of nutritional status that is predictive of mortality in CKD,[34] was associated with higher muscle NRLP3, suggesting a role of inflammasome activation in wasting. On the other hand, a low-fibre CSA, an expression of muscle atrophy, was associated with higher muscle Nox 4 mRNA and nitrotyrosine, suggesting that oxidative/nitrative stress is a major determinant of muscle protein content in uraemia. This is not surprising, because high levels of ROS/RNS cause lipid peroxidation, protein oxidation/nitration, inactivation of enzymes, and DNA damage and long-lived, differentiated muscle fibres are highly susceptible to oxidative damage.[35] As shown also in previous studies, nitrotyrosine levels may be independent of inflammatory markers like CRP.[36]

It has been suggested that ameliorating the inflammatory response through anti-cytokine therapies could improve cardiovascular risk factors and potentially survival in CKD.[18] Because the whole sequence of NLRP3 inflammasome activation requires the involvement of priming and activation signals, all of them can be targeted to control the inflammation response. Current treatments for NLRP3-related diseases include DAMP-inhibiting molecules or biological agents that target IL-1β, IL-18, or their receptors.[18,37] With this regard, IL-1β inhibition has been shown to reduce major adverse cardiovascular event rates among high-risk atherosclerosis patients with CKD.[38] There is a lack of therapeutic interventions that are safe and effective in preventing or treating protein-energy wasting, a condition that is commonly observed associated with atherosclerosis and inflammation. Mounting evidence suggests that TLR-mediated inflammatory reactions embrace relevance to cardiovascular diseases, including diabetic cardiomyopathy, atherosclerosis, and hypertension.[39] Consistent with our hypothesis, both NLPR3 inflammasome and atrogin gene expression were improved when the TLR4 inhibitor, TAK-242, was used in myotubes. TAK-242 (resatorvid) is a small-molecule inhibitor of TLR4 signalling, which binds selectively to TLR4 and interferes with interactions between TLR4 and its adaptor molecules.[23] Resatorvid has been previously used with success to prevent muscle wasting induced by sepsis in mice[40]; however, no human study is so far available.

We are aware of the limitations of our study. So, although we observed NLRP3 inflammasome activation in the skeletal muscle of CKD patients, the lack of functional tests, such as IL1b secretion, casp1 cleavage, and NLRP3 oligomerization, did not allow us to evaluate the actual effects of this activation. Moreover, because both ageing and inactivity may concur with inflammation and muscle atrophy, a potential link between CKD and muscle inflammation may exist simply because CKD is a condition commonly found in older adults who are also inactive. Finally, even if the control subjects were matched by age and gender and had inactive behaviour, segregating the impact of CKD per se from that of lifestyle habits and age-associated conditions on muscle pathophysiology poses a relevant challenge.

Nevertheless, we think the data here provided are sound enough to support the conclusion that the activation of the NLRP3 inflammasome, an intracellular sensor that recognizes many endogenous danger signals leading to caspase 1-dependent release of pro-inflammatory cytokines, occurs in the skeletal muscle of patients with advanced-stage CKD.

Our data also reveal TLR4/NLRP3 inflammasome activation as an upstream target for approaches, either dietary or pharmacological, to disrupt the feed-forward loop of inflammation and accelerated muscle catabolism in uraemia.

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Conflict of interest

The authors declare no conflict of interest regarding the content of this manuscript.

Ethics statement

The study was approved by the Ethical Committee of the Department of Internal Medicine of the University of Genoa and the Ethical Committee Regione Liguria IRCCS Ospedale Policlinico San Martino, Genoa, Italy. All subjects were informed about the nature, purposes, procedures, and possible risks of the study, before their informed consents were obtained. The procedures were in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.[41]

Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Supplemental Table 1. Clinical characteristics of controls and chronic kidney disease (CKD) subjects.

Supplemental Table 2. Primer sequences for RT–PCR analyses.

Supplemental Table 3. Product number and dilution ratio of the antibodies used for western blot and immunohistochemical staining.

Supplementary Figure 1. The effects of inhibition of TLR4 signaling on the NLRP3 inflammasome activation induced by US or IS. C2C12 myotubes were pre-treated for 1 hour with TAK-242 (1 μM), before US (Panel A) or IS (Panel B) treatment. TLR4 blocking had an inhibitory action on NLRP3, Il-1β (A, B) and Caspase −1 (A) expressions. In addition, the US-induced atrogin-1 increase was blunted by TAK-242 (A, B). mRNAs were evaluated by rt-PCR and protein by western blot and immunofluorescence (only NLRP-3). Blots were stripped and reprobed with anti-β actin antibody. Magnification x400; Bar = 35 μm. All results represent means ± SEM obtained from three/four independent experiments and are expressed as fold change to NS or IS. $*P < 0.05$, $***P < 0.001$; TLR-4 = Toll like Receptor- 4; US = Uremic serum; IS = Indoxyl sulfate; NLRP3 = NOD-, LRR- and pyrin domain-containing protein 3; IL-1β = Interleukin-1 β; rt-PCR = real time PCR.

Supplementary Figure 2. The effects of inhibition of TLR4 by TAK-242 (1 μM) on the NLRP3 inflammasome and IL-1β expression in C2C12 myotubes exposed to NS. mRNAs were measured by rt-PCR (A) and NLRP3 protein expression was evaluated by immunofluorescence (B). All results represent means ± SEM obtained from three independent experiments and are expressed as fold change to NS; $p > 0.05$; NS = Normal serum; NLRP3 = NOD-, LRR- and pyrin domain-containing protein 3; IL-1β = Interleukin-1 β; rt-PCR = real time PCR; Magnification x400; Bar = 35 μm.

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