

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^{nu}/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 [¹⁸F]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)₁₂₋₁₈ primers (Invitrogen, Life Technologies GmbH, Germany) and SuperScriptIII Reverse Transcriptase (Invitrogen, Life Technologies GmbH, Germany). Quantitative real-time PCR was performed with the iCycler iQTM Real-Time PCR Detection System (Bio-Rad Laboratories GmbH, Germany) and iQTM 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: TACCACGGAAACTTGCCATC;

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: AGGATCAGACACTTCAAAATCTAGG;

Ahsg rev: GGTTGCAACTGTTTATATGAGGTC;

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^{-ΔΔCt} method using GAPDH as internal reference [37, 38].

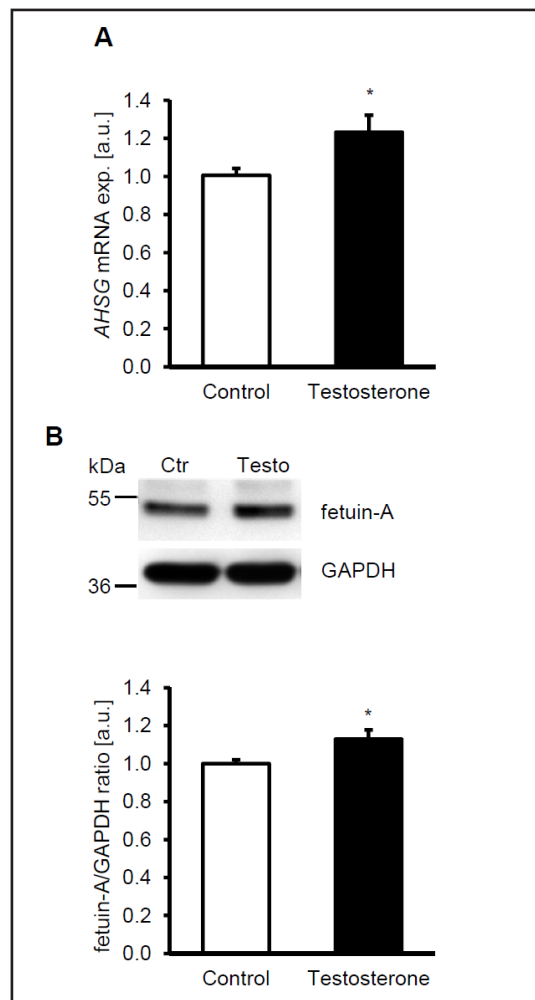
Western blot analysis

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

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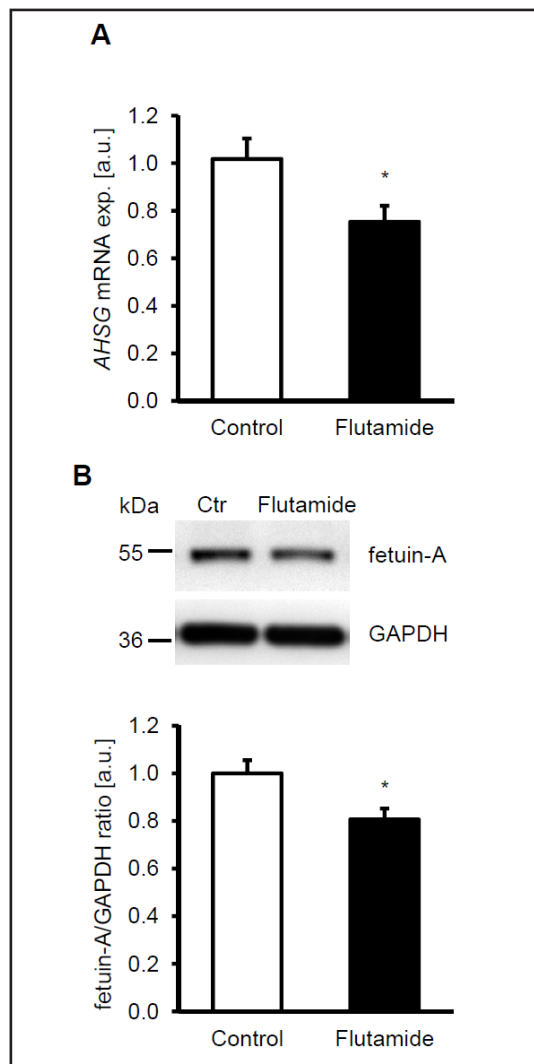


Fig. 3. Down-regulation of fetuin-A expression by the androgen receptor antagonist flutamide in HepG2 cells. A. Arithmetic means \pm SEM ($n = 6$, arbitrary units) of alpha-2-HS-glycoprotein (*AHSG*) relative mRNA levels in HepG2 cells grown in normal FBS medium and treated for 24 hours with vehicle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

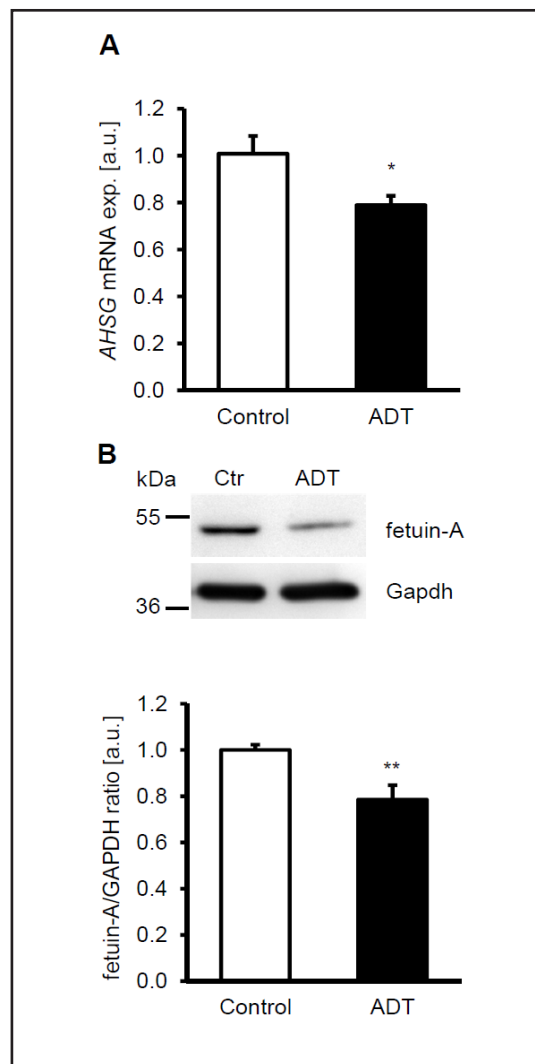


Fig. 4. Decreased hepatic fetuin-A expression following androgen deprivation therapy in male mice. A. Arithmetic means \pm SEM ($n = 4$, arbitrary units) of alpha-2-HS-glycoprotein (*Ahsg*) relative mRNA expression in hepatic tissues from control treated mice (white bar) and following androgen deprivation 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 μ 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.

References

- 1 Mori K, Emoto M, Inaba M: Fetuin-A and the cardiovascular system. *Adv Clin Chem* 2012;56:175-195.
- 2 Herrmann M, Kinkeldey A, Jahnen-Dechent W: Fetuin-A function in systemic mineral metabolism. *Trends Cardiovasc Med* 2012;22:197-201.
- 3 Kuro-o M: Klotho, phosphate and FGF-23 in ageing and disturbed mineral metabolism. *Nat Rev Nephrol* 2013;9:650-660.
- 4 Jahnen-Dechent W, Schinke T, Trindl A, Muller-Esterl W, Sablitzky F, Kaiser S, Blessing M: Cloning and targeted deletion of the mouse fetuin gene. *J Biol Chem* 1997;272:31496-31503.
- 5 Schafer C, Heiss A, Schwarz A, Westenfeld R, Ketteler M, Floege J, Muller-Esterl W, Schinke T, Jahnen-Dechent W: The serum protein alpha 2-Heremans-Schmid glycoprotein/fetuin-A is a systemically acting inhibitor of ectopic calcification. *J Clin Invest* 2003;112:357-366.
- 6 Ketteler M, Vermeer C, Wanner C, Westenfeld R, Jahnen-Dechent W, Floege J: Novel insights into uremic vascular calcification: role of matrix Gla protein and alpha-2-Heremans Schmid glycoprotein/fetuin. *Blood Purif* 2002;20:473-476.
- 7 Moe SM, Chen NX: Pathophysiology of vascular calcification in chronic kidney disease. *Circ Res* 2004;95:560-567.
- 8 Westenfeld R, Schafer C, Kruger T, Haarmann C, Schurgers LJ, Reutelingsperger C, Ivanovski O, Drueke T, Massy ZA, Ketteler M, Floege J, Jahnen-Dechent W: Fetuin-A protects against atherosclerotic calcification in CKD. *J Am Soc Nephrol* 2009;20:1264-1274.
- 9 Shroff R, Long DA, Shanahan C: Mechanistic insights into vascular calcification in CKD. *J Am Soc Nephrol* 2013;24:179-189.
- 10 Shroff RC, McNair R, Figg N, Skepper JN, Schurgers L, Gupta A, Hiorns M, Donald AE, Deanfield J, Rees L, Shanahan CM: Dialysis accelerates medial vascular calcification in part by triggering smooth muscle cell apoptosis. *Circulation* 2008;118:1748-1757.
- 11 Staude H, Jeske S, Schmitz K, Warncke G, Fischer DC: Cardiovascular Risk and Mineral Bone Disorder in Patients with Chronic Kidney Disease. *Kidney Blood Press Res* 2013;37:68-83.
- 12 Pal D, Dasgupta S, Kundu R, Maitra S, Das G, Mukhopadhyay S, Ray S, Majumdar SS, Bhattacharya S: Fetuin-A acts as an endogenous ligand of TLR4 to promote lipid-induced insulin resistance. *Nat Med* 2012;18:1279-1285.
- 13 Singh M, Sharma PK, Garg VK, Mondal SC, Singh AK, Kumar N: Role of fetuin-A in atherosclerosis associated with diabetic patients. *J Pharm Pharmacol* 2012;64:1703-1708.

- 14 Mathews ST, Singh GP, Ranalletta M, Cintron VJ, Qiang X, Goustin AS, Jen KL, Charron MJ, Jahnen-Dechent W, Grunberger G: Improved insulin sensitivity and resistance to weight gain in mice null for the Ahsg gene. *Diabetes* 2002;51:2450-2458.
- 15 Mathews ST, Rakhade S, Zhou X, Parker GC, Coscina DV, Grunberger G: Fetuin-null mice are protected against obesity and insulin resistance associated with aging. *Biochem Biophys Res Commun* 2006;350:437-443.
- 16 Rasul S, Wagner L, Kautzky-Willer A: Fetuin-A and angiopoietins in obesity and type 2 diabetes mellitus. *Endocrine* 2012;42:496-505.
- 17 Li W, Zhu S, Li J, Huang Y, Zhou R, Fan X, Yang H, Gong X, Eissa NT, Jahnen-Dechent W, Wang P, Tracey KJ, Sama AE, Wang H: A hepatic protein, fetuin-A, occupies a protective role in lethal systemic inflammation. *PLoS One* 2011;6:e16945.
- 18 Wang H, Li W, Zhu S, Li J, D'Amore J, Ward MF, Yang H, Wu R, Jahnen-Dechent W, Tracey KJ, Wang P, Sama AE: Peripheral administration of fetuin-A attenuates early cerebral ischemic injury in rats. *J Cereb Blood Flow Metab* 2010;30:493-504.
- 19 Hennige AM, Staiger H, Wicke C, Machicao F, Fritsche A, Haring HU, Stefan N: Fetuin-A induces cytokine expression and suppresses adiponectin production. *PLoS One* 2008;3:e1765.
- 20 Guillory B, Sakwe AM, Saria M, Thompson P, Adhiambo C, Koumangoye R, Ballard B, Binhabazim A, Cone C, Jahnen-Dechent W, Ochieng J: Lack of fetuin-A (alpha2-HS-glycoprotein) reduces mammary tumor incidence and prolongs tumor latency via the transforming growth factor-beta signaling pathway in a mouse model of breast cancer. *Am J Pathol* 2010;177:2635-2644.
- 21 Vlachopoulos C, Ioakeimidis N, Miner M, Aggelis A, Pietri P, Terentes-Printzios D, Tsekoura D, Stefanadis C: Testosterone deficiency: A determinant of aortic stiffness in men. *Atherosclerosis* 2014;233:278-283.
- 22 Yilmaz MI, Sonmez A, Qureshi AR, Saglam M, Stenvinkel P, Yaman H, Eyileten T, Caglar K, Oguz Y, Taslipinar A, Vural A, Gok M, Unal HU, Yenicesu M, Carrero JJ: Endogenous testosterone, endothelial dysfunction, and cardiovascular events in men with nondialysis chronic kidney disease. *Clin J Am Soc Nephrol* 2011;6:1617-1625.
- 23 Kyriazis J, Tzanakis I, Stylianou K, Katsipi I, Moisiadis D, Papadaki A, Mavroeidi V, Kagia S, Karkavitsas N, Daphnis E: Low serum testosterone, arterial stiffness and mortality in male haemodialysis patients. *Nephrol Dial Transplant* 2011;26:2971-2977.
- 24 Hermans MM, Brandenburg V, Ketteler M, Kooman JP, van der Sande FM, Boeschoten EW, Leunissen KM, Krediet RT, Dekker FW, Netherlands cooperative study on the adequacy of D: Association of serum fetuin-A levels with mortality in dialysis patients. *Kidney Int* 2007;72:202-207.
- 25 Laughlin GA, Cummins KM, Wassel CL, Daniels LB, Ix JH: The association of fetuin-A with cardiovascular disease mortality in older community-dwelling adults: the Rancho Bernardo study. *J Am Coll Cardiol* 2012;59:1688-1696.
- 26 Enli Y, Fenkci SM, Fenkci V, Oztekin O: Serum Fetuin-A levels, insulin resistance and oxidative stress in women with polycystic ovary syndrome. *Gynecol Endocrinol* 2013;29:1036-1039.
- 27 Beato M: Gene regulation by steroid hormones. *Cell* 1989;56:335-344.
- 28 Quigley CA, De Bellis A, Marschke KB, el-Awady MK, Wilson EM, French FS: Androgen receptor defects: historical, clinical, and molecular perspectives. *Endocr Rev* 1995;16:271-321.
- 29 Lee CM, Yen CH, Tzeng TY, Huang YZ, Chou KH, Chang TJ, Arthur Chen YM: Androgen response element of the glycine N-methyltransferase gene is located in the coding region of its first exon. *Biosci Rep* 2013;33:
- 30 Verrijdt G, Haelens A, Claessens F: Selective DNA recognition by the androgen receptor as a mechanism for hormone-specific regulation of gene expression. *Mol Genet Metab* 2003;78:175-185.
- 31 Horie-Inoue K, Bono H, Okazaki Y, Inoue S: Identification and functional analysis of consensus androgen response elements in human prostate cancer cells. *Biochem Biophys Res Commun* 2004;325:1312-1317.
- 32 Read JT, Rahmani M, Boroomand S, Allahveridian S, McManus BM, Rennie PS: Androgen receptor regulation of the versican gene through an androgen response element in the proximal promoter. *J Biol Chem* 2007;282:31954-31963.
- 33 Ikeda H, Serria MS, Kakizaki I, Hatayama I, Satoh K, Tsuchida S, Muramatsu M, Nishi S, Sakai M: Activation of mouse Pi-class glutathione S-transferase gene by Nrf2(NF-E2-related factor 2) and androgen. *Biochem J* 2002;364:563-570.
- 34 Shaffer PL, Jivan A, Dollins DE, Claessens F, Gewirth DT: Structural basis of androgen receptor binding to selective androgen response elements. *Proc Natl Acad Sci USA* 2004;101:4758-4763.

- 35 Wolff M, Jelkmann W, Dunst J, Depping R: The Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT/HIF-1 beta) is Influenced by Hypoxia and Hypoxia-Mimetics. *Cell Physiol Biochem* 2013;32:849-858.
- 36 Alesutan I, Seifert J, Pakladok T, Rheinlaender J, Lebedeva A, Towhid ST, Stournaras C, Voelkl J, Schaffer TE, Lang F: Chorea Sensitivity of Actin Polymerization, Cell Shape and Mechanical Stiffness of Vascular Endothelial Cells. *Cell Physiol Biochem* 2013;32:728-742.
- 37 Alesutan I, Feger M, Pakladok T, Mia S, Ahmed MSE, Voelkl J, Lang F: 25-Hydroxyvitamin D-3 1-Alpha-Hydroxylase-Dependent Stimulation of Renal Klotho Expression by Spironolactone. *Kidney Blood Press Res* 2013;37:475-487.
- 38 Feger M, Fajol A, Lebedeva A, Meissner A, Michael D, Voelkl J, Alesutan I, Schleicher E, Reichetzeder C, Hoher B, Qadri SM, Lang F: Effect of Carbon Monoxide Donor CORM-2 on Vitamin D-3 Metabolism. *Kidney Blood Press Res* 2013;37:496-505.
- 39 Pakladok T, Almilaji A, Munoz C, Alesutan I, Lang F: PIKfyve Sensitivity of hERG Channels. *Cell Physiol Biochem* 2013;31:785-794.
- 40 Voelkl J, Pasham V, Ahmed MSE, Walker B, Szteyn K, Kuhl D, Metzler B, Alesutan I, Lang F: Sgk1-Dependent Stimulation of Cardiac Na⁺/H⁺ Exchanger Nhe1 by Dexamethasone. *Cell Physiol Biochem* 2013;32:25-38.
- 41 Hu MC, Shi M, Zhang J, Quinones H, Griffith C, Kuro-o M, Moe OW: Klotho deficiency causes vascular calcification in chronic kidney disease. *J Am Soc Nephrol* 2011;22:124-136.
- 42 Scialla JJ, Kao WH, Crainiceanu C, Sozio SM, Oberai PC, Shafi T, Coresh J, Powe NR, Plantinga LC, Jaar BG, Parekh RS: Biomarkers of Vascular Calcification and Mortality in Patients with ESRD. *Clin J Am Soc Nephrol* 2014;ahead of print.
- 43 Dousdampanis P, Trigka K, Fourtounas C, Bargman JM: Role of Testosterone in the Pathogenesis, Progression, Prognosis and Comorbidity of Men With Chronic Kidney Disease. *Ther Apher Dial* 2013;10.1111/1744-9987.12101
- 44 Haring R, Nauck M, Volzke H, Endlich K, Lendeckel U, Friedrich N, Dorr M, Rettig R, Kroemer HK, Wallaschofski H: Low serum testosterone is associated with increased mortality in men with stage 3 or greater nephropathy. *Am J Nephrol* 2011;33:209-217.
- 45 Jones TH, Saad F: The effects of testosterone on risk factors for, and the mediators of, the atherosclerotic process. *Atherosclerosis* 2009;207:318-327.
- 46 Ruige JB, Ouwens DM, Kaufman JM: Beneficial and adverse effects of testosterone on the cardiovascular system in men. *J Clin Endocrinol Metab* 2013;98:4300-4310.
- 47 Spitzer M, Huang G, Basaria S, Travison TG, Bhasin S: Risks and benefits of testosterone therapy in older men. *Nat Rev Endocrinol* 2013;9:414-424.
- 48 Yeap BB, Araujo AB, Wittert GA: Do low testosterone levels contribute to ill-health during male ageing? *Crit Rev Clin Lab Sci* 2012;49:168-182.
- 49 Zirkin BR, Tenover JL: Aging and Declining Testosterone: Past, Present, and Hopes for the Future. *J Andrology* 2012;33:1111-1118.
- 50 Tirabassi G, Gioia A, Giovannini L, Boscaro M, Corona G, Carpi A, Maggi M, Balercia G: Testosterone and cardiovascular risk. *Intern Emerg Med* 2013;8:S65-69.
- 51 McGill JJ, Shoskes DA, Sabanegh ES: Androgen deficiency in older men: indications, advantages, and pitfalls of testosterone replacement therapy. *Cleve Clin J Med* 2012;79:797-806.
- 52 Sakwe AM, Koumangoye R, Goodwin SJ, Ochieng J: Fetuin-A ({alpha}2HS-glycoprotein) is a major serum adhesive protein that mediates growth signaling in breast tumor cells. *J Biol Chem* 2010;285:41827-41835.
- 53 Xu Y, Xu M, Bi Y, Song A, Huang Y, Liu Y, Wu Y, Chen Y, Wang W, Li X, Ning G: Serum fetuin-A is correlated with metabolic syndrome in middle-aged and elderly Chinese. *Atherosclerosis* 2011;216:180-186.
- 54 Siegel-Axel DI, Ullrich S, Stefan N, Rittig K, Gerst F, Klingler C, Schmidt U, Schreiner B, Randrianarisoa E, Schaller HE, Stock UA, Weigert C, Konigsrainer A, Haring HU: Fetuin-A influences vascular cell growth and production of proinflammatory and angiogenic proteins by human perivascular fat cells. *Diabetologia* 2014;10.1007/s00125-014-3177-0
- 55 Papadopoulou N, Papakonstanti EA, Kallergi G, Alevizopoulos K, Stournaras C: Membrane androgen receptor activation in prostate and breast tumor cells: molecular signaling and clinical impact. *IUBMB Life* 2009;61:56-61.