Vitamin D receptor agonist doxercalciferol modulates dietary fat-induced renal disease and renal lipid metabolism

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Wang XX, Jiang T, Shen Y, Santamaria H, Solis N, Arbeeny C, Levi M. Vitamin D receptor agonist doxercalciferol modulates dietary fat-induced renal disease and renal lipid metabolism. Am J Physiol Renal Physiol 300: F801–F810, 2011. First published January 5, 2011; doi:10.1152/ajprenal.00338.2010.—Diet-induced obesity (DIO) and insulin resistance in mice are associated with proteinuria, renal mesangial expansion, accumulation of extracellular matrix proteins, and activation of oxidative stress, proinflammatory cytokines, profibrotic growth factors, and the sterol regulatory element binding proteins SREBP-1 and SREBP-2, that mediate increases in fatty acid and cholesterol synthesis. The purpose of the present study was to determine whether treatment of DIO mice with the vitamin D receptor (VDR) agonist doxercalciferol (1α-hydroxyvitamin D2) prevents renal disease. Our results indicate that treatment of DIO mice with the VDR agonist decreases proteinuria, podocyte injury, mesangial expansion, and extracellular matrix protein accumulation. The VDR agonist also decreases macrophage infiltration, oxidative stress, proinflammatory cytokines, and profibrotic growth factors. Furthermore, the VDR agonist also prevents the activation of the renin-angiotensin-aldosterone system including the angiotensin II type 1 receptor and the mineralocorticoid receptor. An additional novel finding of our study is that activation of VDR results in decreased accumulation of neutral lipids (triglycerides and cholesterol) and expression of adipophillin in the kidney by decreasing SREBP-1 and SREBP-2 expression and target enzymes that mediate fatty acid and cholesterol synthesis and increasing expression of the farnesoid X receptor. This study therefore demonstrates multiple novel effects of VDR activation in the kidney which prevent renal manifestations of DIO in the kidney.

diabetes; proteinuria; fatty acid and cholesterol synthesis

DECREASED SERUM CONCENTRATIONS of 1,25-dihydroxyvitamin D (calcitriol), the hormonally active form of vitamin D, and of its precursor 25-hydroxyvitamin D are reported with increasing frequency in subjects with obesity and type 1 or type 2 diabetes (5, 16, 20, 29, 41, 59). Since vitamin D plays a key role in metabolic homeostasis (11), the vitamin D deficiency that is observed in these patients is believed to contribute to increased risk of diabetes complications and mortality.

Treatment with vitamin D receptor (VDR) agonists has been proposed to play an important role in the prevention of diabetic complications, including cardiovascular disease and diabetic nephropathy (7, 37, 56). Recent studies, using mice with type 1 and type 2 diabetes, demonstrated that treatment with a VDR agonist decreases proteinuria and glomerulosclerosis (31, 51, 52). These effects are mediated, in part, by the inhibition of the renin and NF-κB signaling pathways, leading to anti-inflammatory and anti-fibrotic effects in the kidney. In contrast, VDR knockout mice are more susceptible to diabetic kidney injury (25, 62). VDR agonists have also been shown to decrease proteinuria in human subjects with chronic kidney disease (2, 3, 13) and IgA nephropathy (47) and improve survival in patients with late-stage end-stage renal disease (55).

In addition to the well-known effects of diabetes mellitus in causing renal disease, we determined that diet-induced obesity (DIO) and insulin resistance also result in proteinuria. This is associated with renal mesangial expansion, accumulation of extracellular matrix proteins, and activation of oxidative stress, proinflammatory cytokines, and profibrotic growth factors. A direct causative role for SREBPs and renal lipids is demonstrated in SREBP-1c knockout mice where there are no changes in serum glucose or lipids, increased renal expression of SREBP-1a results in activation of proinflammatory cytokines, profibrotic growth factors, and results in mesangial expansion, accumulation of extracellular matrix proteins, and increased proteinuria (46).

Among clinically used VDR agonists, doxercalciferol (1α-hydroxyvitamin D2) and paricalcitol (19-nor-1,25-dihydroxyvitamin D2) demonstrated a lower propensity to cause hypercalcemia and hypercalciuria (15) than calcitriol. This property will make it possible the use of higher tolerable doses for a greater likelihood of benefit. Doxercalciferol is a prohormone, requiring hepatic metabolism to the active metabolite 1,25-dihydroxyvitamin D2. The half-life of 1,25-dihydroxyvitamin D2 is longer than that of calcitriol or paricalcitol in human subjects (4), which provides flexibility in drug administration in animal studies.

The purpose of the present study was to determine whether treatment of mice with DIO with the VDR agonist doxercalciferol prevents renal disease and to assess the underlying mechanisms for this effect, including decreases in oxidative stress, proinflammatory cytokines, and profibrotic growth fac-

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Table 1. Dose effects of doxercalciferol on serum calcium and phosphorus

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>25 ng/kg</th>
<th>125 ng/kg</th>
<th>500 ng/kg</th>
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<tbody>
<tr>
<td>Serum Ca, mg/dl</td>
<td>9.14 ± 1.26</td>
<td>8.93 ± 0.87</td>
<td>8.37 ± 1.37</td>
</tr>
<tr>
<td>Serum Pi, mg/dl</td>
<td>9.89 ± 0.42</td>
<td>10.05 ± 0.79</td>
<td>9.75 ± 0.39</td>
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Data are means ± SE (n = 6 mice in each group).

tors. Furthermore, we asked whether the beneficial effects of doxercalciferol are also associated with modulation of renal expression of SREBPs and renal lipid accumulation.

METHODS AND MATERIALS

Animals and treatments. Male NON/LtJ (NON) mice were obtained from The Jackson Laboratories (Bar Harbor, ME). They were maintained on a 12:12-h light-dark cycle and fed a control low-fat diet [LF; D12450B; 10 kcal% fat (lard)] or a high-fat diet [HF; D12492; 60 kcal% fat (lard)] obtained from Research Diets (New Brunswick, NJ) for 28 wk. They were treated with l) vehicle (80% propylene glycol/20% PBS) or 2) 125 ng/kg body wt doxercalciferol, administered intraperitoneally, 3 times a wk. We studied n = 12 mice in each experimental group for biochemical studies (the kidneys from 2 mice were combined for the RNA and protein studies). In addition, we studied n = 6 mice in each experimental group for the histology and immunofluorescence studies.

In preliminary studies, we determined that this dose of doxercalciferol did not cause increases in serum calcium or phosphorus (Table 1) while it induced an increase in renal VDR gene expression and as expected a decrease in 1-α-hydroxylase and an increase in 24-α-hydroxylase (Fig. 1). The animal studies and the protocols were approved by the Institutional Review Boards at the University of Colorado Denver.

Blood and urine chemistries. Blood glucose levels were measured with a Glucometer Elite XL (Bayer, Tarrytown, NY). Plasma triglyceride and cholesterol were measured with kits from Wako Chemical (Richmond, VA). Urine albumin and creatinine concentrations were determined using kits from Exocell (Philadelphia, PA). Results are expressed as the urine albumin-to-creatinine ratio (μg/mg).

RNA extraction and quantitative real-time PCR. Total RNA was isolated from the kidneys using SV total RNA isolation system from Promega (Madison, WI), and cDNA was synthesized using reverse transcript reagents from Bio-Rad Laboratories (Hercules, CA). The mRNA level was quantified using Bio-Rad iCycler real-time PCR machine. Cyclophilin was used as internal control, and the amount of RNA was calculated by the comparative threshold cycle method as recommended by the manufacturer. All the data were calculated from triplicate reactions. Primer sequences used have been described previously (21–24, 40, 46, 54) and are available from the authors upon request.

Protein electrophoresis and Western blotting. Equal amounts of protein samples were subjected to SDS-PAGE, and they were then transferred to nitrocellulose membranes. After being blocked with 5% fat-free milk powder in 0.1% Tween 20 in Tris-buffered saline (20 mmol/l Tris-HCl and 150 mmol/l NaCl, pH 7.4), the blots were incubated with antibodies against l) podocin (1:200; G20; Santa Cruz Biotechnology, Santa Cruz, CA), or 2) WT-1 (1:200; C19; Santa Cruz Biotechnology), or 3) synaptophysin (1:200; Sigma, St. Louis, MO), or 4) fibronectin (1:200; Sigma). Corresponding secondary antibodies were visualized using enhanced chemiluminescence (Pierce, Bradford, IL). The signals were quantified with a chemiluminescence detector and the accompanying densitometry software (UVP, Upland, CA).

Lipid extraction and measurement of lipid composition. Lipids from the kidneys were extracted by the method of Bligh and Dyer, as we previously described (21–24, 40, 46, 54). Triglyceride and cholesterol content was measured using kits from Wako Chemicals.

NF-κb transcriptional activity assay. Nuclear extracts from kidney tissue were obtained as previously described (21–24, 40, 46, 54) and they were used for the measurement of NF-κb transcriptional activity with the kit from Marligon Biosciences (Rockville, MD).

Oxidized protein analysis. The amount of oxidized proteins in kidney homogenates was determined by using an OxyElisa Oxidized Protein Quantitation Kit (Millipore, Billerica, MA) according to the manufacturer’s instructions.

Histology, oil red O staining, and immunofluorescence microscopy. Sections (4-μm-thick) cut from 10% formalin-fixed, paraffin-embedded kidney samples were used for periodic acid Schiff (PAS) staining. Frozen sections were used for oil red O staining (21–24, 40, 46, 54). Frozen sections were also used for immunostaining for synaptophysin (Sigma), fibronectin (Sigma), and CD68 (Serotec, Raleigh, NC) and

Fig. 1. Treatment of NON/LtJ (NON) mice with doxercalciferol regulates renal vitamin D receptor (VDR) and vitamin D (VidD)-metabolizing enzymes. The mRNA expression level of VDR, 1,25-dihydroxyvitamin D3 24-hydroxylase, 25-hydroxyvitamin D3–1α-hydroxylase, and TRPV5 was determined by quantitative real-time PCR. *P < 0.05 vs. NON mice on low-fat (LF) diet with vehicle (Veh), **P < 0.05 vs. NON mice on high-fat (HF) diet with Veh, #P < 0.05 vs. NON mice on LF diet with doxercalciferol (n = 6 mice per group).

Artificial intelligence is a rapidly advancing field that has the potential to transform many aspects of our lives, from healthcare to transportation and beyond. As with any emerging technology, there are both opportunities and challenges that must be addressed to ensure its responsible development and deployment. In healthcare, for example, AI has the potential to improve diagnosis and treatment by enabling more accurate predictions of patient outcomes and guiding personalized interventions. However, concerns have been raised about the potential for AI to exacerbate existing biases in medical data and decision-making processes. Similarly, in transportation, autonomous vehicles promise increased safety and efficiency, but they also raise questions about liability, privacy, and public acceptance. To address these challenges, it is essential to foster interdisciplinary collaboration among researchers, policymakers, and ethicists, and to engage the public in ongoing dialogue about the implications of AI for society. By doing so, we can work to ensure that the benefits of AI are realized while minimizing its risks and unintended consequences.
imaged with a laser-scanning confocal microscope (LSM 510, Zeiss, Jena, Germany).

Statistical analysis. Results are presented as means ± SE for at least three independent experiments. Data were analyzed by ANOVA and Student-Newman-Keuls tests for multiple comparisons or by Student’s t-test for unpaired data between two groups. Statistical significance was accepted at the P < 0.05 level.

RESULTS

Treatment of NON mice fed a HF diet with doxercalciferol induced upregulation of VDR in the kidney. Treatment of NON mice fed a HF diet with doxercalciferol induced increased expression of VDR mRNA level (Fig. 1). There was a parallel decrease in 25-hydroxyvitamin D3-1α-hydroxylase mRNA and an increase in 1,25-dihydroxyvitamin D3 24-hydroxylase mRNA level (Fig. 1). Doxercalciferol also induced renal expression of TRPV5 (Fig. 1).

Treatment of NON mice fed a HF diet with doxercalciferol improved proteinuria, prevented loss of podocytes, and accumulation of extracellular matrix proteins. Relative to the NON mice fed a LF diet, NON mice fed a HF diet had increased urinary albumin-to-creatinine ratio and proteinuria, which were prevented by treatment with doxercalciferol (Fig. 2A). PAS staining of glomerular sections revealed that NON mice fed a HF diet had mesangial expansion and increase in intensity of PAS staining. Treatment with doxercalciferol decreased mesangial expansion and intensity of PAS staining (Fig. 2B).

Treatment of NON mice fed a HF diet with doxercalciferol prevented podocyte injury. NON mice fed a HF diet had decreases in WT1 and podocin protein abundance. Treatment with doxercalciferol prevented the decreases in WT1 and podocin as well as synaptopodin immunostaining (Supplementary Fig. 1, A–C; the online version of this article contains supplemental data).

Treatment of NON mice fed a HF diet with doxercalciferol prevented accumulation of extracellular matrix proteins. HF diet caused increases in fibronectin mRNA and immunostaining in NON mice. Treatment with doxercalciferol decreased glomerular and tubulointerstitial accumulation of fibronectin immunostaining, which was also con-

**Fig. 2.** A: treatment of NON mice with doxercalciferol significantly decreases albuminuria in NON mice fed a HF diet. Urinary albumin excretion is expressed as urinary albumin-to-creatinine ratio. *P < 0.05 vs. NON mice on LF diet with Veh. **P < 0.05 vs. NON mice on HF diet with Veh (n = 6 mice per group). B: representative periodic acid Schiff (PAS) staining of kidney sections (×400) shows that treatment of NON mice with doxercalciferol decreases mesangial expansion and matrix accumulation in NON mice fed a HF diet.
firmed by Western blotting and real-time quantitative PCR (Supplementary Fig. 2, A–C). Similarly, treatment with doxercalciferol decreased the mRNA abundance of type I collagen, type IV collagen, and α-smooth cell actin (Supplementary Fig. 2, D–F).

Treatment of NON mice fed a HF diet with doxercalciferol prevented expression of profibrotic growth factors. NON mice fed a HF diet had increased expression of transforming growth factor (TGF)-β and plasminogen activator inhibitor-1 (PAI-1) mRNA expression. Treatment with doxercalciferol resulted in decreased expression of TGF-β, PAI-1, and connective tissue growth factor (CTGF) (Supplementary Fig. 3, A–C).

Treatment of NON mice fed a HF diet with doxercalciferol prevented increased expression of the renin-angiotensin-aldosterone system. In agreement with earlier reports in mice with type 1 diabetes mellitus or type 2 diabetes mellitus (31, 51, 52), treatment of NON mice fed a HF diet with doxercalciferol resulted in decreased expression of renin (Fig. 3A). Furthermore, we determined that doxercalciferol also decreased the expression of the angiotensin II type 1 receptor (AT1R; Fig. 3B) and the mineralocorticoid receptor (MR; Fig. 3C). The decrease in MR occurs in association with a decrease in hydroxy steroid 11-β-dehydrogenase isozyme 2 (Hsd11b2; Fig. 3D).

Treatment of NON mice fed a HF diet with doxercalciferol prevented macrophage infiltration, decreased NF-κB activity, and prevented expression of proinflammatory cytokines. NON mice fed a HF diet had increased expression of CD68-positive macrophages. Treatment with doxercalciferol prevented the infiltration of the kidney with CD68-positive macrophages (Fig. 4A). NON mice fed a HF diet also had increased NF-κB transcriptional activity, which was decreased by treatment (Fig. 4B). In addition, NON mice fed a HF diet also had increased MCP-1 expression as well as other proinflammatory cytokines and chemokines including Cox-2, RAGE, and TLR-4, which were also decreased by doxercalciferol (Fig. 4, C–F).

Treatment of NON mice fed a HF diet with doxercalciferol prevented expression of oxidative stress markers and oxidation of proteins. NON mice fed a HF diet had increased expression of NADPH oxidase including Nox-4, p47-phox, and Nox-2. Treatment with doxercalciferol resulted in decreased expression of NADPH oxidase including Nox-4, p47-phox, and Nox-2. These changes were associated with decreased abundance of oxidized proteins in the kidney (Fig. 5).

Treatment of NON mice fed a HF diet with doxercalciferol prevented the increase in renal lipid accumulation. NON mice fed a HF diet had a marked increase in renal oil red O staining, which corresponds to accumulation of neutral lipids including triglycerides and cholesterol esters. Treatment with doxercalciferol prevented the increase in renal lipid accumulation (Fig. 6A). In addition, doxercalciferol also decreased the expression of adipophilin, which is a marker of lipid droplets (Fig. 6B). Studies performed to determine the mechanisms of decreased lipid accumulation revealed that treatment with doxercalciferol decreased mRNA abundance of SREBP-1c and its target enzymes ACL, ACC, and FAS, which are the major mediators of fatty acid and triglyceride synthesis (Fig. 7A). At the same time, doxercalciferol treatment increased PPAR-α, mediator of fatty acid oxidation, and also decreased CD36, mediator of fatty acid uptake (Fig. 7B). In addition, doxercalciferol also decreased the mRNA abundance of SREBP-2 and HMG CoA reductase, which are mediators of cholesterol synthesis and LDL receptor, mediator of cholesterol uptake (Fig. 7C). Doxercalciferol, therefore, has multiple effects on pathways of renal fatty acid and cholesterol metabolism that results in decreased renal lipid accumulation.

Treatment of NON mice fed a HF diet with doxercalciferol resulted in upregulation of renal farnesoid X receptor activation. Since the effects of activation of VDR with doxercalciferol are quite similar to the effects that we saw with treatment of diabetic mice with the bile acid-activated nuclear receptor farnesoid X receptor (FXR) (23, 52, 53), we deter-

Fig. 3. Treatment with doxercalciferol decreases mRNA abundance of renin (A), angiotensin II type 1 receptor (AT1R; B), mineralocorticoid receptor (MR; C), and hydroxysteroid 11-β-dehydrogenase isozyme 2 (Hsd11b2; D) in NON mice. *P < 0.05 vs. NON mice on LF diet with Veh. **P < 0.05 vs. NON mice on HF diet with Veh (n = 6 mice per group).
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Fig. 4. Treatment of NON mice fed a HF diet with doxercalciferol prevents macrophage infiltration, decreases NF-κB activity, and prevents expression of proinflammatory cytokines. A: Immunofluorescence staining of kidney sections for CD68. B: NF-κB DNA binding activity. C–F: Analysis of mRNA expression of renal inflammatory markers by quantitative real-time PCR for Cox-2 (C), receptor for advanced glycation products (RAGE; D), toll-like receptor-4 (TLR-4; E), and monocyte chemotactic protein-1 (MCP-1; F). *P < 0.05 vs. NON mice on LF diet with Veh. **P < 0.05 vs. NON mice on HF diet with Veh (n = 6 mice per group).

Discussion

VDR agonists have been shown to play a protective role in several nondiabetic models of renal disease, including in mice and rats with Heyman Nephritis (6), anti-thy-1 model of mesangial proliferative glomerulonephritis (33, 35, 38), subtotal nephrectomy (18, 28, 44), puromycin aminoglycoside-induced podocyte damage (57), and unilateral ureteral obstruction (48). VDR agonists also are protective in mouse models of diabetes mellitus. The studies in mice with type 1 diabetes mellitus and type 2 diabetes mellitus showed that the renal protective effects of VDR agonists are mediated by inhibition or antagonism of the renin-angiotensin system (14, 27, 32, 39, 58, 61) and NF-κB (9, 50, 63), which are major mediators of increased intraglomerular pressure, podocyte damage, oxidative stress, inflammation, and fibrosis. In addition, VDR agonists activate hepatocyte growth factors and prevent renal interstitial myofibroblast activation (30, 49). In contrast, VDR knockout mice have been shown to be more susceptible to streptozotocin-induced...
diabetic kidney disease in part via activation of the renin-angiotensin system (62). In this regard, VDR agonists in combination therapy with an angiotensin-converting enzyme inhibitor or an angiotensin II type 1 receptor blocker have synergistic effects in development of kidney disease in rats with subtotal nephrectomy (36) and mice with diabetes (10, 60, 64).

NON mice are an inbred strain, originally developed as a control strain for the well-known type 1 diabetes model of NOD/LtJ. NON mice have genes predisposing to type 2 diabetes, evidenced by early impaired glucose tolerance, maturity-onset diabetes and obesity, and development of kidney glomerulosclerotic lesions (1). Therefore, NON mice represent a new DIO model for researching obesity-induced diabetes.

The current study examined the effects of a VDR agonist in a model of DIO, which has been associated with mesangial expansion and proteinuria mediated by increased renal expression of oxidative stress, inflammation, fibrosis, as well as accumulation of lipids (24, 53). Our results in this model of DIO in NON mice are in agreement with previous studies in type 1 and type 2 diabetes mellitus, where VDR-activating agonists have been shown to prevent proteinuria, podocyte injury, mesangial expansion, accumulation of extracellular matrix proteins, infiltration with macrophages, and activation of markers of oxidative stress, inflammation, and fibrosis.

Although in the current study we used doxercalciferol, we would expect to see similar effects with other active VDR agonists such as calcitriol and paricalcitol as was seen in
many parallel studies with different VDR agonists (14, 31, 32, 60 – 64). In the current study, we demonstrated that doxercalciferol treatment increased VDR expression and triggered the feedback regulatory loop to control the renal production of active vitamin D by suppressing 1α-hydroxylase and stimulating deactivating enzyme 24α-hydroxylase. In addition, doxercalciferol induced renal expression of TRPV5, which is an important VDR-dependent transepithelial calcium transport protein (12). These lines of evidence indicate a VDR activation role for doxercalciferol. At present, there is no evidence that VDR agonists including doxercalciferol exert VDR-independent effects. However, this possibility cannot be ruled out without a careful study using VDR-null mice.

A novel finding of our study is that activation of VDR results in decreased accumulation of neutral lipids (triglycerides and cholesterol) and expression of adipophilin in the kidney. Analysis of lipid metabolism pathways in the kidney indicates that the decrease in triglycerides is mediated by vitamin D-induced 1) decrease in fatty acid synthesis as a result of decreases in SREBP-1-dependent ACL, ACC, and FAS, 2) increase in fatty acid oxidation as a result of...
increased PPAR-α, and 3) decrease in fatty acid uptake as a result of decrease in CD36. The decrease in cholesterol is mediated by vitamin D-induced 1) decrease in cholesterol synthesis as a result of decreases in SREBP-2 and HMG CoA reductase and 2) decrease in cholesterol uptake as a result of a decrease in LDL receptor. Whether VDR transactivation is directly involved in the renal lipid-lowering effects is not clear. However, we found that activation of VDR results in increased expression of the bile acid-activated nuclear hormone receptor FXR, which suggests a mechanism whereby VDR may act through FXR to partially mediate the beneficial effects of VDR agonists in the kidney, including the regulation of renal lipid metabolism. Previous studies indicated that bile acids activated VDR (17, 34). Recent studies also indicate that VDR agonists can increase portal bile acid concentration and trigger FXR-like effects in the liver and the intestine in rats (8). In further support of our findings, VDR knockout mice have decreased FXR expression in the ileum, and treatment of mice with calcitriol increased FGF15 expression in the ileum, which is a known FXR target (43). Clearly, our results warrant further investigation into how these two nuclear receptors cross-talk.

As shown for the protective role of FXR in DIO model (53), VDR, as other nuclear receptors studied, exhibits multifaceted effects in the protection of renal disease in the DIO model. Other than its effects on fibrosis, inflammation, and oxidative stress, which are major pathogenic factors in DIO-related renal disease, VDR activation also regulates renin-angiotensin-aldosterone system, which plays a major role in modulation of kidney disease (26, 42, 45).

In summary, our findings in this study with doxercalciferol identify the effects of VDR activation on the prevention of DIO-induced nephropathy and potential metabolic pathways that may mediate the DIO-related alterations in renal lipid metabolism, fibrosis, inflammation, and oxidative stress. The multiple beneficial effects of VDR activation in the kidney are summarized in Fig. 9.

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Fig. 8. Treatment with doxercalciferol increases farnesoid X receptor (FXR) expression in NON mice fed with HF diet. **P < 0.05 vs. NON mice on HF diet with Veh (n = 6 mice per group).

Fig. 9. Summary of effects of VDR activation on the prevention of diet-induced obesity (DIO)-induced nephropathy in the NON mice.
DISCLOSURES

C. Arbeeny is with Genzyme. Other authors declared no competing interests.

REFERENCES


