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► **To cite this version:**

Stanislas Bataille, Nathalie Mckay, Laetitia Koppe, Alice Beau, Bérengère Benoit, et al.. Indoxyl sulfate inhibits muscle cell differentiation via Myf6/MRF4 and MYH2 downregulation. *Nephrology Dialysis Transplantation*, 2024, 39 (1), pp.103-113. 10.1093/ndt/gfad123 . hal-04254193

**HAL Id: hal-04254193**

**<https://amu.hal.science/hal-04254193v1>**

Submitted on 28 Feb 2024

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# Indoxyl sulfate inhibits muscle cell differentiation via *Myf6*/MRF4 and MYH2 downregulation

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## ABSTRACT

**Background.** Chronic kidney disease (CKD) is associated with a significant decrease in muscle strength and mass, possibly related to muscle cell damage by uremic toxins. Here, we studied *in vitro* and *in vivo* the effect of indoxyl sulfate (IS), an indolic uremic toxin, on myoblast proliferation, differentiation and expression of myogenic regulatory factors (MRF)—myoblast determination protein 1 (*MyoD1*), myogenin (*Myog*), Myogenic Factor 5 (*Myf5*) and myogenic regulatory factor 4 (*Myf6*/MRF4)—and expression of myosin heavy chain, *Myh2*.

**Methods.** C2C12 myoblasts were cultured *in vitro* and differentiated in myotubes for 7 days in the presence of IS at a uremic concentration of 200  $\mu$ M. Myocytes morphology and differentiation was analyzed after hematoxylin-eosin staining. MRF genes' expression was studied using reverse transcription polymerase chain reaction in myocytes and 5/6th nephrectomized mice muscle. *Myf6*/MRF4 protein expression was studied using enzyme-linked immunosorbent assay; MYH2 protein expression was studied using western blotting. The role of Aryl Hydrocarbon Receptor (AHR)—the cell receptor of IS—was studied by adding an AHR inhibitor into the cell culture milieu.

**Results.** In the presence of IS, the myotubes obtained were narrower and had fewer nuclei than control myotubes. The presence of IS during differentiation did not modify the gene expression of the MRFs *Myf5*, *MyoD1* and *Myog*, but induced a decrease in expression of *Myf6*/MRF4 and MYH2 at the mRNA and the protein level. AHR inhibition by CH223191 did not reverse the decrease in *Myf6*/MRF4 mRNA expression induced by IS, which rules out the implication of the ARH genomic pathway. In 5/6th nephrectomized mice, the *Myf6*/MRF4 gene was down-regulated in striated muscles.

**Conclusion.** In conclusion, IS inhibits *Myf6*/MRF4 and MYH2 expression during differentiation of muscle cells, which could lead to a defect in myotube structure. Through these new mechanisms, IS could participate in muscle atrophy observed in CKD.

**Keywords:** chronic kidney disease, indoxyl sulfate, myogenesis, uremic toxin

## KEY LEARNING POINTS

### What was known:

- Chronic kidney disease (CKD) is associated with a higher risk of sarcopenia.
- Uremic toxins, including the uremic toxin indoxyl sulfate (IS), have been shown to exert toxic effects on mature myocytes, but the effect of IS on myoblast differentiation in myocytes remains unclear.

### This study adds:

- This study shows that IS inhibits myoblast differentiation in myocytes.
- It demonstrates that IS inhibits *Myf6*/MRF4 and MYH2 expression during differentiation of muscle cells.
- This is a new mechanism by which IS might induce sarcopenia during CKD.

### Potential impact:

- This study describes a new pathophysiological mechanism of sarcopenia during CKD which could be a new therapeutic target.
- It also demonstrates that uremic toxin targeting (by removal, prevention of accumulation and/or decrease in generation) is an important approach in treating sarcopenia in CKD patients, especially when muscle is in a regenerating process (after lesion).

## INTRODUCTION

Chronic kidney disease (CKD) is associated with muscle wasting and decrease in strength, a clinical state named sarcopenia [1–4]. Sarcopenia is associated with a high risk of death and of progression of renal failure [5]. Several factors explain the loss of muscle strength and muscle mass in CKD. Some are directly related to CKD, like uremic toxin accumulation and chronic inflammation; others are due to associated comorbidities, like diabetes, age, obesity or a sedentary lifestyle [6–9]. Nevertheless, the precise pathophysiology of sarcopenia in CKD remains to be understood.

After muscle wounding, muscle repair involves the recruitment of multipotent stem cells named satellite cells (SCs). SCs are quiescent stem cells located at the periphery of the sarcolemma of muscle fibers, under the basement membrane. When activated, these cells can proliferate, differentiate and give rise to new muscle fibers or fuse to pre-existent muscle fibers to increase their size, providing new myonuclei to muscle fibers.

The activation of SCs leads to their transformation into proliferating myoblasts which, in turn, differentiate into mature muscle fibers. After that, a subset of myoblasts returns to a quiescent state. Differentiation requires the sequential transcription of four basic helix–loop–helix transcription factors that regulate myogenesis. These transcription factors are the myogenic regulatory factors (MRF) family [10], which includes myogenic factor 5 (MYF5), myoblast determination protein 1 (MYOD1), myogenin (MYOG) and myogenic regulatory factor 4 (MYF6/MRF4, MYF6 being the gene encoding the protein MRF4).

During embryogenesis, some MRF are essential for a healthy striated muscle development, but others exert a partly redundant role. *Myf5* null mice and *Myod1* null mice have a roughly normal phenotype (*Myf5*<sup>null/null</sup> mice have a retarded trunk muscle and *Myod1*<sup>null/null</sup> mice have delayed early limb and branchial arch muscle development), but *Myf5* and *Myod1* double knock-out mice have a complete absence of skeletal myocytes or myofibers [10]. *Myog*<sup>null/null</sup> mice have a marked reduction in skeletal muscle, indicating its role in muscle cell differentiation. *Myf6*<sup>null/null</sup> mice have a normal phenotype. During post-natal muscle repair and myogenesis, the MRF are sequentially activated in myoblasts and drive the differentiation into muscle fibers [11]. In adults, MRF are not expressed in quiescent SCs, nor are they in mature muscle cells except *Myf6*/MRF4 which could be necessary for mature muscle fiber innervation and maintenance of the SC pool [10, 12, 13].

Beside MRF that are only expressed during regeneration, some structural proteins are permanently expressed in adult muscle, especially proteins involved in the structure and contractibility of muscle cells. Within them, Myosin-heavy chain 2 (MyHCIIa), encoded by the MYH2 gene, is a protein of the sarcomere involved in muscle contraction.

Muscle cells from CKD patients are permanently exposed to uremic toxins. Among them, the indolic toxin indoxyl sulfate (IS) was shown to accumulate in CKD muscle tissue *in vivo* [14] and to be deleterious for muscle cells [15, 16]. IS is an indolic uremic toxin derived from the metabolism of dietary tryptophan by intestinal bacteria. The majority of the harmful effects of IS are related to its ability to activate its cell receptor Aryl Hydrocarbon Receptor (AHR) [17]. AHR is a ligand-activated transcription factor which also possesses an intrinsic E3 ubiquitin ligase function [18]. As an Ahr ligand, IS activates Ahr and increases the expression of the downstream target gene *Cyp1a1* in mouse muscle cells [19]. Hemodialyzed patients with a high level of IS showed a greater loss of handgrip strength, even after adjustment for po-

tential confounders [20]. In CKD mice, the reduction of IS levels by AST-120 treatment rescues the decrease in skeletal muscle weight [21]. Studies have shown that IS could induce muscle atrophy by impairing mitochondrial function [21], inducing metabolic alterations such as upregulation of glycolysis [14], and stimulating reactive oxygen species-mediated production of muscle atrophy-related genes [16]. In the present work, we studied the effect of IS on muscle cell differentiation as well as on MRF and MYH2 expression *in vitro* and *in vivo*.

## MATERIALS AND METHODS

### Culture and differentiation of C2C12 cells

Mouse C2C12 myoblasts were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) containing GlutaMAX™ (Life technologies, ThermoFisher Scientific, Courtaboeuf, France) supplemented with 10% fetal calf serum (Dominique Dutscher, Brumath, France) and 1% penicillin, streptomycin and amphotericin B (PS), under 37°C and 5% CO<sub>2</sub>.

Differentiation in myocytes was obtained after 7 days of culture of C2C12 myoblasts in DMEM high glucose GlutaMAX™ medium with 1% PS added with 2% horse serum. During differentiation, cells were incubated with IS (Sigma-Aldrich, France) at 200 μM, a concentration in the range of those reported in CKD [22]. IS was diluted from a stock solution prepared in water. Because IS was purchased as a potassium salt, IS was compared with potassium chloride (KCl, Sigma-Aldrich, France), used as control. Solutes were diluted 1:1000 in culture medium to reach working concentrations.

### C2C12 cell proliferation assay

The effect of IS on C2C12 proliferation was assessed by measuring 5-Bromo-2'-deoxy-uridine (BrdU) incorporation into cellular DNA. C2C12 at a concentration of 5.10<sup>4</sup> cells/mL were seeded in 96-well culture plates in DMEM medium containing 10% fetal calf serum and 1% PS, and cultured for 24 h. Cells were serum-starved for 2 h, then incubated for 48 h with IS at 200 μM or controls (medium or KCl) in DMEM medium containing 10% fetal calf serum and 1% PS. BrdU was added to the wells during the last 12 h.

Cell incorporation of BrdU was measured by enzyme-linked immunosorbent assay (ELISA) with the Roche's Cell Proliferation ELISA, BrdU (colorimetric) (Sigma-Aldrich, France), according to the manufacturer's instructions. In brief, cells were fixed, cellular DNA was partially digested by nuclease treatment, and incorporated BrdU was detected with anti-BrdU mAb conjugated with peroxidase. The absorbance was measured at 405 nm with a microplate reader (Glomax, Promega, France) and was directly correlated to the level of BrdU incorporation into cellular DNA. Results were expressed in % of BrdU incorporation into cellular DNA versus culture medium alone.

### Evaluation of IS cytotoxicity by a lactate dehydrogenase assay

The cytotoxicity of IS was evaluated by measuring lactate dehydrogenase (LDH) activity in cell supernatants. LDH was measured using the CyQUANT™ LDH Cytotoxicity Fluorescence Assay (Invitrogen), according to the manufacturer's protocol. Fluorescence intensity was measured at 560/590 nm, using a Glomax microplate reader (Promega, France). Results were expressed in % of cytotoxicity.

## Hematoxylin-eosin staining

After 7 days of differentiation in presence of IS 200  $\mu\text{M}$ , the morphology of C2C12 cells was examined by hematoxylin–eosin (HE) staining. Briefly, cells were fixed in methanol for 10 min at  $-20^{\circ}\text{C}$ , washed with PBS, and subjected to HE staining. At least 15 fields were analyzed by microscopy using a DM8 microscope (Leica, France). The number of myotubes per field and the number of nuclei per myotube were counted. The thickness and length of myotubes were measured and expressed in  $\mu\text{m}$ . All counts and measurements were done blindly to the experimenter.

## RNA extraction and RT-qPCR analysis of mRNA expression in C2C12

C2C12 cells were incubated during 7 days of differentiation with IS (Sigma-Aldrich, France) at 200  $\mu\text{M}$ . Total RNA was extracted from C2C12 myoblasts and myocytes by RNeasy mini-kit (Qiagen, Courtaboeuf, France). RNA concentrations were determined using a NanoDrop Spectrophotometer (ThermoFisher Scientific, Wilmington, DE, USA).

Reverse transcription (RT) was performed from 500 ng of total RNA with the Takara PrimeScript RT Reagent Kit (Takara, Saint-Germain-en-Laye, France) followed by quantitative polymerase chain reaction (qPCR) on 10 ng of cDNA using the TaqMan™ Universal Master Mix II from Applied Biosystems™ (ThermoFisher Scientific, Courtaboeuf, France). Taqman probes were purchased from ThermoFisher Scientific (Courtaboeuf, France); references are provided in Supplementary data, Table S1. The housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (*Hprt*) was used to normalize the target gene values.

All qPCRs were performed using an Applied Biosystems StepOnePlus Real-Time PCR System (ThermoFisher scientific, Courtaboeuf, France). The data were acquired and analyzed with the StepOne software (ThermoFisher scientific, Courtaboeuf, France). Normalized mRNA levels were expressed as fold-change versus control, calculated using the  $2^{-\Delta\Delta\text{Ct}}$  method. The gene was considered to have no expression when Ct was  $>35$ .

## Myf6/MRF4 ELISA

To measure Myf6/MRF4 protein expression in C2C12 cells, we used a Myf6/MRF4 colorimetric cell-based ELISA (CB5859) (CytoGlow™—AssayBiotech, Fremont, CA, USA). The assay was performed as recommended by the manufacturer. Protein expression was evaluated by measuring the optical density at 450 nm using a Glomax® Explorer microplate reader (Promega Corporation, Madison, WI, USA). Data were normalized with GAPDH as recommended by the manufacturer.

## Western blot

C2C12 cells were exposed to IS (Sigma-Aldrich, France) at 200  $\mu\text{M}$  or control KCl during 7 days of differentiation. After cell incubation, cells were lysed on ice with cold RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 1 mM EDTA, pH 7.4) containing protease inhibitors (Complete mini, Roche Diagnostics France, Meylan, France). Protein concentrations were determined using the Bicinchoninic Acid Kit BCA1 (Sigma Aldrich, St Quentin Fallavier, France). Total proteins (25  $\mu\text{g}$ ) from cell lysates were separated using 4%–12% NuPAGE™ sodium dodecyl sulfate–polyacrylamide electrophoretic gel (Life

technologies, ThermoFisher Scientific, France) and transferred onto nitrocellulose membranes. Nonspecific binding was blocked with 5% skim milk for 1 h at room temperature. The membranes were incubated with primary antibodies directed against MYH2 (A4.74 Santa Cruz, Clinisciences, France) or the loading control actin (D6A8, Cell Signaling, Ozyme, France), and then with the secondary peroxidase-conjugated goat anti-mouse (ThermoFisher Scientific, France) or anti-rabbit (ThermoFisher Scientific, France) antibodies. Revelations were done by chemiluminescence (ECL Western blotting substrate, Pierce, Courtaboeuf, France). Gel images were captured using the Syngene GBox (Ozyme, Saint Quentin en Yvelines, France) and analyzed with the ImageJ software (NIH, MD, USA). Results were expressed as a ratio between values obtained with IS and values obtained with the control KCl, and then normalized with values obtained with actin.

## CKD model of 5/6th nephrectomized mice

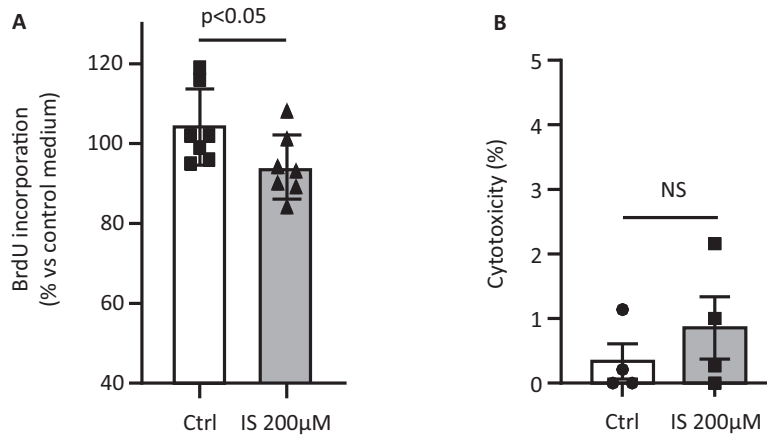
All experimental protocols were approved by the local ethic committee named “comité d’éthique en expérimentation animale de la Région Rhône-Alpes, Lyon, France” (CECAPP # LS\_2019\_004). Male C57BL/6j mice were housed in an air-conditioned room with a controlled environment of  $21 \pm 0.5^{\circ}\text{C}$  and 60%–70% humidity with a 12-h light/dark cycle. CKD was induced by 5/6 nephrectomy with a two-step surgical procedure in 5-week-old male mice as previously described under anesthesia ketamine/xylazine (100 mg/kg and 20 mg/kg, intraperitoneal, respectively) [23]. Sham mice underwent two mock surgeries. Eight weeks later, mice were euthanized with ketamine/xylazine (100/20 mg/kg). Plasma was collected and stored at  $-80^{\circ}\text{C}$ . Gastrocnemius and soleus were removed, weighted, snap-frozen in liquid nitrogen and then stored at  $-80^{\circ}\text{C}$  until analysis.

## RNA extraction and RT-qPCR analysis of mRNA expression in mice

Total RNAs from soleus and gastrocnemius muscles were extracted using TRI Reagent® (Sigma Aldrich, France). Purity and concentration of RNA were determined using NanodropOne (Ozyme) and quality checked using Bioanalyser (Agilent). First-strand cDNAs were synthesized from 1  $\mu\text{g}$  of total RNAs using PrimeScript RT kit reagent kit (Perfect Real Time) (Takara Bio Europe, Saint-Germain-En-Laye, France). Real-time qPCR assays were performed with Rotor-Gene 6000 (Qiagen) using TB Green Premix Ex Taq (Tli RNaseH plus) (Takara Bio Europe) as previously described [24]. TATA-box binding protein (*Tbp*) was used as a reference gene to normalize the results. Results are the ratio of target mRNA levels to *tbp* mRNA levels and are expressed as percentage of the sham group values. Primers sequences are listed in Supplementary data, Table S1.

## Statistical analysis

Statistical analyses were performed with the Prism (GraphPad Software Inc., San Diego, CA, USA). Significant differences in cell culture experiments were revealed by the Wilcoxon signed rank test. *In vitro* data are expressed as mean  $\pm$  standard error of the mean (SEM) of independent experiments performed on different cell preparations. Significant differences in animal model experiments were revealed by the Mann–Whitney test. *In vivo* data are expressed as mean  $\pm$  SEM. In all statistical analyses, *P*-values  $<.05$  were considered as significant.



**Figure 1:** IS inhibits C2C12 myoblast proliferation. Cultured C2C12 myoblasts were subjected to IS (200  $\mu$ M) for 48 h. C2C12 proliferation (A) was investigated by measuring BrdU incorporation. Data are expressed in % of BrdU incorporation versus culture medium alone. Dots represent individual values; column bars represent the mean  $\pm$  SEM of  $n = 7$  independent experiments. The cytotoxicity of IS on C2C12 cells (B) was studied by LDH release. Data are expressed in % of cytotoxicity. Dots represent individual values; column bars represent the mean  $\pm$  SEM of  $n = 4$  independent experiments.

## RESULTS

### IS inhibits myoblast proliferation

C2C12 myoblasts were cultured during 48 h with IS, and myoblast proliferation was studied. By measuring BrdU incorporation into cellular DNA, a 10% decrease in C2C12 proliferation was observed in the presence of IS, compared with the control (Fig. 1A). The cytotoxicity of IS was then analyzed by evaluating LDH release by C2C12 myoblasts cultured during 48 h in presence of IS (Fig. 1B). IS did not mediate a significant cytotoxicity, compared with its control (Fig. 1B).

### IS inhibits myoblast differentiation

C2C12 cells were differentiated into myocytes for 7 days in the presence of IS. The effect of IS on cell differentiation was studied by counting the number of myotubes and the number of nuclei per myotube, and by analyzing two morphological criteria: thickness and length of myotubes. Figure 2A and B displays representative pictures of myocytes after 7 days of C2C12 cell differentiation in presence of control KCl (Fig. 2A) or IS 200  $\mu$ M (Fig. 2B). The presence of IS during differentiation did not affect the number of myotubes (Fig. 2C), but significantly decreased the number of nuclei per myotube (Fig. 2D). In addition, IS reduced myotube thickness (Fig. 2E) without affecting myotube length (Fig. 2F). A kinetic study of IS cytotoxicity during differentiation by measuring LDH release by C2C12 cells was performed. This study demonstrated that the presence of IS during differentiation did not induce cytotoxicity (Fig. 2G).

### IS inhibits the expression of the myogenic regulatory transcription factor Myf6/MRF4 during muscle cell differentiation

The expression of the four MRF genes *Myf5*, *Myod1*, *Myog* and *Myf6* in C2C12 cells exposed to IS during the 7 days of differentiation (Fig. 3) was studied. As expected, and in accordance with the literature, the expression of *Myf5*, which is normally expressed in myoblasts but not in myotubes, decreased during differentiation from Day 4 (Fig. 3A). *Myod1* (Fig. 3B) and *Myog* (Fig. 3C) increased with differentiation from Day 3. *Myf6*, which is the latest MRF to be expressed during differentiation, increased from Day 4 (Fig. 3D).

After 7 days of C2C12 cell differentiation, IS induced a decrease ( $P < .05$  vs control) in *Myf6* mRNA expression (Fig. 3D) without changing the expression of *Myf5* (Fig. 3A), *Myod1* (Fig. 3B) and *Myog* (Fig. 3C). Using a colorimetric Cell-Based ELISA, we found that MRF4 protein expression was also decreased in myocytes exposed to IS during 7 days of differentiation (Fig. 3E and F).

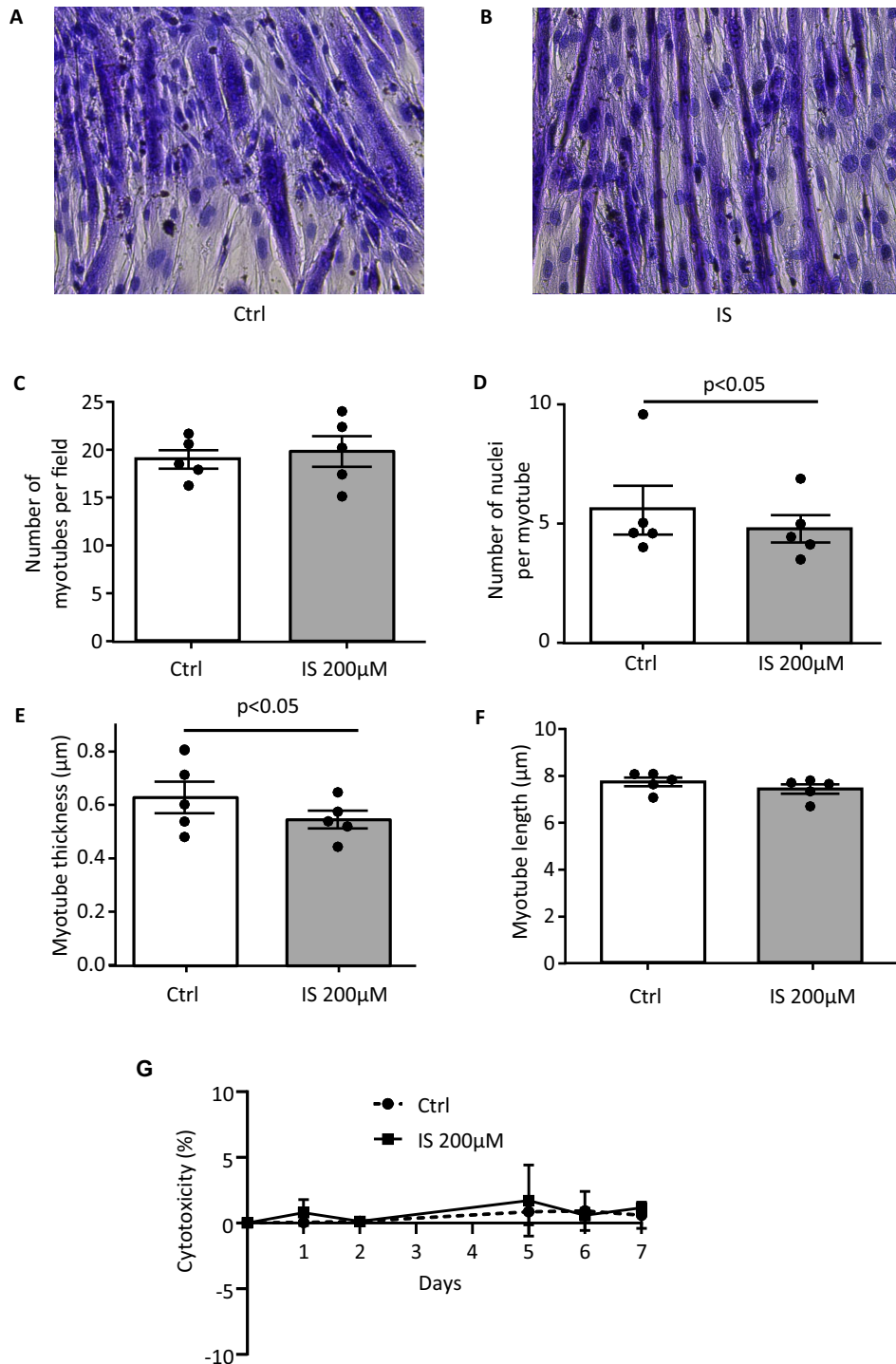
### Myf6/MRF4 expression is decreased in the muscles of CKD mice

We investigated whether CKD modified *Myf6* expression *in vivo*, and measured *Myf6* mRNA expression in *gastrocnemius* and *soleus* muscles in a 5/6th nephrectomy CKD mouse model. CKD mice had higher urea levels than sham mice ( $24.0 \pm 2.4$  mmol/L vs  $9.2 \pm 0.5$  mmol/L, respectively,  $P < .0001$ ). *Myf6* expression in *gastrocnemius* and *soleus* muscles was significantly lower in CKD mice than in sham mice (Fig. 4A and B).

### Role of Ahr in Myf6/MRF4 inhibition during muscle cell differentiation

To analyze the mechanism of *Myf6* downregulation by IS in C2C12 cells, we studied the role of Ahr, the intracellular receptor of IS. We first investigated the activation of Ahr by measuring the mRNA level of the Ahr target genes *Cyp1a1* (Fig. 5A) and *Cyp1b1* (Fig. 5B) in the presence of IS and of the Ahr inhibitor CH223191. Both genes showed an upregulation by IS which was strongly decreased by CH223191, demonstrating the activation of the genomic pathway of Ahr by IS and its inhibition by CH223191 (Fig. 5A and B). The expression of Ahr itself was not modified in the presence of IS, in the presence of CH223191, or both (Fig. 5C).

To study the involvement of Ahr in *Myf6* downregulation induced by IS, C2C12 cells were exposed to IS and CH223191 during the 7 days of differentiation (Fig. 6). CH223191 did not reverse the decrease in *Myf6* mRNA expression induced by IS (Fig. 6A). Moreover, when cells were incubated only with CH223191, it decreased *Myf6* mRNA expression ( $P < .01$  vs control), as IS did (Fig. 6A). As expected, CH223191 had no effect on the mRNA expression of the MRF that were not modified by IS, namely *Myf5*, *Myod1* and *Myog* (Fig. 6B–D).

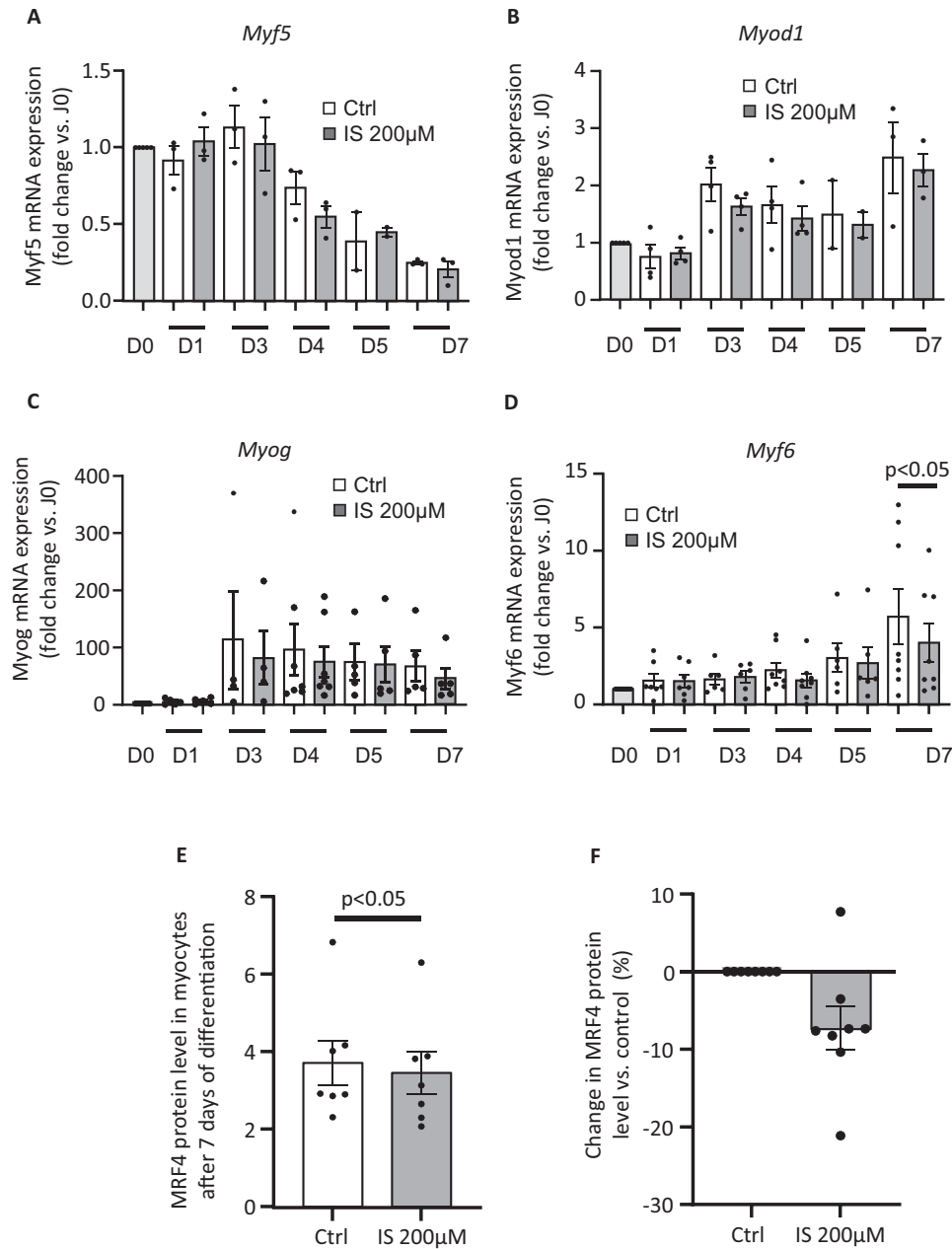


**Figure 2:** IS inhibits C2C12 myoblasts differentiation without inducing cytotoxicity. C2C12 cells were differentiated for 7 days in the presence of IS 200  $\mu$ M and subjected to HE staining. Panels (A) and (B) display representative pictures of C2C12 cells differentiated in presence of KCl (control) and IS 200  $\mu$ M, respectively. At least 15 fields per well were analyzed. C2C12 differentiation was studied by counting the number of myotubes per field (C) and the number of nuclei per myotube (D), and measuring the thickness (E) and length (F) of myotubes (both expressed in  $\mu$ m). (C-F) Dots represent value in each field values, column bars represent the mean  $\pm$  SEM of  $n = 5$  independent experiments. (G) Kinetic study of IS cytotoxicity during C2C12 differentiation, studied by LDH release. Data are expressed in % of cytotoxicity. Data from  $n = 3$  independent experiments are represented as mean  $\pm$  SEM.

### IS down-regulates *Myh2* expression during myoblast differentiation

Because IS induced an impairment of myotube structure, we investigated whether IS affected proteins involved in structural and mechanical integrity of the contractile apparatus of mus-

cle. For this purpose, the expression of myosin heavy chain MyHCIIa (encoded by the *Myh2* gene) was examined in C2C12 cells exposed to IS during 7 days of differentiation (Fig. 7). After 7 days, the presence of IS strongly decreased *Myh2* mRNA expression (Fig. 7A). In addition, the induction of MyHCIIa protein



**Figure 3:** Effect of IS on the expression of the myogenic regulatory factors in muscle cells. C2C12 cells were differentiated for 7 days (named D0 to D7) in the presence of IS 200 µM. The mRNA expression of *Myf5* (A), *Myod1* (B), *Myog* (C) and *Myf6* (D) was studied by RT-qPCR. Data are expressed in mRNA fold change vs control at D0. Dots represent individual values; column bars represent the mean ± SEM of at least  $n = 3$  independent experiments. (E, F) The MRF4 protein level was studied using a colorimetric Cell-Based ELISA. Panel (E) shows the expression value and (F) the percentage variation of protein expression value. Dots represent individual values; column bars represent the mean ± SEM of  $n = 7$  independent experiments.

expression observed after 7 days of differentiation was inhibited by IS (Fig. 7B).

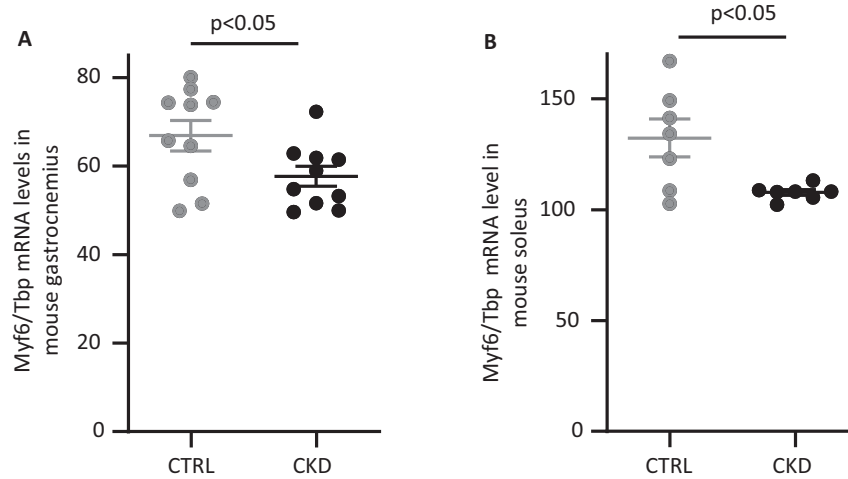
To analyze the involvement of Ahr in *Myh2* mRNA downregulation induced by IS during differentiation, we tested the effect of CH223191 on *Myh2* mRNA expression (Fig. 7C). CH223191 did not reverse the decrease in *Myh2* mRNA expression induced by IS, nor modified *Myh2* mRNA expression in the absence of IS (Fig. 7C).

*In vivo*, we found no difference in *Myh2* mRNA expression in *gastrocnemius* (Fig. 7D) and *soleus* (Fig. 7E) muscles between 5/6th nephrectomized and sham mice.

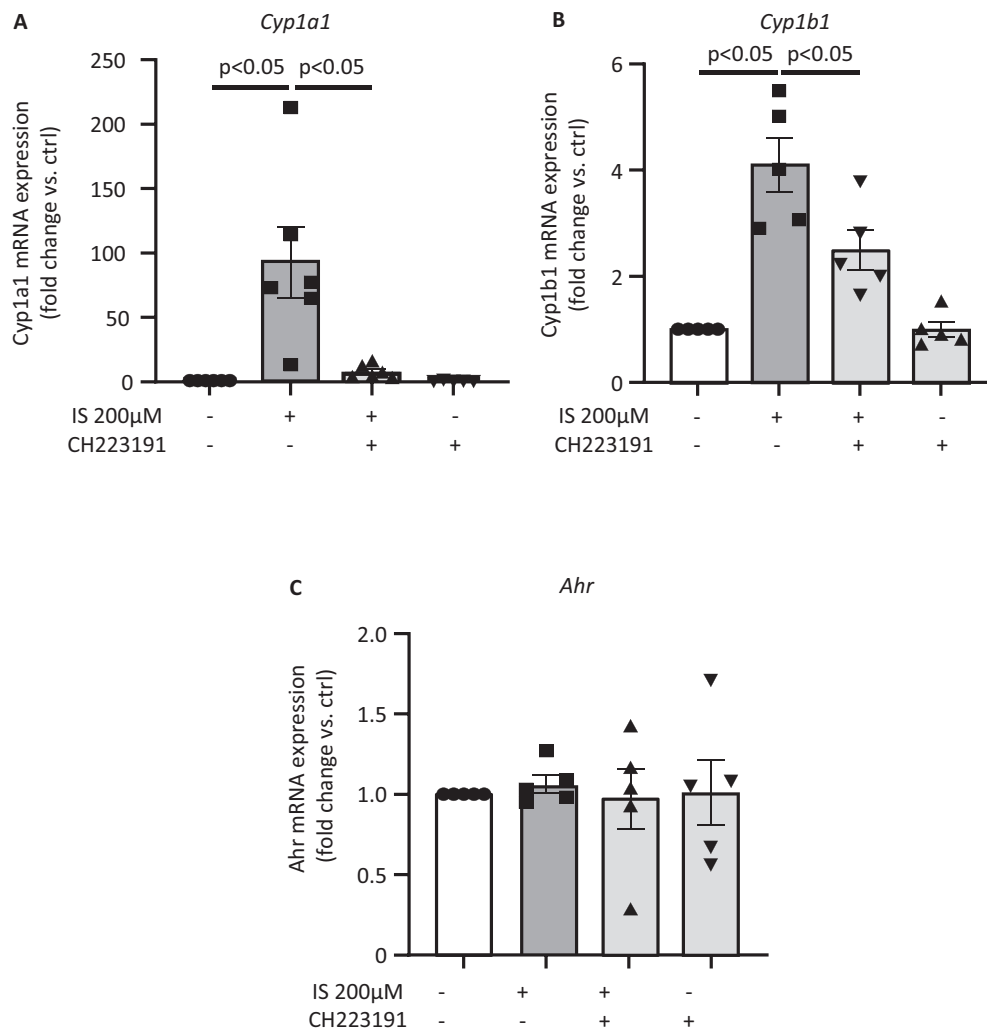
## DISCUSSION

This work shows that the uremic toxin IS inhibits myoblast differentiation by decreasing *Myf6*/MRF4, thereby impairing myotube regeneration. It also suggests that IS could induce muscle atrophy via myosin heavy chain *Myh2* inhibition. These two mechanisms are newly described pathways that could participate in muscle weakness and sarcopenia in CKD patients.

First, we report two important anomalies in C2C12 myotube: that were obtained from myoblasts exposed to IS during differentiation: a reduced myotube thickness and a reduced number of

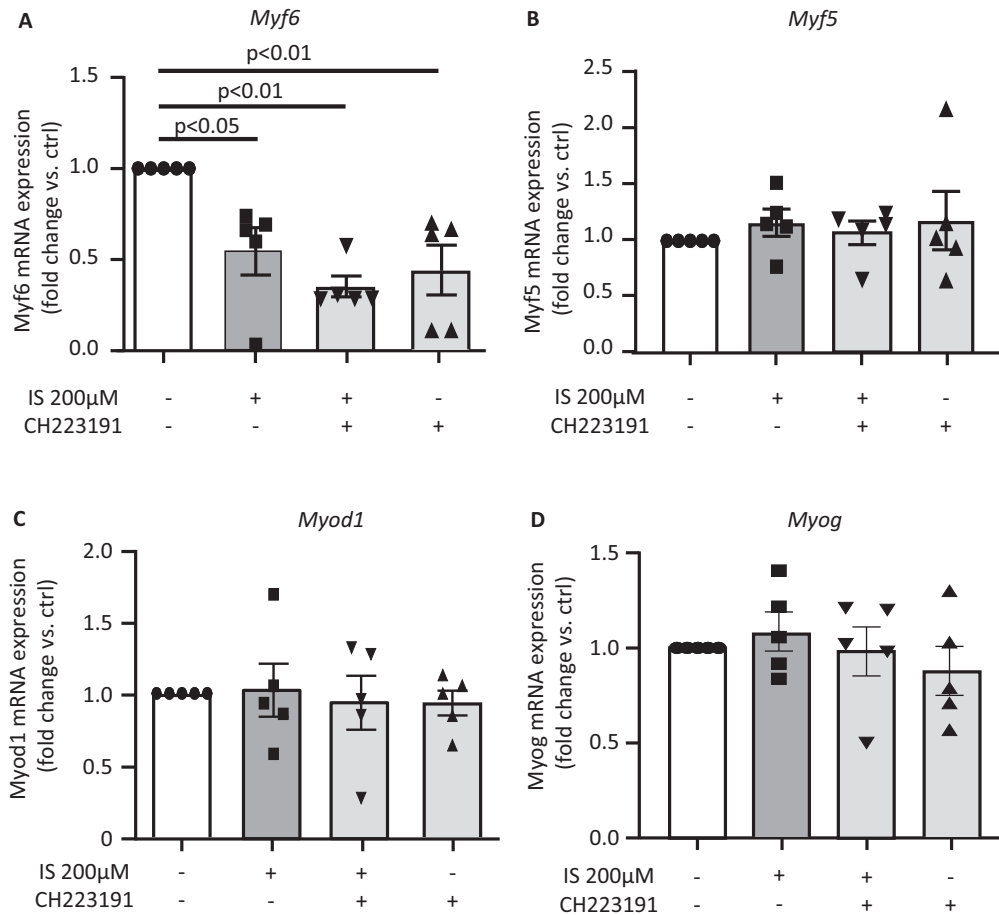


**Figure 4:** *Myf6* expression is decreased in muscles from CKD mice. *Myf6* mRNA expression was studied by RT-qPCR in (A) *gastrocnemius* ( $n = 10$  per group) and (B) *soleus* ( $n = 7$  per group) of 5/6th nephrectomized CKD mice and control (CTRL) mice. Dots represent individual values; bars represent the mean  $\pm$  SEM.



**Figure 5:** Effect of IS on *Ahr* activation and expression in C2C12 cells. C2C12 cells were differentiated for 7 days in the presence of IS 200  $\mu$ M and/or CH223191 10  $\mu$ M. The mRNA expression of *Ahr* target genes *Cyp1a1* (A) and *Cyp1b1* (B) and of *Ahr* (C) was studied by RT-qPCR. Data are expressed in mRNA fold change vs control. Dots represent individual values; column bars represent the mean  $\pm$  SEM of  $n = 5$  independent experiments.





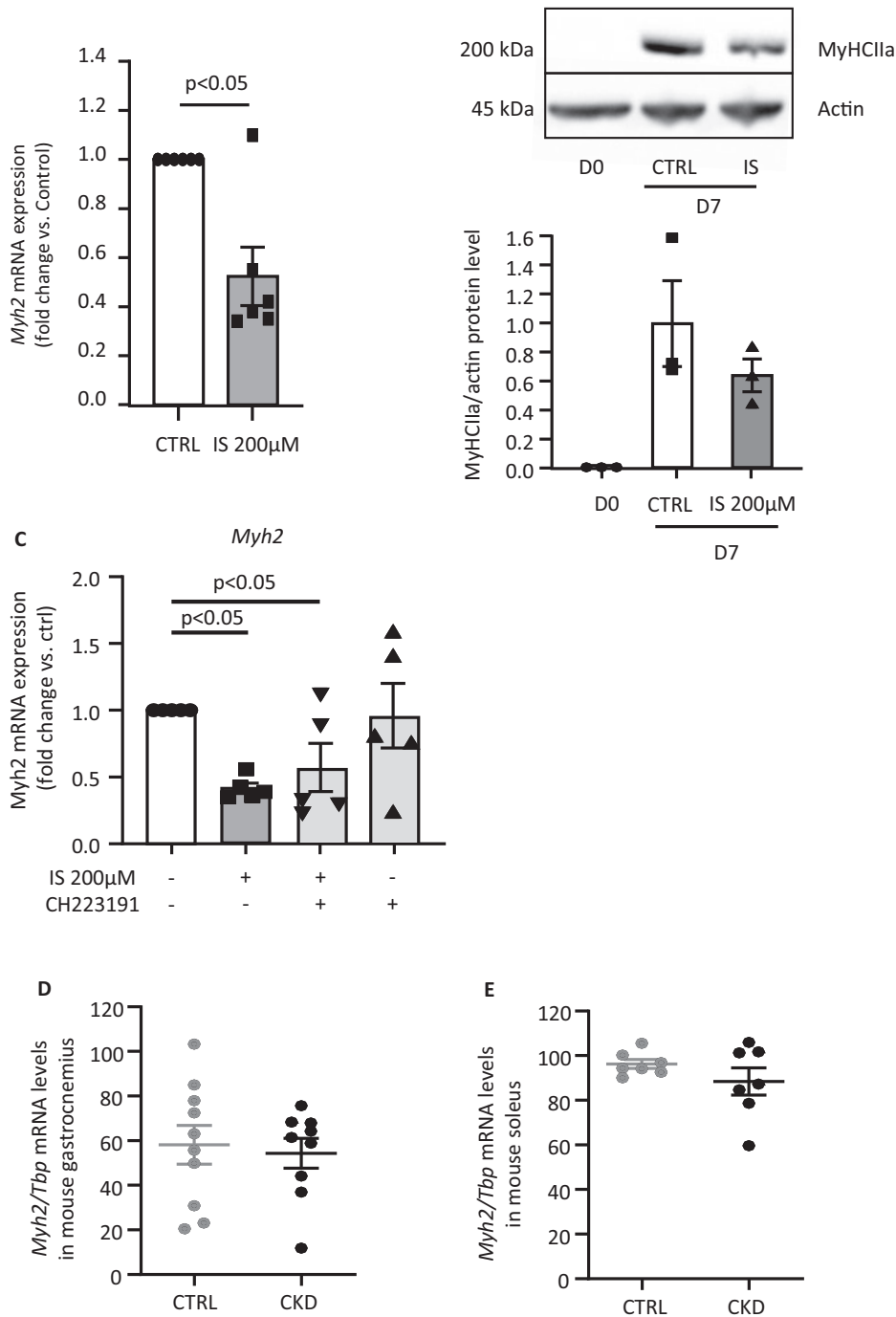
**Figure 6:** Effect of Ahr inhibition on IS-induced *Myf6* expression in C2C12 cells. C2C12 cells were differentiated for 7 days in the presence of IS 200  $\mu$ M and/or CH223191 10  $\mu$ M. The mRNA expression of *Myf6* (A), *Myf5* (B), *Myod1* (C) and *Myog* (D) was studied by RT-qPCR. Data are expressed in mRNA fold change vs control. Dots represent individual values; column bars represent the mean  $\pm$  SEM of  $n = 5$  independent experiments.

nuclei per myotube. In the literature, Enoki and colleagues have already reported that C2C12 cells exposed to IS for 7 days of differentiation produce fewer myotubes (lower number of myotubes per field) which are also narrower [16]. Another study has also reported decreased diameters of myotubes exposed to IS during differentiation [25]. Our data showing that IS exposure during differentiation leads to reduced number of nuclei per myotube is an important finding because we propose that the mechanism leading to a lower number of nuclei in myotubes is fusion inhibition, a different mechanism from myotube growth.

We show for the first time that muscle cells exposed to IS during differentiation display an inhibition of *Myf6*/MRF4 expression—but not the expression of other MRFs. *Myf6*/MRF4 is a central MRF for myocyte–myocyte and myocyte–myotube fusion during embryogenesis and repair and plays a role in the very late states of differentiation [11]. Its expression in regenerating fibers *in vivo* is restricted to the time around and after fusion [26]. Thus, *Myf6*/MRF4 inhibition could explain the lower number of nuclei per myotube obtained in C2C12 cultures exposed to IS. This suggests that IS may impair myocyte fusion during myotube formation. A decrease of 10% of MRF4 protein expression could be interpreted as a small effect, but in hemodialysis patients, muscle strength declines over several years. Thus, a small but long-lasting decrease in the muscle regeneration capacities after lesion could be of clinical relevance in CKD patients.

We investigated the AHR-dependent mechanism of *Myf6* downregulation by IS and showed that the decrease in *Myf6* expression by IS was not reversed by the AHR inhibitor CH223191. AHR can activate several signaling pathways, the first described being the genomic pathway that regulates the expression of AHR target genes such as Cytochrome p450 1A1 (*CYP1A1*) and 1B1 (*CYP1B1*) [18]. Here, the up-regulation of *Cyp1a1* and *Cyp1b1* by IS was reversed by CH223191, supporting that CH223191 does inhibit IS-mediated activation of the AHR genomic pathway in C2C12 in mouse muscle cells. However, CH223191 did not reverse the effect of IS on *Myf6* downregulation, ruling out an involvement of the AHR genomic pathway. The role of AHR via non-genomic pathways, including modulation of signaling pathways like Src, MAPK or Wnt/*b*-catenin, or via its function as an E3 ubiquitin ligase [27] was not studied.

A decrease in *Myf6*/MRF4 expression was observed in striated muscles of the 5/6th nephrectomy mouse model. In this model, high urea serum levels confirm the presence of CKD and we have already reported that 5/6th nephrectomized mice display high IS blood concentrations [28]. Our *in vitro* approach was an IS “single toxin” approach and the decrease in *Myf6*/MRF4 expression observed in the 5/6th nephrectomized model, a CKD model in which all uremic toxins, including IS accumulate, could result from the effect of IS alone, or from a cocktail effect of IS with other uremic toxins. In this model, it has been observed that a decrease of weight and muscle-fiber size



**Figure 7:** IS inhibits *Myh2* expression in muscle cells *in vitro*. (A, B) C2C12 myoblasts were differentiated for 7 days in presence of 200 µM IS. (A) *Myh2* mRNA expression was studied by RT-qPCR. Data are expressed in mRNA fold change vs control. Dots represent individual values, column bars represent the mean ± SEM of  $n = 6$  independent experiments. (B) MyHCIIa protein level was studied by western blot. Dots represent individual values; column bars represent the mean ± SEM of  $n = 3$  independent experiments. (C) C2C12 cells were differentiated for 7 days in the presence of IS 200 µM and/or CH223191 10 µM. Effect of IS and/or CH223191 on *Myh2* expression in myocytes at Day 7 was studied by RT-qPCR. Data are expressed in mRNA fold change vs control. Dots represent individual values; column bars represent the mean ± SEM of  $n = 3$  independent experiments. (D, E) *Myh2* expression was studied in gastrocnemius (D) and soleus (E) of 5/6th nephrectomized CKD and sham mice by RT-qPCR. Dots represent individual values; bar represent the mean ± SEM of  $n = 10$  (C) or  $n = 7$  (D) animals per group.

distribution in skeletal muscle is associated with a shift toward small myofibers [29].

Few data are available on the role of *Myf6*/*MRF4* in adult muscle fibers but it could negatively regulate muscle mass [30, 31]. It has been proposed that the biological effect of *MRF4* is modulated by

its phosphorylation under the control of p38 MAPK [32], whose activation by IS was demonstrated in many cell types, including cardiomyocytes [33]. The effect of IS on the phosphorylation level of *MRF4* in muscle cells might be interesting to study. *MYF6*/*MRF4* is also important to maintain the SC pool in adult skeletal muscle

[13], but CKD patients have a normal number of SCs and a normal PAX7 expression in muscles [34].

Unlike Myf6/MRF4, the expression of other MRFs Myod1 and Myog was not modified by IS exposure during differentiation. In the literature, results are conflicting regarding Myod1 and Myog expression in CKD. Some studies report a down regulation of Myod1 and Myog in animal models of CKD, but others did not confirm such findings [34–36]. Regarding the effect of IS on gene expression in cultured muscle cells, Rodrigues and colleagues did not find any modification in Myod1 and Myog expression in cultured C2C12 myoblasts during differentiation, even at IS concentration of 1 mM [15]. Enoki and colleagues described a slight down-regulation of MyoD protein, but not myogenin in C2C12 myoblasts exposed to 1 mM IS, as well as in half-nephrectomized mice injected intraperitoneally with IS [16].

The effect of IS was studied on a protein playing a key role in structural and mechanical integrity of the contractile apparatus of muscle tissue: myosin heavy chain MyHCIIa, encoded by the MYH2 gene. The MYH2 gene is expressed in fast type 2A and 2B muscle fibers and functions in skeletal muscle contraction. MYH2 mutations are responsible for myopathy, characterized by a lack of or a reduction in the number and size of type 2A fibers, with mild to moderate muscle weakness [37]. Enoki and colleagues reported a down-regulation of MyHCIIa protein expression in mature myocytes exposed to IS [16], which was not found by Rodrigues and colleagues even at high IS concentrations [15]. Here, we found that IS down-regulated MyHCIIa mRNA expression and protein production in C2C12 cells when added during cell differentiation. The down regulation of MyHCIIa during myogenesis could be involved in the myotube atrophy that we observed in presence of IS. However, unlike Myf6, we did not observe an inhibition of Myh2 expression in myocytes exposed to CH223191 and CKD mice displayed a normal Myh2 expression in striated muscles, suggesting that the underlying mechanism by which IS inhibits Myh2 *in vitro* is distinct from the mechanism involved for Myf6.

In addition to its effects on differentiation, IS inhibited C2C12 myoblast proliferation at a uremic concentration of 200  $\mu$ M. Alcalde-Estévez and colleagues reported that a mix of IS and paracresyl sulfate (PCS), each at uremic concentration of 100  $\mu$ M, inhibited the entry of myoblasts in phase M and their proliferation, associated with a slower wound healing [38]. However, it is not clear if this effect was due to IS or to PCS. Using a methylene blue assay, Enoki and colleagues reported an inhibition of 40% of myoblast proliferation when cells were exposed to a high IS concentration of 1 mM [16]. In Enoki's study, IS had the strongest antiproliferative effect compared with other uremic toxins (indole-3-acetic acid, PCS, hippuric acid, kynurenic acid and 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid). Here, we show that the antiproliferative effect of IS is also relevant at a concentration of 200  $\mu$ M which can be found in CKD patients. The inhibition of myoblast proliferation induced by IS was not due to IS cytotoxicity. Indeed, by measuring LDH release, IS at uremic concentration has no cytotoxic effect on myoblasts, nor on myocytes throughout their differentiation. The absence of a cytotoxic effect on myoblasts of IS at a uremic concentration (53.0 mg/L or 211  $\mu$ M) was also described by Rodrigues and colleagues using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. A cytotoxic effect of IS on myoblasts was observed only at a supraphysiological concentration of IS (236.0 mg/L or 940  $\mu$ M) [15], which had no cytotoxic effect on myotubes [25].

In conclusion, IS inhibits myoblast proliferation, down-regulates Myf6/MRF4 and Myh2 expression during muscle cell dif-

ferentiation, and leads to a defect in myotube structure. Through these mechanisms, IS could contribute to the muscle atrophy observed in CKD.

## SUPPLEMENTARY DATA

Supplementary data are available at *ndt* online.

## CONFLICT OF INTEREST STATEMENT

The authors declare they have no disclosure regarding this study.

## AUTHORS' CONTRIBUTIONS

S.B. designed and performed experiments, and wrote the paper. N.M. performed cell culture experiments. L.K., A.B. and B.B. performed animal experiments. M.B. helped in the study design. N.D.S. helped with cell culture methods. S.P. designed and performed animal experiments, participated in data interpretation and wrote the paper. J.A. helped in performing experiments. R.C. helped with cell cultures. S.B. participated in the study design and wrote the paper. L.D. designed and performed experiments and wrote the paper. All authors helped in the writing of the paper.

## FUNDING

The study was performed thanks to a grant of the French nephrology society: Société Francophone de Néphrologie, Dialyse et Transplantation.

## DATA AVAILABILITY STATEMENT

The data underlying this article will be shared on reasonable request to the corresponding author.

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