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Abstract

Aims: Low testosterone in men is associated with increased cardiovascular events and mortality. Testosterone has beneficial effects on several cardiovascular risk factors including cholesterol, endothelial dysfunction and inflammation as key mediators of atherosclerosis. Although evidence suggests testosterone is anti-atherogenic, its mechanism of action is unknown. The present study investigates whether testosterone exerts anti-atherogenic effects by stimulating cholesterol clearance from macrophages via activation of liver X receptor (LXRα), a nuclear master regulator of cellular cholesterol homeostasis, lipid regulation, and inflammation. **Main Methods:** Using human monocyte THP-1 cells differentiated into macrophages, the effect of testosterone (1-10nM) treatment (24-72 h) on the expression of LXRα and LXR- targets apolipoprotein E (APOE), ATP-binding cassette transporter A1 (ABCA1), sterol regulatory element-binding transcription factor 1 (SREBF1) and fatty acid synthase (FAS), was investigated via qPCR and western blotting, with or without androgen receptor blockade with flutamide or LXR antagonism with CPPSS-50. Cholesterol clearance was measured by monitoring fluorescent dehydroergosterol (DHE) cellular clearance and ABCA1 cellular translocation was observed via immunocytochemistry in testosterone treated macrophages. **Key Findings:** Testosterone increased mRNA and protein expression of LXRα, APOE, ABCA1, SREBF1 and FAS. These effects were blocked by flutamide and independently by LXR antagonism with CPPSS-50. Furthermore testosterone stimulated cholesterol clearance from the macrophages and promoted the translocation of ABCA1 toward the cell membrane. **Significance:** Testosterone acts via androgen receptor-dependent pathways to stimulate LXRα and downstream targets to induce cholesterol clearance in human macrophages. This may, in part, explain the anti-atherogenic effects of testosterone frequently seen clinically.

Key Words: Atherosclerosis, Macrophage, Liver X receptor, Androgen Receptor.

Introduction

An increase in cardiovascular mortality has been demonstrated in men with testosterone deficiency in epidemiological studies [1] and also within a population of men with angiographically-proven coronary heart disease [2] indicating low testosterone levels as an independent cardiovascular risk factor. The administration of testosterone therapy (TTh) has been shown to improve risk factors for atherosclerosis, the major underlying cause of cardiovascular disease (CVD), including reducing central adiposity and insulin resistance and improving lipid profiles (in particular, lowering cholesterol), clotting and inflammatory profiles and vascular function (reviewed in [3]). Despite this evidence the impact of testosterone on the cardiovascular system remains controversial with concerns in regard to the safety of TTh in men with cardiovascular disease [4-6]. These studies however have been highly criticised in their methodological approach and interpretation of data. Systematic meta-analyses of TTh clinical trials have not demonstrated an increased risk of adverse cardiovascular events or mortality [7,8]. The majority of large observational studies with extended follow-up report TTh was associated with a significant reduction in all-cause mortality, myocardial infarction, and stroke [9,10].

While there are no definitive randomised clinical trials of TTh that report direct effects on atherosclerosis, some clinical and scientific studies have investigated the mechanistic effects of testosterone on atherogenesis seen in testosterone deficient men. Carotid intimal media thickness (CIMT), considered a surrogate marker of the degree of atherosclerosis, has been shown to be reduced by TTh in some randomised clinical trials [11,12] yet was unchanged in others [13]. Conversely, coronary artery plaque volume was increased as a result of TTh in hypogonadal men considered as a result of increased non-calcified plaque volume and fibrous plaque volume compared to placebo [14]. Animal studies provide consistent evidence that testosterone is atheroprotective. Aortic plaque formation is accelerated following castration in models of atherosclerosis while replacing testosterone abrogates plaque development [15-17]. These studies suggest that testosterone may act via both genomic androgen receptor (AR) -dependent and non-genomic AR-independent mechanisms to protect against atherogenesis.

From their recruitment to the vascular sites of inflammation through to apoptosis and destabilisation/rupture of the plaque, macrophages are considered the 'driving force' behind atherogenesis with the formation of foam cells considered as the first sign of plaque development. Foam cell formation is influenced by increased lipid uptake and the inability of macrophages to efflux sufficient amounts of cholesterol to the reverse cholesterol transport (RCT) pathway. Indeed, macrophage RCT reflects the removal of cholesterol from the arterial wall, and its impairment is associated with increased atherosclerosis in several animal models [18-20]. Stimulation of cholesterol efflux from macrophages therefore has the potential to be atheroprotective.

Testosterone has known immunomodulatory actions which may contribute to its observed atheroprotective effects [21,22]. Although only relatively few studies have investigated the influence of androgens on monocyte or macrophage function relevant to atherosclerosis, the majority of these indicate anti-inflammatory actions [23-25] or improvements in cellular lipid processing [26,27]. Specifically, foam cell induction by oxLDL is inhibited by dihydrotestosterone (DHT) in a cultured macrophage cell line [27] and human monocyte-derived macrophages treated with androgens in vitro demonstrate inhibited cholesterol accumulation [26]. In testosterone deficient mice whereby the primary cellular constituents in the atherosclerotic plaque were identified as macrophages, TTh reduced overall plaque size compared to placebo treatment suggesting a reduction in macrophage content in the arterial wall [16]. The molecular events underpinning this androgen action are not yet known.

Liver X receptor (LXR), a member of the nuclear receptor superfamily, is a key regulator of cholesterol metabolism within the cell and the LXRα isoform is expressed in macrophages[28]. Upon ligand-activation LXRα regulates the expression of proteins involved in RCT, such as ATP-binding cassette transporter A1 (ABCA1) which mediates cellular cholesterol efflux, and apolipoprotein E (APOE) which binds cholesterol to form high density lipoprotein (HDL) subsequently preventing a build-up of cholesterol within the cell [29,28]. LXRα is also known to regulate fatty acid synthesis, glucose metabolism and suppresses inflammatory cytokines [30]. LXR agonists have been shown to significantly

reduce atherosclerotic plaque development in animal models [31]. One study has reported that in Swiss white rabbits the effect of an LXR agonist has an equivalent effect to statin therapy in reducing plaque size and, importantly, when both are administered together there is evidence of plaque regression [32]. The use of LXR agonists as a therapy has been confounded by the concomitant development of hepatic steatosis in many of these studies. Testosterone has however been shown to inhibit enzymes of fatty acid synthesis in the liver in animal studies [33]. In the present study we investigate whether testosterone exerts anti-atherogenic effects through the stimulation of LXRα-mediated cholesterol clearance in macrophages.

Materials and Methods

Cell Culture

Human acute monocytic leukemia, THP-1 (ECACC Cat# 88081201, RRID:CVCL_0006) and Mono Mac 6, MM6 (DSMZ Cat# ACC-124, RRID:CVCL_1426) cell lines utilised in this study were both originally derived from male acute monocytic leukemia patients. Cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and 1% sodium pyruvate and passaged every 3-4 days. Cells were used up to passage 20. Prior to experiments, THP-1 cells were incubated in phorbol-12-myristate-13-acetate (PMA; Sigma-Aldrich) in FBS-free medium for 72 hours to induce differentiation to a M0 macrophage-like phenotype. All experiments were performed under FBS-free conditions. Testosterone (Sigma-Aldrich) and flutamide (Sigma-Aldrich) were solubilised in 100% ethanol and serially diluted in FBS-free media to final concentrations of 1-10 nM. The LXR agonist T0901317 (Sigma-Aldrich) was dissolved in DMSO and diluted to a final concentration of 1 µM. LXR antagonist 5-Chloro-*N*-2'-n-penthylphenyl-1,3-dithiothalimide (5CPPSS-50; Wako) was dissolved in methanol and diluted to a final concentration of 20 µM. Control conditions used relevant vehicle solutions of equivalent volumes to associated treatments.

Cholesterol Clearance Assay

$5x10^4$ THP-1 cells per well were seeded into 96-well plates. Following differentiation, cells were incubated in 10 nM testosterone or vehicle for 24 hours. Dehydroergosterol (DHE; Sigma-Aldrich) was added at a concentration of 320 µM and cells were incubated for a further 18 hours to allow the cholesterol analogue to be taken up by the cells. Cells were then washed in 1x PBS and fresh FBS-free media was added. For direct observation of DHE clearance, cells were viewed using a Leica AF6000LX inverted microscope. A single field of view was imaged every 15 minutes for 6 hours. A FLUOstar plate reader, set to a wavelength of 390/460 nm, was used to measure changes in fluorescence at set time points

following careful media replacement. Fluorescence values were normalized against media only wells and are shown as percentage decrease in cellular fluorescence from baseline.

Quantitative analysis of mRNA

Following treatments, cells were harvested and total RNA extracted using an RNA mini kit (Ambion). RNA concentrations were measured spectrophotometrically. cDNA was synthesised from 250 ng of RNA using a superscript reverse transcription kit (Qiagen) and its quality was checked by performing end point PCR using GAPDH primers and agarose gel electrophoresis. Quantitative PCR was performed on a Stratagene system using SYBR Green (Applied Biosystems) according to the manufacturers' recommendations. Gene-specific primers were designed using PrimerBlast software and synthesised by Invitrogen (Table 1). Each sample was run in triplicate and mRNA levels were normalized to the housekeeping gene GAPDH and assessed relative to time-point and cell-type controls. Melting curve analysis was performed each time to check specificity of primers.

Table 1. Primer sequences.

Gene	NCBI Reference	Direction	Primer sequence	Product length
Androgen Receptor	NM_000044.2	F	AAG GCC TTG CCT GGC TTC CG	141
		R	AGG GGC GAA GTA GAG CAT CCT GG	
CD14	NM_000591.3	F	ACT GTC AGA GGC AGC CGA AGA GT	141
		R	CGC GCG CTC CAT GGT CGA TA	
GAPDH	NM_002046.3	F	GAA GGC TGG GGC TCA TTT GCA GG	150
		R	CAG TTG GTG GTG CAG GAG GCA T	
LXRα	NM_005693.2	F	CTC TGC AGA CCG GCC CAA CG	128
		R	GCA TCC GTG GGA ACA TCA GTC GG	
ApoE	NM_000041.2	F	GCT GGG AAC TGG CAC TGG GTC	124
		R	TCG TCC ATC AGC GCC CTC AGT T	
ABCA1	NM_005502.3	F	GGC CTT GGC CTT TGT CGG GG	122
		R	TGG TGC GGC CTT GTC GGT AT	
FAS	NM_004104.4	F	GCT GGA AGG CGG GGC TCT AC	124
		R	CGC AGC TGA GGG TCC ATC GT	
SREBF1c	NM_001005291.2	F	CGA CAT CGA AGG TGA AGT CGG CG	146
		R	GGC CAG GGA AGT CAC TGT CTT GGT T	

142 ***Western Blot Analysis***

143 Following treatments, cells were harvested and lysed in RIPA buffer containing protease inhibitors.
144 Protein concentration of cell lysates was determined by Bradford Assay and 50 µg protein was separated
145 by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; 150 V, 90 min) and
146 electrophoretically transferred to nitrocellulose membrane (100 V, 120 min). Membranes were blocked
147 with bovine serum albumin (BSA) or milk in TBST, dependent upon the protein of interest (Table 2).
148 Membranes were then incubated in primary antibodies against specific targets LXRα, APOE, ABCA1,
149 SREBF1 and FAS (Table 2). GAPDH was used as a loading control. Subsequent incubation with
150 horseradish peroxidase (HRP)-labelled goat polyclonal secondary antibody was carried out.
151 Immunocomplexes were visualised using chemiluminescence development kit and images were captured
152 and densitometry performed using GeneSnap and GeneTools software.

153 **Table 2. Antibody details.**

Protein	Size (kDa)	Blocking conditions	1° Antibody		2° Antibody
			Host	Dilution	Dilution
ABCA1	220	5% milk	Mouse	1 in 500 in 2.5% milk	1 in 1000 in 2.5% milk
APOE	36	0.5% BSA	Rabbit	1 in 250 in 2.5% BSA	1 in 1000 in 2.5% BSA
CALNEXIN	75	10% milk	Rabbit	1 in 20,000 in 10% milk	1 in 1000 in 10% milk
FAS	273	10% milk	Rabbit	1 in 500 in 10% milk	1 in 1000 in 10% milk
GAPDH	36	5% milk	Mouse	1 in 500 in 2.5% milk	1 in 1000 in 2.5% milk
LXR	50	0.5% milk	Mouse	1 in 250 in 2.5% BSA	1 in 1000 in TBST
SREBF1c	125	5% milk	Mouse	1 in 100 in 2.5% milk	1 in 1000 in 2.5% milk

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Immunocytochemistry

Cells were grown and differentiated on poly-L-lysine coated chamber slides followed by treatments for 6 hours. Cells were fixed using 4% paraformaldehyde, permeabilised with 0.25% Triton X-100, blocked with 1% BSA, 0.3 M glycine and incubated in ABCA1 primary antibody (1/250) at 4°C overnight. AlexaFluor 555 secondary antibody (1/300) was used to visualise ABCA1 staining. Nuclei were counter-stained using DAPI and images were acquired using a Leica DM14000B inverted microscope. Total ABCA1 area was assessed via fluorescence coverage in 5 randomised, blinded images from 3 repeated experimental conditions (n=3) for treated and control cells using image j analysis software. Likewise, mean pixel intensity was assessed via image j analysis. ABCA1 subcellular localisation was also estimated in 5 randomised, blinded images from 3 repeated experimental conditions (n=3) for treated and control cells and percentage of cells expressing perinuclear, cytoplasmic and cell membrane associated ABCA1 staining calculated.

Statistical analysis

Data is presented as mean ± standard error (SEM) unless otherwise stated. Differences between groups with normally distributed data were compared using two-tailed unpaired Student's t tests performed in Excel. Non-normally distributed data was investigated with Mann-Whitney U tests for significance between groups. Significance was accepted at $P \leq 0.05$.

Results

Testosterone increases LXRα, APOE, ABCA1, SREBF1 and FAS gene and protein expression.

Exposing differentiated macrophages to both 1 nM and 10 nM testosterone resulted in a significant increase in *LXRα* mRNA levels compared with control cells, after 24 and 48 hours (n = 6; Fig. 1 *A*). Protein levels of LXRα were raised significantly after 48 hours in 1 nM testosterone (n = 4) and after 24 hours in 10 nM testosterone (n = 3; Fig. 1 *B* and *K*). Both concentrations of testosterone were found to significantly increase *APOE* mRNA at all time points observed (n = 3; Fig. 1 *C*) and APOE protein levels were increased significantly after 24 hours in 10 nM testosterone (n = 3; Fig. 1 *D* and *K*). Testosterone significantly increased *ABCA1* mRNA at both 1 nM and 10 nM after 24 (n = 8) and 48 hours (n = 9; Fig. 1 *E*). This translated to a significant increase in ABCA1 protein seen after 72 hours in 1 nM testosterone and after 48 hours in 10 nM testosterone (n = 3; Fig. 1 *F* and *K*). 10 nM testosterone increased *SREBF1* mRNA levels significantly at all of the time points observed whereas 1nM testosterone significantly increased *SREBF1* mRNA levels after both 24 and 72 hours (n = 3; Fig. 1 *G*). SREBF1 protein was increased significantly after 48 hours in 1 nM testosterone (n = 5) and after 72 hours in the presence of 10 nM testosterone (n = 4; Fig. 1 *H* and *K*). Exposure to 10 nM testosterone caused *FAS* mRNA levels to increase significantly after 48 hours (n = 5) whereas testosterone at 1 nM elicited a small but insignificant increase in *FAS* mRNA (n = 6; Fig. 1 *I*). FAS protein levels were significantly increased after 48 hours in 1 nM testosterone (n = 3; Fig. 1 *J* and *K*). Thus testosterone increases the expression of LXRα and LXRα-target genes in THP-1 monocyte-derived macrophages.

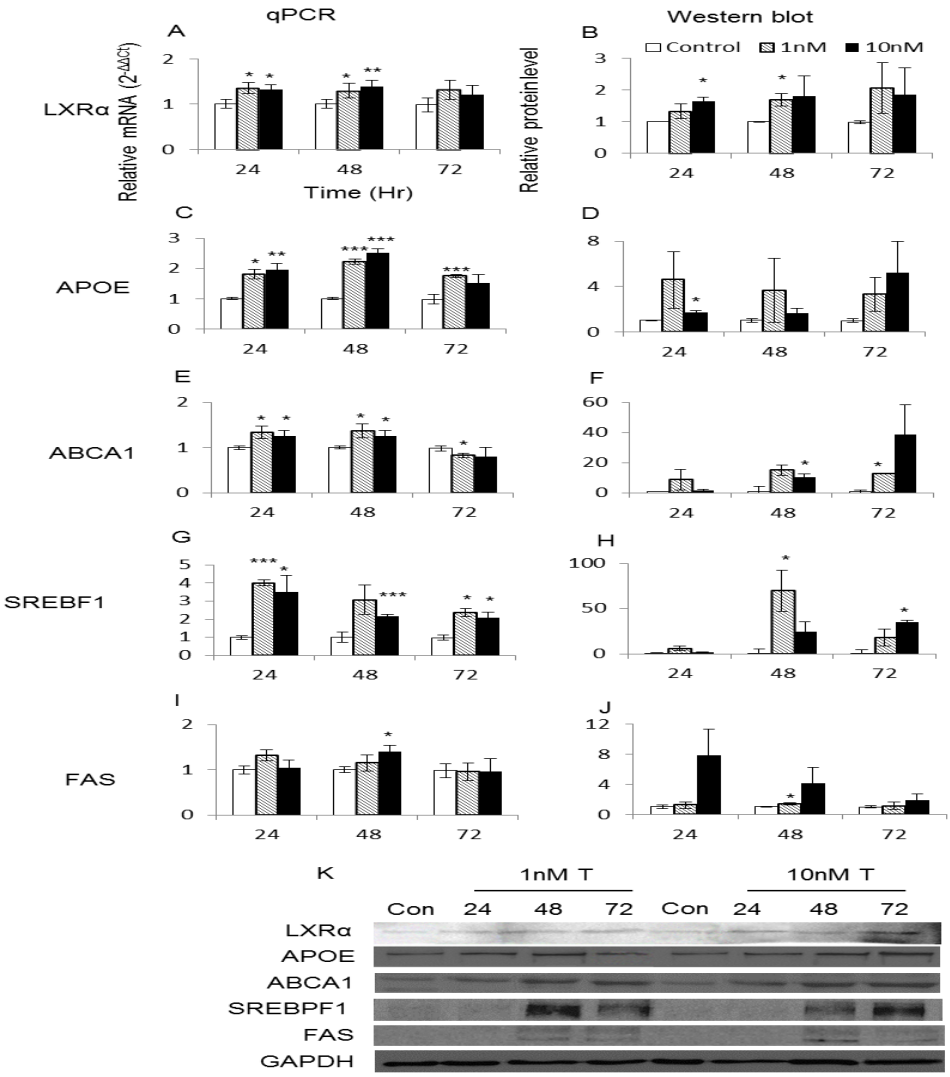


Figure 1: Testosterone increases LXRα, APOE, ABCA1, SREBF1 and FAS gene and protein expression in THP-1 macrophages. Analysis of gene and protein expression in differentiated THP-1 macrophages by quantitative PCR and Western blot followed by densitometry, for LXRα (A and B), APOE (C and D), ABCA1 (E and F), SREBF1 (G and H) and FAS (I and J). THP-1 macrophages treated with testosterone (T) 1 nM (white columns) 10 nM (black columns) over time with data presented as mean ± SEM after normalisation to GAPDH as reference for both mRNA and protein analysis. qPCR values are presented as relative fold change from associated control treatments and western blot data as arbitrary densitometry units (A.U.). (K) Representative western blot images showing protein levels at 24, 48 and 72 hours in response to testosterone 1 nM and 10 nM. n=3, * $p<0.05$, ** $p<0.01$, *** $p<0.005$.

Testosterone increases APOE, ABCA1, SREBF1 and FAS gene expression via LXRα.

THP-1 cells increased *LXRα* expression almost 4-fold in response to LXR agonist T0901317 (n = 6; Fig. 2 *B*). Exposure to T0901317 in combination with testosterone however did not elicit a further increase in *LXRα* gene expression over that observed with T0901317 alone (n = 6; Fig. 2 *B*). Exposure to the LXR antagonist 5CPPSS-50 in combination with testosterone completely abolished any increase in *LXRα* gene expression observed with testosterone alone (n = 6; Fig. 2 *B*). The LXR agonist elicited an increase in the expression of the LXRα target genes *APOE*, *ABCA1*, *SREBF1* and *FAS* (n = 6; Fig. 2 *C-F*). Testosterone in combination with T0901317 did not further increase the expression of these target genes beyond mon-treatment effects (n = 6; Fig. 2 *C-F*). Conversely, when LXR activity is blocked by the antagonist 5CPPSS-50, testosterone did not elicit an increase in the expression of *APOE*, *ABCA1*, *SREBF1* and *FAS* (n = 3; Fig. 2 *C-F*).

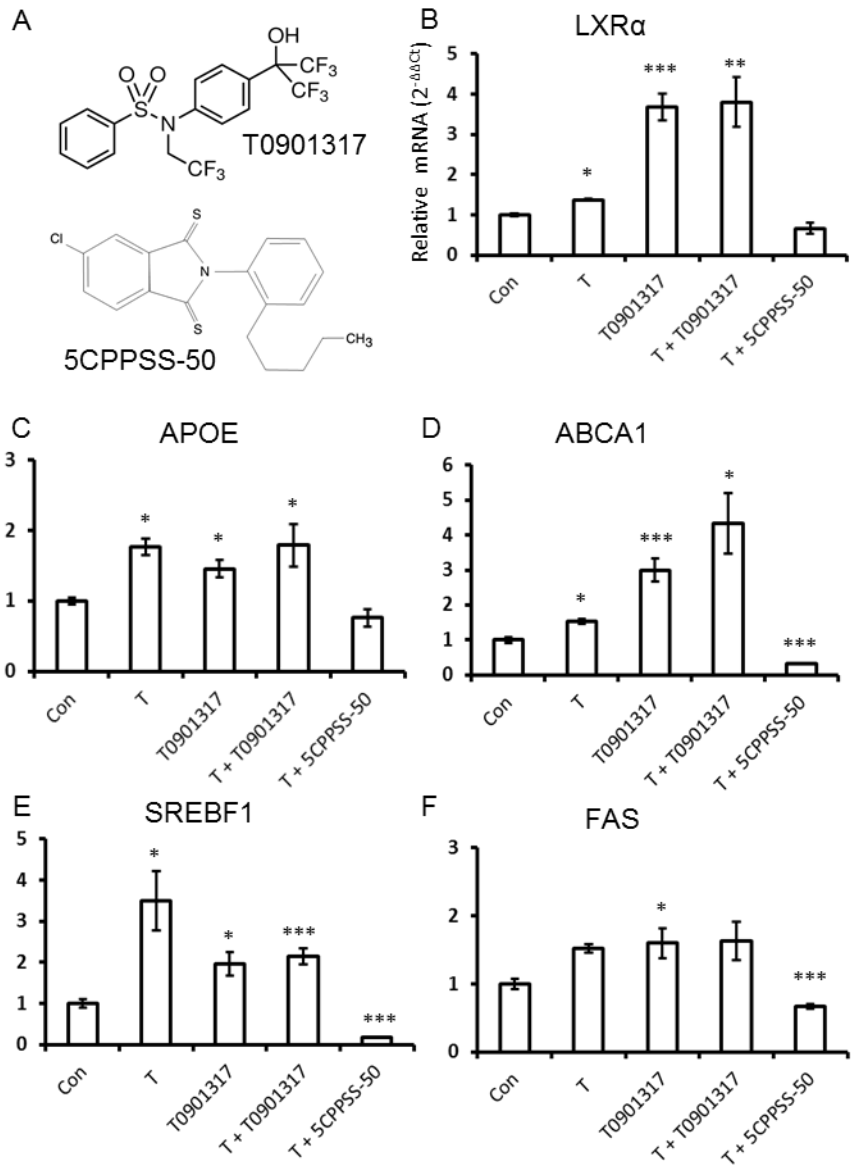
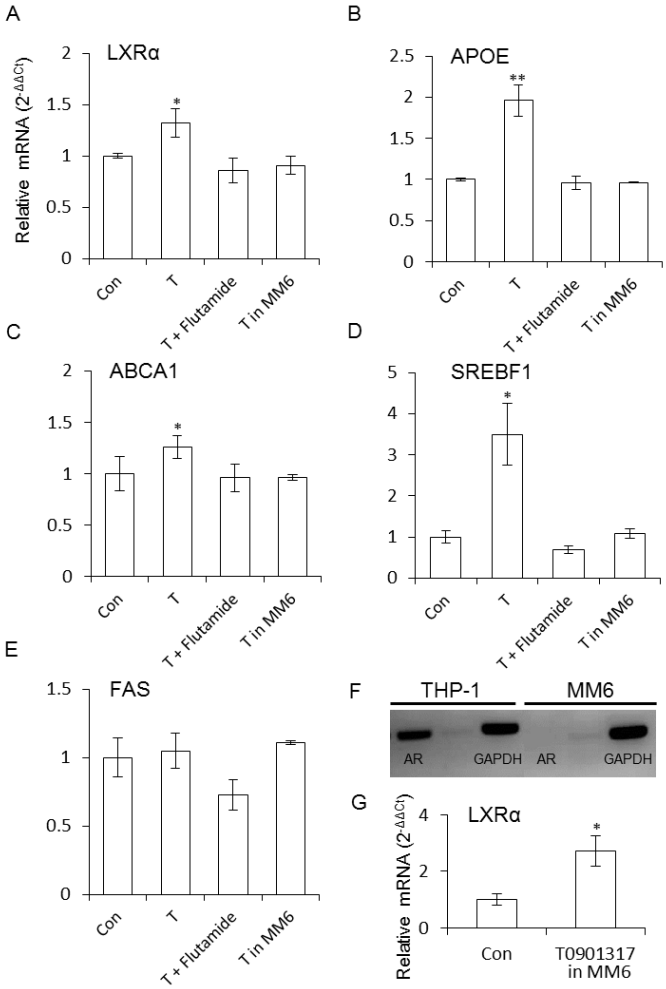


Figure 2: Testosterone increases APOE, ABCA1, SREBF1 and FAS gene expression in THP-1 macrophages via LXRα. (A) Molecular structure of the LXR agonist, T0901317 and LXR antagonist, 5CPPSS-50. Relative mRNA levels of LXRα (B), APOE (C), ABCA1 (D), SREBF1 (E) and FAS (F) in response to 24h treatment with testosterone 10nM (T), T0901317, T0901317 in combination with T, and 5CPPSS-50 in combination with T.

Testosterone acts through the AR to stimulate LXRα.

When the activity of the AR is blocked with flutamide, testosterone did not increase the expression of *LXRα* or any of its target genes (n = 6; Fig. 3 *A*-*E*). The cell line MM6 does not express the AR (Fig. 3 *F*) and exposure of these cells to 10 nM testosterone for 24 hours did not increase the expression of *LXRα* or any of its target genes (n = 3; Fig. 3 *A*-*E*). In order to demonstrate MM6 cells are able to respond to experimental stimuli we applied the LXR agonist T0901317 for 24 hours and observed an increase in the expression of *LXRα* (n = 3; Fig. 3 *G*).



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233 ***Figure 3: Testosterone acts through the androgen receptor to stimulate LXRα actions in THP-1***
234 ***macrophages.*** Relative mRNA levels of LXRα (A), APOE (B), ABCA1 (C), SREBF1 (D) and FAS (E)
235 in response to 24h treatment with testosterone 10 nM (T), T in combination with androgen receptor (AR)
236 blocker flutamide and the response to T in AR deficient MM6 cells. (F) End-point PCR shows AR
237 expression in THP-1 cells but not in MM6 cells, using GAPDH as a positive control. (G) Relative mRNA
238 levels of LXRα in MM6 cells in response to LXR agonist, T0901317. Data presented as mean ± SEM
239 after normalisation to GAPDH as reference. n=3, * p<0.05, ** p<0.01, *** p<0.005.

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Testosterone stimulates cholesterol clearance from human macrophages.

Within 6 hours of 10 nM testosterone treatment the rate of cholesterol clearance was significantly increased with a reduction of 19.1% of the total DHE taken up by the cell (n = 3; black dashed line) compared with a decrease of 14.7% of total DHE in control cells (black unbroken line; Fig. 4 *A*). Using fluorescence microscopy, DHE (white arrow in *B*) was seen leaving the cell much faster in testosterone-treated macrophages (n = 3; Fig. 4 *C, E, G, I* and *K*) than in controls (Fig. 4 *B, D, F, H* and *J*). After 24 hours testosterone-treated cells had cleared 53.2% of the DHE they originally contained (n = 7; black dashed line; Fig. 4 *A* and *M*) compared with controls which had cleared only 23.1% (black unbroken line; Fig. 4 *A*). Control cells at 24 hours can be seen to contain much higher levels of DHE when observed directly using fluorescence microscopy (n = 3; Fig. 4 *L Vs M*). Thus testosterone increases the rate at which THP-1 monocyte-derived macrophages clear cellular cholesterol. In cells treated with both testosterone and the LXR antagonist 5CPPSS-50 or testosterone and flutamide for 6h, an increase in cholesterol clearance was not observed compared to control cells (n = 3; Fig. 4 N).

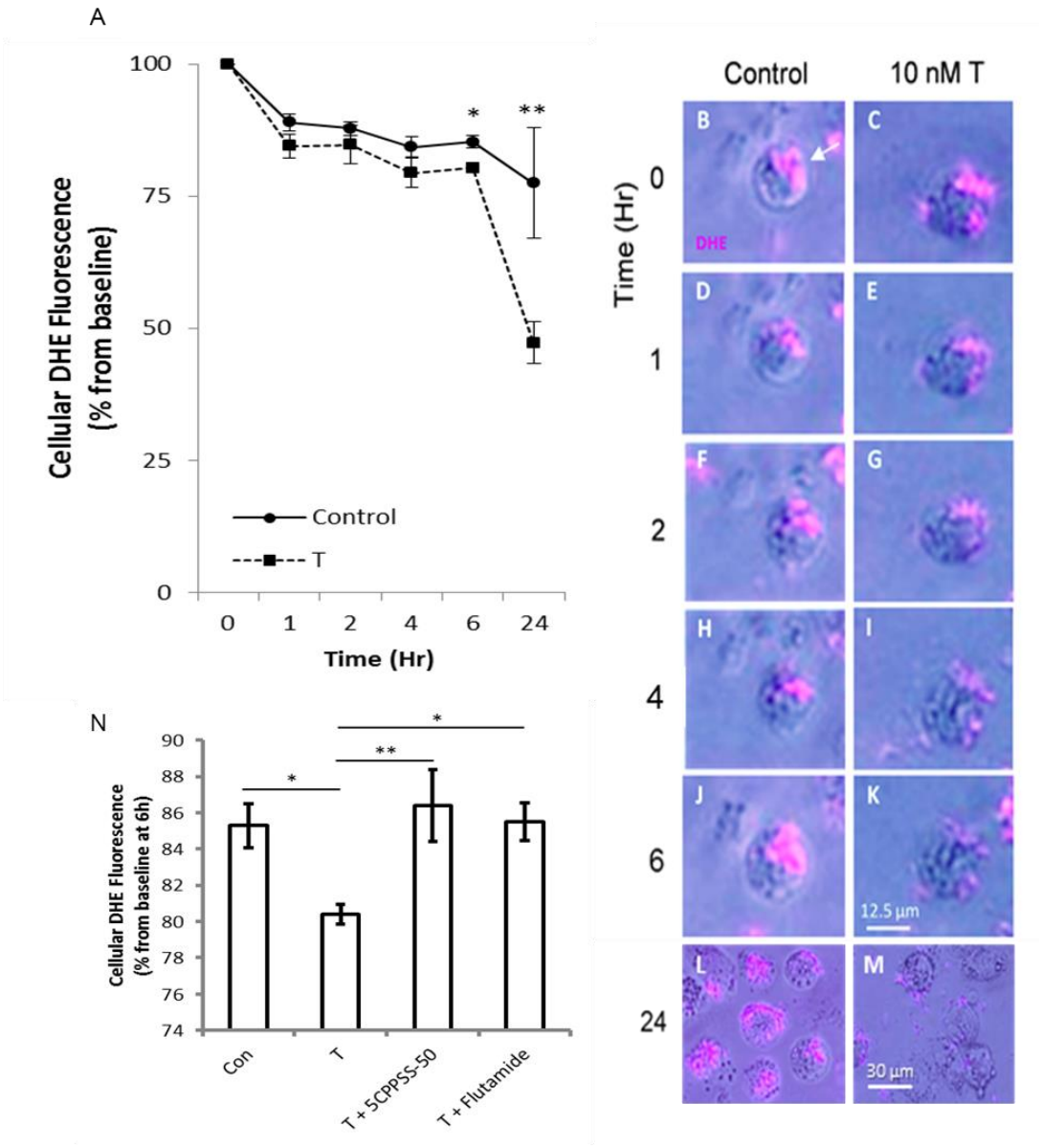

Figure 4: Testosterone stimulates cholesterol clearance from THP-1 macrophages. (A) Line graph shows the percentage cellular fluorescence over 24 hours in control cells (solid line) and cells treated with testosterone 10 nM (T) (dashed line). (B-M) Representative time-lapse fluorescence microscopy reveals dehydroergosterol (DHE: pink) clearance from a single cells over time (B-K, Scale bars, 12.5 µm) and cell populations at 24 hours (L-M, Scale bar 30 µm). (N) 6 hour percentage cellular fluorescence in control cells, treated with testosterone 10nM (T), T in combination with LXR antagonist 5CPPSS-50, or T in combination with flutamide. Data presented as mean ± SD. n=3, * $p<0.05$, ** $p<0.01$.

Testosterone promotes the translocation of ABCA1 protein toward the cell membrane.

Approximately 50% of control cells displayed ABCA1 protein in the perinuclear region (Fig. 5 *D* and representative image in *A*), almost one quarter displayed ABCA1 protein throughout the cell in cytoplasm, and a small percentage displayed ABCA1 protein at the cell membrane (Fig. 5 *D*). Following exposure to 10 nM testosterone there was a significant drop in the number of cells displaying ABCA1 protein within the perinuclear region (n = 8; Fig. 5 *D*) and a significant increase in the number of cells displaying ABCA1 at the cell membrane (n = 8; Fig. 5 *D* and representative image in *C*). A small increase in the number of cells displaying ABCA1 protein throughout the cell in the cytoplasm was also seen in cells treated with testosterone (n = 8; Fig. 5 *D* and representative image in *B*).

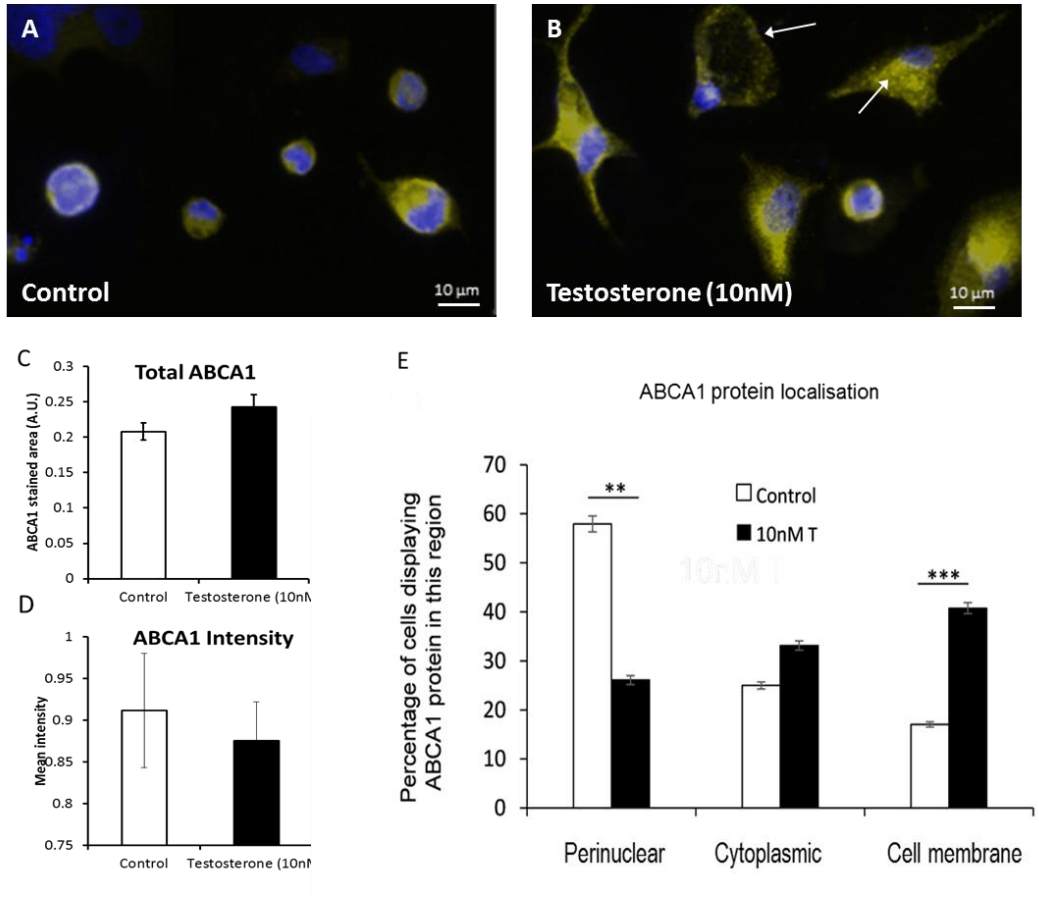

Figure 5: Testosterone promotes the translocation of ABCA1 protein toward the cell membrane in THP-1 macrophages. Representative fluorescence microscopy images reveal predominant localisation of ABCA1 protein (yellow) to the perinuclear region in a control cell (A). Cells treated with testosterone 10 nM (T) demonstrate increased localisation of ABCA1 to the membrane and cytoplasm of the cell with the presence of cytoplasmic vesicles observed (B). White arrows indicate areas of high ABCA1 protein accumulation. Scale bar, 10 µm. Semi-quantitative total ABCA1 expression as indicated by field of view fluorescence area (C) and mean fluorescence pixel intensity (D) compares control (white bars) and T treated (black bars) cells. (E) Percentage of cells displaying ABCA1 protein in the perinuclear region, cytoplasm and at the cell membrane is altered between control and T treated cells. Data presented as mean ± SEM. n=3, * $p<0.05$, ** $p<0.01$, *** $p<0.005$.

Discussion

Low levels of testosterone are associated with increased incidence of CVD in men and TTh has been shown in some studies to reduce several risk factors that contribute to the development of atherosclerosis [21]. Here we provide evidence that testosterone may exert some of its anti-atherogenic properties through the stimulation of LXRα-mediated cholesterol clearance in macrophages. Testosterone stimulates expression of the *LXRα* gene in human THP-1 monocyte-derived macrophages, leading to an increase in LXRα protein within the cell. LXRα functions as a master metabolic regulator controlling the transcription of many genes encoding proteins involved in cholesterol and lipid metabolism. Indeed, the LXRα-target genes ABCA1, APOE, SREBF1, and FAS, were also upregulated. Increased expression of these targets was dependent upon nuclear receptor activity as LXRα antagonism abolished testosterone effects. Furthermore, using the AR blocker flutamide, and a cell line which does not express the AR we demonstrate that testosterone acts genomically via AR to increase LXRα and related target gene expression. Functionally, testosterone promoted cholesterol clearance from cholesterol-loaded THP1 macrophages, an effect we suggest is via increased ABCA1 translocation to the cell membrane and potentially as a result of AR-mediated LXR actions on ABCA1 and ApoE expression.

Within the early atherosclerotic plaque, a key feature of macrophages is their ability to internalise modified low-density lipoprotein and promote removal of excess cholesterol from the developing atheroma. As atherosclerosis progresses, there is a deregulation of cholesterol uptake and reverse transport by macrophages leading to the development of lipid laden foam cells. Analogous to the present study, it has been previously shown that primary human monocyte-derived macrophages treated with testosterone demonstrated reduced cholesterol accumulation, a mechanism considered to be due to increased cholesterol efflux and via upregulation of scavenger receptor-1B (a membrane receptor involved in the binding of HDL) which consequently activates RCT [26]. No effect was observed on the expression of ABCA1 although translocation to the membrane was not investigated. Similarly, the

induction of foam cell formation by oxLDL has been shown to be inhibited by DHT in cultured macrophages [27]. Whether this was due to enhanced cholesterol processing and efflux was not clarified.

Many studies provide strong evidence that ABCA1-mediated cholesterol efflux from macrophages has atheroprotective properties [34-36]. Furthermore in humans, mutations of ABCA1 lead to Tangier disease which is characterised by severe HDL deficiency and severe generalised atherosclerosis [37]. Clinically, testosterone therapy in hypogonadal men is often confounding in studies, either reducing, having no effect or raising circulating HDL levels [38]. Lower HDL may putatively indicate pro-atherogenic actions, but it is conversely considered that testosterone intensifies the RCT process thus reducing circulating HDL, conditionally dependent upon subsequent hepatic uptake and catabolism of HDL cholesteryl esters, and thereby exerting an overall anti-atherogenic rather than a pro-atherogenic effect [26]. Furthermore, HDL efflux capacity can differ markedly among individuals with identical HDL-c concentrations an effect considered via ABCA1 activity [39] and a large population study identified efflux capacity a better predictor of existing coronary artery disease than HDL-c concentration [40]. The present study indicates that testosterone increases ABCA1 expression in macrophages and suggests this may be via activation of LXR as co-treatment with LXR antagonist, 5CPPSS-50, abrogated the effect. Similarly, previous studies have shown that LXR activation time- and dose-dependently increases ABCA1 levels in macrophages [41,29,42]. Due to the known inherent instability of ABCA1, these studies suggest that as well as transcriptional regulation, LXRs may also promote stabilisation of the cholesterol transporter and therefore the potential for prolonged cholesterol efflux. Indeed, we observed testosterone induced upregulation of ABCA1 levels at 48 and 72h beyond gene expression changes suggesting that testosterone or androgen receptor signalling may additionally promote ABCA1 stabilisation via LXR activation, although the underlying mechanism for this remains unclear.

Evidence that LXR activation via alternate nuclear signalling pathways can lead to increased macrophage-cholesterol efflux is apparent in studies utilising agonists of peroxisome proliferator-activated receptor alpha (PPARα) and PPARγ which also upregulate LXR [43,44]. What was apparent in

the present study was that despite no increase in protein expression following 24h testosterone treatment of THP1 macrophages, a translocation of ABCA1 from perinuclear regions occurred increasing ABCA1 density on the plasma membrane to potentially enhance intracellular cholesterol transport towards the membrane ready for efflux. Indeed, testosterone promoted a greater than two-fold induction of cholesterol clearance when compared to control cells within a 24 hour period.

In addition to ABCA1, the present study shows that testosterone increases macrophage APOE expression in an LXRα-dependent manner as a potential mechanism to further promote RCT. Zanotti et al. [45] demonstrate that macrophage, but not systemic, apoE is necessary for macrophage RCT in vivo. Macrophages isolated from apoE−/− mice effluxed significantly less cholesterol than WT macrophages, even in the absence of extracellular acceptors, suggesting that apoE selectively expressed in macrophages may efficiently improve RCT in vivo by enhancing cholesterol availability for transport to liver and faeces [45]. Indeed, reduced macrophage-derived cholesterol was observed in plasma, liver, and faeces with concomitant increased atherosclerosis in healthy mice reconstituted with apoE-deficient macrophages, independently of changes in the plasma lipoprotein profile [46]. Therefore, the increased macrophage ABCA1 and ApoE expression observed in the present study may have resulted in enhanced cholesterol efflux implicating a potentially beneficial effect of testosterone on atherosclerosis.

We have previously shown in vivo that testosterone increases *LXRα* and associated targets *APOE* and ABCA1 expression in the liver of testicular feminized (Tfm) mice, which have a non-functional androgen receptor and low circulating testosterone, following a high-cholesterol diet [47,33]. Marked hepatic lipid accumulation was apparent in the Tfm mouse but not in the wild-type and testosterone treated Tfm mice. The pathophysiology of hepatic steatosis is considered analogous to the processes that occur in atherosclerosis and indeed early atherosclerosis was reduced in testosterone-treated Tfm mice suggesting that testosterone influences lipid and cholesterol metabolism and/or transport at these sites. Contrary to the present study however, this previous investigation suggests that the beneficial influence of

testosterone may act, at least in part, via AR-independent mechanisms indicating differences in either testosterone or LXRα-regulation across different tissues and/or species [47].

Beyond effects on RCT, we show that testosterone partially increases targets of fatty acid metabolism *SREBF1* and *FAS* in THP-1 macrophages via LXR activation. Cellular fatty acid and triacylglycerol biosynthesis, upregulated by SREBF1 and FAS, triggers and is triggered by an inflammatory response. Several studies show that enhanced fatty acid synthesis in macrophages is linked to inflammation, although the dynamic relationship is not fully elucidated [48-50]. Srebf-1a isoform and LXRα are both highly expressed in macrophages and known regulators of cytokine release. Specifically, Srebf-1 KO macrophages exhibit reduced production of anti-inflammatory FAs resulting in late hyper-inflammatory states and inhibited resolution of inflammatory responses [51,52]. While the present study did not specifically investigate inflammation, it could be postulated that by altering macrophage lipid metabolism testosterone may improve the inflammatory environment of the atherosclerotic plaque. Testosterone has been previously shown to alter SREBF-1 expression in liver [53], as well as in subcutaneous adipose tissue [47] and prostate cancer cell lines [54], although the involvement of LXR in these studies was not assessed.

The influence of LXR in testosterone actions in the current study was demonstrated using a pan-LXR antagonist which gives rise to the possibility that the increase observed in target gene expression may be mediated by LXRβ or a combination of both LXR subtypes. Evidence from previous studies however suggests that LXRα plays a more dominant role in the regulation of cholesterol and fatty acid metabolism than LXRβ. For example, deletion of LXRα but not LXRβ in APOE-knockout mice leads to an accumulation of cholesterol as well as atherosclerosis and the expression of genes involved in cholesterol and fatty acid metabolism, such as *SREBF1* and *FAS*, are additionally impaired in LXRα-knockout mice but not in LXRβ-knockout mice [55-57]. In the present study, co-treatment of THP-1 cells with testosterone and pan-LXR agonist, T0901317, did not induce any further response in LXR-dependent gene expression than treatments alone suggesting competing pathways of activation. Furthermore,

testosterone has no effect on gene expression in THP-1 monocyte-derived macrophages when the AR is blocked or deficient demonstrating that testosterone requires a functional AR to stimulate LXRα and LXR-associated target expression in these cells. The exact mechanisms of these nuclear receptor interactions and cross-talk warrant further investigation.

While THP-1 macrophages are frequently used as a model for investigating human-derived macrophage function and have been previously utilised to study LXR ligand responses [58,59], isolated primary human male monocytes should be further investigated to translate current findings to the clinical situation and highlight any potential role of testosterone and LXR in atherosclerosis. Furthermore, caution should be applied when interpreting statistical significance as a representation of true biological meaning in small data sets such as those presented in the current study. Additionally, the present study assessed cholesterol clearance from DHE loaded macrophages as a potential indication of cholesterol efflux in the absence of cholesterol acceptors as demonstrated previously [45]. However, further investigation is required to confirm detailed mechanisms of testosterone action on cholesterol efflux pathways.

Conclusion

This study suggests that testosterone can stimulate cholesterol clearance from macrophages potentially through membrane translocation of ABCA1 and via LXRα. Testosterone additionally influenced the expression of cholesterol and lipid regulating proteins including ApoE via AR-dependent mechanisms, which warrants further investigation to uncover the underlying mechanisms of action. This study therefore highlights some important potential actions of testosterone which may explain how TTh reduces vascular lipid accumulation in animal models and surrogate markers of atherosclerosis in some clinical studies to improve cardiovascular risk in hypogonadal men.

Conflict of interest statement

ELK has received research funding from Bayer Healthcare AG (Bayer) was supported by BayerHealthcare, UK and Barnsley Hospital Endocrinology Research Fund for the current study. DMK has received research grants from Bayer, speaker honoraria from Bayer and funding to attend conferences from Bayer and Novo Nordisk. THJ has received a research grants from Bayer Pharma AG and Besins Healthcare, consultancy for Clarus, Merck, speaker honoraria for educational lectures and advisory boards and travel grants for Bayer, Besins Healthcare and Prostrakan.

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