

## Original Paper

# Up-Regulation of Hepatic Alpha-2-HS-Glycoprotein Transcription by Testosterone via Androgen Receptor Activation

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## Key Words

Fetuin-A • Alpha-2-HS-glycoprotein • Testosterone • Androgen receptor • ADT • AREs

## Abstract

**Background/Aims:** Fetuin-A (alpha-2-HS-glycoprotein, AHSG), a liver borne plasma protein, contributes to the prevention of soft tissue calcification, modulates inflammation, reduces insulin sensitivity and fosters weight gain following high fat diet or ageing. In polycystic ovary syndrome, fetuin-A levels correlate with free androgen levels, an observation pointing to androgen sensitivity of fetuin-A expression. The present study thus explored whether the expression of hepatic fetuin-A is modified by testosterone. **Methods:** HepG2 cells were treated with testosterone and androgen receptor antagonist flutamide, and were silenced with androgen receptor siRNA. To test the *in vivo* relevance, male mice were subjected to androgen deprivation therapy (ADT) for 7 weeks. *AHSG* mRNA levels were determined by quantitative RT-PCR and fetuin-A protein abundance by Western blotting. **Results:** In HepG2 cells, *AHSG* mRNA expression and fetuin-A protein abundance were both up-regulated following testosterone treatment. The human alpha-2-HS-glycoprotein gene harbors putative androgen receptor response elements in the proximal 5 kb promoter sequence relative to TSS. The effect of testosterone on *AHSG* mRNA levels was abrogated by silencing of the androgen receptor in HepG2 cells. Moreover, treatment of HepG2 cells with the androgen receptor antagonist flutamide in presence of endogenous ligands in the medium significantly down-regulated *AHSG* mRNA expression and fetuin-A protein abundance. In addition, ADT of male mice was followed by a significant decrease of hepatic *Ahsg* mRNA expression and fetuin-A protein levels. **Conclusions:** Testosterone participates in the regulation of hepatic fetuin-A expression, an effect mediated, at least partially, by androgen receptor activation.

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## Introduction

Fetuin-A or alpha-2-HS-glycoprotein (AHSG), a protein produced in the liver and released into plasma [1], mediates the formation of calciprotein particles thus preventing tissue calcification [2, 3]. Along those lines gene-targeted mice lacking fetuin-A [4] develop severe tissue calcification following mineral and vitamin D-rich diet [5]. Fetuin-A deficiency further contributes to the pathogenesis of cardiovascular calcifications in dialysis patients [6-8]. Vascular calcification and subsequent cardiovascular events are decisive pathophysiological mechanisms in renal insufficiency limiting the life span of affected patients [9-11].

Fetuin-A further causes insulin resistance [12, 13]. Accordingly, insulin sensitivity is enhanced in mice lacking fetuin-A, which are protected against weight gain under high-fat diet [14] and ageing [15]. Along those lines, fetuin-A may contribute to type II diabetes [16]. Fetuin-A has further been shown to modify the inflammatory response [17-19] and to foster tumor growth [20].

Total testosterone levels are a marker for aortic stiffness in men [21]. Furthermore, in male chronic kidney disease patients, decreased testosterone levels correlate with endothelial dysfunction and enhanced risk of cardiovascular events [22]. Along those lines, testosterone deficiency is in hemodialysis patients associated with increased vascular stiffness and mortality [23]. Low fetuin-A levels are in dialysis patients similarly predictive of mortality [24]. Low serum fetuin-A levels are further associated with higher cardiovascular disease mortality in older adults without diabetes, but associated with lower cardiovascular disease mortality in older adults with diabetes [25].

In woman suffering from polycystic ovary syndrome, a positive correlation was observed between serum fetuin-A levels and free androgens, an observation possibly reflecting the participation of androgen receptors in the regulation of fetuin-A expression [26].

However, to the best of our knowledge nothing is known about an influence of androgens on fetuin-A expression. The present study thus explored, whether fetuin-A expression in hepatocytes is influenced by testosterone. Physiological effects of testosterone are mediated by activating the androgen receptor (AR) [27, 28]. After interacting with androgen, the activated AR is translocated into the nucleus where it binds to androgen receptor response elements (AREs) present in different target genes [29, 30]. AR recognizes and binds to 15-bp palindromic androgen response element (ARE) sequences which consists of two hexameric half-site arranged as inverted repeats with a 3-bp spacer (GGTACA-nnn-TGTTCT). Near-consensus ARE sequences have been identified in the transcriptional regulatory regions of androgen-responsive genes [29, 31-34]. The promoter of the alpha-2-HS-glycoprotein gene has thus been analyzed to possibly identify putative ARE's.

## Materials and Methods

### *Cell culture and silencing of HepG2 cells*

Human liver hepatocellular carcinoma (HepG2) cells [35] were routinely cultured in Dulbecco's Modified Eagle Medium DMEM GlutaMAX-I containing 1 g/l glucose (Gibco, Life Technologies GmbH, Germany), 10% FBS (Gibco, Life Technologies GmbH, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Life Technologies GmbH, Germany). The medium was changed to 10% charcoal stripped FBS medium (Gibco, Life Technologies GmbH, Germany) 24 hours prior to each experiment to reduce the effects of endogenous ligands, unless stated otherwise. The HepG2 cells were subsequently transfected with 10 nM validated androgen receptor AR siRNA (ID no. s1539, Ambion, Life Technologies GmbH, Germany), or with 10 nM negative control siRNA (ID no. 4390843, Ambion, Life Technologies GmbH, Germany) using siPORT amine transfection agent (Ambion, Life Technologies GmbH, Germany) according to the manufacturer's instructions. The cells were used 72 hours after transfection. The efficiency of silencing was verified by quantitative RT-PCR [36]. HepG2 cells were treated in charcoal stripped FBS medium for 24 hours with 100 nM testosterone (Sigma-Aldrich, Germany) dissolved in ethanol. HepG2 cells were treated in normal growing medium for 24 hours with 1 µM flutamide (Sigma-Aldrich, Germany) dissolved in ethanol. Equal amounts of vehicle were used as control.

### Animal experiments

All animal experiments were performed according to the guidelines for the care and use of research animals of the German Animal Protection Law. BALB/c-nude (CAnN.Cg-Foxn1<sup>nu</sup>/Crl) male mice (Charles River Laboratories, Germany) were randomly divided into control and androgen deprivation therapy (ADT) groups at the age of nine weeks through surgical castration. Briefly, a small incision was made on the sterile scrotum after the initiation of anesthesia (Fentanyl: 0.05 mg/kg BW (Actavis, Germany); Midazolam: 5 mg/kg BW (Ratiopharm, Germany); Medetomidine: 0.5 mg/kg BW (Pfizer, Germany)). In the ADT group, the testes were removed after the spermatic vessels were tied with sterile absorbable sutures. Afterwards, the wound was closed with sterile absorbable sutures. The control group was sham operated with similar procedure without testis excision. Seven weeks post ADT, the animals were fasted and sacrificed, and the livers were excised for evaluation of fetuin-A. Part of the mice were used within another more complex imaging study applying PET and MRI with [<sup>18</sup>F]FDG as PET tracer before they were used at the end point for fetuin-A quantification.

### Quantitative RT-PCR

Total RNA was isolated from HepG2 cells and from murine liver tissues using Trifast Reagent (Peqlab Biotechnologie GmbH, Germany) according to the manufacturer's instructions. Reverse transcription of 2 µg RNA was performed using oligo(dT)<sub>12-18</sub> primers (Invitrogen, Life Technologies GmbH, Germany) and SuperScriptIII Reverse Transcriptase (Invitrogen, Life Technologies GmbH, Germany). Quantitative real-time PCR was performed with the iCycler iQ<sup>TM</sup> Real-Time PCR Detection System (Bio-Rad Laboratories GmbH, Germany) and iQ<sup>TM</sup> SybrGreen Supermix (Bio-Rad Laboratories GmbH, Germany) according to the manufacturer's instructions. The following human primers were used (5'-3' orientation) for quantitative RT-PCR measurements:

*AHSG* fw: TCCTTGGGGATACAAACACACC;

*AHSG* rev: TACCACGGAAAACCTGCCATC;

*AR* fw: GACGACCAGATGGCTGTCATT;

*AR* rev: GGGCGAAGTAGAGCATCCT;

*GAPDH* fw: GAGTCAACGGATTTGGTCGT;

*GAPDH* rev: GACAAGCTTCCCGTTCTCAG.

The following mouse primers were used (5'-3' orientation) for quantitative RT-PCR measurements:

*Ahsg* fw: AGGATCAGACACTTCAAATCTAGG;

*Ahsg* rev: GGTTGCAACTGTTTTATATGAGGTC;

*Gapdh* fw: AGGTCGGTGTGAACGGATTTG;

*Gapdh* rev: TGTAGACCATGTAGTTGAGGTCA.

The specificity of the PCR products was confirmed by analysis of the melting curves. All PCRs were performed in duplicate, and mRNA fold changes were calculated by the 2<sup>-ΔΔCt</sup> method using GAPDH as internal reference [37, 38].

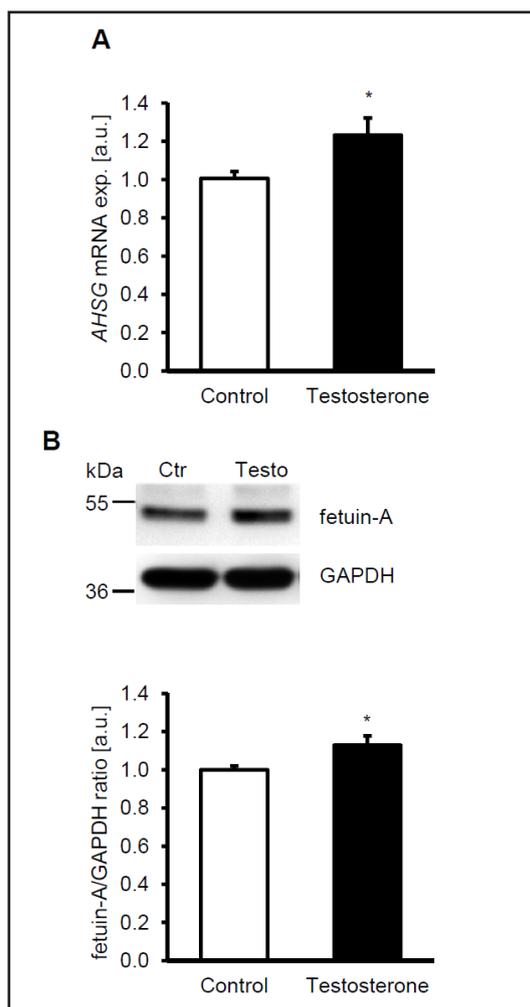
### Western blot analysis

HepG2 cells were washed with PBS and lysed with ice-cold RIPA lysis buffer (Cell Signaling, USA) supplemented with complete protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, USA). After centrifugation at 10000 rpm for 5 min, protein concentration was determined by Bradford assay (Biorad Laboratories GmbH, Germany). Proteins were boiled in Roti Load1 protein loading buffer (Carl Roth, Germany) at 100°C for 10 min, separated on SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were incubated overnight at 4°C with rabbit anti-fetuin-A antibody (1:1000, Cell Signaling, USA) or rabbit anti-GAPDH antibody (1:1000; Cell Signaling, USA) and then with secondary anti-rabbit HRP-conjugated antibody (1:1000; Cell Signaling, USA) for 1 hour at RT. For loading controls, the membranes were stripped in stripping buffer (Thermo Fisher Scientific, USA) at RT for 10 min. Antibody binding was detected with the ECL Western Blotting Substrate (Pierce, USA). Bands were quantified using Quantity One Software (Bio-Rad, Germany) and results are shown as the ratio of total protein to GAPDH normalized to the control treated group [39, 40].

### Statistics

Data are provided as means ± SEM, n represents the number of independent experiments. All data were tested by ANOVA followed by post hoc analysis (Tukey test), unpaired Student t-test (normally distributed

**Fig. 1.** Up-regulation of fetuin-A expression by testosterone in HepG2 cells. A. Arithmetic means  $\pm$  SEM (n = 9, arbitrary units) of alpha-2-HS-glycoprotein (AHSG) relative mRNA expression in HepG2 cells grown in charcoal stripped FBS medium and treated for 24 hours with vehicle alone (white bar) or with 100 nM testosterone (black bar). B. Representative original Western blots and arithmetic means  $\pm$  SEM (n = 7, arbitrary units) of normalized fetuin-A to GAPDH protein ratio in HepG2 cells grown in charcoal stripped FBS medium and treated for 24 hours with vehicle alone (white bar) or with 100 nM testosterone (black bar). \*(p<0.05) indicates statistically significant differences from HepG2 cells treated with vehicle alone.

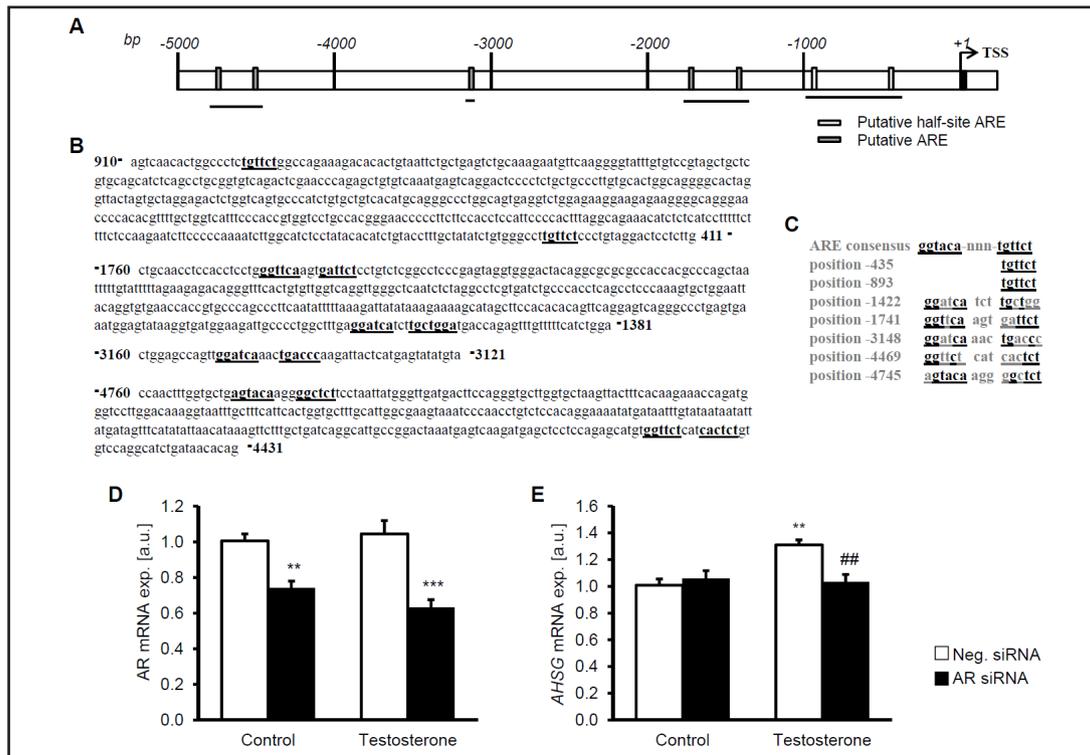


data) or Mann-Whitney test (non-normally distributed data) according to Shapiro-Wilk test. Only results with  $p < 0.05$  were considered statistically significant.

## Results

The present study explored the impact of testosterone on fetuin-A (encoded by the alpha-2-HS-glycoprotein gene) expression. To this end, human liver hepatocellular carcinoma (HepG2) cells have been treated for 24 hours with 100 nM testosterone. To avoid potential effects of endogenous ligands in the medium, the experiments were performed using charcoal-stripped FBS medium. As illustrated in Fig. 1A, testosterone treatment was followed by a statistically significant increase of alpha-2-HS-glycoprotein (AHSG) mRNA expression. Similar observations were made for fetuin-A protein abundance: in HepG2 cells, fetuin-A protein expression was significantly increased following testosterone treatment (Fig. 1B).

Further experiments addressed the mechanisms underlying testosterone sensitive alpha-2-HS-glycoprotein gene transcription. The physiological effects of testosterone are mediated by activating the androgen receptor (AR) [27, 28]. To determine whether alpha-2-HS-glycoprotein transcription is regulated by AR directly, we tried to identify potential androgen receptor response elements (AREs) in the alpha-2-HS-glycoprotein gene promoter. The nucleotide sequence subjected to analysis represents the proximal 5000 bp relative to the transcription start site (TSS) of the human alpha-2-HS-glycoprotein gene promoter. The results showed that there are two putative half-site AREs located in the proximal 1 kb upstream of the TSS

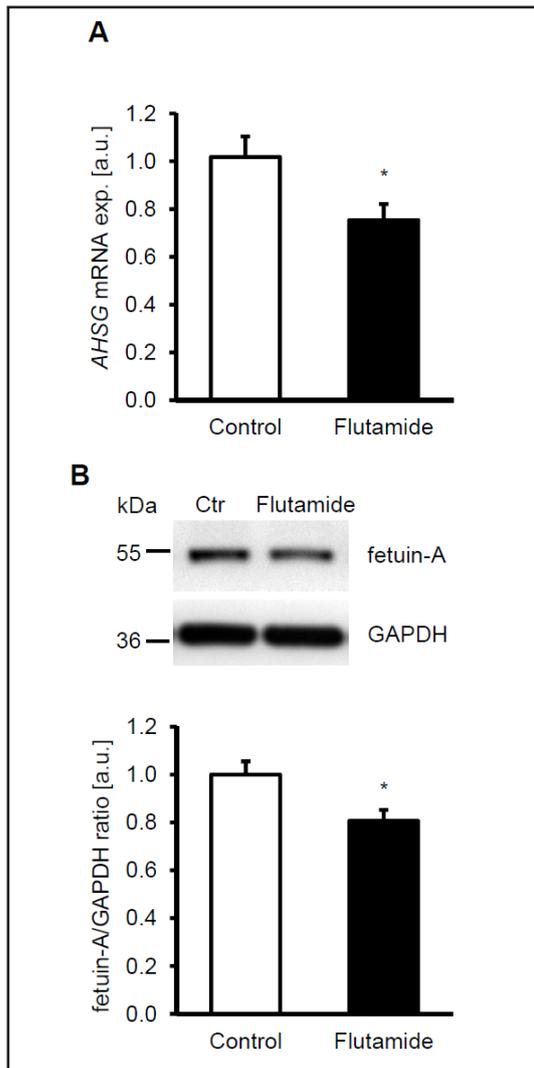


**Fig. 2.** Activation of the alpha-2-HS-glycoprotein gene promoter by testosterone in an androgen receptor-dependent manner. A. Graphic representation of the putative half-site androgen response elements (white bars) and complete androgen response elements (grey bars) in the proximal 5000 bp DNA sequence of the human alpha-2-HS-glycoprotein gene promoter relative to the transcription start site (TSS). B. DNA sequences of the human alpha-2-HS-glycoprotein gene promoter with highlighted putative androgen response elements (AREs). C. Alignment of the indicated potential AREs to the consensus sequence for androgen receptor AR binding site. Arithmetic means  $\pm$  SEM (n = 8, arbitrary units) of androgen receptor (AR, D) and alpha-2-HS-glycoprotein (AHSG, E) relative mRNA expression in HepG2 cells grown in charcoal stripped FBS medium following 72 hours silencing with 10 nM negative control siRNA (white bars) or with 10 nM androgen receptor siRNA (black bars) and 24 hours treatment with vehicle alone (Control, left columns) or with 100 nM testosterone (Testosterone, right columns). \*\*( $p < 0.01$ ), \*\*\*( $p < 0.001$ ) indicate statistically significant differences from HepG2 cells silenced with negative control siRNA and treated with vehicle alone; ##( $p < 0.01$ ) indicates statistically significant differences from HepG2 cells silenced with negative control siRNA and treated with testosterone.

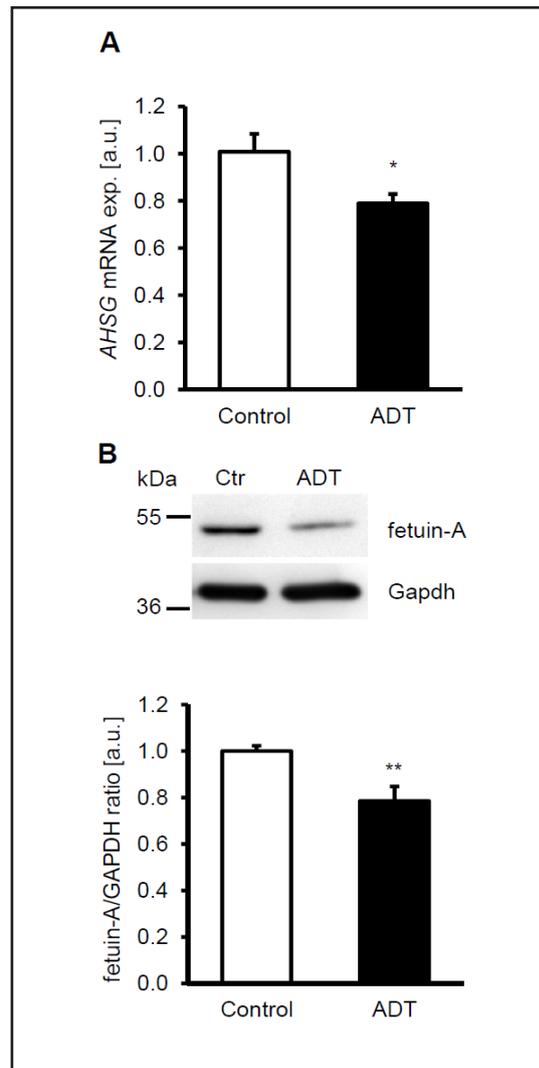
and, in addition, five putative imperfect AREs in the proximal 5 kb upstream of the TSS of alpha-2-HS-glycoprotein gene promoter (Fig. 2A,B). As illustrated in Fig. 2C, alignment of the putative AREs identified in the promoter of alpha-2-HS-glycoprotein gene showed sequence similarity to the consensus ARE. Functional incomplete AREs in the proximal promoter region of other genes have previously been reported [32-34].

To further define the impact of the androgen receptor on testosterone-induced alpha-2-HS-glycoprotein mRNA expression, RNA interference was used to suppress the endogenous androgen receptor (AR) gene in HepG2 cells. Silencing efficiency was verified by quantitative RT-PCR (Fig. 2D). As shown in Fig. 2E, AHSG mRNA expression was significantly increased following testosterone treatment in negative control siRNA silenced HepG2 cells, an effect significantly blunted in AR siRNA silenced HepG2 cells. Thus, testosterone up-regulates alpha-2-HS-glycoprotein mRNA expression in an androgen receptor-dependent manner.

In view of the role of androgen receptor in activation of the alpha-2-HS-glycoprotein gene promoter, further experiments were performed to explore the effects of the androgen receptor antagonist flutamide on fetuin-A expression. To this end, HepG2 cells were treated for 24



**Fig. 3.** Down-regulation of fetuin-A expression by the androgen receptor antagonist flutamide in HepG2 cells. A. Arithmetic means  $\pm$  SEM ( $n = 6$ , arbitrary units) of alpha-2-HS-glycoprotein (*AHSG*) relative mRNA levels in HepG2 cells grown in normal FBS medium and treated for 24 hours with vehicle alone (white bar) or with 1  $\mu$ M flutamide (black bar). B. Representative original Western blots and arithmetic means  $\pm$  SEM ( $n = 6$ , arbitrary units) of normalized fetuin-A to GAPDH protein ratio in HepG2 cells grown in normal FBS medium and treated for 24 hours with vehicle alone (white bar) or with 1  $\mu$ M flutamide (black bar). \*( $p < 0.05$ ) indicates statistically significant differences from HepG2 cells treated with vehicle alone.



**Fig. 4.** Decreased hepatic fetuin-A expression following androgen deprivation therapy in male mice. A. Arithmetic means  $\pm$  SEM ( $n = 4$ , arbitrary units) of alpha-2-HS-glycoprotein (*Ahsg*) relative mRNA expression in hepatic tissues from control treated mice (white bar) and following androgen deprivation therapy (ADT, black bar). B. Representative original Western blots and arithmetic means  $\pm$  SEM ( $n = 10$ , arbitrary units) of normalized fetuin-A to Gapdh protein ratio in hepatic tissues from control treated mice (white bar) and following androgen deprivation therapy (ADT, black bar). \*( $p < 0.05$ ), \*\*( $p < 0.01$ ) indicates statistically significant differences from control treated mice.

hours with 1  $\mu$ M flutamide in normal FBS medium, in the presence of endogenous ligands in the medium. As illustrated in Fig. 3A, flutamide treatment was followed by a significant decrease of *AHSG* mRNA expression in HepG2 cells. Furthermore, the decrease in *AHSG*

mRNA levels was paralleled by a significant decrease of fetuin-A protein expression in HepG2 cells (Fig. 3B).

Additional experiments were performed to elucidate whether testosterone sensitivity of fetuin-A expression is relevant *in vivo*. To this end, male mice were subjected to androgen deprivation therapy (ADT) for 7 weeks. As shown in Fig. 4A, alpha-2-HS-glycoprotein (*Ahsg*) mRNA expression was significantly decreased in hepatic tissue of male mice in response to ADT as compared to control treated male mice. Similarly, the hepatic fetuin-A protein abundance was significantly decreased following ADT in male mice (Fig. 4B).

## Discussion

The present study reveals a novel action of testosterone, i.e. the up-regulation of alpha-2-HS-glycoprotein gene transcription through activation of the androgen receptor. The proximal promoter of the human alpha-2-HS-glycoprotein gene harbors putative half-site and imperfect androgen receptor response elements (AREs) which presumably account for the testosterone sensitivity of alpha-2-HS-glycoprotein transcription. Functional half-site AREs [34] and near-consensus ARE sequences [29, 31-34] have been identified in the transcriptional regulatory regions of androgen-responsive genes. Our results suggest that alpha-2-HS-glycoprotein is a direct target gene of AR in HepG2 cells, as *AHSG* mRNA expression induced by testosterone is blunted by silencing of AR. Along those lines, treatment with the androgen receptor antagonist flutamide down-regulated alpha-2-HS-glycoprotein mRNA expression and fetuin-A protein levels. Of note, following culture in charcoal stripped FBS medium deprived of androgen receptor ligands, flutamide did not significantly modify fetuin-A expression (data not shown).

Up-regulation of fetuin-A may be particularly important in disorders associated with excessive vascular calcification, such as chronic kidney disease (CKD) [9-11, 41]. Vascular calcification and mortality of patients in end-stage renal disease are fostered by low levels of fetuin-A [42]. Along those lines low testosterone levels are associated with poor prognosis of end-stage renal disease [22, 43, 44]. In view of the present observations, it is tempting to speculate that decreased stimulation of fetuin-A expression contributes to the negative effect of testosterone deficiency in end-stage renal disease. Vascular calcification is further fostered by advanced age [3], which is associated with declining testosterone levels [45-49]. Even in the absence of end-stage renal disease, testosterone deficiency is associated with cardiovascular disease [45, 50]. Testosterone deficiency is associated with endothelial dysfunction, increased blood pressure, dyslipidemia, atherosclerosis and thrombosis [46]. To which extent reduced fetuin-A levels contribute to the risk of cardiovascular disease in the aging population remains, however, to be shown. Clearly, additional mechanisms contribute to cardiovascular disease in testosterone deficiency in the elderly [46]. Moreover, testosterone replacement therapy may increase the risk of cardiovascular disease [51]. Beyond that, fetuin-A is associated with metabolic syndrome, insulin resistance and enhanced risk of tumor growth [12, 52, 53]. In perivascular fat cells, fetuin-A fosters inflammatory responses [54].

The present observations reveal genomic regulation of fetuin-A by testosterone, an effect presumably mediated by the intracellular androgen receptor. Beyond that androgens activate the membrane androgen receptor (mAR) [55], which may, at least in theory, contribute to the regulation of fetuin-A expression and/or release. Future studies will be required to decipher the contribution of the intracellular and the membrane androgen receptor.

In conclusion, alpha-2-HS-glycoprotein is an androgen receptor-target gene and its transcription is stimulated by testosterone. Thus, testosterone sensitivity of alpha-2-HS-glycoprotein expression may contribute to the known impact of testosterone deficiency on cardiovascular disease.

## Abbreviations

ADT (androgen deprivation therapy ); *AHSG* ( alpha-2-HS-glycoprotein); AR (androgen receptor); AREs (androgen receptor response elements); CKD (chronic kidney disease); TSS (transcription start site).

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## Disclosure Statement

All authors disclose that they have no potential conflict of interest and that the results presented in this paper have not been published previously in whole or part, except in abstract format.

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