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Original Article

Farnesoid X receptor (FXR) inhibits coagulation process via inducing hepatic antithrombin III expression in mice

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Abstract: Farnesoid X receptor (FXR) has been identified as an inhibitor of platelet function and an inducer of fibrinogen protein complex. However, the regulatory mechanism of FXR in hemostatic system remains incompletely understood. In this study, we aimed to investigate the functions of FXR in regulating antithrombin III (AT III). C57BL/6 mice and FXR knockout (FXR KO) mice were treated with or without GW4064 (30 mg/kg per day). FXR activation significantly prolonged prothrombin time (PT) and activated partial thromboplastin time (APTT), lowered activity of activated factor X (FXa) and concentrations of thrombin-antithrombin complex (TAT) and activated factor II (FIIa), and increased level of AT III, whereas all of these effects were markedly reversed in FXR KO mice. *In vivo*, hepatic AT III mRNA and protein expression levels were up-regulated in wild-type mice after FXR activation, but down-regulated in FXR KO mice. *In vitro* study showed that FXR activation induced, while FXR knockdown inhibited, AT III expression in mouse primary hepatocytes. The luciferase assay and ChIP assay revealed that FXR can bind to the promoter region of AT III gene where FXR activation increased AT III transcription. These results suggest FXR activation inhibits coagulation process via inducing hepatic AT III expression in mice. The present study reveals a new role of FXR in hemostatic homeostasis and indicates that FXR might act as a potential therapeutic target for diseases related to hypercoagulation.

Key words: farnesoid X receptor (FXR); antithrombin III (AT III); anticoagulation; GW4064

法尼醇X受体(FXR)通过诱导肝脏抗凝血酶III的表达抑制小鼠凝血过程

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摘 要：法尼醇X受体(farnesoid X receptor, FXR)已被发现可在凝血系统中发挥重要作用，包括抑制血小板功能、促进纤维蛋白原表达等。然而至今，FXR在凝血系统中的调节机制尚未完全阐明。本研究旨在探讨FXR对抗凝血酶III (antithrombin III, AT III)的调节作用。用FXR特异性激动剂GW4064 (每天30 mg/kg)处理野生型(WT)和FXR基因敲除(FXR KO) C57BL/6小鼠1和3 天，结果显示，在WT小鼠上，FXR激动可显著延长凝血酶原时间和活化部分凝血活酶时间，降低活化凝血因子X (activated factor X, FXa)活性，降低活化凝血因子II (activated factor II, FIIa)和凝血酶-抗凝血酶复合物(thrombin-antithrombin complex,

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TAT)浓度，升高血浆AT III浓度，使血液处于低凝状态；当FXR敲除时，以上指标全部逆转，血液处于高凝状态。激动FXR后，WT小鼠肝脏AT III表达增加；而FXR KO小鼠肝脏中AT III表达较WT小鼠明显降低。体外研究结果显示，GW4064和FXR过表达腺病毒均可显著上调小鼠原代肝细胞中AT III的表达，相反，siRNA敲减FXR可明显抑制AT III表达。AT III启动子区含有FXR结合位点，GW4064可显著上调Luc-AT III荧光素酶活性并增加FXR与AT III启动子区的结合。以上结果提示，FXR可通过直接转录调控AT III的表达而抑制凝血过程。本研究揭示了FXR在凝血平衡中的新作用，提示FXR可能成为血液高凝状态相关疾病的潜在治疗靶点。

关键词：法尼醇X受体(FXR)；抗凝血酶III (AT III)；抗凝；GW4064
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As a process of clot formation, hemostasis is vital for all mammals. Unbalanced hemostasis causes thrombosis or hemorrhage, either of which may cause severe diseases such as stoke, acute myocardial infarction and cerebral hemorrhage, even death.

Hemostasis consists of four stages, creation of a platelet plug, propagation of clots by activation of various enzymes, clot formation by proper antithrombin III (AT III) control and removal of the clot by fibrinolysis [1]. In the first stage, Von Willibrand factor (VWF) is released by the injured endothelial cells and megakaryocytes which mediate platelet adhesion to damaged vascular surface and aggregation of platelets. There are two regulatory processes of propagation of clots, extrinsic and intrinsic pathways [2]. The extrinsic pathway is initiated by factor III (FIII) which is activated by exposure from vascular disruption or damage. Then the activated FIII binds to factor VII (FVII) and calcium, which converts factor X (FX) to activated FX. The intrinsic pathway starts from activation of factor XI (FXI) by factor XII (FXII), HMW kininogen, and prekallikrein. Activated factor XI (FXIa) then makes factor IX (FIX) activation. In conjunction with its cofactor factor VIII (FVIII), FXIa leads to the activation of FX [3]. Activated factor X (FXa), as a central hub of the coagulation cascade, converts prothrombin to thrombin in conjunction with calcium, tissue and platelet phospholipids. After that, anticoagulants control the balance of coagulation and anticoagulation delicately. Among them, antithrombin III (AT III) is a major physiological anticoagulant, which inhibits clot formation mainly by forming enzymatically inactive complexes with the active coagulation factors thrombin and FXa [4]. In the last stage of hemostasis, the organized clot is removed by fibrinolysis.

As a ligand-dependent transcriptional factor, farnesoid X receptor (FXR, NR1H4) belongs to the nuclear receptor superfamily. It forms a heterodimer with retinoid X receptor and binds to the FXR response element (FXRE) located in the promoter regions of FXR target genes. Bile acids are the endogenous ligands of FXR, so it is also known as bile acid receptor (BAR) [5, 6]. FXR is a multi-functional transcriptional factor which was reported to work as an anticoagulant recently. In the first stage of hemostasis, activation of FXR by its selective ligands GW4064 and 6-ECDCA inhibits platelet aggregation and secretion [7]. In the last stage of hemostasis, fibrinogen-α, -β, -γ were all induced by FXR, indicating that FXR modulates fibrinolytic activity [8]. It has been recently reported that FXR may influence AT III expression in cultured hepatocytes [9]. We therefore hypothesized that FXR may be involved in the regulation of coagulation process through regulating AT III expression and activity. In this study, we utilized both *in vivo* and *in vitro* models to determine the role of FXR in hepatic AT III expression. We provide evidence that FXR may be a potential target for the prevention and treatment of thrombotic disorders.

1 MATERIALS AND METHODS

1.1 *Animals*

All the animal experiments in this study were reviewed and approved by the Ethical Committee of Dalian Medical University, and followed the Guide for the Care and Use of Laboratory Animals (https://www.nap.edu/read/12910/chapter/1). FXR knockout (KO) mice (Fig. S1, see https://www.actaps.com.cn/supplement/193Sfig1.pdf), purchased from the Jackson Laboratory, were maintained on standard mouse chow and housed on a 12-hour light/black cycle under controlled temperature (22–24 °C) and humidity (50%–65%) in the animal facility of Dalian Medical University Laboratory Animal Center. Experiments were performed using male 4-month-old C57BL/6 wild-type and FXR KO littermates.

1.2 *Animal treatment*

Mice were intraperitoneally treated with GW4064 (Sigma-

Aldrich, St Louis, MO, USA, a selective agonist of FXR, 30 mg/kg per day, dissolved in DMSO). Fifteen male wild-type mice were divided into 3 groups randomly: one was treated with DMSO as vehicle for 3 days (n = 5), and the others were treated with GW4064 for 1 day or 3 days (n = 5 per group). Fifteen FXR KO mice were treated the same way.

1.3 *Coagulation parameters tests*

Citrated whole blood (800 μL) was drawn (blood: citrate = 9:1), and platelet poor plasma was prepared by centrifugation (3 000 r/min, 5 min). Activated partial thromboplastin time (APTT, s) and prothrombin time (PT, s) were assessed by automatic coagulation analyzer (CS-5100, Sysmex, Japan). The activity of FXa (ab204711, Abcam, USA), concentration of activated factor II (FIIa) (ab230933, Abcam, USA), thrombin-antithrombin complex (TAT) (ab137994, Abcam, USA), AT III (ab108800, Abcam, USA), Protein C (PROC) (ab137988, Abcam, USA) and Protein S (PROS) (ab190808, Abcam, USA) were detected by commercial ELISA kits.

1.4 *Primary hepatocytes culture and treatment*

Primary hepatocytes were cultured according to a two-step collagenase perfusion procedure to isolate hepatocytes as described by Wang *et al* [10]. Cells were treated with different concentrations and durations of GW4064. FXR was overexpressed by infection with an adenovirus carrying a cDNA encoding a full-length mouse FXR (FXR-ad) or GFP (CON-ad) (Hanbio Biotechnology Co., Ltd, Shanghai, China) for 24 h.

1.5 *RNA extraction and real-time quantitative PCR (qPCR)*

Mouse liver tissue (100 mg) or 2 × 10^6 cells were collected for RNA extraction by TRIzol (Invitrogen, USA), and cDNA was prepared with reverse transcriptase from Tian Gen Biotech (Beijing, China). qPCRs were performed as described previously [10]. In brief, qPCR was performed in 7300 Plus Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA, USA) with reagents obtained from TransGen Biotech (Beijing, China). β-actin was used as the internal control. The primers specific to mouse AT III, PROC, PROS and β-actin cDNAs were listed in Table S1 (see https://www.actaps.com.cn/supplement/193Stab1.pdf).

1.6 *Western blotting analysis*

Protein extracted from mouse liver or primary hepatocytes was measured by BCA assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Total protein (40 μg) was separated by 10% SDS-PAGE and transferred onto PVDF membranes. Antibodies against AT III (1∶1 000, ab126598, Abcam, USA) and β-actin (1∶1 000, AC026, Abclonal, Wuhan, China) were used for incubation at 4 °C overnight. After HRP-conjugated secondary antibodies (1∶5 000, ABclonal Technology, Woburn, MA, USA), the membranes were transferred to ECL. Images were captured on Tanon-5200 (Tanon, Shanghai, China) and the densitometry was performed with ImageJ software (NIH, Maryland, USA).

1.7 *Immunohistochemistry assay*

Sections cut from 10% formalin-fixed, paraffin-embedded liver samples were used for immunostaining. Sections were incubated with an anti-AT III antibody (ab126598, Abcam, USA) at dilution 1:100 at 4 °C overnight. The other steps followed the secondary antibody kit (PV-6000 ZSGB-Bio Beijing, China).

1.8 *siRNA transfection*

Mouse primary hepatocytes were transfected with FXR siRNA (siRNA-FXR) or scramble siRNA (siRNA-NC) (100 nmol/L) by Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). The siRNA oligonucleotides for FXR were designed and synthesized by Suzhou GenePharma Co., Ltd (Suzhou, China). The sequences were as follows: 1) siRNA-FXR: sense: 5′-GCC GUG UAC-AAG UGU AAG ATT-3′, antisense: 5′-UCU UAC-ACU UGU ACA CGG CTT-3′; 2) siRNA-NC: sense: 5′-UUC UCC GAA CGU GUC ACG UTT-3′, antisense: 5′-ACG UGA CAC GUU CGG AGA ATT-3′. Total RNA and protein were extracted 24 h after transfection.

1.9 *Luciferase reporter assay*

Mouse AT III gene promoter-driven luciferase reporter was constructed. Briefly, mouse AT III promoter region was amplified from the genomic DNA isolated from the liver of a C57BL/6 mouse. The mouse AT III promoter region containing the fragment −2 000 bp – +100 bp was amplified by PCR with the oligonucleotides 5′-TTT-CTC TAT CGA TAG CTT CAC AGA GAA ACC CTG-TCT CG-3′ (forward primer) and 5′-GCA GAT CTC-GAG CCC CTC TCC CCT TAA AGT CTT CAG G-3′ (reverse primer). The amplified fragment was cloned into the luciferase reporter gene vector PGL3.0 Basic (Promega, Madison, WI, USA), and the resultant construct designated as LUC-AT III was sequenced to validate the orientation and sequence. Mouse FXR expression plasmid (250 ng, OriGene Technologies, Beijing, China), 250 ng AT III gene promoter-driven

luciferase reporter construct (AuGCT Biotechnology Co., Ltd., Beijing, China) and 25 ng internal reference PRL-CMV plasmid (Promega, Madison, WI, USA) were transfected into HEK293T cells. After incubation for 6 h, 1 μmol/L GW4064 or DMSO were added into medium. Following another 3-hour incubation, the cells were harvested in the luciferase lysis buffer for the detection of luciferase activity, which was normalized with renilla luciferase activity.

1.10 *Chromatin immunoprecipitation (ChIP) assay*

ChIP assay was performed by using a ChIP-IT® Express Chromatin Immunoprecipitation Kit (Active Motif, Carlsbad, CA, USA). Mouse primary hepatocytes were treated with DMSO or GW4064 (1 μmol/L) for 3 h. Immunoprecipitation was performed with anti-FXR/NR1H4 antibody (R&D Systems, Minneapolis, MN, USA) and normal mouse IgG as a control at 4 °C overnight. Precipitated DNA was analyzed by PCR using the following primers: 5′-CAT TGG GAA GAA AGT-GGT TAG G-3′ (sense); 5′-GCT TCC AAC TCT TGT-TCA CC-3′ (antisense). Amplified product with 221 bp (−1 392 bp – −1 173 bp) was then examined by electrophoresis and sequenced.

1.11 *Statistical analysis*

Statistical analysis was performed using PRISM (GraphPad Software, USA). Results were presented as means ± standard error of the mean (SEM). Comparisons between two groups were performed by *t*-test, and when comparing more than 2 independent groups, one-way ANOVA was used. Statistical significance was set at a P value of < 0.05.

2 RESULTS

2.1 Effect of FXR in the anticoagulation process

To investigate the involvement of FXR in the anticoagulation process, we measured PT, APTT, concentration of TAT and FIIa and the activity of FXa in mice treated with the FXR activator GW4064 or deficient for FXR gene. The results showed that APTT was increased after GW4064 treatment for 1 d, and PT and APTT were both significantly prolonged following GW4064 treatment for 3 d (Fig. 1*A*&*B*). The activity of FXa and concentrations of FIIa were both down-regulated after GW4064 treatment for 1 and 3 d, and the concentration of TAT was decreased after GW4064 treatment for 3 d (Fig. 1*C*–*E*). All the alterations of these parameters important in the anticoagulation process were markedly reversed in FXR KO mice (Fig. 1*F*–*J*).

2.2 Effect of FXR on concentrations of anticoagulant factors

We also examined the concentrations of the three major anticoagulant factors, AT III, PROC and PROS. Among these factors, only the AT III levels were increased after

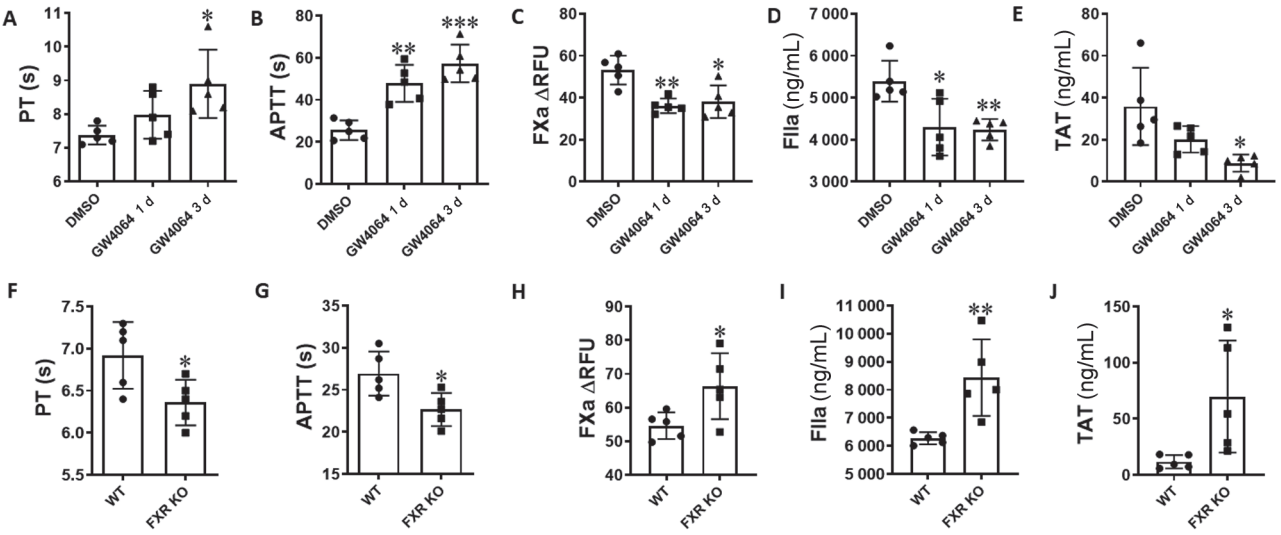

Fig. 1. Role of FXR in the anticoagulation process. Wild-type (WT) and FXR knockout (KO) mice on C57BL/6 background were treated with GW4064 for 1 or 3 d. The plasma was prepared, and the prothrombin time (PT) (*A*, *F*), activated partial thromboplastin time (APTT) (*B*, *G*), the activity of FXa (*C*, *H*), the concentration of FIIa (*D*, *I*), and the concentration of thrombin-antithrombin complex (TAT) (*E*, *J*) were measured. Data were presented as mean ± SEM, $n = 5$ in each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* DMSO (*A*–*E*) or WT (*F*–*J*).

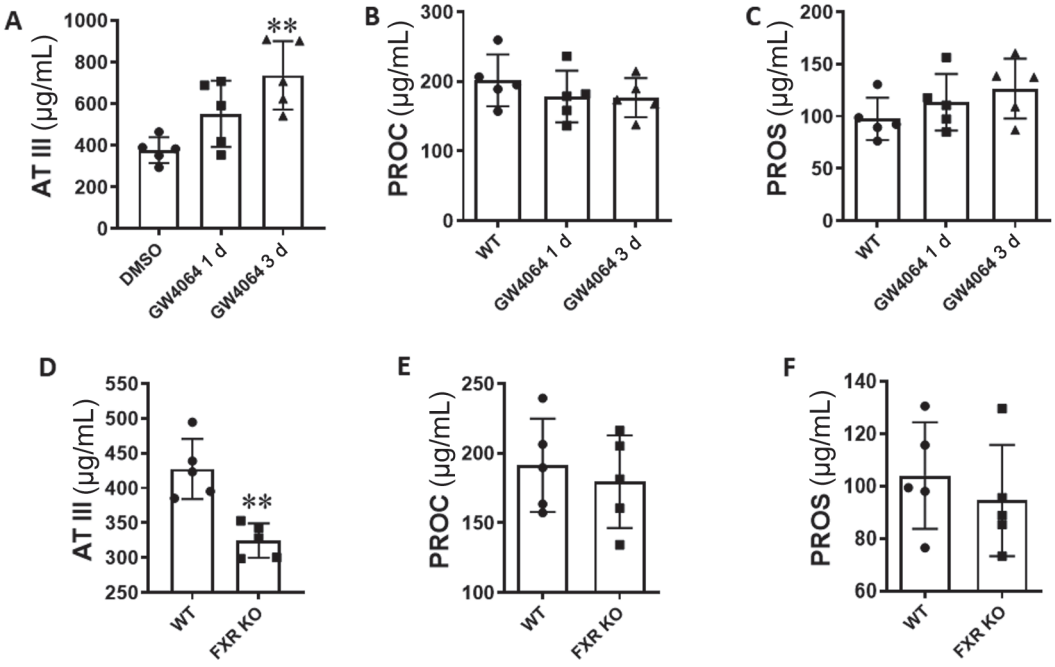

Fig. 2. Effect of FXR on major anticoagulant factors. WT C57BL/6 mice and FXR KO mice were treated with GW4064 for 1 or 3 d, and the major anticoagulant factors were examined in plasma. *A*, *D*: The concentration of AT III in plasma; *B*, *E*: The concentration of PROC in plasma; *C*, *F*: The concentration of PROS in plasma. Data were presented as mean ± SEM. $n = 5$ in each group. $^{**}P < 0.01$ *vs* DMSO (*A*–*C*) or WT (*D*–*F*).

GW4064 treatment (Fig. 2*A*). In contrast, the AT III levels were decreased in FXR KO mice compared to WT mice (Fig. 2*D*). Levels of PROC (Fig. 2*B*&*E*) and PROS (Fig. 2*C*&*F*) were unaltered after FXR activation and deficiency.

2.3 Induction of hepatic AT III in mice treated with GW4064

To further investigate the induction of AT III after FXR activation, the AT III expression was checked in mouse liver after GW4064 treatment. Hepatic AT III mRNA levels were slightly increased after GW4064 treatment for 1 d and significantly upregulated after GW4064 treatment for 3 d (Fig. 3*A*). In consistent, Western blot assay showed that GW4064 treatment also remarkably increased the AT III protein levels in mouse livers (Fig. 3*B*&*C*). The immunostaining results also revealed an up-regulated expression of AT III in mouse livers (Fig. 3*D*). In parallel, we found that in two published RNA-Sequencing Datasets, hepatic AT III expression was also induced by FXR activation (GSE70296) and was reduced in FXR deficiency (GSE54557) (Fig. S2, see https://www.actaps.com.cn/supplement/193Sfig2.pdf). Together, these findings suggest that treatment of mice with the FXR selective agonist GW4064 markedly increases hepatic AT III expression.

2.4 Down-regulation of hepatic AT III expression in mice deficient for FXR gene

We also determined the hepatic levels of AT III in FXR KO mice. The mRNA levels of AT III were significantly down-regulated in the livers of FXR KO mice (Fig. 3*E*). Consistently, the protein levels of AT III were decreased in the livers of FXR KO mice (Fig. 3*F*&*G*).

2.5 Induction of AT III expression in cultured hepatocytes with FXR activation and overexpression

The primary mouse hepatocytes were cultured and treated with GW4064 at multiple doses (0.5, 1, 2, 5 and 10 μmol/L) for 12 h or at 1 μmol/L for various time periods (1, 3, 6, 12 and 24 h). The results showed that activation of FXR by GW4064 significantly increased AT III expression at both mRNA (Fig. 4*A*&*D*) and protein levels (Fig. 4*B*, *C*, *E*&*F*) as compared with the vehicle (DMSO) control in dose- and time course-dependent manner. Moreover, an adenovirus carrying a cDNA encoding the full-length mouse FXR (FXR-ad) was used to overexpress FXR in cultured hepatocytes. Hepatocytes were infected with 10 MOI of FXR-ad or a control GFP adenovirus (CON-ad) for 24 h. Adenovirus-mediated FXR overexpression led to a significant

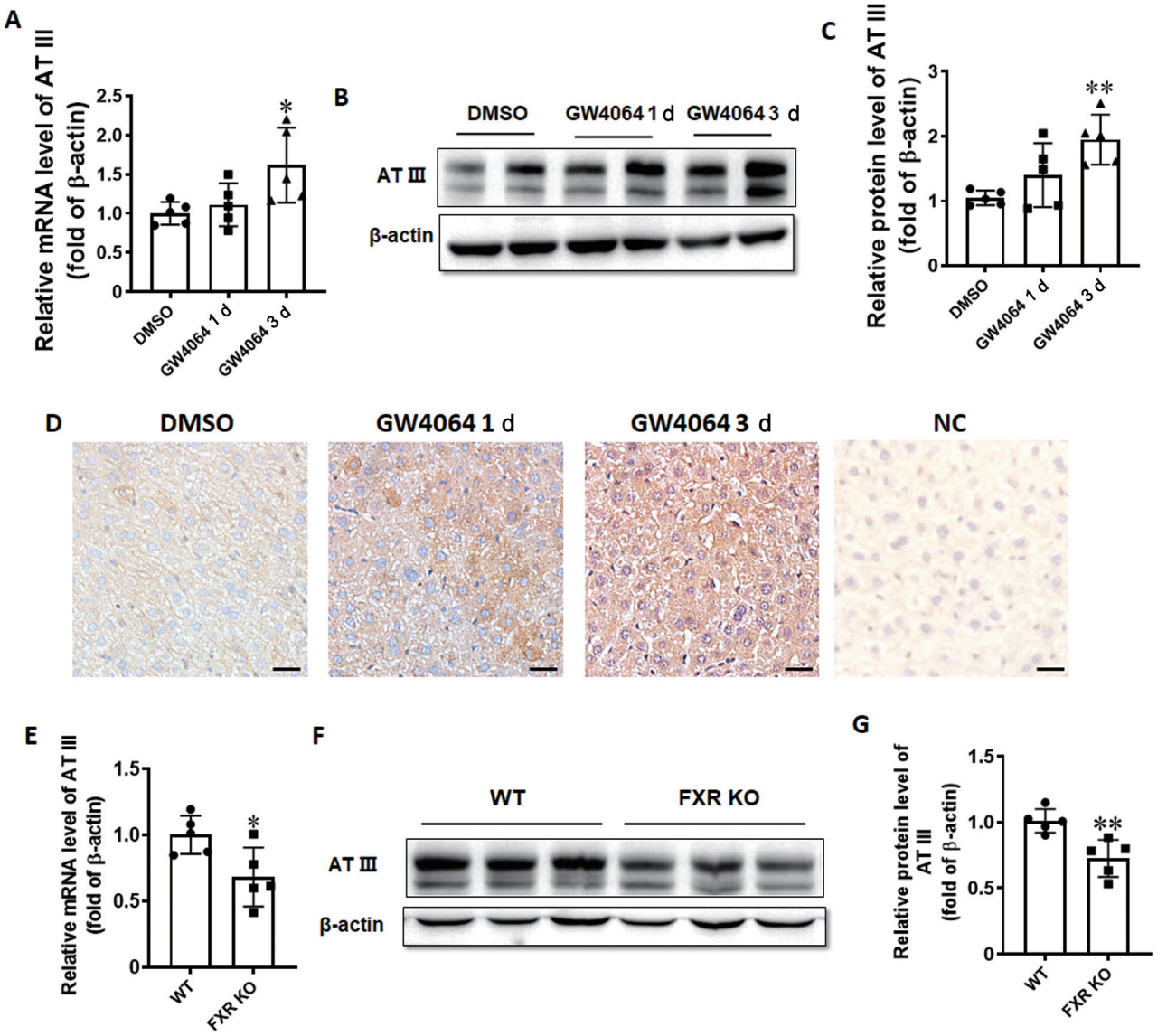

Fig. 3. Regulation of hepatic AT III expression by GW4064 in mouse liver. Expression levels of mRNA and protein of AT III in the livers were measured. *A*: qPCR analysis showing the induction of AT III mRNA levels in the livers of mice treated with GW4064 for 1 and 3 d. *B*, *C*: Western blot and quantitative analysis demonstrating a significant up-regulation on AT III protein levels in livers of mice treated with GW4064 for 1 and 3 d. *D*: Immunohistochemistry staining showing that AT III was widely expressed in liver and increased after GW4064 treatment. Scale bar, 50 μm. *E*: qPCR analysis showing the reduction of AT III mRNA levels in the livers of FXR KO mice. *F*, *G*: Western blot and quantitative analysis demonstrating a significant inhibition on AT III protein levels in livers of FXR KO mice. Data were presented as mean ± SEM. $n = 5$ in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* DMSO (*A*–*C*) or WT mice (*E*–*G*).

up-regulation of AT III expression at both mRNA (Fig. 5*A*) and protein levels (Fig. 5*B*&*C*). These data indicate that FXR activation and overexpression significantly induce the AT III level in liver cells.

2.6 Reduced AT III expression after FXR knockdown in cultured hepatocytes

To further determine the effect of reduced FXR expression on AT III expression, siRNA was applied to knockdown the FXR level in cultured primary hepatocytes. As shown in Fig. 5*D*, siRNA against FXR effectively suppressed AT III mRNA expression in cultured hepatocytes. In consistent, AT III protein expression was also significantly reduced by FXR knockdown (Fig. 5*E*&*F*). These findings demonstrate that inhibition of FXR expression results in a marked reduction of AT III abundance in liver cells.

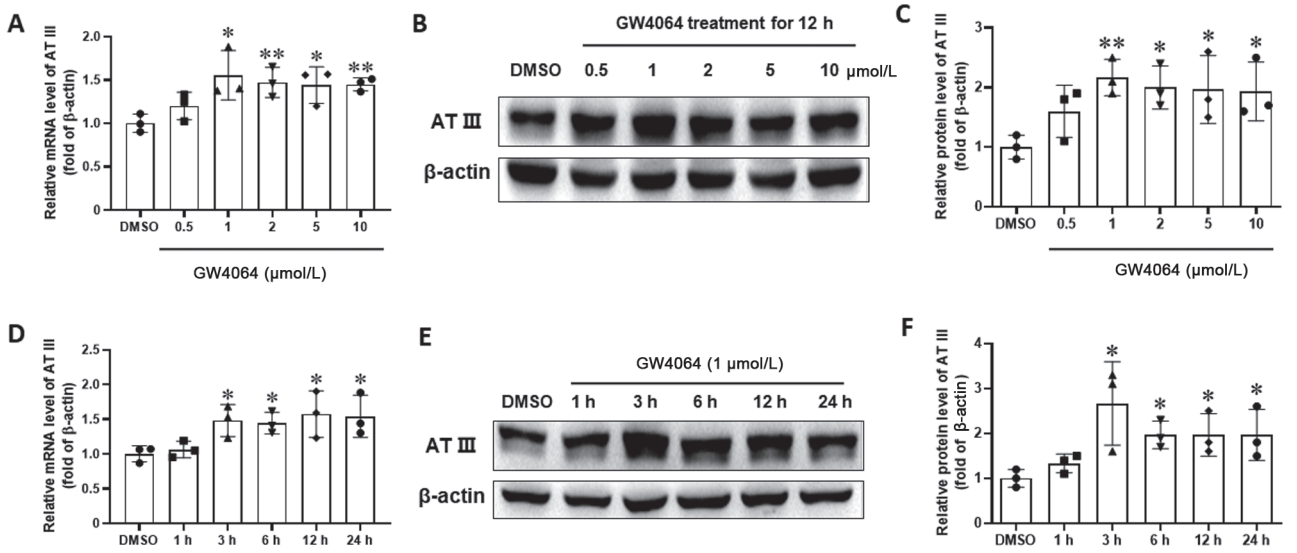

Fig. 4. Induction of AT III expression by GW4064 in cultured hepatocytes. Primary cultured hepatocytes were treated with GW4064 in dose- (0.5 μmol/L, 1 μmol/L, 2 μmol/L, 5 μmol/L and 10 μmol/L) and time course-dependent (1 h, 3 h, 6 h, 12 h and 24 h) manner. *A*: Dose-dependent induction of AT III mRNA level by GW4064. *B*: Dose-dependent induction of AT III protein level by GW4064. *C*: Quantitative analysis of AT III protein levels in inset *B*. *D*: Time course-dependent induction of AT III mRNA levels by GW4064. *E*: Time course-dependent induction of AT III protein levels by GW4064. *F*: Quantitative analysis of AT III protein levels in inset *E*. Data were presented as mean ± SEM. $n = 3$ in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* DMSO.

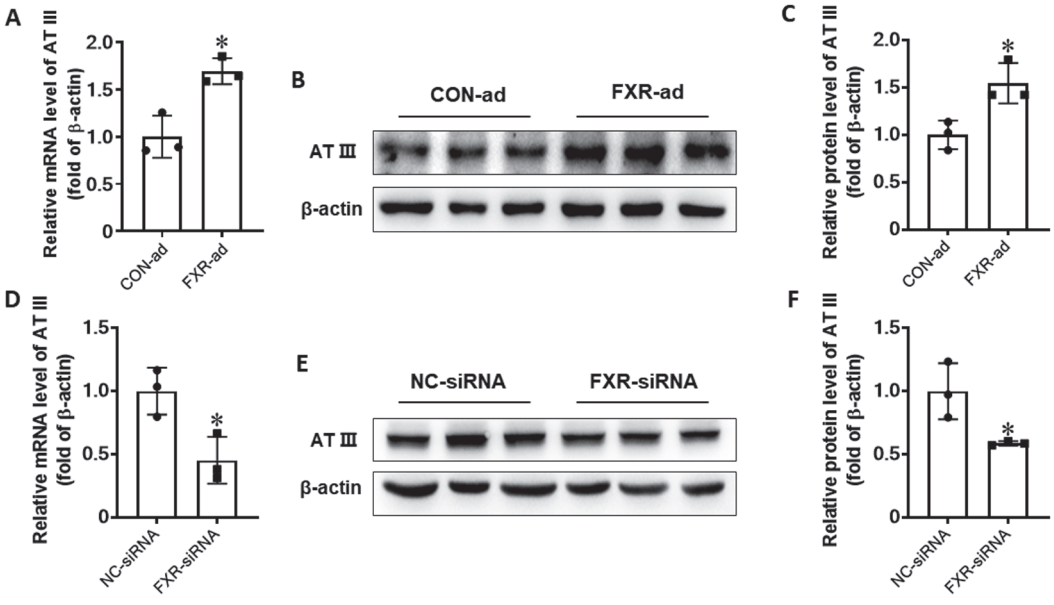

Fig. 5. Effect of FXR overexpression or knockdown on AT III expression in cultured hepatocytes. Primary cultured hepatocytes were infected with an adenovirus expressing a full-length mouse FXR cDNA or transfected with FXR siRNA. *A*: qPCR analysis showing the induction of AT III mRNA levels after adenovirus-mediated FXR overexpression. *B*: Western blot demonstrating the up-regulation of AT III protein levels after adenovirus-mediated FXR overexpression. *C*: Quantitative analysis of AT III protein levels in inset *B*. *D*: qPCR analysis showing the inhibition of AT III mRNA expression after FXR knockdown by FXR siRNA. *E*: Western blot demonstrating the down-regulation of AT III protein levels after FXR knockdown by FXR siRNA. *F*: Quantitative analysis of AT III protein levels in inset *E*. Data were presented as mean ± SEM. $n = 3$ in each group. $^{*}P < 0.05$ *vs* CON-ad (*A*–*C*) or NC-siRNA (*D*–*F*).

2.7 AT III is a downstream target gene of FXR

To determine the molecular mechanism by which FXR regulates AT III level in the liver, the PROMO 3.0 software was used (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) for analyzing potential FXR binding sites on mouse AT III gene promoter region. A putative FXRE sequence between −1 339 bp – −1 328 bp upstream to the transcription start site was identified (Fig. 6*A*). Luciferase and ChIP assays were performed to determine the possible binding of FXR to this potential FXRE. The constructed LUC-AT III reporter was transfected into HEK293T cells, and the luciferase activity was significantly induced by GW4064 compared to DMSO (Fig. 6*B*). ChIP assay further revealed a single band with estimated size which perfectly matched the predicted FXRE sequence (−1 339 bp – −1 328 bp) in the mouse AT III promoter (Fig. 6*C*). These results suggest that AT III gene may represent as a novel downstream target gene of FXR.

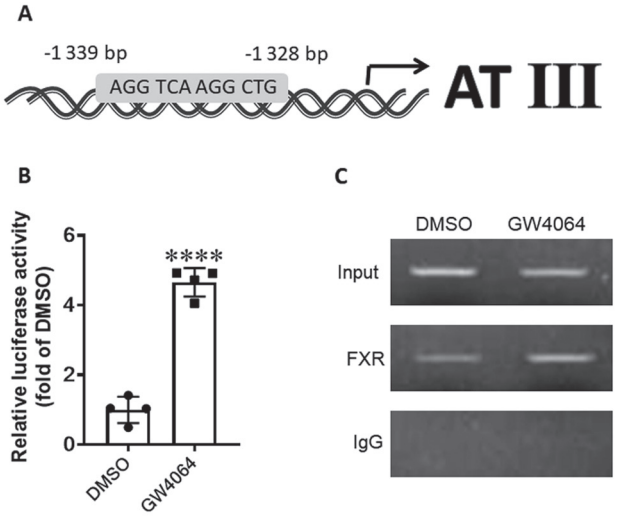

Fig. 6. Activation of FXR significantly enhances the promoter activity of mouse AT III gene. *A*: A schematic structure of mouse AT III gene promoter with predicted FXRE sequence between −1 339 bp – −1 328 bp upstream to the transcription start site. *B*: Luciferase assay showing that GW4064 treatment for 3 h significantly increased the luciferase activity of LUC-AT III. Data were presented as mean ± SEM. $n = 4$ in each group. $^{****}P < 0.000\ 1$ *vs* DMSO. *C*: ChIP assay showing that FXR may bind to the putative FXRE sequence in mouse AT III promoter. A predicted PCR amplification band was evident and confirmed by sequencing. Input, positive control; FXR, anti-FXR antibody precipitated DNA; IgG, IgG precipitated DNA as negative control.

3 DISCUSSION

The present study demonstrates that FXR activation causes a hypocoagulable state, while FXR deficiency leads to a hypercoagulable state, in mice. The underlying mechanism is related to the induction of AT III expression by FXR in the liver cells. FXR is a nuclear receptor highly expressed in the liver, intestine and kidney [5]. Activation of FXR was found to play a critical role in maintaining the hemostatic homeostasis of cholesterol and bile acid *in vivo* [11, 12]. By suppressing sterol regulatory element binding protein 1c (SREBP-1c) and activating peroxisome proliferator-activated receptor alpha (PPARα), FXR inhibits the synthesis of triglycerides, accelerates the removal of triglycerides and regulates the reversal of cholesterol transport, so as to reduce blood lipid levels [13, 14]. Due to its role in inhibiting two rate-limiting enzymes of the synthesis of bile acid, cholesterol 7α-hydroxylase (CYP7A1) and sterol 12α-hydroxylase (CYP8B1), FXR has already become a pharmaceutical target for several major metabolic diseases. Obeticholic acid, a synthetic agonist of FXR, has been proved to be effective to treat primary biliary cholangitis (PBC) and non-alcoholic fatty liver disease (NAFLD) [15–17]. Therefore, further investigation on FXR for developing new medicine and avoiding side effect is of clinical importance.

RNA-sequencing technique has been used for clarifying the hepatic function of FXR. Zhan *et al.* treated primary human hepatocytes and wild-type mice with GW4064 and analyzed the mRNA expression profile. They found that the differentially expressed genes were enriched in complement and coagulation cascades at the percentage of 21% and 11%, respectively [18], suggesting an involvement of FXR activation in regulating coagulation cascades. In support, it has been previously reported that activation of FXR inhibits the platelet aggregation [7] and induces the expression of fibrinogen-α, -β, -γ [8]. In the present study, we further discovered that activation of FXR can promote hypocoagulation, while FXR deficiency leads to hypercoagulation by regulating hepatic AT III expression, indicating that FXR may play an important role in maintaining hemostatic homeostasis.

AT III is one of the most potent natural anticoagulants in blood and serves as up to 80% of the inhibitory components to thrombin formation [19]. Therefore, AT III has been considered as a therapeutic target for various hemostatic disorders. For example, an RNAi therapy

has been developed to degrade AT III mRNA to prevent its synthesis. This therapy can promote hemostasis and reduce the bleeding phenotype in hemophilia patients [20]. In contrast, treatment with AT III is one of the essential therapies for patients with portal vein thrombosis (PVT) in patients with liver disease and lower concentration levels of AT III [21]. The supplementation of AT III is also a potential therapeutic approach for patients with septic-associated disseminated intravascular coagulation (DIC) [22]. In addition, AT III activity can be used as a prognostic marker for the survival of patients with COVID-19-associated acute respiratory distress syndrome (ARDS) [23]. However, up to date, little has been known regarding transcriptional regulation of AT III. The present study provides evidence that hepatic expression of AT III is under direct control of the nuclear receptor FXR.

The FXR-AT III axis we discovered may be clinically relevant. It is well known that hyperlipidemia is a major risk of cardiovascular disease including atherosclerosis and thrombotic complications [24]. FXR, as a key regulator of lipid homeostasis, directly controls the expression of AT III in the liver. Thus, it is reasonably to speculate that the agonists of FXR may act as a new therapy for thrombotic diseases. Whether FXR agonists such as obeticholic acid can be used for the treatment of thrombotic disorders is worth further clinical investigation.

In conclusion, in the present study we found that FXR activation inhibits coagulation process via inducing hepatic AT III expression in mice. Our results reveal a new role of FXR in hemostatic homeostasis and indicate that FXR might act as a potential therapeutic target for diseases related to hypercoagulation.

REFERENCES

1 Doutremepuich C. Anticoagulation. New York: Springer-Verlag, 1994.

2 Mackman N, Tilley RE, Key NS. Role of the extrinsic pathway of blood coagulation in hemostasis and thrombosis. Arterioscler Thromb Vasc Biol 2007; 27(8): 1687–1693.

3 Gailani D, Renne T. Intrinsic pathway of coagulation and arterial thrombosis. Arterioscler Thromb Vasc Biol 2007; 27(12): 2507–2513.

4 Reda S, Muller J, Pavlova A, Pezeshkpoor B, Oldenburg J, Potzsch B, Ruhl H. Functional characterization of antithrombin mutations by monitoring of thrombin inhibition kinetics. Int J Mol Sci 2021; 22(4): 2119.

5 Zhang X, Huang S, Gao M, Liu J, Jia X, Han Q, Zheng S, Miao Y, Li S, Weng H, Xia X, Du S, Wu W, Gustafsson JA, Guan Y. Farnesoid X receptor (FXR) gene deficiency impairs urine concentration in mice. Proc Natl Acad Sci U S A 2014; 111(6): 2277–2282.

6 Xu S, Huang S, Luan Z, Chen T, Wei Y, Xing M, Li Y, Du C, Wang B, Zheng F, Wang N, Guan Y, Gustafsson JA, Zhang X. Farnesoid X receptor is essential for the survival of renal medullary collecting duct cells under hypertonic stress. Proc Natl Acad Sci U S A 2018; 115(21): 5600–5605.

7 Moraes LA, Unsworth AJ, Vaiyapuri S, Ali MS, Sasikumar P, Sage T, Flora GD, Bye AP, Kriek N, Dorchies E, Molendi-Coste O, Dombrowicz D, Staels B, Bishop-Bailey D, Gibbins JM. Farnesoid X receptor and its ligands inhibit the function of platelets. Arterioscler Thromb Vasc Biol 2016; 36(12): 2324–2333.

8 Anisfeld AM, Kast-Woelbern HR, Lee H, Zhang Y, Lee FY, Edwards PA. Activation of the nuclear receptor FXR induces fibrinogen expression: a new role for bile acid signaling. J Lipid Res 2005; 46(3): 458–468.

9 Barettino D, Masia S, Monto F, Perez P, D'Ocon P, Moreno L, Muedra V. Glucocorticoids as modulators of expression and activity of Antithrombin (At): potential clinical relevance. Thromb Res 2015; 135(1): 183–191.

10 Wang B, Zhang H, Luan Z, Xu H, Wei Y, Zhao X, Xing M, Huo X, Zhang J, Su W, Guan Y, Zhang X. Farnesoid X receptor (FXR) activation induces the antioxidant protein metallothionein 1 expression in mouse liver. Exp Cell Res 2020; 390(1): 111949.

11 Modica S, Gadaleta RM, Moschetta A. Deciphering the nuclear bile acid receptor FXR paradigm. Nucl Recept Signal 2010; 8: e005.

12 Calkin AC, Tontonoz P. Transcriptional integration of metabolism by the nuclear sterol-activated receptors LXR and FXR. Nat Rev Mol Cell Biol 2012; 13(4): 213–224.

13 Watanabe M, Houten SM, Wang L, Moschetta A, Mangelsdorf DJ, Heyman RA, Moore DD, Auwerx J. Bile acids lower triglyceride levels via a pathway involving FXR, SHP, and SREBP-1c. J Clin Invest 2004; 113(10): 1408–1418.

14 Zhang Y, Edwards PA. FXR signaling in metabolic disease. FEBS Lett 2008; 582(1): 10–18.

15 Xu Y, Li F, Zalzala M, Xu J, Gonzalez FJ, Adorini L, Lee YK, Yin L, Zhang Y. Farnesoid X receptor activation increases reverse cholesterol transport by modulating bile acid composition and cholesterol absorption in mice. Hepatology 2016; 64(4): 1072–1085.

16 Li H, Xi Y, Xin X, Tian H, Hu Y. Gypenosides regulate farnesoid X receptor-mediated bile acid and lipid metabolism in a mouse model of non-alcoholic steatohepatitis. Nutr Metab (Lond) 2020; 17: 34.

17 Zhang Y, Jackson JP, St Claire RL 3rd, Freeman K, Brouwer

KR, Edwards JE. Obeticholic acid, a selective farnesoid X receptor agonist, regulates bile acid homeostasis in sandwich-cultured human hepatocytes. Pharmacol Res Perspect 2017; 5(4): e00329.

18 Zhan L, Liu HX, Fang Y, Kong B, He Y, Zhong XB, Fang J, Wan YJ, Guo GL. Genome-wide binding and transcriptome analysis of human farnesoid X receptor in primary human hepatocytes. PLoS One 2014; 9(9): e105930.

19 Bae J, Desai UR, Pervin A, Caldwell EE, Weiler JM, Linhardt RJ. Interaction of heparin with synthetic antithrombin III peptide analogues. Biochem J 1994; 301(Pt 1): 121–129.

20 Sehgal A, Barros S, Ivanciu L, Cooley B, Qin J, Racie T, Hettinger J, Carioto M, Jiang Y, Brodsky J, Prabhala H, Zhang X, Attarwala H, Hutabarat R, Foster D, Milstein S, Charisse K, Kuchimanchi S, Maier MA, Nechev L, Kandasamy P, Kel'in AV, Nair JK, Rajeev KG, Manoharan M, Meyers R, Sorensen B, Simon AR, Dargaud Y, Negrier C, Camire RM, Akinc A. An RNAi therapeutic targeting antithrombin to rebalance the coagulation system and promote hemostasis in hemophilia. Nat Med 2015; 21(5): 492–497.

21 Okuno M, Kimura Y, Taura K, Nam NH, Li X, Ogiso S, Fukumitsu K, Ishii T, Seo S, Uemoto S. Low level of postoperative plasma antithrombin III is associated with portal vein thrombosis after liver surgery. Surg Today 2021; 51(8): 1343–1351.

22 Iba T, Levy JH, Aihara K, Kadota K, Tanaka H, Sato K, Nagaoka I. Newly developed recombinant antithrombin protects the endothelial glycocalyx in an endotoxin-induced rat model of sepsis. Int J Mol Sci 2020; 22(1): 176.

23 Anakli I, Ergin Ozcan P, Polat O, Orhun G, Alay GH, Tuna V, Celiksoy E, Kilic M, Mercan M, Ali A, Besisik S, Esen F. Prognostic value of antithrombin levels in COVID-19 patients and impact of fresh frozen plasma treatment: A retrospective study. Turk J Haematol 2021; 38(1): 15–21.

24 Porez G, Prawitt J, Gross B, Staels B. Bile acid receptors as targets for the treatment of dyslipidemia and cardiovascular disease. J Lipid Res 2012; 53(9): 1723–1737.