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Mechanisms of myostatin and activin A accumulation in chronic kidney disease

Stanislas Bataille, Laetitia Dou, Marc Bartoli, Marion Sallée, Julien Aniort, Bohrane Ferkak, Rania Chermiti, Nathalie Mckay, Nathalie da Silva, Stéphane Burtey, et al.

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Abstract: Background

Myostatin induces muscle wasting by activating the ubiquitin proteasome system and inhibiting the Akt/mTOR pathway. In chronic kidney disease (CKD), myostatin concentrations in plasma are increased, but it is not clear whether there is an increased production by muscle cells or a decreased myostatin renal clearance. We studied myostatin production by muscle in CKD and analyzed the relationship between myostatin blood levels and estimated glomerular filtration rate (eGFR).

Methods

We studied myostatin blood concentration in 232 CKD patients and its correlation with eGFR. We analyzed the myostatin gene (MSTN) expression in hemodialysis (HD) patients muscle biopsies. We measured circulating myostatin and the Mstn expression in muscles in two mouse models of CKD (adenine model and 5/6th nephrectomy model). Finally, we analyzed whether indoxyl sulfate (IS) increased Mstn expression in mice and in cultured muscle cells.

Results

In CKD patients, myostatin was inversely correlated with eGFR, even after adjustment with age, gender and diabetes. MSTN expression was lower in HD patients' muscles (vastus lateralis) than in controls. In mice with CKD, myostatin blood concentrations were increased, but myostatin mRNA and protein expressions in gastrocnemius and tibialis anterior were similar to control animals. The exposure to the uremic toxin IS did not induce Mstn up-regulation in mice and in cultured myoblasts and myocytes.

Conclusions

In CKD, myostatin blood concentrations are increased without an increase in myostatin production from muscle. Our study suggests an impaired renal clearance of myostatin. We propose to add myostatin to the list of uremic toxins.

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3 **Significance statement (max 120 words)**
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6 Sarcopenia is a complication of chronic kidney disease (CKD), but its pathophysiology is not
7 fully understood. Myostatin is a chalone protein that induces proteolysis in muscle cells. Data
8 have shown that myostatin blood concentrations are increased in CKD. Here, we studied
9 whether this increase is due to myostatin overproduction by muscle or to reduced clearance by
10 the kidney. We showed an elevation of myostatin blood concentrations in human and mice with
11 CKD, but no increased transcription of *Mstn* gene in striated muscles. In CKD patients,
12 myostatin plasma levels were correlated with eGFR, arguing for myostatin accumulation rather
13 than overproduction.
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25 This finding is important in the context of targeting myostatin as a therapeutic to prevent
26 sarcopenia in patients with CKD.
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Origin of myostatin in CKD

Original Article

Myostatin accumulates and is not overproduced by skeletal muscle in chronic kidney disease.

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Running title: Origin of myostatin during CKD

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Origin of myostatin in CKD

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Origin of myostatin in CKD

Significance statement (max 120 words)

Sarcopenia is a complication of chronic kidney disease (CKD), but its pathophysiology is not fully understood. Myostatin is a chalone protein that induces proteolysis in muscle cells. Data have shown that myostatin blood concentrations are increased in CKD. Here, we studied whether this increase is due to myostatin overproduction by muscle or to reduced clearance by the kidney. We showed an elevation of myostatin blood concentrations in human and mice with CKD, but no increased transcription of *Mstn* gene in striated muscles. In CKD patients, myostatin plasma levels were correlated with eGFR, arguing for myostatin accumulation rather than overproduction.

This finding is important in the context of targeting myostatin as a therapeutic to prevent sarcopenia in patients with CKD.

Origin of myostatin in CKD

Abstract (max 250 words)

Background

Myostatin induces muscle wasting by activating the ubiquitin proteasome system and inhibiting the Akt/mTOR pathway. In chronic kidney disease (CKD), myostatin concentrations in plasma are increased, but it is not clear whether there is an increased production by muscle cells or a decreased myostatin renal clearance. We studied myostatin production by muscle in CKD and analyzed the relationship between myostatin blood levels and estimated glomerular filtration rate (eGFR).

Methods

We studied myostatin blood concentration in 232 CKD patients and its correlation with eGFR. We analyzed the myostatin gene (*MSTN*) expression in hemodialysis (HD) patients muscle biopsies. We measured circulating myostatin and the *Mstn* expression in muscles in two mouse models of CKD (adenine model and 5/6th nephrectomy model). Finally, we analyzed whether indoxyl sulfate (IS) increased *Mstn* expression in mice and in cultured muscle cells.

Results

In CKD patients, myostatin was inversely correlated with eGFR, even after adjustment with age, gender and diabetes. *MSTN* expression was lower in HD patients' muscles (*vastus lateralis*) than in controls. In mice with CKD, myostatin blood concentrations were increased, but myostatin mRNA and protein expressions in *gastrocnemius* and *tibialis anterior* were similar to control animals. The exposure to the uremic toxin IS did not induce *Mstn* up-regulation in mice and in cultured myoblasts and myocytes.

Conclusions

Origin of myostatin in CKD

In CKD, myostatin blood concentrations are increased without an increase in myostatin production from muscle. Our study suggests an impaired renal clearance of myostatin. We propose to add myostatin to the list of uremic toxins.

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Origin of myostatin in CKD

Introduction

Sarcopenia is frequently observed in patients with chronic kidney disease (CKD) and grieves physical function, quality of life and survival¹⁻³. Many factors have been proposed to explain the loss of muscle strength and muscle mass in CKD. Some of them are in direct relationship to CKD like uremic toxins accumulation and chronic inflammation; others are linked to comorbidities such as diabetes, age, obesity or a sedentary lifestyle. Myostatin (Mstn) has recently been proposed as an important player in the CKD associated sarcopenia^{4,5}.

Mstn, a transforming growth factor β (TGF- β) family member also called growth differentiation factor 8 (GDF-8), is a chalone protein for muscle, which means Mstn is secreted by mature muscle cells and inhibits muscle overgrowth that would be deleterious for muscle functioning. Its molecular weight is 25 kDa. Mstn inhibits satellite muscle cells recruitment, induces proteolysis by activating the ubiquitin proteasome system and inhibits the anabolic Akt/mTOR signaling pathway in muscle cells⁶. It thus leads to loss of muscle mass. Inhibition of Mstn or Mstn pathway has been proposed as a potential therapeutic target in genetic myopathies and many pathophysiological states associated with sarcopenia such as cancer⁶.

Mstn concentrations in plasma of patients with CKD are higher than in subjects with a normal renal function but data remain controversial^{5,7,8}. Mstn concentrations have been correlated to muscle mass and muscle strength in CKD^{9,10}. Nevertheless, whether high Mstn concentrations are due to Mstn accumulation or overproduction in CKD is not clear at this point.

Some authors, but not all, have reported increased expression of Mstn mRNA and protein in muscles from CKD patients¹¹⁻¹³. Moreover, studies have shown that some factors elevated in CKD, like uremic toxins or inflammatory mediators, increase Mstn production by muscle cells. Experimental studies have proposed that indoxyl sulfate (IS), a protein-bound uremic toxin, induces *Mstn* gene transcription by activating the Aryl Hydrocarbon Receptor (AHR)^{14,15}. AHR

Origin of myostatin in CKD

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3 is a transcription factor activated by IS in mouse and human with CKD, which regulates the
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5 cell physiology, notably cell proliferation and differentiation^{16,17}.
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8 In the perspective of finding new targets of treatment for CKD-associated sarcopenia,
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10 identifying the underlying mechanisms leading to high Mstn concentrations is crucial¹⁸. Using
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12 animal models of CKD and cultured skeletal muscle cells, we explored whether high level of
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14 circulating Mstn in CKD is due to Mstn accumulation related to decreased renal clearance or
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16 Mstn overproduction by skeletal muscle.
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Origin of myostatin in CKD

Methods

Human cohort of patient with CKD

We measured Mstn blood concentrations in two previous cohorts of CKD patients from the CHU Conception in Marseille, France. The first cohort came from the REDOX clinical trial (ClinicalTrials.gov NCT00684034) that included non-diabetic CKD patients¹⁹. The second cohort was a prospective cohort named CPinMRC aiming to analyze the correlation between CD146 concentration and proteinuria in CKD patients (ClinicalTrial.gov NCT03341949). We analyzed which parameters were associated with Mstn concentration within age, gender, estimated Glomerular Filtration rate (eGFR) calculated using the CKD Epidemiology Collaboration creatinine equation (CKD-EPI)²⁰ and body mass index (BMI). The two studies were approved by the local ethic committee (Comité consultatif de protection des personnes Sud-Méditerranée II) and by the scientific board (Centre d'Investigation Clinique, Marseille) and were in compliance with the Helsinki Declaration. All the patients provided a written inform consent to participate.

Human HD muscle biopsies

Human muscle biopsy samples were obtained from another previously described study through a collaboration with the Nephrology, Dialysis and Transplantation of Clermont-Ferrand, France. Data of patients and muscle biopsies have already been described ²¹. Briefly, 7 HD patients and 7 gender and age-matched controls were included. Exclusion criteria were acute or chronic infection, diabetes mellitus, corticosteroid or hormone therapy, and pregnancy. Surgical *vastus lateralis* biopsy was performed and directly snap frozen in liquid nitrogen and stored at -80°C. Reverse transcription of total mRNA was performed using the QuantiTect® Reverse Transcription kit (Qiagen®). The study (DGS-2008-A00479-46) was approved by the Sud Est

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VI Research Ethics Committee and was conform to the standards set by the Declaration of Helsinki.

Mice

C57BL/6J wild-type (WT) mice were purchased from the Jackson Laboratory and maintained as a breeding colony in the animal care facility at the faculty of Medicine of Marseille. Male and female mice were used in this study and comparisons were made between sex-and age-matched groups.

Kidney failure was induced in 10-week-old mice as previously described by alternating 0.25% adenine-enriched (A04+0.25% adenine, SAFE Augy, France) or a regular chow diet (A04 standard, SAFE) every other week for 6 weeks. Mice were sacrificed at 16 weeks after induction of CKD²². Control mice were fed with regular chow diet for 6 weeks. As a second model of CKD, we performed 5/6th nephrectomy (5/6th Nx) using a 2-step procedure, as previously described^{15,17,23}. Briefly, mice (9-week-old) underwent 5/6th Nx or sham surgery under general anesthesia (ketamine 125 mg/kg, xylazine 12.5 mg/kg, atropine 0.25 mg/kg; i.p.). The left kidney was exposed through a flank incision, and we applied cortical electrocautery to the upper and lower poles (2/3 of the left kidney). One week later, we performed right total nephrectomy through a similar incision. All the surgical experiments were done by the same operator (SP). Mice were sacrificed at 28 weeks after induction of CKD¹⁵.

To study the specific effect of the uremic toxin indoxyl sulfate (IS), 10-week-old mice were injected intraperitoneally (*i.p.*) daily with IS (400 mg/kg) or KCl as control, for 5 days. Mice were sacrificed 4h after the last injection¹⁷.

The experiments were performed in compliance with the Directive 2010/63/EU of the European Parliament and were approved by the local Ethic Committee (“Comité d’Ethique en Expérimentation Animale de Marseille”, C2EA-14; ethics approval numbers:

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2017091414144363v6; 2018090715512181v3 and approval dates: 20th November 2018, 14th March 2019).

Measurement of Mstn concentrations

Mstn concentrations, in human plasma or serum, mouse plasma or serum and muscle, were measured by ELISA (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

Biological assays in mice

Mouse blood was obtained by puncture either of the heart or of the *vena cava* under general anesthesia. Serum was obtained from blood drawn from cardiac puncture that was allowed to clot and then centrifuged. Platelet free plasma was obtained from blood drawn from *vena cava* puncture anticoagulated with sodium heparin and then centrifuged.

Urea and creatinine were measured with an Olympus AU400 autoanalyzer at the Biochemistry Laboratory of the Centre de Recherche sur l'inflammation (UMR 1149 Inserm, Université Paris Diderot, ERL CNRS 8252, Paris, France). IS was measured by high-performance liquid chromatography, according to Calaf *et al*²⁴.

Mice muscles

At sacrifice, mice *gastrocnemius* or *tibialis anterior* were surgically removed and stored in RNAlater® (Sigma-Aldrich) for quantitative Polymerase Chain Reaction (qPCR) experiments or snap frozen in liquid nitrogen-cooled isopentane for later protein extraction.

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Culture and differentiation of C2C12 cells

Mouse C2C12 myoblasts were purchased from the American Type Culture Collection (ATCC, Manassas, Virginia, USA) and cultured in high glucose DMEM medium containing Glutamax™ (Life technologies, Saint Aubin, France) supplemented with 10% fetal calf serum (Dominique Dutscher, Brumath, France) and 1% penicillin, streptomycin and amphotericin B (PS), and maintained under 37 °C and 5% CO₂. Differentiation in myocytes was obtained after 5 days of culture of C2C12 myoblasts in DMEM high glucose GlutaMAX™ medium with 1% PS added with 2% horse serum. C2C12 myoblasts and myocytes were incubated during 24h and 48h with IS (Sigma-Aldrich, France) at 200µM or 1mM. Because IS was purchased as a potassium salt, KCl was used as control.

Ribonucleic acid (RNA) extraction

Total RNA was extracted from C2C12 myoblasts and myocytes by RNeasy mini-kit (Qiagen, Courtaboeuf, France). Mouse *gastrocnemius* and *tibialis anterior* were lysed in TRIzol and total RNA was purified by chloroform extraction and isopropanol precipitation. RNA concentrations were determined using a NanoDrop Spectrophotometer (Thermoscientific, Wilmington, DE).

RT-qPCR experiments

Reverse transcription (RT) was performed from 500 ng of total RNA with the Takara PrimeScript RT Reagent Kit (Takara, France). Primers and MGB-Taqman probes were purchased from ThermoFisher Scientific (Courtaboeuf, France); references for Taqman probes are provided in Supplementary Table S1. Polymerase chain reactions (PCR) was Performed using the Brilliant II QPCR Master Mix (Agilent Technologies, Les Ulis, France) and target gene expression was normalized on the basis of the hypoxanthine-guanine phosphoribosyl transferase (*Hprt*) content of each sample. We considered that gene was not expressed when Ct

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was >35. qPCR results were expressed as N-target for human and mouse *in vivo* experiments and as fold-change versus control for *in vitro* cell culture experiments.

Statistical analysis

In CKD patients, categorical variables were described by their number and associated frequency. Quantitative variables were described by the mean and standard deviation. For the analytical statistics, Student's t tests were used to compare two means. To compare frequencies, Chi-square tests were performed. A multiple linear regression analysis was performed according to the top-down Wald method. Also, a Pearson correlation was performed. All tests used were with an alpha risk at 0.05, to determine the significant p-value. Statistical analyses were performed with SPSS software (IBM SPSS Statistics 20).

For other statistical analysis, data were expressed as mean \pm standard deviation (SD) or median \pm interquartile range (IQR) when appropriate. Monovariate analysis were performed by the Mann–Whitney U-test or the linear regression test performed with the Prism software (GraphPad Software Inc, California, United States). In all statistical analyses, p-values <0.05 were considered as significant.

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Results

Elevation of circulating Mstn in patients with CKD

The CKD cohort included 232 individuals. Baseline characteristics of the entire cohort and according to eGFR subgroups are shown in Table 1. In monivariate analysis, plasma Mstn concentration was negatively correlated with age ($r = -0.169$; $p = 0.005$), female gender ($r = -0.197$; $p = 0.001$), diabetes ($r = -0.152$; $p = 0.011$), and eGFR ($r = -0.144$; $p = 0.012$), but not with BMI (Table 2, supplementary data Figure S1). In multivariate linear regression, younger age, male gender, diabetes, and lower eGFR were independently associated with higher Mstn concentration (Table 3).

To support the stability of the model, the group of patients was divided into two subgroups according to the median plasma Mstn concentration. The results of the comparison between these two subgroups confirm the stability of the model (supplementary data Table S2).

MSTN mRNA expression is not increased in *vastus lateralis* from HD patients

We analyzed *MSTN* gene expression in human *vastus lateralis*. In the *vastus lateralis* of HD patients, *MSTN* mRNA expression was lower than in matched controls without kidney disease (median N-target [IQR] 15.9 [11.9-62.2] and 73.4 [41.8-130.8], respectively $p = 0.02$) (Figure 1). So *MSTN* was less expressed in muscle of patients undergoing hemodialysis compared to healthy controls.

Mouse models of CKD had elevated myostatin blood concentrations, but no increase in muscle myostatin expression

In the adenine model (Figure 2A), median serum concentrations of urea, creatinine, IS and Mstn were higher in adenine mice than in control mice (Figure 2B-E). *Cyp11a1* expression, the marker of the activation of AHR in muscle, was up-regulated in *gastrocnemius* of adenine mice

Origin of myostatin in CKD

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3 compared to control mice (Figure 2F). The *Mstn* gene expression and Mstn protein
4 concentration were similar in the *gastrocnemius* of adenine-treated and control mice (Figure
5 2G and 2H).
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10 In mice from the 5/6th Nx model (Figure 3A), serum urea and creatinine levels correlated with
11 Mstn concentrations (Figure 3B-C). From that point, to ensure that our model was a CKD
12 model, in the 5/6th Nx group only mice with urea >20mmol/L were considered. In the 5/6th Nx
13 mice, median serum urea and creatinine were higher than in control (22.5 [20.4-24.6] vs 9.1
14 [7.8-10.9] mmol/L, p<0.001 and 45.5 [42.0-51.5] vs 27.0 [25.5-30.5] μmol/L, p<0.001,
15 respectively). Serum IS and Mstn were higher in 5/6th Nx mice (Figure 3D-E). *Mstn* mRNA
16 expression as well as Mstn protein concentration in *gastrocnemius* was similar in 5/6th Nx mice
17 and control mice (Figure 3F and 3G).
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Muscle *Mstn* expression was not up-regulated in IS-injected mice

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32 To analyze whether high IS concentration exposure could up-regulate *Mstn* in muscle *in vivo*,
33 we analyzed the *Mstn* mRNA expression in mice injected intraperitoneally with IS (IS-IP
34 model, Figure 4A). In this model, IS median serum concentration was increased to 27.9 [21.2-
35 1369] μmol/L compared to 8.7 [5.4-9.4] μmol/L in control mice (Figure 4B). In the IS-IP
36 model, *Cyp1a1* mRNA expression in *gastrocnemius* was up-regulated after IS exposure,
37 confirming Ahr activation by IS (Figure 4C). However, we found no *Mstn* up-regulation in
38 *gastrocnemius* (Figure 4D).
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IS does not induce Mstn expression in C2C12 myoblasts and myocytes

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50 We exposed C2C12 myoblasts and myocytes to IS at 200 μM or 1 mM during 24h or 48h. Ahr
51 target gene *Cyp1a1* had a very low basal expression in myoblasts and myocytes (Ct 34.8 ± 0.8
52 and 35.0 ± 0.8 respectively; mean ± SD, n=5) but *Cyp1b1* had a high basal expression (Ct 25.1
53 ± 0.5 and 24.6 ± 0.6 respectively; mean ± SD, n=5). IS increased *Cyp1a1* and *Cyp1b1* mRNA
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Origin of myostatin in CKD

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3 expression in myoblasts and myocytes at both times and both concentrations, in a dose-
4 dependent manner (Figure 5A-D). As expected, *Mstn* mRNA was not expressed in C2C12
5 myoblasts (supplementary data Figure S2), but was expressed in differentiated C2C12
6 myocytes, which also expressed the *Myh2* gene (data not shown). In C2C12 myocytes, *Mstn*
7 expression was not up regulated by IS, whatever the time of incubation or the IS concentration
8 (Figure 5E).
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Oat1 and *Oat3* are not expressed in muscle cells

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20 Because IS enters into kidney tubular cells via the organic anion transporters 1 and 3 (*Oat1* and
21 *Oat3*) in mice and humans²⁵, we searched for *Oat1* and *Oat3* expression in C2C12 myoblasts,
22 C2C12 myocytes and in mice *gastrocnemius*, and found no mRNA expression of *Oat1* and
23 *Oat3* in these muscle cells (supplementary data Figure S3A-B). As expected, we found a high
24 expression of *Oat1* and *Oat3* in mice kidneys (supplementary data Figure S3C).
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Origin of myostatin in CKD

Discussion

In human and mice, CKD is associated with high circulating Mstn levels correlated to renal function. We found no Mstn overproduction in muscle, so accumulation of Mstn in CKD is related to a defect in elimination of Mstn rather than an increased production in relation with uremia.

This study is the largest to demonstrate a correlation between circulating Mstn concentrations and renal function^{5,7,26}. In this field, only few studies have been published and we confirm that CKD is associated with high Mstn concentrations independently from age, gender or diabetes.

Studies have reported conflicting data on *Mstn* mRNA regulation in muscle in CKD patients. On the one hand, Wang *et al.* reported that *Mstn* mRNA was not up-regulated in *vastus lateralis* of 51 HD patients compared to 21 healthy individuals¹², on the other hand, Verzola *et al.* found an up-regulation of *Mstn* in *rectus abdominis* of 22 stage 5 CKD patients compared to 12 healthy subjects¹³. In the present study, we found *Mstn* down-regulation in *vastus lateralis* of HD patients compared to matched controls, in agreement with the study of Wang *et al.* *Mstn* can be stimulated by many triggers including obesity, diabetes, chronic inflammation, lack of muscle mechanical load and sedentary lifestyle, nutritional parameters, ageing, and angiotensin II^{4,27-29}. Thus, because CKD patients have many comorbidities, the role of uremia by itself is difficult to determine by clinical investigations. The *MSTN* down-regulation in our cohort of HD patients which were matched on comorbidities to healthy controls, does not favor the hypothesis that uremia increases Mstn production.

Some studies have suggested that the uremic toxin IS could up-regulate Mstn expression in muscle cells^{14,15}. We tested this hypothesis *in vitro* in C2C12 myoblasts and myocytes, and in mice *in vivo*. In C2C12 myoblasts, we found no *Mstn* mRNA expression, even after exposure to IS. The absence of *Mstn* expression was expected in myoblasts, which do not express genes

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3 of differentiated muscle cells, such as *Myh2*. In C2C12 myocytes, which are differentiated
4 muscle cells, *Mstn* mRNA was expressed but IS did not up-regulate *Mstn* expression, neither at
5 concentrations usually found in HD patients (200 μ M)^{17,30}, nor at higher dose (1mM). AHR
6 target genes *Cyp1a1* and *Cyp1b1* were strongly upregulated by IS in a dose dependent manner
7 in both C2C12 myoblasts and myocytes at 24 and 48h confirming that AHR was activated by
8 IS in these cells. In the adenine-treated and 5/6thNx mouse models, although blood IS
9 concentrations were high, *Mstn* was not up regulated in two different muscles: *gastrocnemius*
10 and *tibialis anterior*. To assess if exposure to high IS concentrations could upregulate *Mstn*, we
11 carried out the IS-IP model in which IS concentrations were even higher than in CKD models.
12 In this latter model, although IS activated AHR target gene *Cyp1a1*, we found no up-regulation
13 of *Mstn* gene.

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29 AHR target genes activation by IS in muscle cells has never been reported before to our best
30 knowledge. Interestingly, *Cyp1a1*, which was poorly expressed in cultured C2C12 myoblasts
31 and myocytes and in control mice, was highly upregulated by IS in cultured cells, in adenine
32 mouse model, as well as in the IS IP mouse model. On the other hand, *Cyp1b1* which basal
33 expression was high in muscle cells was only up-regulated *in vitro* in C2C12 myoblasts and
34 myocytes, but not *in vivo* in CKD or IS IP mice models (data not shown).

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43 OAT1 (*Slc22a6*) and OAT3 (*Slc22a8*) are organic anion transporters allowing IS to enter the
44 kidney tubular cells²⁵. Here, we found a high expression of these genes in mouse kidneys,
45 consistent with the high expression in kidney tubular cells reported in the literature^{25,31}. Studies
46 analyzing C2C12 myoblasts and myocytes treated with probenecid -a classical inhibitor of
47 OAT1 and 3- proposed that these proteins could also be the transporters of IS in muscle cells.
48 We searched for mRNA expression of *Oat1* and *Oat3* in C2C12 myoblasts, myocytes and in
49 mouse muscles but did not find any expression. No data on the expression of these genes in
50 muscle cells *in vivo* are available to our knowledge. In our study, we show that IS activates Ahr
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Origin of myostatin in CKD

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3 pathway in muscle cells, arguing for the existence of another transporter than OAT1 or OAT3
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5 in these latter.
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8 Mstn production has been reported in many cells and tissues like smooth muscle cells, heart,
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10 adipocytes, mammary glands and hematopoietic stem cells under physiological conditions^{5,32}.
11
12 Mstn expression was also found in tubulo-interstitial compartment of kidneys in humans with
13
14 diabetic nephropathy, where it was associated with glomerulosclerosis, interstitial inflammation
15
16 and fibrosis³³. Mstn production can be stimulated by high glucose concentrations in HK-2 cells
17
18 in vitro; but the precise causes of Mstn up-regulation in diabetic nephropathy kidneys is not
19
20 totally understood³³. We searched the expression of *Mstn* in kidneys of mice from adenine-
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22 treated model and we did not detect any mRNA expression in both control and CKD mice (Ct
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24 35, data not shown). In the adenine-fed mouse model, mice develop a tubulointerstitial
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26 nephropathy associated with interstitial fibrosis^{22,23}. Circulating myostatin do not come from
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28 kidney as extra muscular source of production in CKD mice.
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34 As all members of the TGF- β family, Mstn is synthesized as a large inactive protein including
35
36 a small amino-terminal signal peptide for extracellular secretion, a large pro-domain which
37
38 represents almost 70% of the non-mature protein and finally a small active peptide of 110
39
40 amino-acid³². Mstn circulates in the plasma as a dimer of the small active peptide linked non-
41
42 covalently to the pro-domain. The proteolytic cleavage of the pro-domain by BMP-1/TLD
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44 proteases is necessary to release the active Mstn dimer³⁴. The circulating Mstn dimer is of ~90
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46 kDa³² and might not be filtered by the renal tubule, but the small active peptide, which is of 25
47
48 kDa in its dimeric form³⁵ and of 12 kDa in its monomeric form might be cleared by the kidney
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50 under physiological conditions and could accumulate in CKD. It is not clear which form of
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52 Mstn is measured by the ELISA test used in our study, but it requires a denaturation step and
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54 might measure the two Mstn forms. Nevertheless, the strong correlation between creatinine or
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Origin of myostatin in CKD

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3 urea and Mstn concentrations in our 5/6th Nx mice argues for a mechanism of accumulation due
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5 to decreased renal clearance.
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8 The potential harm of Mstn accumulation in CKD remains to be studied. Several association
9
10 studies have reported a positive correlation between Mstn concentrations and muscle mass or
11
12 strength^{5,9}. In these studies, Mstn concentration must be interpreted as surrogate marker of
13
14 muscle mass and does not only reveal the lack of elimination. Importantly, the
15
16 pathophysiological role of Mstn cannot be interpreted without studying the other players of the
17
18 pathway: inhibitors as follistatin, and other activators of the Mstn receptor ACTR1B: growth
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20 derived factor 11 (GDF11) and activin A.
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25 We acknowledge our study has limitations. First of all, the biological measurement of Mstn
26
27 concentration is difficult due to a lack of specificity with other TGF- β family members^{9,36}.
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29 Nevertheless, the assay used in our study is now the most frequently used in studies⁵. The
30
31 unique measurement and the cross-sectional design of the human CKD cohort are other
32
33 limitations and prospective studies need to be designed in CKD patients. To the best of and
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35 some . Also, we do not show directly a decrease in Mstn renal clearance but as we found no
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37 overproduction of Mstn, which is mostly secreted by striated muscle cells, our conclusion is
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39 that Mstn is not cleared by the dysfunctional kidneys in CKD.
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44 In conclusion, high circulating Mstn levels in CKD could be due to Mstn accumulation because
45
46 of decreased renal clearance rather than Mstn overproduction by muscle cells. We propose to
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48 add myostatin to the list of uremic toxins.
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Origin of myostatin in CKD

Authors' Contributions

Stanislas Bataille designed and performed experiments, wrote the paper. Laetitia Dou performed experiments and wrote the paper. Marc Bartoli helped in the study design and cell culture methods. Marion Sallée provided human serum data. Julien Aniort provided the human muscle data. Bohrane Ferkak performed the statistical analysis of the human CKD cohort. Rania Chermiti helped for cell cultures. Nathalie McKay performed experiments. Nathalie Da Silva helped for cell culture methods. Stéphane Burtey participated to the study design and wrote the paper. Stéphane Poitevin designed and performed animal experiments, participated in data interpretation and wrote the paper. All authors helped writing the paper.

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None

Disclosures

None

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Tables

Table 1: Clinical and biological features of patients according to eGFR. eGFR: estimated Glomerular Filtration Rate using the CKD

Epidemiology Collaboration (CKD-EPI) creatinine equation²⁰. BMI: body mass index. Data are expressed as mean \pm standard deviation or percentages.

Variable	TOTAL n=232	eGFR ≥ 60 mL/min/1.73m ² n=90	eGFR 30-59 mL/min/1.73m ² n=48	eGFR 15-29 mL/min/1.73m ² n=52	eGFR <15 mL/min/1.73m ² n=42
Age (years)	57.7 \pm 16.6	50.1 \pm 15.4	60.6 \pm 13.3	67.3 \pm 14.0	58.9 \pm 18.1
Men (%)	54.3%	33.3%	75.0%	73.1%	52.4%
Diabetes (%)	24.7%	11.1%	38.3%	34.6%	26.2%
BMI (kg/m ²)	26.6 \pm 5.5	25.7 \pm 5.7	28.5 \pm 5.0	26.6 \pm 4.4	26.6 \pm 6.2
Urea (mmol/L)	14.1 \pm 11.7	5.6 \pm 1.7	11.0 \pm 4.0	18.4 \pm 9.9	30.6 \pm 12.4
Creatinine (μ mol/L)	213 \pm 218	77 \pm 21	148 \pm 31	238 \pm 55	549 \pm 315
eGFR (mL/min/1.73m ²)	49.4 \pm 34.1	87.1 \pm 18.2	42.4 \pm 9.0	22.8 \pm 4.5	9.4 \pm 3.0
Myostatin (pg/mL)	3034 \pm 1478	2806 \pm 1199	3239 \pm 1791	3075 \pm 1488	3237 \pm 1598

Origin of myostatin in CKD

Table 2: Monovariate analysis of factors associated with myostatin concentrations (Pearson correlation).

	Pearson correlation coefficient	p-value
Age	-0.169	0.005
Gender (Women)	-0.197	0.001
Diabetes	-0.152	0.011
BMI	-0.083	0.104
Urea	0.049	0.231
Creatinine	0.131	0.023
eGFR	-0.149	0.012

Table 3: Multivariate linear regression of factors associated with Mstn concentration in patients.

	adj. coeff. (95%CI) for Mstn (pg/mL)	p-value
Age (per year)	-21.11 (-33.25 ; -8.98)	0.01
Gender (women)	-604.40 (-991.44 ; -217.36)	0.002
Diabetes	-525.23 (-971.13 ; -79.34)	0.02
eGFR (per mL/min)	-8.63 (-14.58 ; -2.69)	0.005

Origin of myostatin in CKD

Legends to figures

Figure 1 Expression of myostatin mRNA was significantly decreased in vastus lateralis muscle of hemodialyzed patients (HD) compared to controls. Results represent the median and the values from lower to upper quartile (25th to 75th percentile). * p-value ≤ 0.05

Figure 2. Circulating myostatin (Mstn) is increased in adenine-fed CKD mice. (A) Timeline and design of the CKD mouse model based on adenine diet. The reduction of kidney function in adenine-treated (Ade) mice was shown by elevated serum urea (B) and creatinine (C). (C) Serum concentrations of IS (D) and myostatin (E) were increased in Ade CKD mice. mRNA expression levels of Cyp11a1 (F) and Mstn (G) in gastrocnemius muscle of mice. (H) Mstn protein expression was not modified in gastrocnemius muscle of CKD mice. Results represent the median, and the values from lower to upper quartile (25th to 75th percentile) are indicated. *P ≤ 0.05 ; **P ≤ 0.01 .

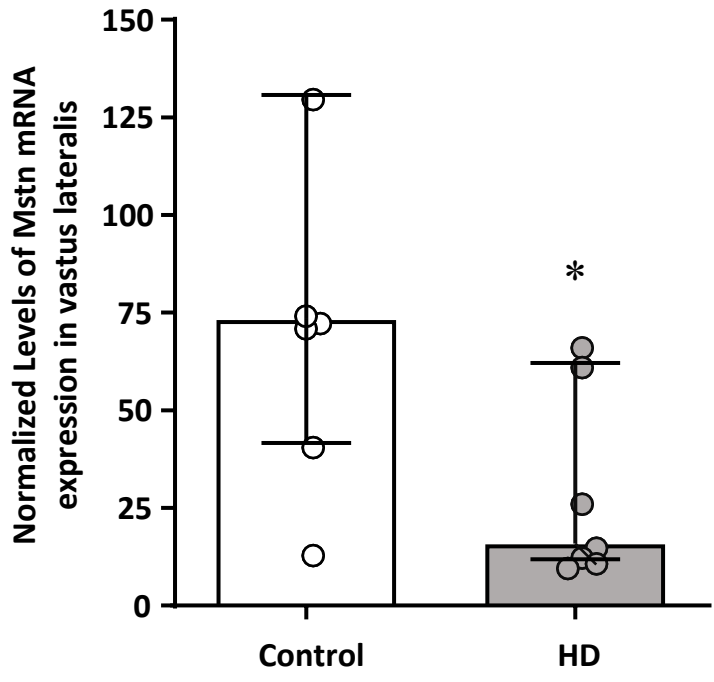
Figure 3. Elevated circulating myostatin is correlated to the renal insufficiency level, without modification of Mstn muscle expression in 5/6th Nx CKD mice. (A) Timeline and design of the CKD mouse model based on the 5/6th nephrectomy (5/6th Nx). Correlation between serum urea (B) or creatinine (C) and Mstn in sham- and 5/6th Nx mice. Serum concentrations of IS (D) and myostatin (E) were increased in 5/6th Nx mice with blood urea nitrogen (BUN) ≥ 20 nmol/L. Mstn mRNA (F) and protein (G) expression was not modified in gastrocnemius muscle of CKD mice (BUN ≥ 20 nmol/L). Results represent the median, and the values from lower to upper quartile (25th to 75th percentile) are indicated (D-G). **p-value ≤ 0.01 ; ***p-value ≤ 0.001 .

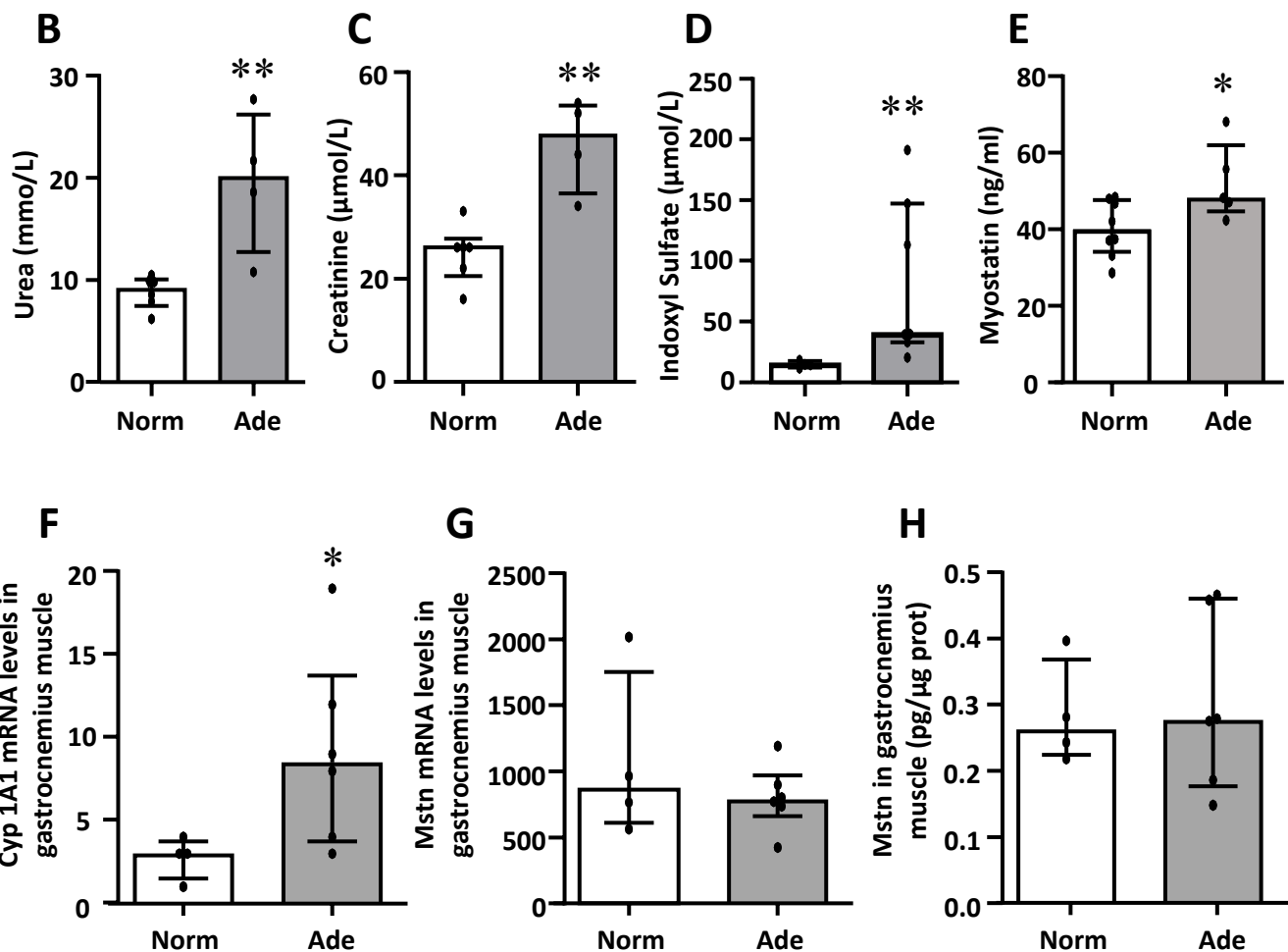
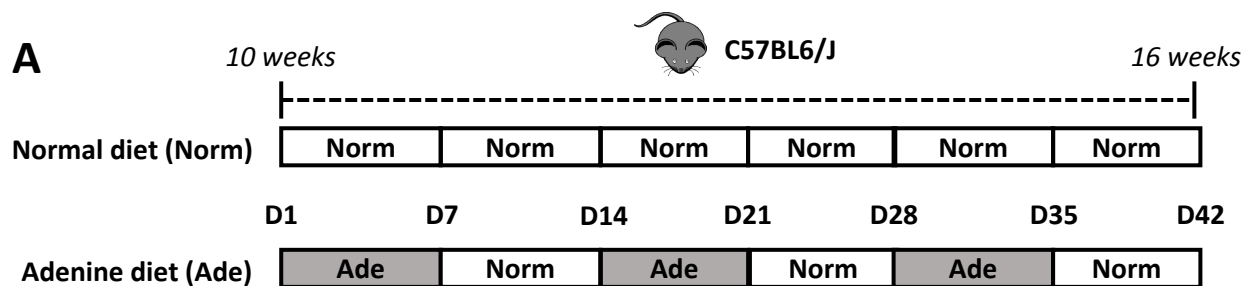
Origin of myostatin in CKD

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2
3 **Figure 4. Indoxyl sulfate (IS) does not induce myostatin mRNA expression in mouse**
4 **muscles.** (A) Timeline and design of the IS-injected mouse model. Serum concentrations of IS
5 (B) and Cyp1a1 mRNA expression in gastrocnemius muscle (C) were increased in IS-treated
6 mice. (D) Mstn mRNA expression was not modified in gastrocnemius muscle of IS-treated
7 mice. Results represent the median and the values from lower to upper quartile (25th to 75th
8 percentile) are indicated. *p-value ≤ 0.05 ; **p-value ≤ 0.01 .
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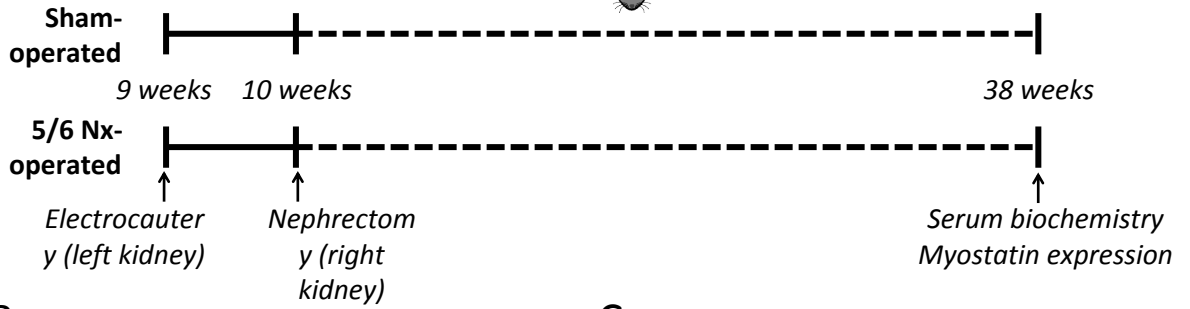
19 **Figure 5. IS increases the expression of AhR-target genes in cultured muscle cells.** C2C12
20 myoblasts (A, B) and myocytes (C, D) were incubated with IS for the indicated incubation times
21 and mRNA expression of Cyp1a1 (A, C) and Cyp1b1 (B, D) was quantified by quantitative
22 comparative RT-PCR. The expression of Mstn mRNA is not increased by IS in C2C12
23 myocytes (E). Results are expressed as mean \pm SD of 6 independent experiments. *p-value \leq
24 0.05; **p-value ≤ 0.01 ; ***p-value ≤ 0.001 .
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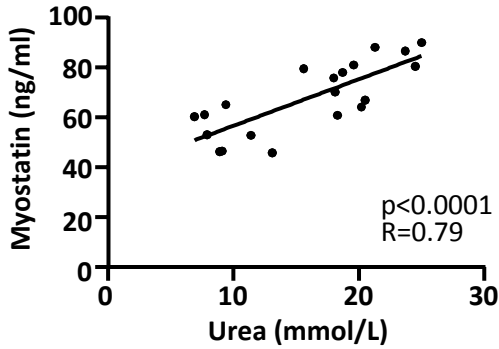




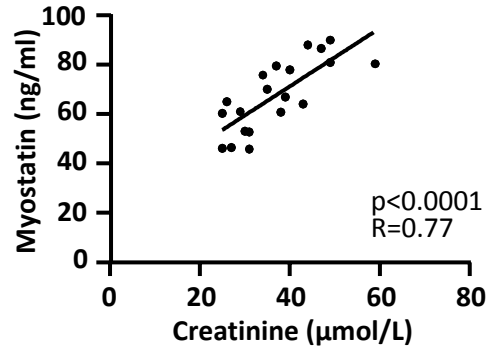
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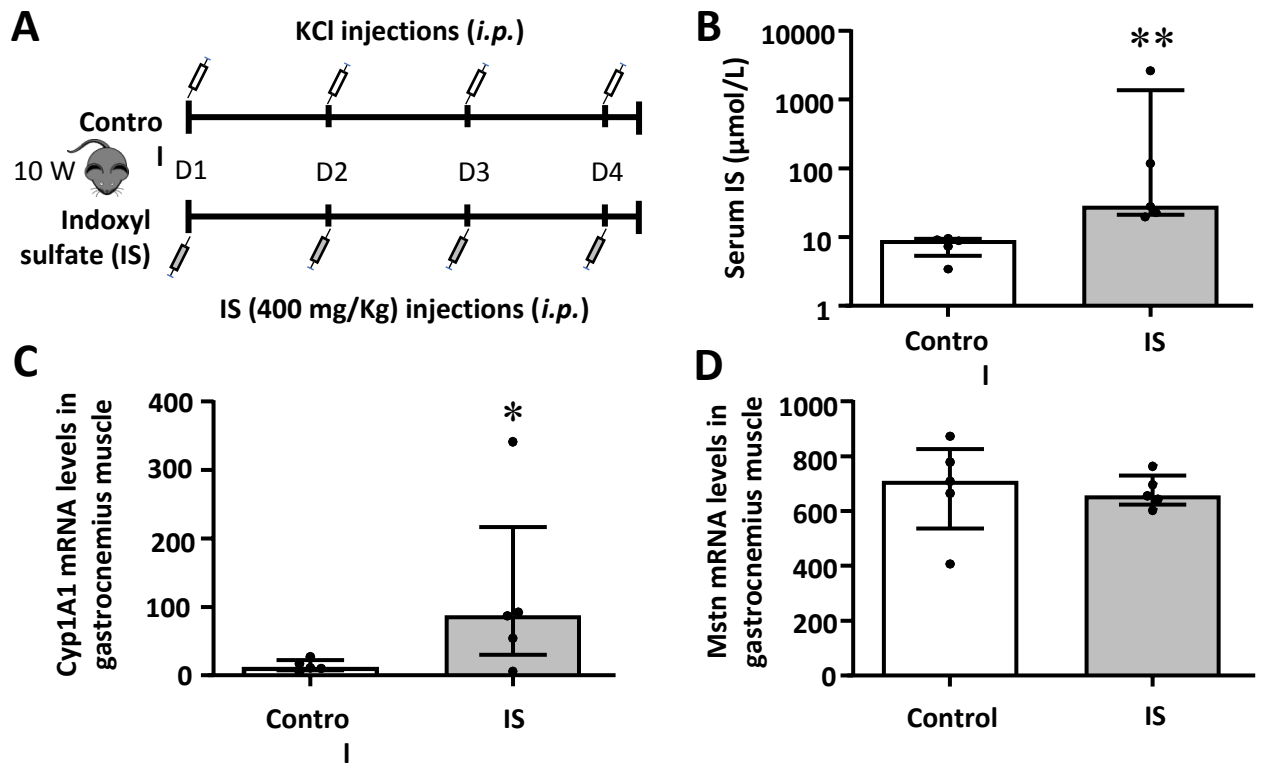


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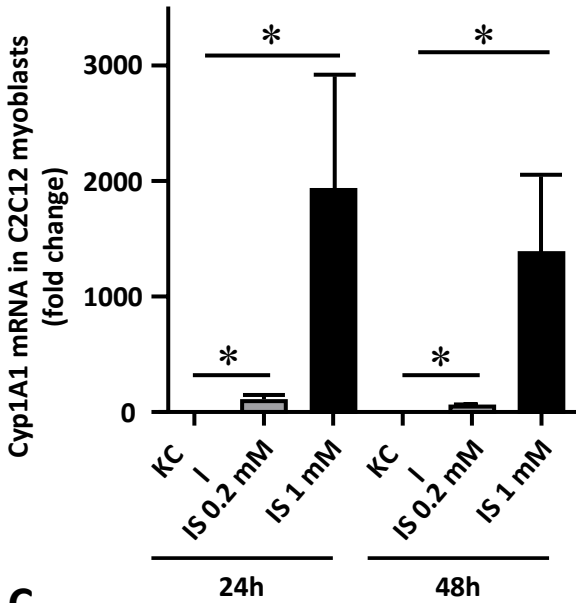
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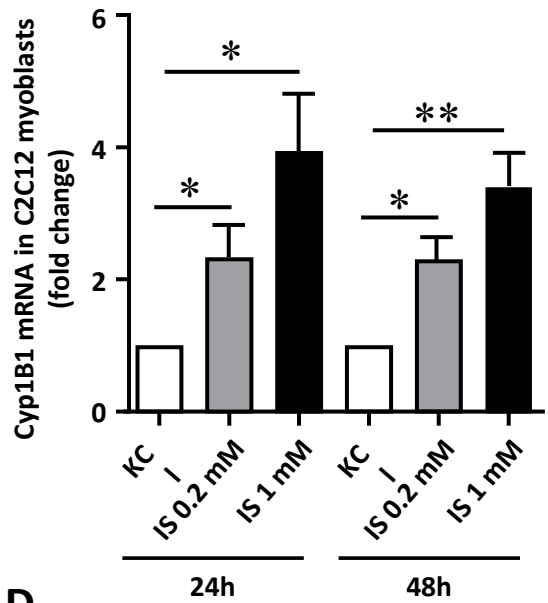


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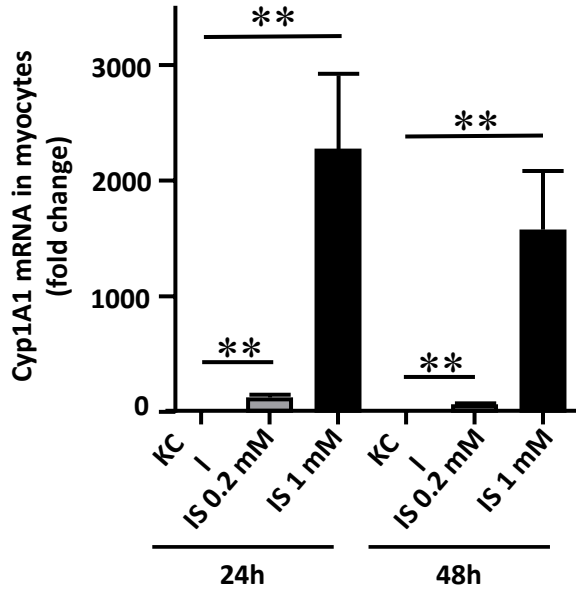
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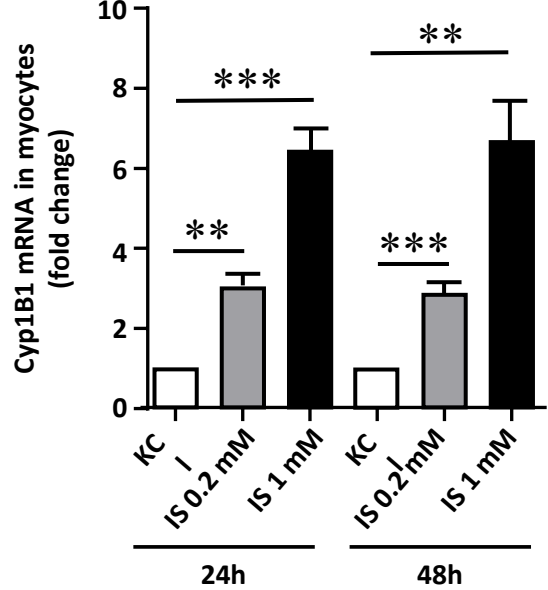
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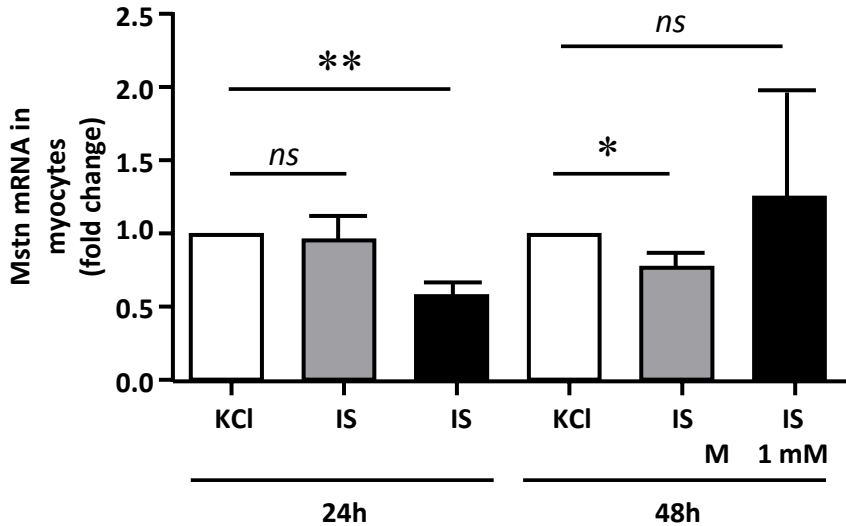
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Supplementary Material

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Table S2: Monovariate linear regression of factors associated with Mstn concentration in patients according to median myostatin concentration in the human CKD.

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Figure S2: Myostatin is not expressed in C2C12 myoblasts.

Figure S3: Expression of Slc22a6 and Slc22a8 in C2C12 myoblasts, myocytes, and mice muscle and kidney.

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Table S1: List of primer references used in qPCR experiments

	Target genes	TaqMan probes ID
Human	<i>HPRT</i>	Hs02800695_m1
	<i>MSTN</i>	Hs00976237_m1
Mouse	<i>Hprt</i>	Mm03024075_m1
	<i>Mstn</i>	Mm03024050_m1
	<i>Cyp1a1</i>	Mm00487218_m1
	<i>Cyp1b1</i>	Mm00487229_m1
	<i>Slc22A6</i>	Mm00456258_m1
	<i>Slc22A8</i>	Mm00459534_m1

Table S2: Monovariate linear regression of factors associated with Mstn concentration in patients according to median myostatin concentration in the human CKD. Data are expressed as mean \pm standard deviation or percentages.

Variable	Myostatin ≤ 2783.33 pg/mL	Myostatin > 2783.33 pg/mL	p-value
Age (years)	60.3 \pm 17.5	55.1 \pm 15.2	0.05
Gender (female)	52.6%	38.8%	0.04
Diabetes	31.0%	18.3%	0.02
eGFR (mL/Min/1.73m²)	53.7 \pm 35.8	45.1 \pm 31.9	0.01

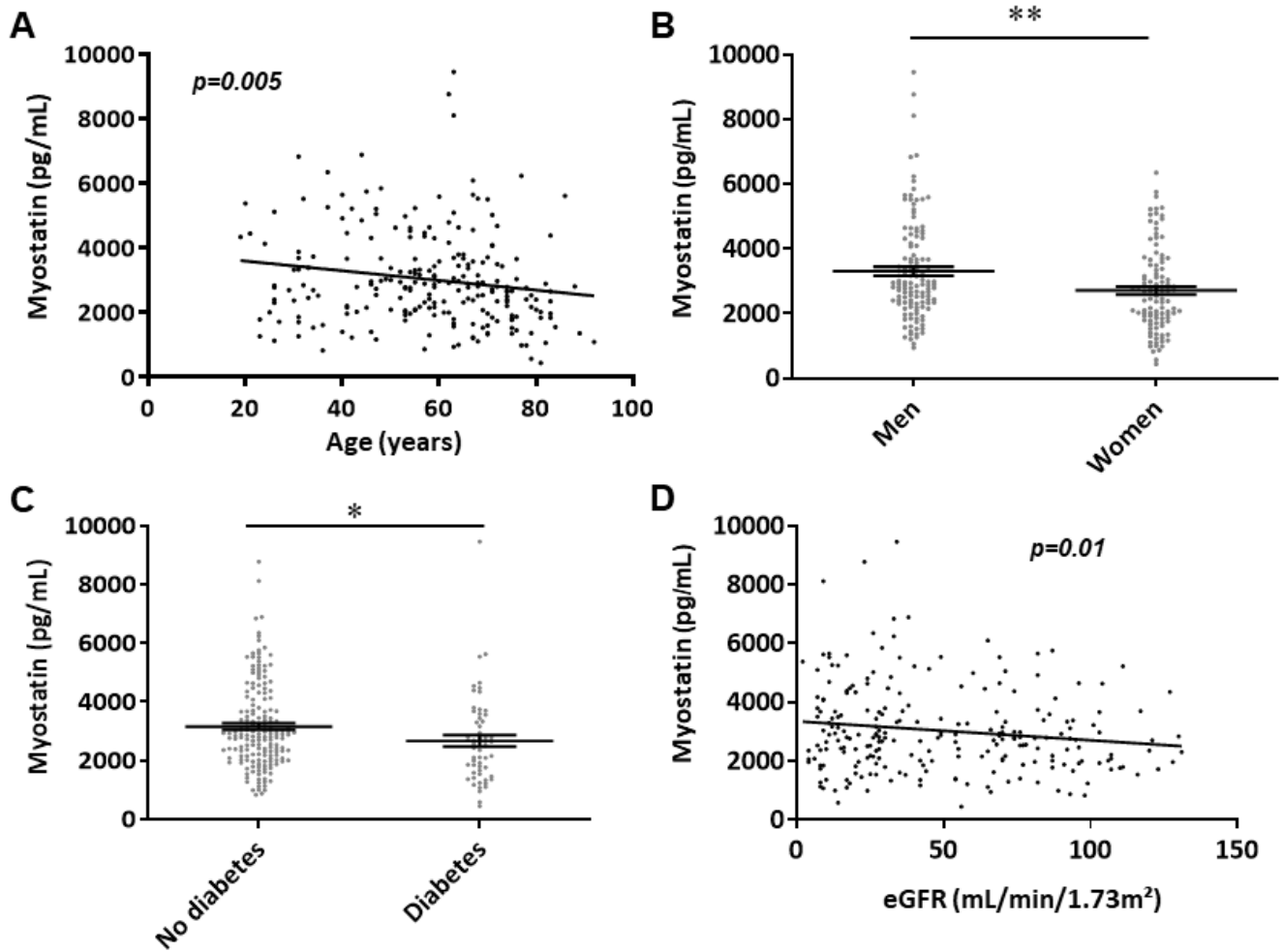


Figure S1: Increased serum myostatin is correlated with the decrease of renal function. (A)

Serum myostatin in the subjects from the human CKD cohort was correlated with age (B-C)

Serum myostatin in subjects was lower in women compared to men and lower in diabetics patients versus non-diabetic subjects. Results are expressed as mean \pm SD; *p-value \leq 0.05;

**p-value \leq 0.01. (D) Serum myostatin was correlated eGFR calculated according the MDRD

formula

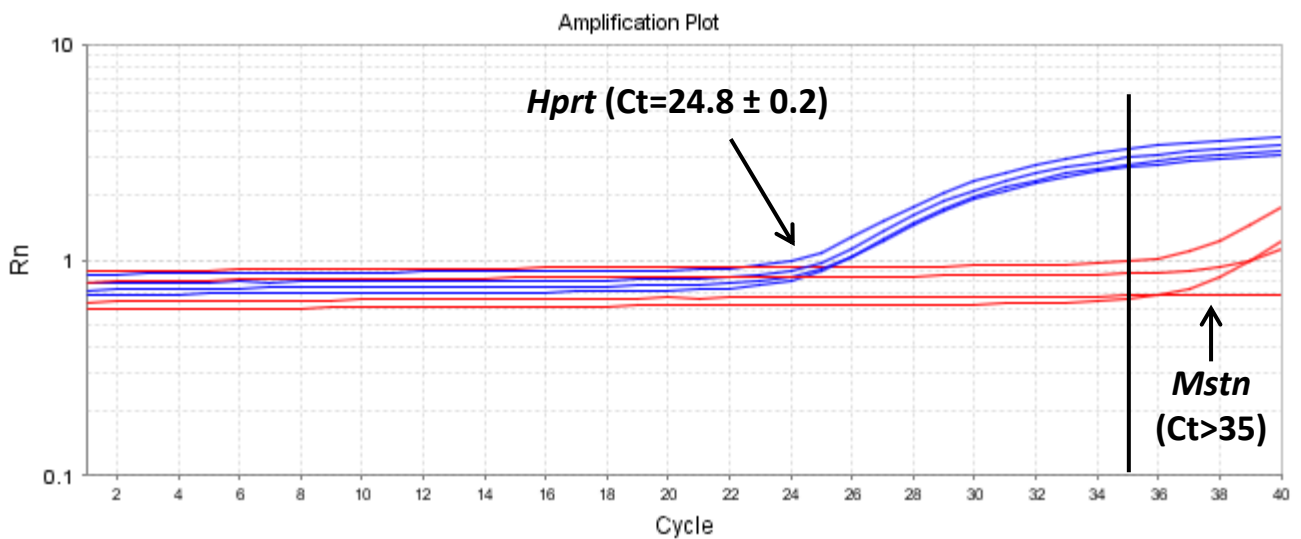


Figure S2: Myostatin is not expressed in C2C12 myoblasts. Results show amplification plots of the housekeeping gene HPRT and of myostatin (*Mstn*) obtained by RT-qPCR. Mean threshold cycles (Ct) for HPRT is provided (mean ± SD, n= 4)

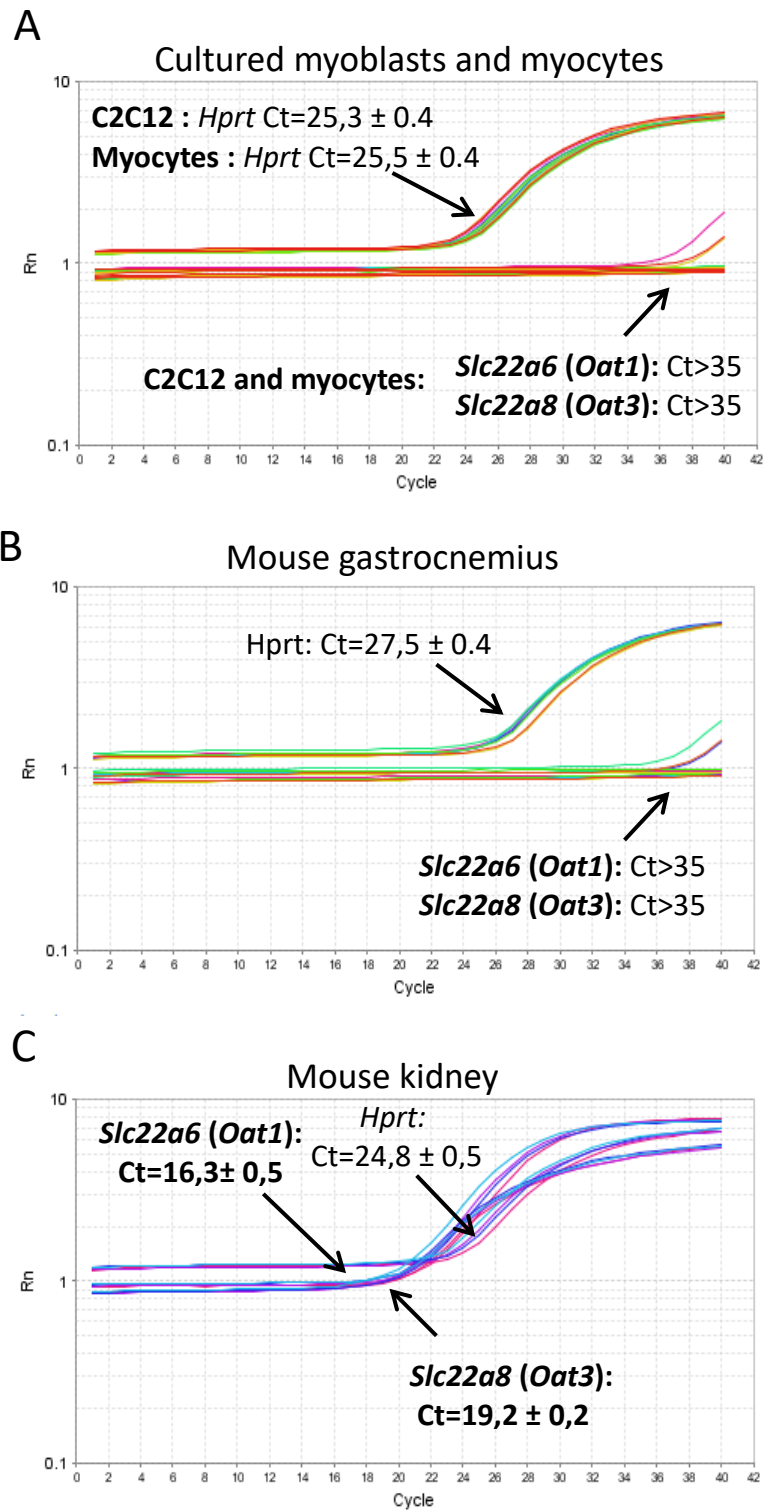


Figure S3: Expression of *Slc22a6* and *Slc22a8* in C2C12 myoblasts, myocytes, and mice muscle and kidney. (A-B) *Slc22a6* and *Slc22a8* are not expressed in C2C12 myoblast, in cultured myocytes and in mouse gastrocnemius muscle. (C) Expression of *Slc22a6* and *Slc22a8* in mouse

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kidney. Results show amplification plots of studied genes obtained by RT-qPCR. Mean threshold cycles (Ct) are provided (mean \pm SD, n= 4)