

# Myostatin in the Arterial Wall of Patients with End-Stage Renal Disease

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**Aim:** Myostatin (Mstn) has been described as a trigger for the progression of atherosclerosis. In this study, we evaluated the role of Mstn in arterial remodeling in patients with end-stage renal disease (ESRD).

**Methods:** Vascular specimens were collected from 16 ESRD patients ( $56.4 \pm 7.9$  years) undergoing renal transplant (recipients) and 15 deceased kidney non-uremic donors ( $55.4 \pm 12.1$  years). We studied gene and protein expression of Mstn, ubiquitin ligases, Atrogin-1, and muscle ring finger protein-1 (MuRF-1), inflammatory marker CCL2, cytoskeleton components, and Klotho by reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry. Moreover, we assessed vascular calcification and collagen deposition. Finally, we studied the effects of recombinant Mstn on rat vascular smooth muscle cells (VSMCs, A7r5) and evaluated the effects of uremic serum (US) on primary human VSMCs.

**Results:** Myostatin mRNA was upregulated in the arterial vascular wall of recipients compared with donors ( $\sim 15$ -folds,  $p < 0.05$ ). This response was accompanied by the upregulation of gene expression of Atrogin-1 and MuRF-1 ( $+2.5$ - and  $+10$ -fold) and CCL2 ( $+3$ -fold). Conversely, we found downregulation of protein expression of Smoothelin,  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), vimentin, and Klotho ( $-85\%$ ,  $-50\%$ ,  $-70\%$ , and  $-80\%$ , respectively;  $p < 0.05$ ) and gene expression of vimentin and Klotho. Exposition of A7r5 to Mstn induced a time-dependent SMAD 2/SMAD 3 phosphorylation and expression of collagen-1 and transforming growth factor  $\beta$  (TGF $\beta$ ) mRNA, while US induced overexpression of Mstn and Atrogin-1 and downregulation of Smoothelin and Klotho.

**Conclusions:** Our data suggest that uremia might induce vascular Mstn gene expression together with a complex pathway of molecular and structural changes in the vascular wall. Myostatin, in turn, can translate the metabolic alterations of uremia into profibrotic and stiffness inducing signals.

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**Key words:** Myostatin, Uremic vasculopathy, Vascular fibrosis, Klotho, Vascular smooth muscle cells

## Introduction

Cardiovascular disease accounts for more than 50% of total chronic kidney disease (CKD) mortality and is the most prevalent complication observed in CKD patients, especially in those affected by end-stage renal disease (ESRD) requiring dialysis<sup>1</sup>. Uremic vascular disease presents unique features, including accelerated aging, abnormal resistance vessel remodel-

ing<sup>2, 3</sup>, endothelial dysfunction, arterial stiffness, and vascular calcification<sup>4, 5</sup>. The development of vascular disease involves the response to a number of insults and a complex interplay between cells resident in the vascular wall, including vascular smooth muscle cells (VSMCs), endothelial cells, and infiltrating cells of the innate and adaptive immune system<sup>6</sup>. In particular, in atherosclerosis, VSMCs undergo a shift from a contractile to a synthetic phenotype, accompanied by

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reduced expression of VSMC marker proteins, such as  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), smooth muscle 22-alpha (TAGLN), smooth muscle myosin heavy chain (MYH11), the transcription factor myocardin, and Smoothelin. These changes result in increased proliferative and migratory rate of VSMCs and release of a number of chemo- and cytokines that, interacting with infiltrating cells, boost inflammation and cause loss of arterial elasticity and deterioration of the vessel wall<sup>7, 8</sup>. This condition might be further aggravated in uremia, where VSMCs acquire a calcifying phenotype, characterized by the upregulation of osteochondrocytic genes, finally leading to vascular calcification<sup>9</sup>. It is commonly retained that this process is regulated by the combination of several factors, such as hyperphosphatemia, inflammation, Klotho deficiency, reduced levels of calcification inhibitors, and expression of pro-fibrotic molecules<sup>10-12</sup>.

Myostatin (Mstn), a 375-amino acid peptide member of the transforming growth factor  $\beta$  (TGF $\beta$ ) superfamily, is primarily expressed in skeletal muscle cells, where it limits muscle growth and promotes protein breakdown<sup>13</sup>. Myostatin exerts its effects in both autocrine and paracrine ways by binding a cell-bound receptor activin type 2B<sup>14</sup>. The complex Mstn receptor assembles with either the type I receptor ALK4 or ALK5 leading to the activation of intracellular signaling pathways including SMAD 2/SMAD 3 and Akt, which finally upregulate ubiquitin-proteasome system (UPS), causing protein degradation and muscle atrophy<sup>15</sup>. Recent evidence indicates that, besides its effects on skeletal muscle atrophy, Mstn is involved in the pathogenesis of heart failure and fibrosis<sup>16</sup>. Myostatin attenuates cardiomyocyte proliferation in models of cardiac hypertrophy and promotes myocardial fibrosis by blocking myofibroblast differentiation<sup>17</sup>. Very recently, we observed that Mstn is upregulated in VSMC and in infiltrating macrophages of the abdominal aorta and that its expression progressively increases along with the severity of atherosclerotic damage<sup>18</sup>. In additional experiments, we observed that Mstn downregulates cell proliferation and stiffness-related genes in rat VSMCs and acts as a chemoattractant in monocytes, raising the MCP-1-dependent chemotaxis. Overall, these studies indicate that Mstn actively participates in the progression of atherosclerotic damage. In CKD patients, Mstn is upregulated by inflammation in the skeletal muscle<sup>19, 20</sup> and plays a major role in uremic wasting and muscle fibrosis<sup>21, 22</sup>. Moreover, recent observations indicate that vessel activin receptor is upregulated in CKD<sup>23</sup>. However, no information is available on the presence and localization of Mstn in the arterial wall of uremic patients and about its potential relationship with the

different vascular regulatory pathways.

## Materials And Methods

### Patients and Vascular Samples

Vascular specimens were obtained from consecutive uremic patients with ESRD undergoing renal transplant (i.e., recipients) and paired deceased kidney non-uremic donors (considered as control group) at the time of surgery at “San Matteo” Hospital of Pavia, Italy. We enrolled patients aged 18–70 years, at their first transplant, with a dialysis vintage of at least 12 months. We collected demographic and clinical data, such as presence of comorbidities, and measured albumin, calcium, phosphorus, PTH, C-reactive protein (CRP), lipids, and lymphocyte count, while for recipients we also collected data on dialytic history. Informed written consent was obtained from all patients, and the study was approved by the local ethics committee.

### Tissue mRNA Expression

Total RNA was extracted from vascular tissues using the guanidine-based RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany). A total of 1  $\mu$ g of RNA per condition was reversely transcribed by iScript<sup>™</sup> cDNA Synthesis Kit (Bio-Rad Laboratories, Segrate, Italy). The primers were obtained from TIB Molbiol (Genoa, Italy). The sequences are reported in **Table 1**. PCR amplification was carried out in a total volume of 10  $\mu$ L, containing 1  $\mu$ L cDNA solution, 5  $\mu$ L SYBR solution PrecisionPLUS qPCR MasterMix (Primerdesign, Southampton, Great Britain), 0.5  $\mu$ L each primer, and 3.5  $\mu$ L of nuclease-free water.  $\beta$ -actin was used for the normalization of expression values of the other genes. The  $\Delta\Delta CT$  method of relative quantification was used to determine the fold change in expression<sup>24</sup>. Assays were run in triplicate by a Universal PCR Master Mix on MasterCycler realplex (Eppendorf, Hamburg, Germany) PCR system.

### Histological Preparation and Immunohistochemical Staining

Paraffin sections (5  $\mu$ m) of 2% paraformaldehyde-fixed tissue were deparaffinized, hydrated, and treated with 3% H<sub>2</sub>O<sub>2</sub> in methanol. Each sample was analyzed for the detection of Mstn (polyclonal antibody; Proteintech, DBA Italia s.r.l., Segrate, Italy), CCL2/MCP-1 (polyclonal antibody; Novus Biologicals, DBA Italia s.r.l., Segrate, Italy), Atrogin-1 (polyclonal antibody; ECM Biosciences, DBA Italia s.r.l., Segrate, Italy), Klotho (polyclonal antibody; Abcam, Prodotti Gianni, Milan, Italy), Smoothelin, vimentin (monoclonal antibodies; Santa Cruz Biotechnology,

**Table 1.** Primers for RT-PCR

PRIMERS	FORWARD	REVERSE
$\beta$ Actin (Human)	CATCCCCCAAAGTTCACAAT	AGTGGGGTGGCTTTTAGGAT
Myostatin (Human)	CCAAAGCTCCTCCACTCCG	GGGAGTACAGCAAGGGCC
Vimentin (Human)	GCAAAGATTCCACTTTGCGT	GAAATTGCAGGAGATGC
$\alpha$ SMA (Human)	AAGCAGAGAAAAGAGGAAT	ATGTGCTCCCAGTTGGTGAT
Atrogin1/MAFbx (Human)	TTTCCTGGAAGGGCACTGAC	ACGACTGACCTCTCGACCCTTAT
Murf1 (Human)	GTGCAAGGTGTTTGGGATCCAC	AGGGACTGGATGGCAGTTTCCA
CCL2 (Human)	ACCGAGAGGCTGAGACTAAC	AATGAAGGTGGCTGCTATGAG
Smoothelin (Human)	TGAGGAGCTGATGACTATT	CTGATCCAGCATCTTGTC
Klotho (Human)	GATAGAGAAAAATGGCTTCCCTCC	GGTCGGTAAACTGAGACAGAGTGG
GAPDH (Rat)	CTCTCTGCTCCTCCCTGTTCT	ATACGGCCAAATCCGTTTACA
Collagen 1 (Rat)	TCACCTACAGCACGCTTG	GGTCTGTTTCCAGGGTTG
TGF $\beta$ (Rat)	TGGAAGTGGATCCACGCGCCCAAGG	GCAGGAGCGCACGATCATGTTGGAC

DBA Italia s.r.l., Segrate, Italy),  $\alpha$ -SMA (Dako Italia, Milan, Italy), and CD45 (Novocastra, Leica Microsystem, Milan, Italy). Staining was performed by immunohistochemistry after microwave oven treatment in 0.1 M sodium citrate. Slides were incubated with primary antibody overnight, followed by incubation with biotinylated antibody for 30 minutes. Immunostaining was completed with streptavidin-peroxidase method, performed as previously described<sup>25</sup>. In order to evaluate the co-distribution of two different antigens in the same sample, a double immunohistochemistry procedure was carried out as previously described<sup>18</sup>. The protein expression was examined by image analysis and expressed as positive areas.

### Tissue Calcification and Collagen Analysis

For tissue calcification, Von Kossa staining was performed, while Picrosirius red was used to stain collagen in vascular tissue. Slides were examined through a polarizing microscope. All samples were evaluated by image analysis as previously described<sup>26</sup>.

### Rat VSMC Cultures

Rat VSMCs (A7r5) were obtained from the European Collection of Cell Cultures (ECACC, UK). A7R5 cells were maintained in DMEM (EuroClone, Milan, Italy) culture medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, and 100 U penicillin-streptomycin. Sub-confluent cell cultures were treated with Mstn (PeproTech, DBA Italia s.r.l., Segrate, Italy) (500 ng/ml) for 5 and 24 hours.

### Western Blot Analysis

A7r5 cell layers 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  $\mu$ g/L

aprotinin, leupeptin, pepstatin A, 1 mM PMSF, and Na<sub>3</sub>VO<sub>4</sub>). Protein concentration was determined by using the Bicinchoninic Protein Assay Kit (Merck Group, Vimodrone, Italy), and 10–20  $\mu$ g of protein concentration were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred to a PVDF membrane (Merck Group, Vimodrone, Italy). Blots were probed using anti-phospho-SMAD 2, anti-phospho-SMAD 3, and SMAD 2/SMAD 3 polyclonal antibody (Cell Signaling Technology, EuroClone, Pero, Italy) and incubated in horseradish peroxidase secondary antibodies (Cell Signaling Technology, EuroClone, Pero, Italy). Immunoblots were developed with Immobilon Western Chemiluminescent HRP Substrate (Merck Group, Vimodrone, Italy). Band intensities were determined using Alliance system (Uvitec, Cambridge, UK).

### cDNA Reverse Transcription and Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted by A7r5 cells using QIAzol Lysis Reagent (QIAGEN Italia, Milan, Italy) according to the manufacturer's instructions. Total RNA (1  $\mu$ g) was reverse transcribed into cDNA by iScript<sup>™</sup> RT Supermix (Bio-Rad Laboratories, Segrate, Italy). The gene expression of collagen-1 was quantified by real-time PCR as above described. The corresponding primers are reported in **Table 1**.

### Primary Human VSMC Cultures

Aortic tissue was explanted by donors, cleaned of fatty tissue, and separated from intima and adventitia layers, leaving the tunica media alone. Subsequently, the tunica media was cut into 1–2 mm cubes, digested by collagenase and elastase (Sigma Aldrich, Milan, Italy), and transferred to 100 mm culture plates. After

**Table 2.** Clinical and biochemical characteristics of patients at the enrollment

	Recipients (ESRD pts)	Donors
<i>N</i>	16	15
Age (years)	56.4 ± 7.9	55.4 ± 12.1
Sex, male/female	9/7	8/7
BMI (kg/m <sup>2</sup> )	24.2 ± 2.9	26.9 ± 4
Hypertension, <i>n</i> (%)	14 (87.5%)*	5 (33%)
Diabetes, <i>n</i> (%)	2 (12.5%)*	0
Active smokers, <i>n</i> (%)	2 (12.5%)	4 (26%)
Dialysis vintage (months)	68.7 ± 40.3	-
Serum albumin (g/dl)	3.7 ± 0.4*	2.8 ± 0.7
CRP (mg/dl)	0.36 ± 0.6	0.9 ± 0.6
Phosphate (mg/dl)	4.3 ± 1.3	3.7 ± 2.3
Corrected calcium (mg/dl)	9.5 ± 0.9	9.7 ± 0.8
PTH (pg/ml)	405 (149-543)	-

Abbreviations:

ESRD=End-stage renal disease, BMI=Body mass index, CRP=C-Reactive Protein, HD=Hemodialysis, PD=Peritoneal Dialysis, \* =  $p < 0.05$  vs donors.

their adhesion at 37°C for 45 min in the incubator, aortic media small cubes were gently covered with 4 mL of 231 culture medium (Gibco, Life Technology, Monza, Italy) supplemented with 25 mL smooth muscle growth supplement from Gibco, 100 mg/mL streptomycin, and 100 U/mL penicillin. Cell cultures were maintained at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Explants were left undisturbed for six days to prevent detachment, and half the medium was replaced every three days. VSMCs migrated out from the explants within 1–2 weeks. Then, after removing the explants from the flask surface, the cells were trypsinized. Primary cultures have limited expansion and were used for experiments between passages P3 and P5<sup>27</sup>.

Finally, to explore whether circulating factors progressively retained in blood in uremia directly influenced vascular cell biology, we treated human VSMC with 10% uremic serum (US) or normal serum (NS) for 24–48 hours<sup>28</sup>.

### Statistical Analysis

Quantitative variables were represented by mean ± standard deviation (SD) or median-interquartile ranges (IQR) if they were not normally distributed (Shapiro test). Differences among groups were assessed by Student *t*-test or nonparametric Mann-Whitney test when appropriated. Correlations were analyzed using Spearman-Rho rank test. All tests were two-sided, and  $p < 0.05$  was considered statistically significant. Data analysis was performed with GraphPad Prism statistical package (version 5.00, GraphPad Software, San Diego, California, USA).

## Results

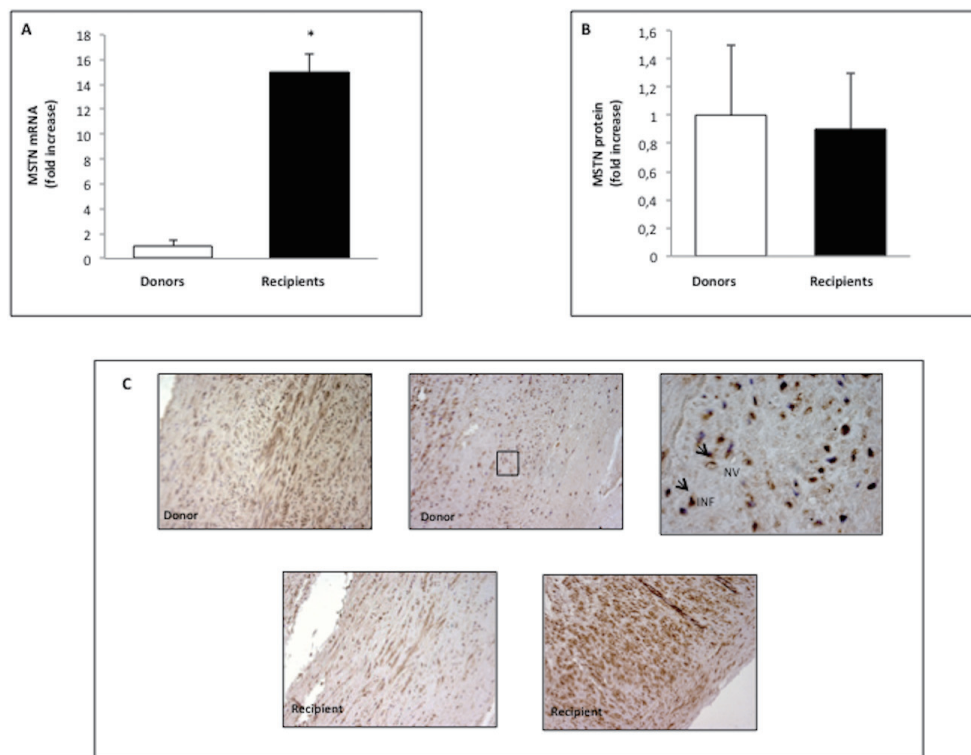
### Patient Characteristics

At the time of kidney transplant, iliac artery patches were collected from 16 recipients (56.4 ± 7.9 years, nine males, dialysis vintage 68.7 ± 40.3 months) (Table 2). Fourteen patients were hypertensive, two patients were diabetic, and two were active smokers. Main underlying nephropathies included glomerulonephritis, nephroangiosclerosis, and adult dominant polycystic disease. As a control group, we considered aortic artery samples collected from 15 (55.4 ± 12.1 years, eight males) deceased kidney donors. Causes of deaths were as follows: intracerebral hemorrhage (eight patients), stroke (three patients), trauma (three patients), and brain tumor (one patient). Five subjects were hypertensive and four were active smokers. Their mean serum creatinine at time of the donation was 0.7 ± 0.23 mg/dl. Donors presented lower albumin levels than recipients (2.8 ± 0.7 vs. 3.7 ± 0.4 g/dl,  $p < 0.05$ ), while there were not significant differences in age and sex distribution.

### Arterial Mstn Expression and Localization in Uremic Patients

There was a greater than 15-fold increase in Mstn mRNA levels in the vascular wall of recipients compared to donors ( $p < 0.05$ ) (Fig. 1A). The Mstn immunohistochemical signal in recipients was not different from donors (Fig. 1B).

The immunohistochemical signal showed that Mstn was mainly localized in the tunica media, in infiltrating cells, and neovasa both in recipients and



**Fig. 1.**

Expression of Mstn mRNA (A) and protein (B) in human aorta of donors ( $N=16$ ) and the iliac artery of recipients ( $N=13$ ). Myostatin mRNA expression level was determined by real-time PCR. Myostatin protein expression was evaluated by immunohistochemistry and image analysis. (C) Representative pictures of immunohistochemistry staining in donor and recipient arteries. Myostatin positivity was detectable in the tunica media, infiltrating cells (INF), and neovasa (NV) (magnification:  $\times 100$ – $1000$ ). Data are expressed as fold change  $\pm$  SEM to donor aortas.  $*p < 0.05$  vs. donors.

donors (Fig. 1C).

Finally, vascular protein and gene expression of Mstn did not significantly correlated with any basal clinical and laboratory characteristics of both recipients and donors.

### Myostatin Localization in Arterial Resident VSMCs and Bloodstream-Derived Cells

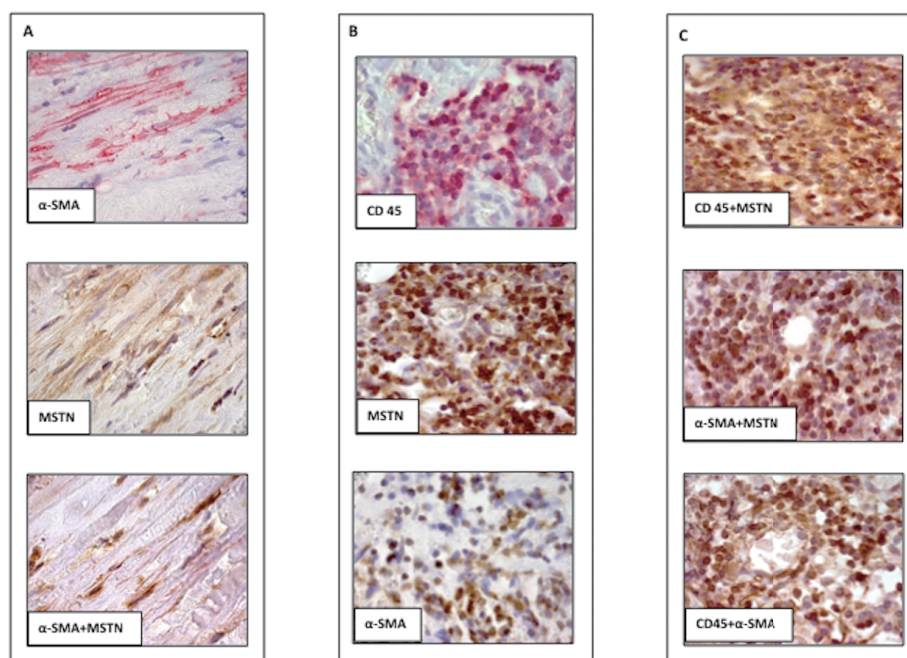
To gain information on the cell types responsible for Mstn enrichment within the vessel wall, we performed colocalization studies of Mstn with  $\alpha$ -SMA and CD45, markers of VSMCs, and cells from hematopoietic lineage, respectively. Myostatin staining colocalized with  $\alpha$ -SMA in VSMC (Fig. 2A) and with infiltrating CD45+ cells (Fig. 2C). Interestingly, some CD45+/Mstn+ cells were also positive for  $\alpha$ -SMA (Fig. 2B, C), marker of fibrosis, leading to hypothesize the commitment of bloodstream-derived cells toward a fibroblast-like differentiation within the vessel wall. The colocalization was assessed only in donor specimens.

### Vascular Cytoskeleton Protein Expression is Downregulated in Uremic Patients

In arterial samples of recipients, VSMCs showed an altered morphology, characterized by loss of their elongated and spindle shape and appearance of a rounded phenotype (Fig. 3A). Coherently, we found that protein expression of cytoskeleton components, such as Smoothelin and  $\alpha$ -SMA, was blunted or significantly decreased (by 50%–85% vs. donors,  $p < 0.001$ – $0.05$ , respectively), while no significant differences were observed in their mRNAs (Fig. 3B, C). On the opposite, both vimentin mRNA and its protein were downregulated (by 40%–70%,  $p < 0.05$ ) (Fig. 3D).

### Atrogin-1 and MuRF-1 are Upregulated in the Vascular Wall of Uremic Patients

Consistent with the previous evidence that showing in skeletal muscle an upregulation of atrophic signaling elements of the UPS<sup>29</sup>, we observed that Atrogin-1 and muscle ring finger protein-1 (MuRF-1) mRNAs significantly increased (2.5-fold Atrogin-1



**Fig. 2.** Aortic cellular components associated with Mstn by immunohistochemistry double staining in donors

(A) Staining with Mstn (brown) and  $\alpha$ -SMA (red). In merged image, Mstn colocalizes with  $\alpha$ -SMA. (B, C) Staining with Mstn (brown), CD 45 (red), and  $\alpha$ -SMA (red/brown). Merged images show that Mstn colocalized with CD45 and  $\alpha$ -SMA and the latter with CD45 (magnification: x 1000).

and 10-fold MuRF-1,  $p < 0.05$ ) (**Fig. 3E**). In addition, Atrogin-1 protein levels also rose up (+ 2.6-fold).

### CCL2/MCP-1 is Expressed in Arterial Wall of Donors and Recipients

Previously, we have shown that Mstn induces pro-inflammatory changes in VSMC cell line by increasing CCL2<sup>18</sup>, and it has been demonstrated that a chronic inflammation could be a possible trigger of vascular damage<sup>30</sup>. Then, we evaluated the expression of CCL2 in the vascular wall of recipients compared to donors. Interestingly, as depicted in **Fig. 4A–B**, we found out that CCL2 gene expression had the same trend as Mstn; indeed, CCL2 mRNA was overexpressed in recipients (threefold in donors,  $p < 0.05$ ), while protein expression has no difference.

### Calcification, Klotho Expression, and Collagen Accumulation

Both recipients and donors showed extensive positive calcification areas in the tunica media (**Fig. 5A**). Klotho mRNA was detectable in donor arteries (**Fig. 5B**), while in recipients Klotho mRNA was markedly downregulated (-88%,  $p < 0.001$ ). Klotho protein expression was present in 5/15 donors (33%) and was very weakly expressed in only 1/16

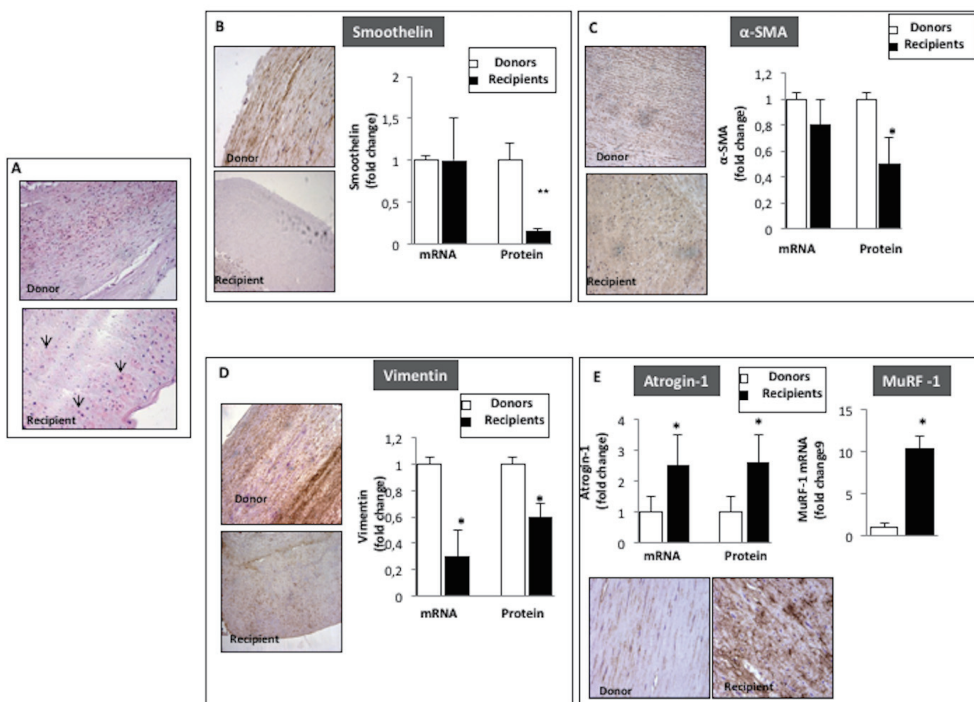
recipients (6%) (**Fig. 5B**). Collagen-1 gene expression was similar in recipients and donors; in addition, by Picosirius red staining, no differences in collagen deposition were observed (**Fig. 6A, B**).

### Myostatin Has Profibrotic Effects on A7r5 VSMC

Exposure of A7r5 rat VSMCs to Mstn induced a time-dependent increase of collagen-1 mRNA (by 12- to 7-folds vs. untreated cells,  $p < 0.05$ ) (**Fig. 7A**). Myostatin also induced TGF $\beta$  mRNA expression (**Fig. 7B**). Lastly, Mstn activated SMAD 2/SMAD 3 by phosphorylation, an effect that was observed after 45 minutes (+ 55%–60% vs. T0,  $p < 0.01$ ) and persisted during the whole time course (**Fig. 7C**).

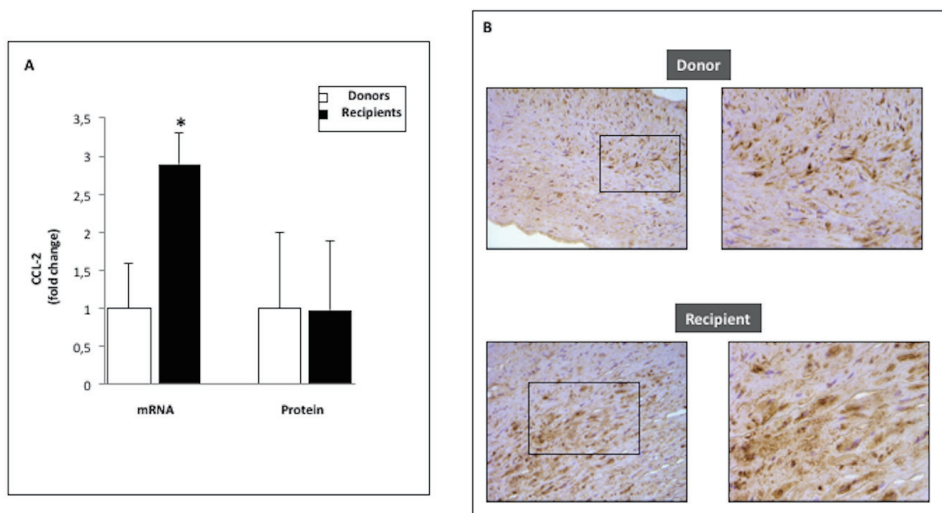
### Effect of US on Human VSMC

To explore the effects of potential uremic toxins retained in blood in uremia, human VSMCs were treated with 10% US for 24 hours. US induced a 60%–70% ( $p < 0.05$ – $0.01$ , respectively), increase in Mstn and Atrogin-1 mRNAs (**Fig. 8A, B**). Also, US exposure downregulated Smoothelin expression (-30%,  $p < 0.05$ ) (**Fig. 8C**) and heavily blunted Klotho mRNA at 24 hours ( $p < 0.05$ ) and 48 hours ( $p < 0.01$ ) (**Fig. 8D**).



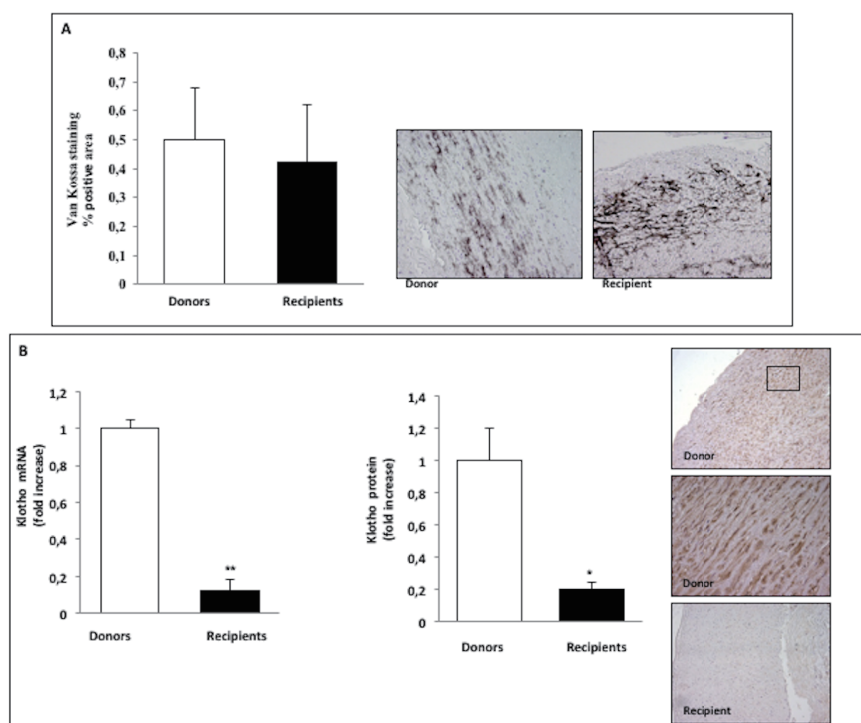
**Fig. 3.**

(A) Tissues were stained by hematoxylin and eosin staining. In recipient arteries, arrows indicate VSMC that lost elongated and spindle shape and showed a rounded phenotype (magnification: x 100). (B) Expression of Smoothelin mRNA and protein in human aorta of donors ( $N=16$ ) and the iliac artery of recipients ( $N=13$ ). Smoothelin mRNA expression level was determined by real-time PCR. Photos are representative of Smoothelin distribution in donor and recipient vasa. (C) Expression of  $\alpha$ -SMA mRNA and protein in human aorta of donors and the iliac artery of recipients. Note that recipient vasa are very scarcely positive for  $\alpha$ -SMA. (D) Expression of vimentin mRNA and protein. Both were significantly downregulated in recipient arteries. Pictures show a strong positivity in donor VSMC and a faintly immunostaining in recipients. (E) Atrogin-1 and MuRF-1 expression. mRNA levels were evaluated by real-time PCR and protein expression by immunohistochemistry and image analysis (magnification: x 100–400). Data are expressed as fold change  $\pm$  SEM to donor aortas. \* $p < 0.05$ , \*\* $p < 0.001$  vs. donors.

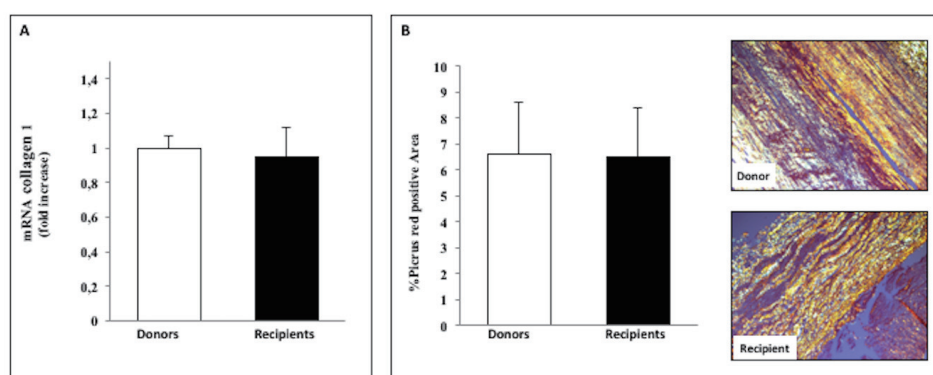


**Fig. 4.**

(A) Expression of CCL2 mRNA and protein in human aorta of donors ( $N=16$ ) and the iliac artery of recipients ( $N=13$ ). CCL2 mRNA expression level was determined by real-time PCR. CCL2 protein expression was evaluated by immunohistochemistry and image analysis. (B) Representative pictures of immunohistochemistry staining in donor and recipient arteries (magnification: x 200–400). Data are expressed as fold change  $\pm$  SEM to donor aortas. \* $p < 0.05$  vs. donors.

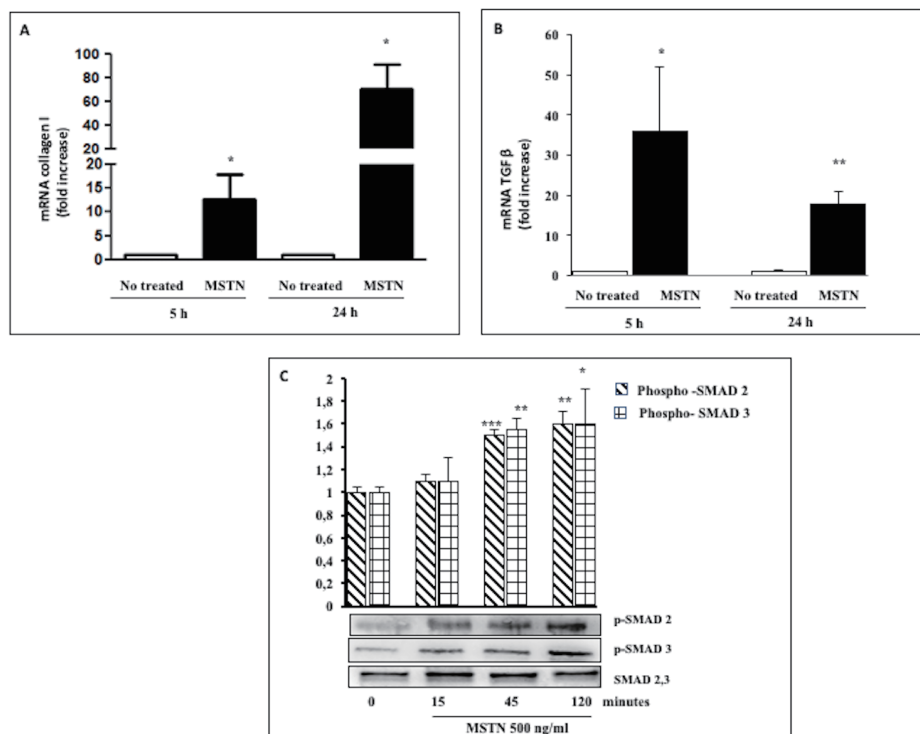
**Fig. 5.**

(A) Von Kossa staining on donor and recipient vasa. Positive areas, which appeared as thin granules or coarse deposits with focal or diffuse distribution, were evaluated by image analysis. Note that there are no differences in intensity staining. (B) Expression of Klotho mRNA and protein in human aorta of donors ( $N=16$ ) and the iliac artery of recipients ( $N=13$ ). Klotho mRNA and protein were strongly downregulated in recipients. Pictures show Klotho staining in donor media as also highlighted in box. mRNA level was evaluated by real-time PCR and protein expression by immunohistochemistry and image analysis (magnification:  $\times 100$ – $1000$ ). Data are expressed as fold change  $\pm$  SEM to donor aortas. \* $p < 0.01$ , \*\* $p < 0.001$  vs. donors.

**Fig. 6.**

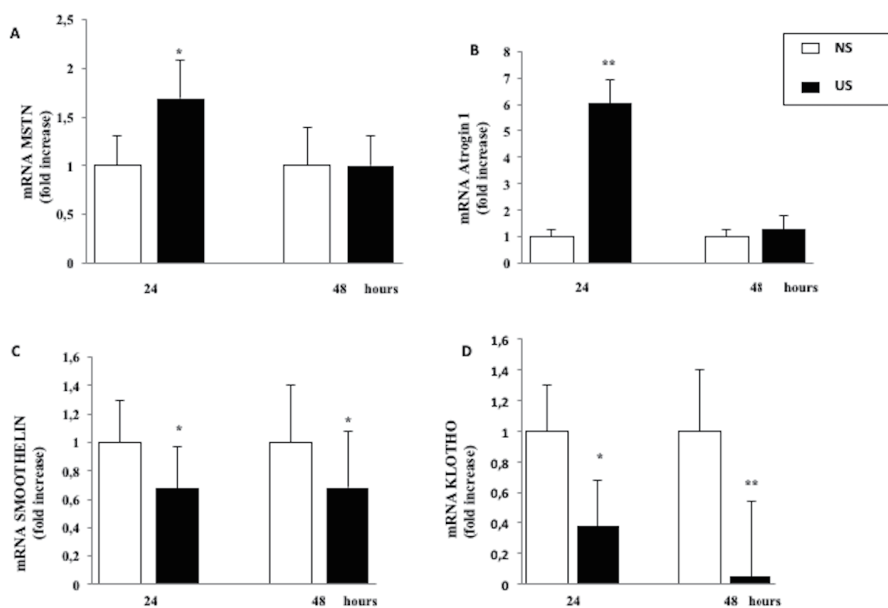
(A) Expression of collagen-1 mRNA in human aorta of donors ( $N=16$ ) and the iliac artery of recipients ( $N=13$ ). No differences were observed between recipient and donor tissues as indicated by Picrosirius red staining (B). Slides were examined through a polarizing microscope. The larger collagen fibers were bright yellow or orange. Positivity was assessed by image analysis. Data are expressed as fold change  $\pm$  SEM to donor aortas \* $p < 0,001$  (magnification:  $\times 100$ ).





**Fig. 7.**

Effects of Mstn on A7r5 VSMC mRNA levels for collagen-1 (A) and TGFβ (B) were analyzed by real-time RT-PCR after 5- and 24-hour treatment. All data were firstly normalized to GAPDH mRNA, and gene expression was calculated relative to corresponding levels in no treated cells. (C) Effects of Mstn on SMAD 2/SMAD 3 phosphorylation. Cells were exposed to Mstn for 15, 45, 60 minutes, and cell lysates were analyzed by Western blot. Membranes were stripped and reprobed for non-phosphorylated SMAD. All results represent mean±SEM obtained from three independent experiments. \* $p < 0,05$ , \*\* $p < 0,01$ , \*\*\* $p < 0,001$  vs. no treated cells.



**Fig. 8.**

The effect of NS and US on Mstn (A), Atrogin-1 (B), Smoothelin (C), and Klotho (D) mRNAs. Cells were incubated with 10% serum for 24–48 hours. mRNA expression was determined by real-time PCR. All results represent mean ± SEM obtained from three independent experiments. \* $p < 0,05$ , \*\* $p < 0,01$ , vs. NS treated cells.

## Discussion

Myostatin is upregulated by inflammation in skeletal muscle of patients with CKD and is a major contributor to the increase in protein degradation and muscle atrophy in this condition<sup>29</sup>). However, if and to what extent Mstn is upregulated in uremic vascular tissue and what could be its role in promoting vascular fibrosis in uremia are still unknown.

Therefore, we focused our study on these topics, allowing us to make three major observations.

First, we identified in the vascular wall of ESRD patients an overexpression of Mstn mRNA, accompanied by the upregulation of Atrogin-1 and MuRF-1, members of E3 ligases and major effectors of protein degradation; increased expression of CCL2/MCP-1, described as a mediator of vascular inflammation; and decreased expression of cytoskeleton proteins and Klotho. As a second new finding, we observed that Mstn induced SMAD 2/SMAD 3 phosphorylation and upregulated TGF $\beta$  and collagen-1 mRNA in cultured VSMCs, indicating that Mstn has profibrotic effects in VSMCs. Finally, we observed that many of the changes observed in the uremic arterial wall were reproduced by US in human VSMCs.

These data suggest that uremia might induce a complex pathway of molecular and structural changes in vascular wall, including upregulation of protein degradation and inflammation, while Mstn might have a role in uremic-related vascular remodeling.

As first step, we investigated the expression of Mstn in the uremic vessels, finding that, such as in atherosclerosis (an inflammatory setting), also in ESRD, Mstn gene expression overexpressed in the arterial tunica media, infiltrating cells, and neovasa, namely, in cells of both muscular and hematopoietic lineage, suggesting that inflammation may upregulate Mstn in different organ systems and settings<sup>18</sup>).

Interestingly, this hypothesis was supported by the evidence that, in the uremic vessels, when compared with donor vascular samples, there was a significant increase of CCL2 expression, which is a potent promoter of monocyte adhesion and consequent inflammation.

In addition, considering that in skeletal muscle of uremic patients inflammation and high IL-6 may activate p-Stat3 to stimulate a pathway from C/EBP $\delta$  to Mstn and increase activity of proteasome system, we also decided to study vascular expression of MAFbx/Atrogin-1 and MuRF-1<sup>31</sup>). These molecules are E3 major ubiquitin ligases that control the degradation of both skeletal and cardiac muscle by exerting inhibitory effects on Akt-dependent cardiac hypertrophy<sup>32-34</sup>).

Our results showed that, in uremic vessels, there was an upregulation of Atrogin-1 and MuRF-1 paralleling the upregulation of Mstn, thus confirming the potential role of UPS activation in the pathogenesis of vascular alterations in ESRD.

Remarkably, similar data were found in experimental studies on rabbit aortic smooth muscle cells, that, when exposed to US, presented increased expression of ubiquitin and ubiquitin-activating enzymes (E1) and an enhanced proteasome activity<sup>35</sup>).

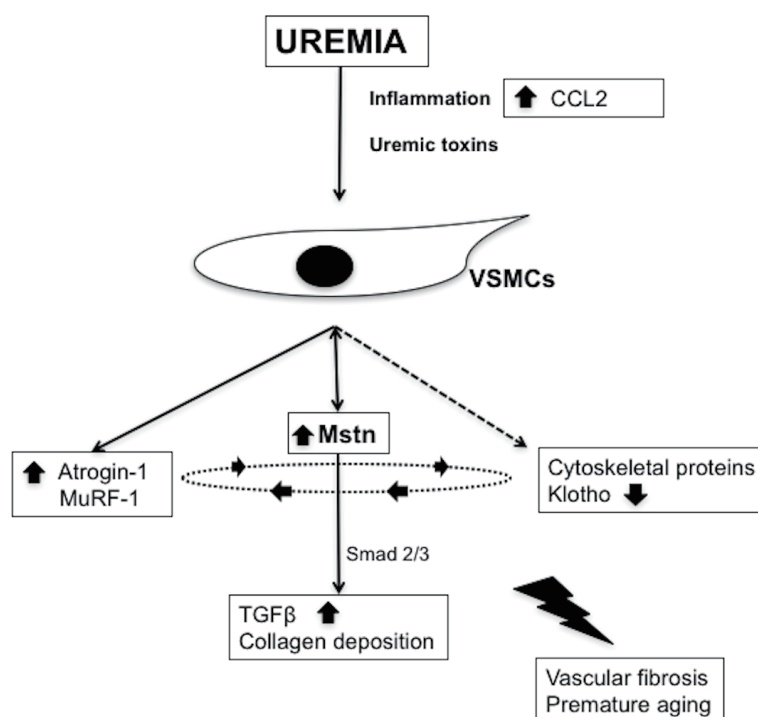
Due to the complexity of vascular biology in uremia, we also evaluated the expression of Klotho and VSMC-specific cytoskeletal proteins, such as  $\alpha$ -SMA, vimentin, and Smoothelin, a structural protein that promotes contractile activity and myogenic tone in VSMCs. Then, we observed a significant reduction of the protein expression of these molecules in vascular tissue of ESRD patients, a finding that might be considered a signature of the phenotype change occurring in uremia. Notably, it has to be underlined that for all the molecules we studied, we found a general discrepancy between gene and protein expression. Indeed, while Mstn and CCL2 gene expression upregulated in uremic transplant recipients, their protein expression did not increase.

Conversely, we found that in spite of a nonsignificant difference in gene expression, in recipient vessels, there was a significant lower protein expression of Smoothelin and  $\alpha$ -SMA, when compared with donor vessels. So, it seems that it is specifically the protein expression to be impaired in uremia, a result that could be explained, at least in part, by the activation of vascular protein degradation systems, as we observed in our patients.

Interestingly, such discrepancy between gene expression and protein content has already been reported in different experimental settings in uremic conditions<sup>36, 37</sup>). In these cases, the authors suggested that either diminished protein production or increased protein degradation (mediated by an enhanced ubiquitin-dependent activity) might concur to reduce the protein levels, while the increased gene expression could also be interpreted as compensatory.

However, the finding of a not increased Mstn tissue protein expression cannot rule out the potential role of Mstn in inducing vascular alterations in uremic patients, also considering that current evidence suggests that Mstn acts mainly at local level in paracrine or endocrine manner<sup>17, 38</sup>).

Among cytoskeleton proteins, the study of gene and protein expression of vimentin, which we found both downregulated, deserves a special attention. Indeed, vimentin, a component of intermediate filaments, is thought to reflect specific differentiation



**Fig. 9.** Putative role of Mstn in uremic vascular biology

In uremic condition, inflammation and accumulation of uremic toxins can stimulate Mstn and ubiquitin ligase expression and downregulate cytoskeletal proteins and Klotho. Moreover, *in vitro* data indicate that in VSMCs Mstn, *per se*, is a profibrotic trigger and could contribute to vascular remodeling. The possible direct interactions among Mstn, Atrogin-1, MuRF-1, and expression of cytoskeletal proteins and Klotho in uremic vessels are still unknown (dotted circle). Continuous and dotted lines represent positive or negative interactions, respectively.

pathways of VSMCs<sup>39</sup>).

In particular, in animal models, it has been described that vimentin deficiency induces oxidative stress and vascular inflammation, while in the uremic setting the decreased vimentin is associated with decreased Klotho expression, configuring a novel specific feature of uremic-related vascular alterations<sup>40, 41</sup>. Interestingly, we made a similar observation, finding that Klotho expression also markedly downregulated in the vascular wall of ESRD patients, when compared to non-uremic donors. These data are of particular interest, because while the association of Klotho deficiency with vascular calcifications and cardiovascular events has been proven, the actual expression of Klotho in vascular tissue is quite controversial and a matter of debate<sup>42, 43</sup>. So, although different studies showed that Klotho is present at low level in arterial wall of CKD patients, claims of total absence of Klotho expression in vasculature have also been reported<sup>44-46</sup>. Thus, our evidence confirms that uremia, at least in some cases, can affect vascular Klotho expression.

However, beyond studies on the whole vascular

tissue, we also performed *in vitro* studies on cell cultures, aiming to better characterize the single contribution of uremia and Mstn in inducing vascular changes. First, considering the autocrine/paracrine activity of Mstn, we exposed rat VSMC cells (A7r5) to Mstn showing a time-dependent activation of intracellular signaling with increased expression of profibrotic genes, such as TGF $\beta$  and collagen-1. Then, we added US to primary human VSMC cultures observing an increase in Mstn and Atrogin-1 production, with a significant decrease of Klotho and Smoothelin synthesis.

Overall, our data suggest that uremia increases Mstn gene expression and promotes vascular inflammation and UPS activation, inducing modifications of VSMC phenotype in the arterial wall, while the upregulated Mstn response might translate the metabolic alterations of uremia into profibrotic and stiffness inducing signals (Fig. 9).

Once the upregulation Mstn gene expression was proven, we wondered which sensing and signaling mechanisms are involved in Mstn activation in the uremic vascular wall. The hypothesis that alterations

in mineral metabolism, which increase the risk for vascular calcification, may act as a nidus for a local inflammatory response<sup>47)</sup> seems excluded by the evidence that Mstn vascular expression was not associated with serum phosphate, calcium, or PTH levels in patients studied here. Another possibility is that some circulating molecules are sensed by muscle as danger signal to mount an inflammatory response. A number of uremic retention solutes, such as guanidine compounds, exert pro- and anti-inflammatory effects on monocyte/macrophage function<sup>48)</sup>. In addition, Zhang *et al.* observed that the upregulation of Mstn in skeletal muscle of patients with CKD is dependent on the pro-inflammatory IL-6/Jak/Stat3 pathway<sup>20)</sup>.

Coherently, our finding that CCL2 gene expression is upregulated in uremic vascular wall confirms that inflammation could have a prominent role in inducing Mstn expression and perpetuating vascular damage in uremic setting.

A limitation in this study is that the control group was made of age-matched, but not comorbidity-free, kidney donors who included hypertensive and active smoker subjects, with low albumin levels. So, it is conceivable that some difference in calcification or fibrosis could have been more evident if healthy subjects had been compared to ESRD patients studied here.

Moreover, because of technical and ethical issues, we compared vascular samples from different sources (aortic artery from donors vs. iliac artery from recipients). Therefore, we cannot rule out that this disparity has impacted on the results, mainly on the study of vascular calcification, even if clinical evidence suggests that there is no difference among the distinctive site of vascular calcification sites in predicting mortality in ESRD patients<sup>49)</sup>.

Anyway, the inclusion of a cohort of nonselected ESRD patients with large dialytic vintage and incident for kidney transplant is one of the strength of the current study, even though it is possible that our cohort may not be indicative of uremia *per se* but may rather express the combined events occurring in aging and renal disease. In this regard, it is interesting that both cell senescence and Mstn are upregulated in sarcopenia of aging, suggesting that the findings observed in patients studied herein may represent an acceleration of processes “naturally” leading to sarcopenia in elderly subjects.

Finally, enrollment of a small number of patients did not allow a complete analysis of the correlations among vascular alterations, Mstn expression, and patient clinical characteristics.

Nevertheless, despite these weaknesses, our results can help to understand the Mstn as a new

player involved in vascular aging and arterial stiffness in CKD patients.

## Conclusions

In conclusion, we think that the characterization of Mstn in the vascular wall of uremic patients and the study of its potential relationship with cellular and tissue alterations present in this specific setting not only can contribute to improve our knowledge on the pathogenesis of vascular diseases in ESRD but also might also provide new therapeutic targets. This is particularly true for Mstn, that is currently object of many researches, studying the potentiality of its pharmacological inhibition on promoting increased muscle mass and improving metabolic profile and frailty in different disease conditions<sup>50-52)</sup>.

## Conflict of Interest Statement

None declared.

## Financial Disclosures

Nothing to declare.

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