

## Fibronectin Type III Domain–Containing Protein 5 rs3480 A>G Polymorphism, Irisin, and Liver Fibrosis in Patients With Nonalcoholic Fatty Liver Disease

Salvatore Petta,<sup>1</sup> Luca Valenti,<sup>2</sup> Gianluca Svegliati-Baroni,<sup>3,4</sup> Massimiliano Ruscica,<sup>5</sup> Rosaria Maria Pipitone,<sup>1</sup> Paola Dongiovanni,<sup>2</sup> Chiara Rychlicki,<sup>3,4</sup> Nicola Ferri,<sup>6</sup> Calogero Cammà,<sup>1</sup> Anna Ludovica Fracanzani,<sup>2</sup> Irene Pierantonelli,<sup>3,4</sup> Vito Di Marco,<sup>1</sup> Marica Meroni,<sup>2</sup> Debora Giordano,<sup>3,4</sup> Stefania Grimaudo,<sup>1</sup> Marco Maggioni,<sup>7</sup> Daniela Cabibi,<sup>8</sup> Silvia Fargion,<sup>2</sup> and Antonio Craxi<sup>1</sup>

<sup>1</sup>Section of Gastroenterology, Di.Bi.M.I.S., University of Palermo, 90100 Palermo, Italy; <sup>2</sup>Department of Pathophysiology and Transplantation, Università degli Studi, Internal Medicine, Fondazione Ca' Granda IRCCS Ospedale Maggiore Policlinico, 20100 Milan, Italy; <sup>3</sup>Department of Gastroenterology, Polytechnic University of Marche, 60100 Ancona, Italy; <sup>4</sup>Obesity Center, Polytechnic University of Marche, 60100 Ancona, Italy; <sup>5</sup>Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli Studi di Milano, 20100 Milan, Italy; <sup>6</sup>Department of Pharmacological Sciences, Padova University, 35100 Padua, Italy; <sup>7</sup>Pathology, Fondazione Ca' Granda IRCCS Ospedale Maggiore Policlinico, 20100 Milan, Italy; and <sup>8</sup>Department of Science for Promotion of Health and Mother and Child Care, Section of Human Pathology, University of Palermo, 90127 Palermo, Italy

**Context:** Contrasting data have been reported on the role of irisin, a novel myokine encoded by the fibronectin type III domain-containing protein 5 (*FNDC5*) gene, in nonalcoholic fatty liver disease (NAFLD) pathogenesis. We tested in patients with suspected nonalcoholic steatohepatitis (NASH) the association of *FNDC5* variants, hepatic expression, and circulating irisin with liver damage (F2 to F4 fibrosis as main outcome). We also investigated whether irisin modulates hepatocellular fat accumulation and stellate cell activation in experimental models.

**Methods:** We considered 593 consecutive patients who underwent liver biopsy for suspected NASH and 192 patients with normal liver enzymes and without steatosis. *FNDC5* rs3480 and rs726344 genotypes were assessed by 5' nuclease assays. Hepatic irisin expression was evaluated in mice fed a high-fat diet or treated with CCl<sub>4</sub>. The effect of irisin was evaluated in fat-laden HepG2 hepatocytes and in hepatic stellate cells (HSCs).

**Results:** In patients at risk for NASH [odds ratio (OR) = 0.64, 95% confidence interval (CI), 0.47 to 0.87; *P* = 0.005], and more so in the high-risk subgroup of those with impaired fasting glucose/diabetes (OR = 0.44, 95% CI, 0.26 to 0.74; *P* = 0.002), the rs3480 A>G variant was independently associated with protection from F2 to F4 fibrosis. Irisin is expressed in human activated HSC, where it mediated fibrogenic actions and collagen synthesis, and is overexpressed in NAFLD patients with F2 to F4 fibrosis and CCl<sub>4</sub>-treated mice. However, Irisin does not affect fat accumulation in HepG2 and is not induced by high-fat-diet–inducing NAFLD.

**Conclusions:** The *FNDC5* rs3480 variant is associated with protection from clinically significant fibrosis in patients with NAFLD, while irisin expression is correlated with the severity of NAFLD and may be

involved in extracellular matrix deposition. These data suggest that irisin is involved in regulation of hepatic fibrogenesis. (*J Clin Endocrinol Metab* 102: 2660–2669, 2017)

**D**ue to epidemic of obesity and diabetes, nonalcoholic fatty liver disease (NAFLD) is becoming the first cause of chronic liver disease worldwide (1). This picture accounts for the dramatic increase in the proportion of hepatocellular carcinoma related to cirrhotic NAFLD (2) and for data in the last 10 years reporting NAFLD as a growing indication for liver transplantation due to end-stage liver disease (3). Two recent large-cohort studies exploring the natural history of NAFLD patients demonstrated that the severity of fibrosis is the main predictor of not only hepatic, but also extrahepatic morbidity and mortality in this clinical setting (4, 5).

The pathogenesis of NAFLD and its progression to severe stages of fibrosis is complex and not fully understood. Obesity, insulin resistance (IR), diabetes, and liver necroinflammation, as well as an imbalance in pro- and anti-inflammatory cytokines/adipocytokines (6), are believed to represent the most relevant drivers (7).

Growing evidence suggests that inherited factors (8), and in particular single-nucleotide polymorphisms (SNPs) in genes involved in lipid metabolism, as well as IR, inflammation, oxidative stress, and fibrogenesis, are involved in the susceptibility to development and progression of NAFLD (8).

Irisin is a myokine encoded by the fibronectin type III domain-containing protein 5 (*FNDC5*) gene that seems able to exert health benefits via “browning” of white adipose tissue (9). Available data suggest that irisin may also be involved in the pathogenesis of NAFLD. However, contrasting observations reported higher (10) or lower (11) serum irisin levels in NAFLD compared with individuals without steatosis. Muscle irisin expression has been directly related to IR in humans (12), whereas recombinant irisin improved steatosis in hepatocytes *in vitro* (13). Finally, whereas some variants in the *FNDC5* gene, such as rs726344, have been associated with improved insulin sensitivity, the noncoding rs3480 A>G SNP may enhance fatty deposition in human liver (12).

Having this in mind, we tested in patients with suspected nonalcoholic steatohepatitis (NASH), the progressive form of NAFLD, whether the *FNDC5* rs3480 and rs726344 variants influence histological damage (clinically significant fibrosis was main outcome). As we detected an association between the *FNDC5* genotype and liver damage, to investigate the mechanism, we examined the 1) hepatic expression and serum irisin levels in patients with NAFLD, 2) whether irisin expression is modified in experimental models of liver injury, and 3) whether irisin participates in hepatocellular fat

accumulation and has any fibrogenic actions in hepatic stellate cells (HSCs).

## Patients and Methods

### Patients

We analyzed data from 593 prospectively recruited Italian patients who underwent liver biopsy for suspected NASH—including those with F4 fibrosis—and had blood samples available for genetic analyses. Other causes of liver disease were ruled out. Patients with body mass index (BMI)  $\geq 40$  kg/m<sup>2</sup>, advanced cirrhosis (Child B and Child C), hepatocellular carcinoma, and current use of steatosis inducing drugs were excluded.

The control group included 192 Italian subjects: 149 cases were referred from general practitioners from Southern Italy. They were part of an ongoing project aimed at assessing cardiovascular risk and liver damage in the general population, according to the presence of NAFLD at ultrasounds. They had no history of chronic liver disease, no evidence of viral infection (anti-hepatitis C virus, anti-HIV, and HBsAg negativity), alcohol consumption  $< 20$  g/d during the previous year (evaluated by interview of patients on amount, frequency and type, and confirmed by at least one family member), normal alanine aminotransferase values ( $< 37$  IU/L), no ultrasonographic evidence of steatosis, and a liver stiffness value  $< 6$  kPa. Forty-three cases were obese subjects from Northern Italy with normal liver biopsy (steatosis  $< 5\%$  of hepatocytes) at routine liver biopsy performed at the time of bariatric surgery (gastric banding).

The study was carried out in accordance with the principles of the Declaration of Helsinki and with local and national laws. Approval was obtained from the hospital Internal Review Boards and their Ethics Committees, and written informed consent for the study was obtained from all controls and patients.

### Clinical and laboratory assessment

Clinical and anthropometric data were collected at the time of liver biopsy. A 12-hour overnight fasting blood sample was drawn at the time of biopsy to determine biochemical parameters.

Irisin was measured on a serum aliquot collected after overnight fasting and stored at  $-80^{\circ}\text{C}$  by a commercial enzyme-linked immunosorbent assay kit (EK-067-29, Phoenix Pharmaceuticals, Germany) (14).

### Genetic analyses

DNA was purified using the QIAmp Blood Mini Kit (Qiagen, Mainz, Germany) and DNA samples were quantified using spectrophotometric determination. Genotyping for patatin-like phospholipase domain-containing protein 3 (*PNPLA3*; rs738409), transmembrane 6 superfamily 2 (*TM6SF2*; rs58542926), and *FNDC5* (rs3480; rs726344) was carried out using the TaqMan SNP genotyping allelic discrimination method (Applied Biosystems, Foster City, CA).

### Histological evaluation

Slides were coded and read at each clinical center by one expert pathologist, who was unaware of patients' identity and

history. Kleiner classification (15) was used to compute steatosis, ballooning and lobular inflammation, and to stage fibrosis from 0 to 4. NASH was diagnosed in presence of steatosis >5%, lobular inflammation, and ballooning.

### **Hepatic expression of Irisin messenger RNA in NAFLD patients**

Liver samples were stored at  $-80^{\circ}\text{C}$  and homogenized using the TissueRuptor apparatus (Qiagen) immediately before nucleic acid extraction. The levels of irisin messenger RNA (mRNA) in the samples were determined using a real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR; ABI PRISM 7500 Fast Real Time System, Applied Biosystems).

### **In vitro experiments**

**Cell culture and treatment.** HepG2 cells were exposed to a mixture of 350  $\mu\text{M}$  oleic acid and 350  $\mu\text{M}$  palmitic acid, with or without 200 ng/mL recombinant irisin (Phoenix Pharmaceuticals, Burlingame, CA). After 24 hours, cells were harvested for analysis. Experiments were repeated in triplicates.

**Triglycerides quantification.** Triglyceride quantification in HepG2 cells was performed by a colorimetric kit, according to manufacturer's instructions (BioVision, Milpitas, CA) (Catalog Number K622-100).

**HSC isolation, purification, and culture.** Mouse HSCs were isolated from 6- to 8-week-old male C57BL/6 mice. Quiescent HSCs were incubated with 100 or 500 ng/mL recombinant Irisin for 5 days. To evaluate the effect of irisin on already activated HSCs (AChSCs), they were serum-starved overnight and subsequently incubated with 100 or 500 ng/mL recombinant Irisin for 6 days with or without 1 ng/mL transforming growth factor (TGF)- $\beta$  (Merk Millipore, Darmstadt, Germany). Data are generated from three independent isolations.

### **In vivo experiments**

**Animals and treatment.** To assess the expression of irisin in chronic liver disease, two different murine models of liver injury were used: a dietary NAFLD model of C57BL/6 mice (Charles River Laboratories International, Inc., New York) fed a high-fat diet (16) and a centivenular injury and liver fibrosis model induced by  $\text{CCl}_4$  injection (17).

**Real-time qRT-PCR.** Total RNA was extracted for HepG2 cells and primary cultured HSCs; qRT-PCR was performed. Relative abundance of the target genes was normalized to Cyclophilin A as internal control.

### **Statistics**

Continuous variables were summarized as mean  $\pm$  standard deviation or as median  $\pm$  interquartile range according to whether they were approximately normal distributed or they showed some grade of skewness or kurtosis. Accordingly, continuous variables not showing a normal distribution were tested for differences among different categories of patients using a Kruskal-Wallis test instead of an analysis of variance test. Categorical variables were summarized as frequencies and

percentages, and the Pearson's  $\chi^2$  test was used to assess differences. Univariate and multiple logistic regression models were fitted to assess factors independently associated with F2 to F4 fibrosis and NASH, whereas a proportional odds ordinal logistic regression model was used for the severity of steatosis. Association structure of the chosen predictors was evaluated with different association measures depending on the measurement scales of each variable (Supplemental Table 1).

Regression analyses were performed using SPSS version 18. The full methods can be found in the supplemental material.

## **Results**

### **Patient features**

The prevalence of FNDC5 rs3480 AA, AG, and GG genotypes was 38.7%, 42.2%, and 19.1%, respectively, in the entire cohort. The frequency distribution of the G variant showed a nonsignificant trend for a higher prevalence in subjects with steatosis compared with those without ( $P = 0.08$ ; Supplemental Fig. 1). After excluding the 43 controls who underwent bariatric surgery, similar results were observed ( $P = 0.08$ ). The prevalence of FNDC5 rs726344 GG, GA, and AA genotypes was 84.1%, 12.7%, and 3.2%, respectively, in the entire cohort, and its frequency distribution was similar when compared with controls without steatosis ( $P = 0.23$ ). The frequency distribution of the PNPLA3, TM6SF2, and FNDC5 rs3480 variants fitted with Hardy-Weinberg equilibrium, whereas the FNDC5 rs726344 did not ( $P = 0.008$ ). Therefore, the rs726344 variant was not further considered in the analyses.

The baseline features of the 593 NAFLD patients stratified according to the FNDC5 rs3480 genotype are shown in Supplemental Table 2. No associations were found between FNDC5 rs3480 and anthropometric, metabolic, and biochemical parameters in the entire cohort of NAFLD patients (Supplemental Table 1).

### **FNDC5 rs3480 A>G is not associated with severity of steatosis and NASH**

The FNDC5 rs3480 G allele was associated with severity of steatosis [odds ratio (OR) = 1.25, 95% confidence interval (CI), 1.02 to 1.54;  $P = 0.02$ ], even if this association was lost after adjusting for age, sex, BMI, impaired fasting glucose (IFG)/diabetes, and both PNPLA3 and TM6SF2 variants (OR = 1.18, 95% CI, 0.95 to 1.46;  $P = 0.13$ ). Notably, severity of steatosis remained independently linked to BMI (OR = 1.05, 95% CI, 1.01 to 1.09;  $P = 0.003$ ), IFG/diabetes (OR = 2.32, 95% CI, 1.61 to 3.35;  $P < 0.001$ ), and both PNPLA3 (OR = 1.87, 95% CI, 1.49 to 2.35;  $P < 0.001$ ) and TM6SF2 (OR = 1.96, 95% CI, 1.26 to 3.06;  $P = 0.003$ ) variants (Supplemental Table 3).

The prevalence of NASH was not different according to FNDC5 rs3480 status (41.7% in AA, 49.7 in AG, and

46.9% in GG; OR = 1.14, 95% CI, 0.92 to 1.43;  $P = 0.22$ ). Similarly, no association was found between NASH and the *FNDC5* rs726344 variant (OR = 0.81, 95% CI, 0.55 to 1.18;  $P = 0.28$ ). NASH was independently associated with BMI (OR = 1.05, 95% CI, 1.01 to 1.09;  $P = 0.009$ ), IFG/diabetes (OR = 1.98, 95% CI, 1.34 to 2.91;  $P < 0.001$ ), and *PNPLA3* variant (OR = 1.38, 95% CI, 1.08 to 1.75;  $P = 0.009$ ; Supplemental Table 4).

As for NASH, the *FNDC5* rs3480 variant was not linked to grade 2 to 3 lobular inflammation (OR = 1.07, 95% CI, 0.82 to 1.39;  $P = 0.60$ ) or to presence of ballooning (OR = 1.11, 95% CI, 0.89 to 1.39;  $P = 0.33$ ).

### ***FNDC5* rs3480 A>G is associated with protection from significant fibrosis**

Supplemental Fig. 2 shows the prevalence of F2 to F4 fibrosis according *FNDC5* rs3480 A>G in the entire cohort and in subgroups of patients with/without diabetes.

In the entire cohort, after correction for sex, BMI, and *TM6SF2* and *PNPLA3* status, *FNDC5* rs3480 variant (OR = 0.64, 95% CI, 0.47 to 0.87;  $P = 0.005$ ) was negatively associated with F2 to F4 fibrosis, together with age (OR = 1.03, 95% CI, 1.01 to 1.05;  $P < 0.001$ ), IFG/diabetes (OR = 1.88, 95% CI, 1.17 to 3.02;  $P = 0.009$ ), severity of steatosis (OR = 1.52, 95% CI, 1.15 to 2.01;  $P = 0.003$ ), and NASH (OR = 8.48, 95% CI, 5.18 to 13.8;  $P < 0.001$ ; Table 1 and Supplemental Table 5). When considering a dominant model instead of an additive model for *FNDC5* rs3480 variant, the rs3480 GG genotype was confirmed independently associated with F2 to F4 fibrosis (OR = 0.49, 95% CI, 0.27 to 0.88;  $P = 0.01$ ). Notably, after stratification for IFG/diabetes, the independent association between *FNDC5* rs3480 variant and F2 to F4 fibrosis was maintained in IFG/diabetic patients (OR = 0.44, 95% CI, 0.26 to 0.74;  $P = 0.002$ ; Table 1 and Supplemental Tables 6 and 7). These data suggest that rs3480 variant is protective against fibrosis in NAFLD.

### **Serum irisin levels are associated with liver damage in NAFLD**

In a subgroup of 112 patients with available serum samples and with characteristics similar to the entire cohort (67.9% male, mean age =  $45.3 \pm 12.8$  years, mean BMI =  $29.3 \pm 3.8$  kg/m<sup>2</sup>, rs3480 AA = 32.9%, AG = 46.6%, GG = 20.5%, 65.2% with grade 2 to 3 steatosis, 83.9% with NASH, and 44.6% with F2 to F4 fibrosis), we assessed irisin serum levels. Irisin levels were not influenced by the *FNDC5* rs3480 (AA =  $21.7 \pm 3.9$  vs AG =  $21.9 \pm 3.2$  vs GG =  $21.7 \pm 5.1$ ;  $P = 0.95$ ) genotype, nor were they associated with demographic, anthropometric, or metabolic features ( $P > 0.10$  for all). However, higher serum irisin was detected in patients with grade 2 to 3 steatosis ( $22.3 \pm 4.3$  vs  $20.8 \pm 2.8$ ;  $P = 0.03$ ), in those with NASH ( $22.1 \pm 4.1$  vs  $20.5 \pm 2.3$ ;  $P = 0.03$ ), and in those with F2 to F4 fibrosis ( $22.7 \pm 4.6$  vs  $21.1 \pm 3.1$ ;  $P = 0.04$ ) when compared with their counterparts. Notably, the association between serum irisin and F2 to F4 fibrosis did not change when adjusting for steatosis. These data suggest that circulating irisin concentration is associated with the severity of NAFLD.

### **Hepatic irisin mRNA levels are associated with liver damage in NAFLD**

In a subgroup of 67 Southern Italy patients with available frozen liver samples and with characteristics similar to the entire cohort (63% male, mean age =  $47.7 \pm 11.7$  years, mean BMI =  $29.6 \pm 4.2$  kg/m<sup>2</sup>, rs3480 AA = 32.9%, AG = 46.6%, GG = 20.5%, 67% with grade 2 to 3 steatosis, 78% with NASH, and 41% with F2 to F4 fibrosis), we assessed the hepatic expression of irisin mRNA. The expression of irisin was not influenced by the *FNDC5* rs3480 genotype, nor was it associated with demographic, anthropometric, or metabolic features ( $P > 0.10$  for all). A significant association was found between liver irisin expression and grade 2 to 3 steatosis [ $P = 0.01$ , Supplemental Fig. 3(A)], NASH [ $P = 0.01$ ,

**Table 1. Association of the *FNDC5* rs3480 Genotype With F2 to F4 Fibrosis by Adjusted Model in 593 Patients Who Underwent Liver Biopsy for Suspected NAFLD, Discriminated According to IFG/Diabetes**

Variable	Significant Fibrosis: OR (95% CI), $P$ Value		
	Entire Population, N = 593	IFG/Diabetes, N = 203	No IFG/Diabetes, N = 390
Mean age, y	1.03 (1.01 to 1.05), <0.001	1.01 (0.98 to 1.05), 0.31	1.04 (1.01 to 1.06), <0.001
Female	1.06 (0.67 to 1.70), 0.77	1.05 (0.50 to 2.19), 0.89	1.09 (0.58 to 2.05), 0.78
BMI, kg/m <sup>2</sup>	0.98 (0.93 to 1.03), 0.62	0.98 (0.90 to 1.07), 0.79	0.97 (0.91 to 1.04), 0.48
IFG/type 2 diabetes	1.88 (1.17 to 3.02), 0.009	—	—
<i>PNPLA3</i> CC vs CG vs GG	0.98 (0.72 to 1.33), 0.90	0.86 (0.53 to 1.40), 0.55	1.01 (0.66 to 1.54), 0.94
<i>TM6SF2</i> CC vs CT vs TT	1.01 (0.55 to 1.86), 0.95	1.43 (0.51 to 4.01), 0.49	0.87 (0.39 to 1.93), 0.73
<i>FNDC5</i> rs3480 AA vs AG vs GG	0.64 (0.47 to 0.87), 0.005	0.44 (0.26 to 0.74), 0.002	0.81 (0.54 to 1.22), 0.32
Steatosis	1.52 (1.15 to 2.01), 0.003	1.09 (0.70 to 1.71), 0.68	1.99 (1.36 to 2.90), <0.001
NASH	8.48 (5.18 to 13.8), <0.001	16.7 (7.05 to 39.5), < 0.001	5.74 (3.06 to 10.7), <0.001

Data are given as mean  $\pm$  standard deviation or as percentage of cases.

Supplemental Fig. 3(B)], and F2 to F4 fibrosis [ $P = 0.02$ , Supplemental Fig. 3(C)]. Notably, the association between hepatic irisin mRNA and F2 to F4 fibrosis did not change when adjusting for steatosis. Moreover, in a subgroup of 31 patients with availability of both serum and hepatic irisin, these last were not significantly associated. Therefore, in keeping with circulating concentration, hepatic irisin expression is associated with NAFLD severity.

### Irisin does not increase lipid accumulation in hepatocytes

To evaluate whether irisin could contribute to hepatocellular fat accumulation, HepG2 cells were incubated with oleic acid and palmitic acid, with or without recombinant irisin. As expected, fatty acids treatment significantly increased neutral lipid content in hepatocytes [Supplemental Fig. 4(A)]. However, the addition of recombinant irisin to the culture medium did not influence lipid storage compared with fatty acids alone [Supplemental Fig. 4(A)].

To further examine whether irisin influences hepatic lipid metabolism, we measured the expression of genes encoding enzymes involved in lipogenesis and lipid catabolism. In our conditions, gene expression of fatty acid synthase was not changed by fatty acids supplementation, although we could observe an increased expression of stearoyl-CoA desaturase, the key enzyme in the conversion of saturated into monounsaturated

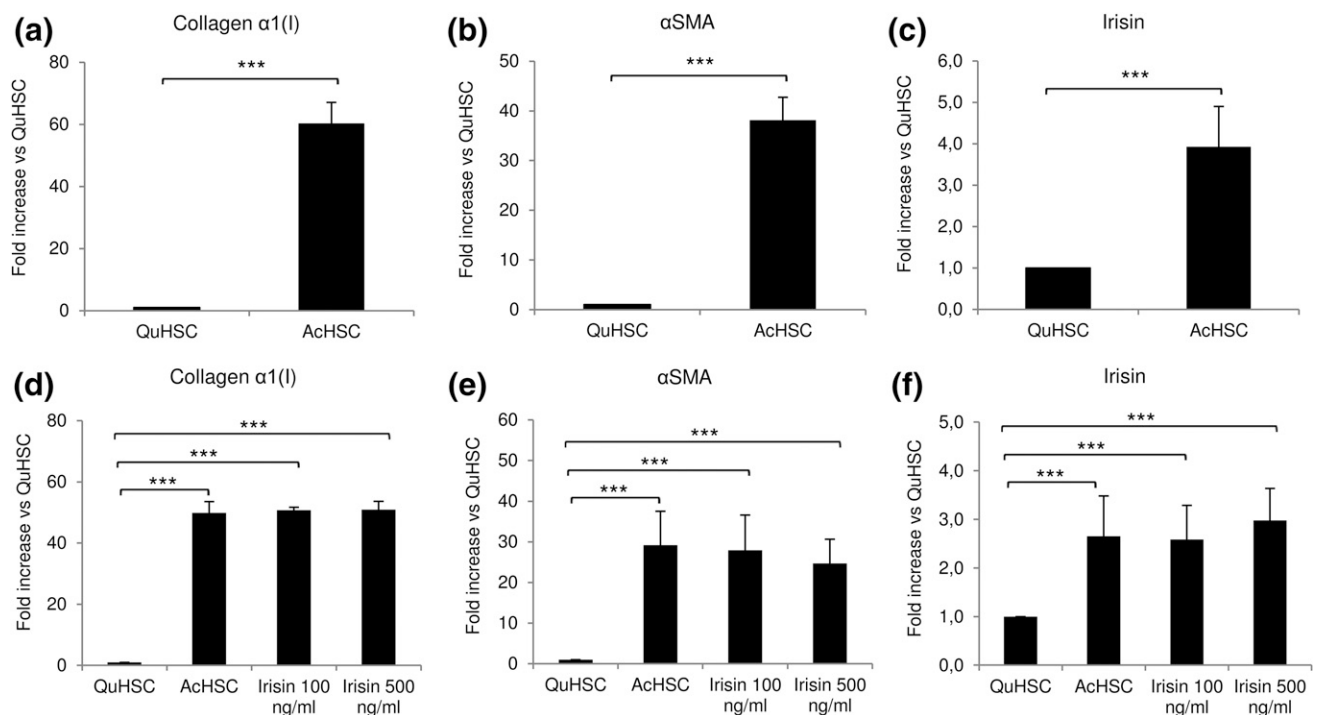
fatty acids [Supplemental Fig. 4(B) and 4(C)]. Finally, to evaluate the role of irisin on fatty acids oxidation, mRNA expression of peroxisome proliferator-activated receptor- $\alpha$  and its downstream target, carnitine palmitoyltransferase I, was quantified. Both genes were significantly upregulated by fatty acids incubation independently from the presence of irisin [Supplemental Fig. 4(D) and 4(E)]. These results suggest that irisin is not involved in the regulation of fat storage and metabolism in cultured hepatocytes.

### Irisin is expressed by activated human HSC

HSC activation is the main process responsible for collagen deposition and liver fibrosis progression. Therefore, we evaluated whether irisin might affect the process of HSCs activation *in vitro*. Irisin expression increased in parallel with HSC activation, as demonstrated by collagen  $\alpha 1(I)$  and  $\alpha$ -smooth muscle actin gene expression [Fig. 1(a–c)]. To investigate the role of irisin in the activation process, quiescent HSCs were incubated with recombinant irisin for 5 days. No differences in collagen  $\alpha 1(I)$  and  $\alpha$ -smooth muscle actin gene expression were observed between control and irisin-incubated HSCs [Fig. 1(d–f)].

### Irisin stimulates fibrogenesis in AchSCs

To analyze the role of irisin in hepatic fibrogenesis, the expression of fibrogenic markers collagen  $\alpha 1(I)$ , tissue



**Figure 1.** Incubation of quiescent HSCs (QuHSCs) with recombinant Irisin. (a–c) Expression of collagen  $\alpha 1(I)$ ,  $\alpha$ -smooth muscle actin ( $\alpha$ SMA) and irisin in AchSCs vs QuHSCs. (d–f) Expression of collagen  $\alpha 1(I)$ ,  $\alpha$ SMA and irisin in QuHSCs after 5 days incubation with recombinant irisin (100 or 500 ng/mL). Mean  $\pm$  standard error (SE). \*\*\*  $P < 0.001$ .

inhibitors of metalloproteinases-1 (TIMP-1), and monocyte chemoattractant protein-1 (MCP-1) was measured in AchSCs. Low irisin concentration upregulated TIMP-1 and MCP-1 expression, whereas 500 ng/mL also induced increased expression of collagen  $\alpha 1(I)$ , indicating higher collagen synthesis [Fig. 2(a–c)]. Next we incubated AchSCs with 1 ng/mL TGF- $\beta$ , 500 ng/mL irisin, or a combination of both to evaluate for a potential synergistic effect. As shown in Fig. 2, both irisin and TGF- $\beta$  increased mRNA expression of all fibrogenic markers, which was not further enhanced by their combination [Fig. 2(d–g)].

### ***Irisin is upregulated in a murine model of fibrosis, but not in experimental NAFLD without significant fibrosis***

We then evaluated irisin expression in two different models of liver injury and fibrosis. No increased hepatic expression of irisin was observed in high-fat-diet-treated mice, a dietary model of NAFLD, which is not characterized by fibrosis deposition [Fig. 3(a)]. On the other hand, irisin expression was upregulated after 8 weeks of CCl<sub>4</sub> treatment (16 injections), a pure model of centrilobular liver injury and hepatic fibrosis, although no changes were observed after 4 weeks of treatment [Fig. 3(b)]. These data suggest that hepatic irisin expression is associated with activation of fibrogenesis but not hepatic fat accumulation.

## **Discussion**

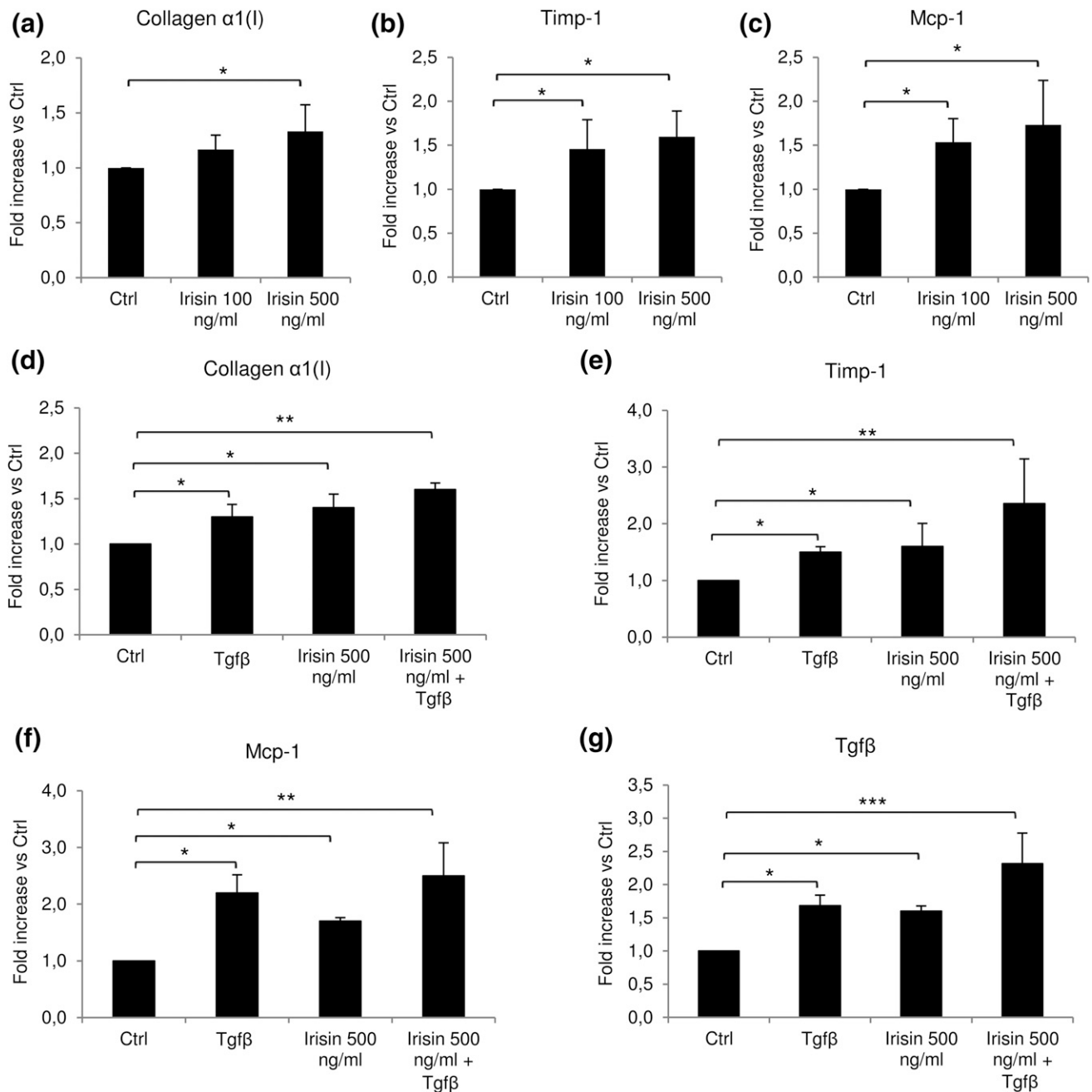
In this study, in a cohort of 593 patients at risk for NASH, characterized by a high prevalence of severe liver damage, we found that *FNDC5* rs3480 A>G was associated with a lower prevalence of clinically significant fibrosis. We also showed that irisin expression increases with activation process of HSCs, where it induces profibrogenic genes, and is overexpressed in an experimental model of hepatic fibrosis, as well as in the liver and the serum of patients with NASH and F2 to F4 fibrosis.

Irisin, a recently discovered “protective” myokine, seems able to modulate IR (9), but little and inconclusive data are available on its effect on liver damage. An important finding of the current study lies in the independent association between *FNDC5* rs3480 variant and a lower risk of significant fibrosis in NAFLD. This was confirmed after adjusting not only for clinicometabolic and genetic (*PNPLA3* and *TM6SF2* variants) confounders, but also for presence of NASH and severity of steatosis. Noteworthy is that when stratifying the population according to the presence or absence of IFG/diabetes, a strong risk factor for NAFLD and its severity, we showed that the main association of the *FNDC5* rs3480 variant with fibrosis was maintained

only in patients with hyperglycemia, suggesting that the effect of G variant in NAFLD is more pronounced in patients at higher metabolic risk. We also observed a direct relationship between both serum levels and liver expression of irisin and F2 to F4 fibrosis. These results are in line with data reporting a correlation between muscle irisin expression and IR in humans (12), and with evidence, on a very small cohort of biopsy-proven NAFLD/NASH, of a link between higher serum irisin and portal inflammation with also a nonsignificant trend for steatosis and fibrosis (18). Finally, we report for the first time, to our knowledge, that irisin is expressed in activated human HSC, where promotes the expression of proinflammatory (MCP-1) and fibrogenic (TIMP-1 and type I collagen) markers. These data suggest that irisin is involved in the fibrogenic process in the liver during NAFLD and that the *FDNC* genotype might affect fibrogenesis by modulating irisin secretion.

However, when looking at an association between the rs3480 variant and irisin serum levels/hepatic expression, we did not find any link, keeping with Tanisawa and colleagues (19), who observed a protective effect of the rs3480 variant on dysmetabolism in the absence of modulation of serum irisin levels. These last are the sum of irisin arising from different tissues (20), and the lack of association of rs3480 with both hepatic and serum irisin would seem to rule out an effect of this variant on liver and perhaps muscle irisin expression. It could therefore be speculated that irisin directly modulates hepatic fibrogenesis and that the rs3480 could indirectly protect from liver fibrosis by modulating irisin expression specifically in HSCs or in other sites like adipose tissue. Further knowledge about irisin receptor(s) and its signaling could add relevant insights about the role of irisin in liver fibrogenesis. Figure 4 resumes how irisin and *FNDC5* rs3480 could affect fibrosis in NAFLD. However, the sample size could have been underpowered to detect an association between rs3480 and both hepatic and serum irisin.

Another finding of our study is the lack of association of the *FNDC5* rs3480 A>G not only with presence of steatosis, but also with a higher risk of severity of steatosis in patients with NAFLD. These data are consistent with current genome-wide studies (21–23), and with data from Staiger and colleagues (12), who assessed, in a cohort of German subjects at high risk for diabetes, the putative association between *FNDC5* variants, including the rs3480, and the presence of steatosis evaluated by magnetic resonance spectroscopy. Consistently, we observed that irisin does not affect triglyceride accumulation and expression of genes modulating steatogenesis in HepG2 cells exposed to fatty acids. In contrast, Park and colleagues (13) showed an apparently protective effect of



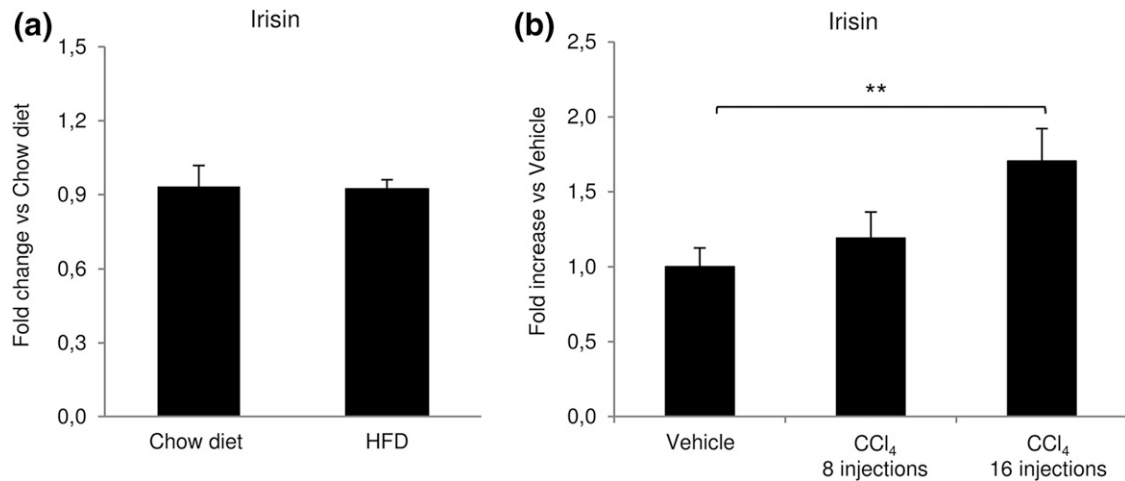
**Figure 2.** Effect of Irisin in AChSCs. (a–c) mRNA expression of fibrogenic markers, such as collagen  $\alpha$ 1(I), TIMP-1, and MCP-1 in AChSCs treated with irisin 100 or 500 ng/mL. (d–g) Expression of collagen  $\alpha$ 1(I), TIMP-1, MCP-1 and TGF- $\beta$  in AChSCs incubated with TGF- $\beta$  1 ng/mL, irisin 500 ng/mL or TGF- $\beta$ +irisin. Mean  $\pm$  SE. \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ .

irisin against steatosis in a different experimental model in mouse hepatocytes incubated with high levels of the toxic palmitic acid alone *in vitro*. The direct association between severity of steatosis and irisin expression that we observed in human NAFLD could be an epiphenomenon of the relationship between higher irisin and severity of fibrosis.

We did not identify any association between both FND5 rs3480 and rs726344 and indexes of glucose balance as reported in literature. However, the current study was not designed to evaluate this outcome, and

indeed, Staiger and colleagues (12) found a link between rs726344 and improved insulin sensitivity in a population without diabetes and characterized by a higher prevalence of females.

Finally, we did not find any association between the PNPLA3 genotype and significant fibrosis as reported in literature (24), even if not always confirmed (25). However, the PNPLA3 genotype was independently associated with NASH, which is the main risk factor for fibrosis. Differences in baseline characteristics of the studied population in terms of metabolic disturbances,

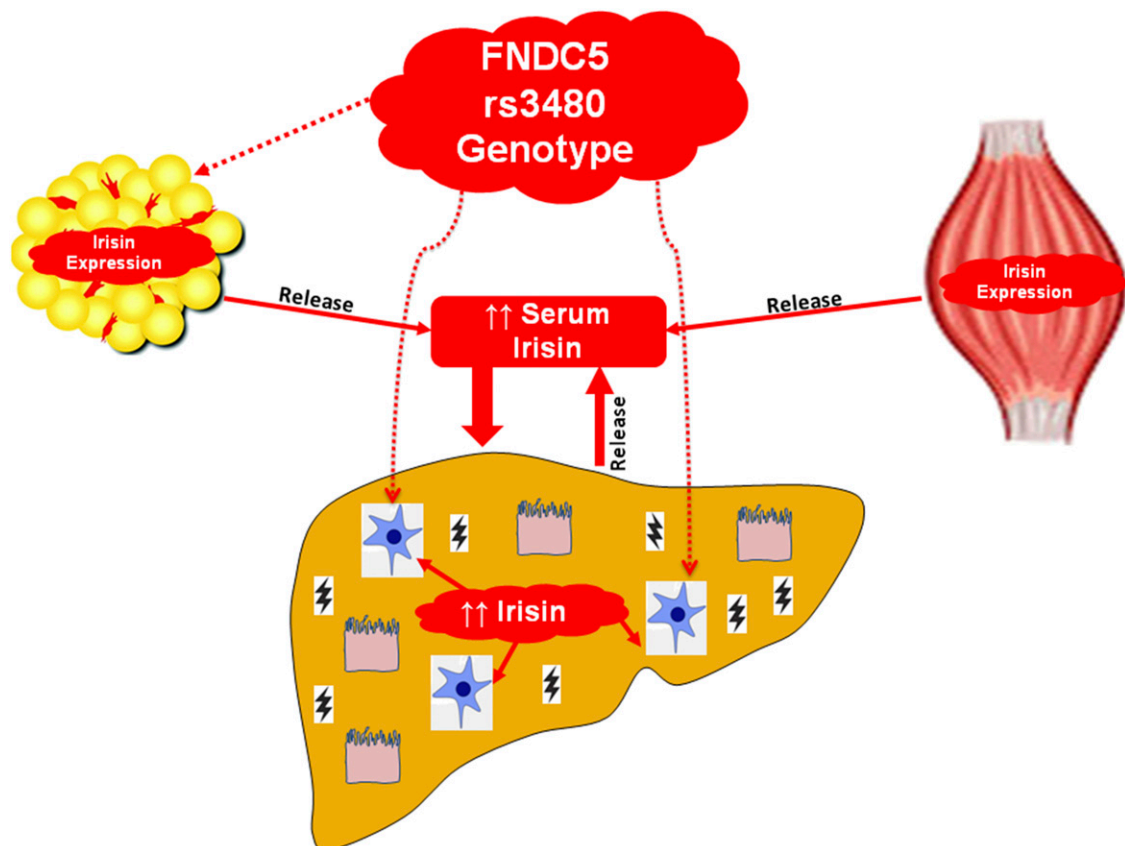


**Figure 3.** Hepatic Irisin expression in animal models of liver fibrosis. (a) Hepatic Irisin expression in mice fed a high-fat diet for 12 weeks. (b) Irisin levels in CCl<sub>4</sub>-treated animals for 4 or 8 weeks (8 or 16 injections, respectively). Mean  $\pm$  SE. \*\*  $P < 0.0$ .

like diabetes and obesity, and of prevalence of fibrosis should explain the different results.

A limitation of the study is that we cannot exclude that variants in other genes in linkage with the FNDC5 rs3489 A>G, or other variants in FNDC5 itself are responsible of the observed associations. A further methodological question is the potentially limited external validity of the

results for different populations and settings. Our study included a cohort of Italian patients enrolled at two tertiary care centers who may be different, in terms of both metabolic features and severity of liver disease, from the majority of prevalent cases of NAFLD in the general population. Finally, the inclusion as controls of some morbidly obese individuals who underwent bariatric



**Figure 4.** Irisin, FNDC5 rs3480, and fibrosis in NAFLD. FNDC5 rs3480 can affect liver fibrosis by modulating the expression of irisin in HSCs and in other sites like perhaps adipose tissue, but not in the muscle. High serum irisin levels as result of irisin from different sites, and hepatic irisin promote the expression in HSCs of proinflammatory (MCP-1) and fibrogenic (TIMP-1 and type I collagen) markers leading to hepatic fibrogenesis.



surgery, and the possibility in some cases of underestimated hidden alcohol intake, could affect the interpretation of results. In spite of these limits, strengths of this study are the large sample size, the confirmation of results after adjusting for multiple clinicometabolic and genetic confounders, and the corroboration of the genetic association between irisin and fibrosis severity by experimental data *in vitro* and *in vivo*.

From a clinical point of view, to depict a genetic map for NAFLD and its severity in the already complex and partially understood genetic background our study suggests additional consideration of the *FNDC5* rs3480 variant that may represent a new actor. Further studies, however, are necessary to independently replicate our findings and to ascertain mechanisms underlying this clinical association.

In conclusion, this study on a large cohort of patients with histological diagnosis of NAFLD showed that *FNDC5* rs3480 A>G is associated with a lower prevalence of significant liver fibrosis, especially in IFG/diabetic patients, and that irisin, overexpressed in both serum and liver of NAFLD patients with significant liver damage, is expressed in AchSCs, where it mediates profibrogenic actions. Further experimental studies are necessary to fully clarify the mechanism linking the *FNDC5* rs3480 genotype, irisin, and liver damage.

## Acknowledgments

Address all correspondence and requests for reprints to: Salvatore Petta, MD, PhD, Sezione di Gastroenterologia Di.Bi.M.I.S Università di Palermo, Piazza delle Cliniche, 2, 90127 Palermo, Italy. E-mail: [petta@inwind.it](mailto:petta@inwind.it).

This study was funded by grants from the Progetti di Rilevante Interesse Nazionale 2010-2011 (Prot. N. 2010C4JJWB\_001), My First AIRC Grant, Associazione Italiana per la Ricerca sul Cancro n. 16888.

Author contributions: S.P., L.V., and G.S.-B. designed the study and revised the article. M.R., R.M.P., P.D., C.R., N.F., A.L.F., I.P., M.M., D.G., and S.G. were involved in data acquisition. D.C. and M.M. read liver histology. S.P. was involved in data analysis and preparation of article. C.C., V.D.M., S.F., and A.C. critically revised the article. The final draft article was approved by all the authors.

Disclosure Summary: The authors have nothing to disclose.

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