Hellenic Society of Cardiology (2016) 57, 417–425

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: http://www.journals.elsevier.com/hellenic-journal-of-cardiology/

ORIGINAL ARTICLE

The role of exercise training and the endocannabinoid system in atherosclerotic plaque burden and composition in Apo-E-deficient mice

Michalis Katsimpoulas [a,b,*], Nikolaos E. Kadoglou [a,b], Petros Moustardas [a,b], Alkistis Kapelouzou [b], Eleni Dede [b], Nikolaos Kostomitsopoulos [b], Panayotis E. Karayannacos [b], Alkiviadis Kostakis [b], Christos D. Liapis [a]

[a] *Department of Vascular Surgery, Medical School, University of Athens, Greece*
[b] *Center of Clinical, Experimental Surgery and Translational Research, Biomedical Research Foundation, Academy of Athens, Greece*

Received 25 June 2015; accepted 1 September 2016
Available online 16 November 2016

KEYWORDS
Cannabinoid Receptor 1;
matrix metalloproteinases;
atherosclerosis

Abstract *Introduction:* We investigated the effect of combining exercise training and treatment with an endocannabinoid receptor 1 inhibitor (Rimonabant) on atherosclerosis burden and composition.
Methods: Forty-eight apolipoprotein E-deficient (ApoE-/-) mice were kept on a 16-week high-fat diet. Mice were then placed on a normal diet and were randomized to the following groups with n = 12 mice for 6 more weeks: 1) Control (Co) - no intervention; 2) Exercise (Ex) - exercise training on treadmill; 3) Rimonabant (Ri) - oral administration of rimonabant (10 mg/kg/day); or 4) Rimonabant+Exercise (RiEx) - combination of Ri and Ex groups treatment. At the end, all animals were sacrificed, and blood samples, as well as aortic root specimens, were obtained for histomorphometric analysis and quantification of the serum and plaque content of matrix metalloproteinases (MMPs).
Results: The mean plaque area was significantly smaller (RiEx: 43.18 ± 1.72%, Ri: 44.66 ± 3.1%, Ex: 49 ± 4.10%, Co: 70.43 ± 2.83%) in all active treatment groups relative to the Co group ($p < 0.01$). Conversely, the relative concentrations of collagen and elastin were increased significantly across all treatment groups compared to Co ($p < 0.05$). Immunohistochemical analysis revealed significantly reduced macrophage content within plaques after all

* Corresponding author. Michalis Katsimpoulas DVM, PhD, Staff Research Scientist (Assistant Professor Level), Center for Experimental Surgery, Biomedical Research Foundation of the Academy of Athens, 4, Soranou Ephessiou st, Athens 11527, Greece. Tel.: +30 2106597367.
E-mail address: mkatsiboulas@bioacademy.gr (M. Katsimpoulas).
Peer review under responsibility of Hellenic Society of Cardiology.

http://dx.doi.org/10.1016/j.hjc.2016.11.013

interventions, with the most pronounced effect observed after combined treatment (RiEx: 9.4 ± 3.92%, Ri: 15 ± 2.45%, Ex: 19.78 ± 2.79%, Co: 34.25 ± 4.99%; $p < 0.05$). Within plaques, the TIMP-1 concentration was significantly upregulated in exercise-treated groups. MMP-3 and MMP-9 concentrations were equivalently decreased in all three active treatment groups compared to controls ($p < 0.001$).

Discussion: Both exercise and rimonabant treatments induced plaque regression and promoted plaque stability. The combined treatment failed to show additive or synergistic benefits relative to either intervention alone.

1. Introduction

Atherosclerosis-related cardiovascular diseases (CVDs) remain the leading cause of mortality globally.[1] They are primarily characterized by chronic inflammation and complex, slow-progressing vascular dysfunction.[2] There are a plethora of data supporting the involvement of zinc proteolytic enzymes, known as matrix metalloproteinases (MMPs), and their inhibitors (TIMPs) in inflammatory pathways, atherogenesis and atherosclerotic plaque destabilization.[3–5] The MMPs/TIMP-1 ratio determines whether extracellular matrix (ECM) is degraded and is thus an essential part of plaque development and disruption.[6,7]

Systemic exercise training has become the cornerstone of effective prevention of atherosclerotic CVDs in humans.[8–10] Although the precise mechanisms are not fully understood, there are several studies documenting its beneficial multivariate action in targeting plaque burden and composition.[11–13] In parallel, a growing body of pharmaceutical agents aims to reduce the development of atherosclerosis, these agents include statins[14] and other novel pharmaceutical drugs.[15,16] Cannabinoid receptors, endocannabinoids and the enzymes that catalyze their synthesis and degradation constitute the endocannabinoid system, which plays an important role in the cardiovascular system.[17] An inhibitor of the endocannabinoid receptors 1 (CB1), rimonabant, emerged as a promising treatment for obesity, metabolic disorders and CVDs.[18] Despite the positive results from animal studies where rimonabant reduced atherosclerotic lesions and inflammation by decreasing MMP-9,[19] CB1-inhibition failed to demonstrate such beneficial effects in humans and was discontinued due to adverse side effects.[20]

Since there are several studies that demonstrate a link between exercise and the endocannabinoid system, [21–23] we investigated the possibility of a complementary action of rimonabant and exercise training on atherosclerotic plaque burden and plaque stability using a well-studied animal model of atherosclerosis (Apolipoprotein E-deficient mice, ApoE-/-).

2. Methods

2.1. Animal study design

Forty-eight male ApoE-/- mice (C57BL/6 J background, 20—25 g), backcrossed for ten generations (Charles Rivers Laboratories, Milan, Italy), were maintained in rooms under a 12/12 h light/dark cycle with lights on at 07:00 am, a temperature of 24 ± 2°C, and relative humidity of 55 ± 10%. Food and water were provided ad libitum. At the age of eight weeks, body weight was recorded and the diet was switched to a Western-type diet (42% of total calories from milk fat and 0.15% from cholesterol; Harlan Teklad TD 88137; Harlan, Boxmeer, Netherlands) for 16 weeks. Then, blood samples were drawn and all mice were placed on a normal diet and randomly assigned to one of the following groups for an additional six weeks: 1) Control (Co, n = 12) mice were kept in their cage without any other intervention; 2) Exercise (Ex, n = 12) - this group underwent exercise training (described below); 3) Rimonabant (Ri, n = 12) - mice received 10 mg/kg/day p.o. rimonabant (Sanofi-Aventis, Paris, France) via gavage; or 4) Rimonabant+Exercise (RiEx, n = 12) - the combined treatment was performed as for groups Ex and Ri. At the end of the 6-week period all animals were sacrificed under isoflurane (Forenium, Abbot, Italy) anesthesia. The study design is shown in Figure 1.

All experiments were performed in accordance with the guidelines of the Veterinary Service of the Prefecture of Athens, as required by the Greek legal requirements for animal experimentation to the European Union Directive 86/609 of the Council of European Communities and were approved by the Ethics Committee for Animal Experimentation of the Foundation of Biomedical Research of the Academy of Athens.

2.2. Exercise training

All animals in groups Ex and RiEx were trained on a motor treadmill (Exer-6M Open Treadmill; Columbus Instruments, Columbus, Ohio, USA) with a rubber belt for a total of 6 weeks, 5 days/week, 60 min/day, with a 2-minute intermediate rest interval, and a slope of 5^0. For exercise acclimation, the treadmill speed was initially set to 7 m/min and was then increased to a maximum speed of 15 m/min by the end of the second week; thereafter the speed was kept constant. All mice tolerated the exercise protocol well throughout the study.

2.3. Tissue processing

At euthanasia, the heart and the aorta were washed thoroughly with phosphate-buffered saline (PBS) via cardiac

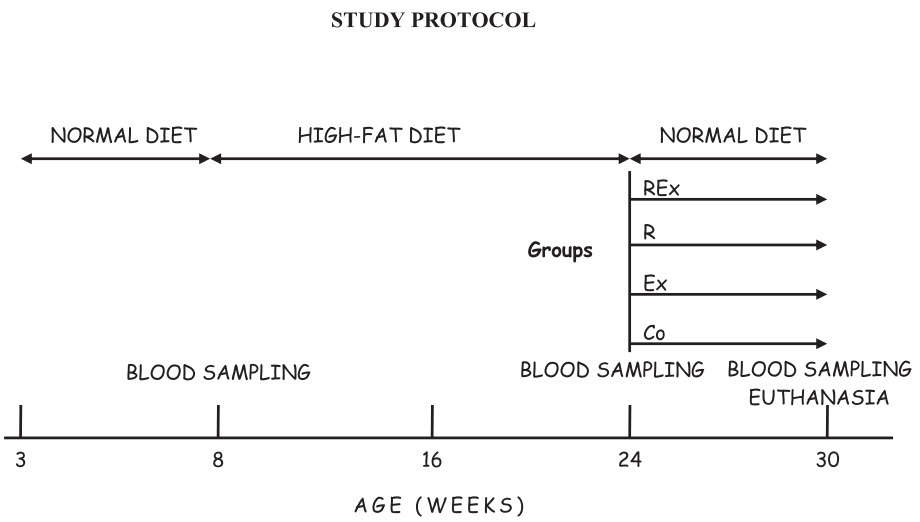

Figure 1 Study design. Groups: RiEx, Rimonabant + Exercise; Ri, Rimonabant; Ex, Exercise; Co, Control.

puncture. Thereafter, the aortic root was excised and fixed in 10% buffered formalin for 24 h and then embedded in paraffin blocks. Only sections containing aortic leaflets were used; 3 sections of 5-μm thickness, equally separated ≃ 100 μm distance, were harvested per slice following a previously described procedure.[13]

2.4. Plaque morphometry and composition

A single observer blinded to group allocation performed all analyses. Three sections per slice from 6 slices per animal were stained with hematoxylin/eosin for morphometric analysis.[12,13] Images obtained using a microscope (CX31, Olympus, UK) were analyzed with Image-Pro Plus software (Version 4.1; Media Cybernetics, USA). The total lumen area and the total plaque area were measured in all sections of the aortic arch, and the mean plaque area (±SD) was then calculated for each mouse and each treatment group.

Histochemical analyses of the plaques were performed using Sirius Red and Orcein stains for collagen and elastin, respectively (Figure 2). Immunohistochemical analyses were performed to detect macrophages (antibodies against Mac-3 antigen, dilution 1:50, Pharmingen, USA), vascular smooth muscles cells (antibodies against the α-smooth muscle isoform of actin, dilution 1:100; Biocare Medical, LLC, USA), MMP-2 and MMP-3 (dilution 1:300; MBL, USA), MMP-9 (dilution 1:300; AbD Serotec, UK) and TIMP-1 (dilution 1:300; Triple Point Biologics Inc., USA) (Figure 3). To quantify the relative concentrations of stained molecules by histochemistry or immunohistochemistry, the segmental stained plaque area was expressed as the percentage of the whole atherosclerotic plaque area, as previously described.[12,13]

2.5. Blood assays

Blood samples were obtained at 24 and 30 weeks of age for all groups. Plasma levels of glucose, triglycerides (TG), and total cholesterol (TC) were determined using an enzymatic technique (Chemwell 2910; Awareness Technology Inc., USA). Serum levels of MMP-2, MMP-3, MMP-9 and TIMP-1 were assayed using commercially available ELISA kits for mice (R&D Systems Inc., Minneapolis, USA) according to the manufacturer's protocol.

2.6. Statistical analysis

All data were expressed as the mean ± SD and analyzed by SPSS (version 16.0; SPSS Inc., Chicago, USA). Data analyses was performed using a paired t-test to compare the differences within groups or a one-way ANOVA with Tukey's post hoc test to compare the differences between groups at the end of the study. A p value <0.05 was considered statistically significant.

3. Results

3.1. Body weight, fasting glucose and lipids

During the entire experiment, no local or systemic adverse effects were observed. There were no significant changes in body-weight within groups or between groups at the end of the study as shown in Table 1 ($p > 0.05$). Fasting glucose only changed significantly within the control group (130 ± 15 mg/dl vs 174 ± 57 mg/dl, $p = 0.045$). Total cholesterol was significantly reduced within the RiEx group (from 612 ± 189 mg/dl to 443 ± 123 mg/dl, $p = 0.031$), while the RiEx and Ex groups had lower total cholesterol levels than the Co group before euthanasia ($p < 0.05$). In parallel, triglycerides were considerably reduced only in the Ex group relative to the RiEx ($p = 0.030$) and Co ($p < 0.001$) groups (Table 1).

3.2. Serum MMPs and TIMP-1

Serum MMPs and TIMP-1 levels are shown in Table 1. At baseline, serum concentrations of MMPs and TIMP-1 did not differ between groups ($p > 0.05$). Statistical analysis revealed significant changes in MMP-3, MMP-9 and TIMP-1 concentrations within all groups ($p < 0.05$). At the end of the study, MMP-3, MMP-9, and TIMP-1 levels improved in all active treatment groups relative to the Co group ($p < 0.05$).

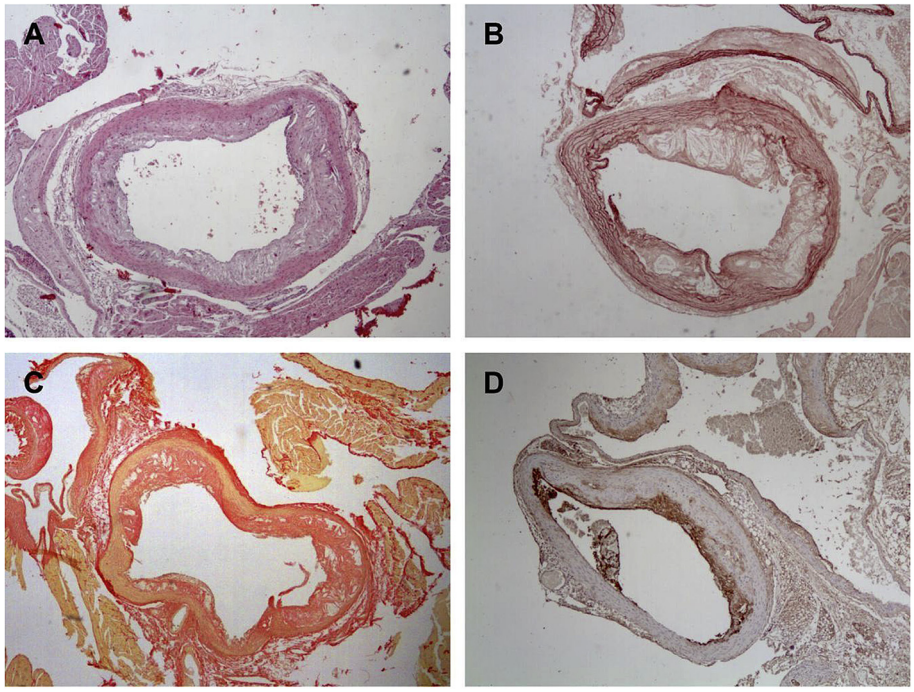

Figure 2 Representative photos of histochemical and immumohistochemical stains of aortic arch sections, for the analysis of plaque morphology and composition. A: Haematoxylin & Eosin stain B: Orcein stain (Elastin molecules) C: Sirius red stain (Colagen) D: Immunohistochemical anti-MMP3 stain. Original magnification: 40x.

Compared to the rest of the groups ($p < 0.05$), RiEx and Ri interventions had a greater effect on MMP-3 levels and RiEx and Ex interventions had a greater effect on MMP-9 levels. No significant differences were observed within or between groups for MMP-2 serum levels by one-way ANOVA ($p > 0.05$).

3.3. Plaque morphometry and composition

In all treatment groups, the mean atherosclerotic plaque area was significantly smaller than that of the Co group ($p < 0.05$). Between group comparisons revealed greater reductions in the atherosclerotic plaque burden in rimonabant-treated groups (RiEx, Ri) than for mice treated with exercise alone ($p < 0.010$). Conversely, the collagen and elastin concentrations were significantly increased across all treatment groups relative to the Co group ($p < 0.05$). There was a trend towards plaques in the Ri group having greater elastin content than those in the Ex group ($p = 0.051$) (Table 2).

Based on immunohistochemical analysis, all interventions significantly reduced the macrophage content of plaques (overall one-way ANOVA, $p < 0.001$), while the most pronounced effect was observed with combined treatment ($p < 0.05$). However, none of the treatment modalities had a statistically significant effect on the VSMC content (overall one-way ANOVA, $p = 0.115$) (Table 2).

Table 3 shows the relative concentrations of the stained molecules by histochemistry or immunohistochemistry. Unlike MMP-2, both MMP-3 and MMP-9 were significantly reduced in all intervention groups relative to the control group ($p < 0.001$). For MMP-3, exercise alone showed higher efficacy than Ri intervention ($p = 0.010$). Finally, TIMP-1 concentrations were significantly increased in exercise-treated groups (RiEx, Ex) compared to controls ($p < 0.001$).

4. Discussion

In the present study, the 6-week intervention with rimonabant administration and exercise training, either alone or combined, reduced the size of atherosclerotic lesions and induced a more stable plaque phenotype relative to untreated hypercholesterolemic mice, even in the absence of significant changes in body-weight. Among the examined parameters, rimonabant and exercise training exerted only complementary effects on macrophages, while they improved collagen and elastin content, MMP-3 concentrations, and MMP-9 concentrations equivalently, with no additional benefit from the combination.

The anti-atherosclerotic actions of systemic exercise have been well-documented in animal studies.[12] However, most but not all previous studies have shown suppressed atherosclerosis development in hypercholesterolemic mice treated with rimonabant.[24–26] In our study, all therapeutic modalities (Ri, Ex, and RiEx) significantly decreased the extent of the aortic atherosclerotic lesions compared to controls. The clinical extrapolation of this result should be done with caution; in the AUDITOR trial, the 30-month treatment with rimonabant led to 5% loss of body weight, but it did not attenuate early carotid atherosclerosis progression relative to placebo.[27] The observed reduction in the mean plaque area after combined treatment in our study was not statistically different from either intervention

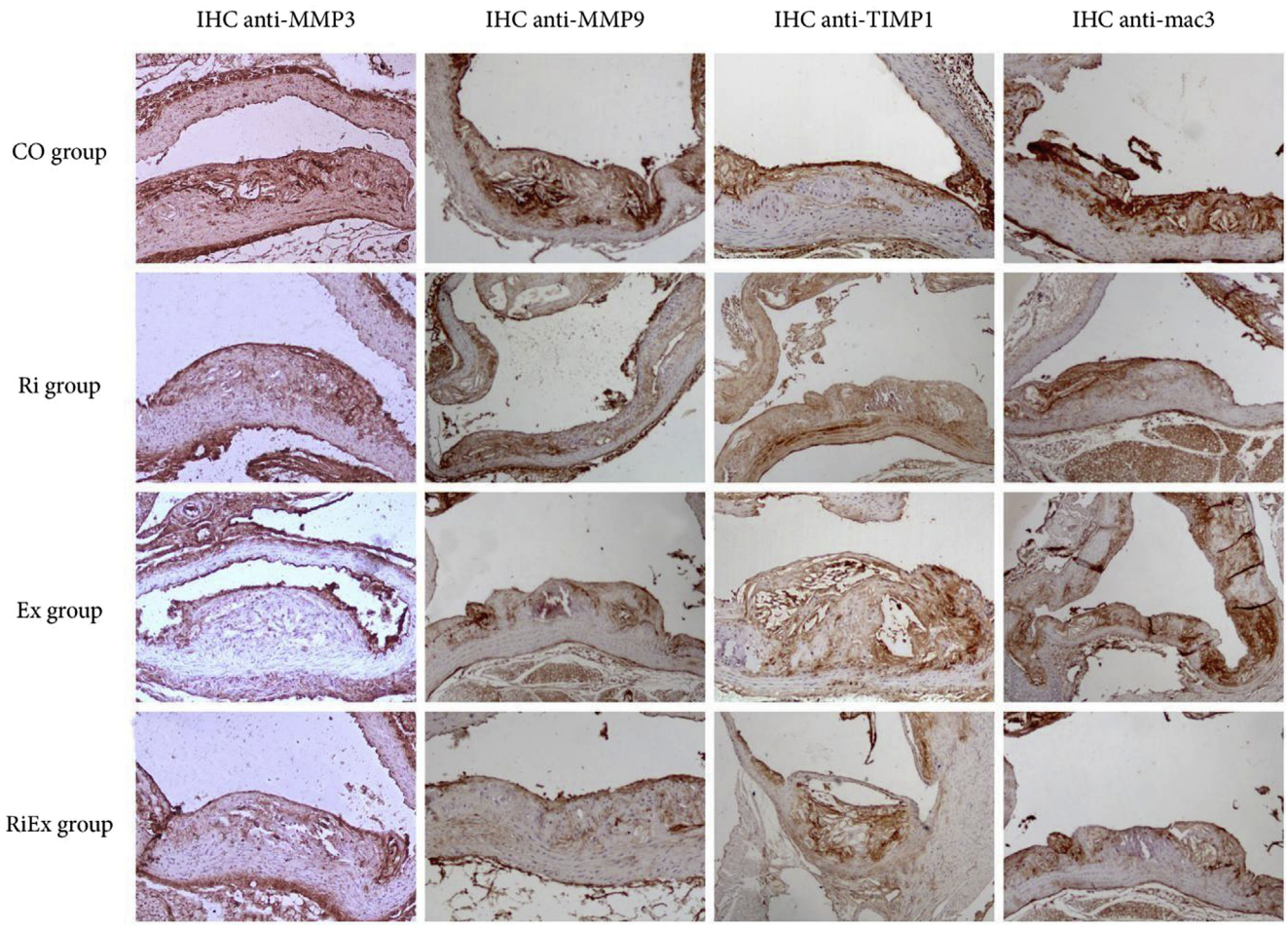

Figure 3 Representative photos of immunohistochemical stains for the visualization of MMP-3, MMP-9, TIMP-1 and mac-3 antigens in sections of the aortic arch of experimental groups. CO: Control group; Ri: Rimonabant group; Ex: Exercise group; RiEx: Combined treatment group. Original magnification: 100x.

alone; thus no complementary anti-atherosclerotic effects were detected. It is possible that these two interventions might act simultaneously on a common pathway, which should be investigated further in future studies.

It is worth mentioning that the aforementioned results occurred despite the absence of significant weight-loss following either rimonabant or exercise treatment. Since the regulation of atherogenesis is multifactorial, perhaps, other non-traditional mechanisms have mediated atherosclerosis regression. Mohapatra et al.[28] reported an anti-inflammatory effect of rimonabant secondary to weight loss. Previously published studies have demonstrated a complicated interplay between the endocannabinoid system and atherosclerosis. In these studies, the direct inhibition of cannabinoids counteracted pro-inflammatory, lipid-mediated, and oxidative mechanisms that are potentially associated with atherogenesis.[25,29,30]

One of the most striking findings of the present study was the exercise-related and rimonabant-related stable phenotype of the atherosclerotic plaques. Pathological studies have indicated that ECM proteins and macrophages within atherosclerotic plaques are the predominant determinants of plaque stability.[31,32] In our study, collagen and elastin increased, while the relative concentration of macrophages decreased within plaques of exercise- and rimonabant-treated mice. A previous study has confirmed the effect of exercise on mouse macrophages,[33] but previous work has not investigated macrophages after CB1 inhibition. Accumulating clinical evidence highlights the importance of plaque texture in CVDs, especially in cases of moderate artery narrowing.[34] Pharmaceutical modalities targeting plaque stability rather than interventions targeting lumen patency have emerged as promising therapeutic approaches for atherosclerosis-related CVDs.

It is well-known that MMPs regulate ECM remodeling and inflammatory cell infiltration,[35] the mainstays of atherosclerosis development.[3,36] Thus, the pharmaceutical inhibition of MMPs and inflammation may yield important anti-atherosclerotic benefits.[37] A complex interplay between MMPs and endocannabinoids via their binding to transmembrane receptors (CB1, CB2)[38,39] could affect plaque development and intraplaque vulnerability.[40] Montecucco et al.[39] assayed CB2 expression and activity within carotid plaques obtained from patients with or without ipsilateral ischemic stroke. CB2 was significantly downregulated in the former group. Moreover, treatment of high-fat fed ApoE$^{-/-}$ mice with CB2 antagonists further decreased MMP-9 content in their aortic root and carotid plaques. In the present

Table 1 Body weight, lipid levels, plasma MMP-2, MMP-3, MMP-9 and TIMP-1 concentrations at baseline (24 weeks) and at the end (30 weeks) of the study in apoE −/− mice.

Groups	Rimonabant + Exercise(n = 12)	Rimonabant(n = 12)	Exercise(n = 12)	Control(n = 12)	*P*	*P1*	*P2*	*P3*	*P4*	*P5*	*P6*
Weight (g)											
Baseline	32.3 ± 4.55	32.3 ± 7.98	33.3 ± 8.08	32.85 ± 7.99	0.854	0.712	0.923	0.449	0.795	0.732	0.634
End	29.7 ± 5.05	33.1 ± 4.55	31.84 ± 3.27	34.66 ± 4.29							
Glucose (mg/dl)											
Baseline	137 ± 30	128 ± 42	131 ± 33	130 ± 15	0.809	0.960	0.998	0.773	0.978	0.907	0.993
End	174 ± 58	189 ± 45	154 ± 48	174 ± 57*							
TC (mg/dl)											
Baseline	612 ± 189	798 ± 219	664 ± 198	612 ± 243	0.048	0.498	0.589	0.021	0.672	0.082	0.037
End	443 ± 123*	712 ± 223	506 ± 110	779 ± 311							
TG (mg/dl)											
Baseline	242 ± 23	264 ± 133	289 ± 32	254 ± 36	0.044	0.334	0.030	0.991	0.128	0.434	<0.001
End	295 ± 29	255 ± 114	196 ± 21*	366 ± 32*							
MMP-2 (ng/ml)											
Baseline	119.11 ± 73.65	119.52 ± 82.47	104.81 ± 23.86	121.51 ± 44.18	0.671	0.590	0.821	0.143	0.901	0.427	0.884
End	158.2 ± 32.72	132.1 ± 20.24	116.66 ± 69.21	123.62 ± 45.15							
MMP-3 (ng/ml)											
Baseline	64.67 ± 15.46	51.02 ± 9.9	62.21 ± 10.93	52.72 ± 12.04	0.021	0.113	0.047	<0.001	0.035	<0.001	0.003
End	20.78 ± 23.63*	26.44 ± 8.7*	53.99 ± 34.62*	70.89 ± 37.98*							
MMP-9 (ng/ml)											
Baseline	29.44 ± 9.46	26.64 ± 8.8	28.7 ± 9.25	24.2 ± 11.01	0.005	0.222	0.885	<0.001	0.028	<0.001	<0.001
End	10.48 ± 6.91*	19.85 ± 5.99*	12.96 ± 8.82*	29.51 ± 2.09*							
TIMP-1 (pg/ml)											
Baseline	1871.3 ± 575.72	1881.08 ± 301.28	1783.44 ± 443.54	1765.12 ± 479.31	<0.001	0.901	<0.001	<0.001	<0.001	<0.001	<0.001
End	2275.51 ± 287.5	2132.39 ± 498.32	3115.46 ± 720.56	1434.7 ± 94.56							

TC, total cholesterol; TG, triglycerides; *P*, one-way ANOVA; *P1*, RiEx vs R; *P2*, RiEx vs Ex; *P3*, RiEx vs Co; *P4*, Ri vs Ex; *P5*, Ri vs Co; *P6*, Ex vs Co; *p < 0.05, within groups (paired samples t-test within groups).

Table 2 Effects on the plaque area, percentage of luminar stenosis, collagen, elastin, VSMC, macrophage content of the atherosclerotic plaques at the end (30 weeks) of the study in ApoE −/− mice.

Groups	Rimonabant + Exercise	Rimonabant	Exercise	Control	*P*	*P1*	*P2*	*P3*	*P4*	*P5*	*P6*
Plaque area/ Lumen area(%)	43.18 ± 1.72	44.66 ± 3.1	49 ± 4.10	70.43 ± 2.83	<0.001	0.995	0.246	<0.001	0.681	0.001	0.006
Collagen (% plaque)	43.5 ± 5.99	34 ± 3.53	35.73 ± 7.39	24.46 ± 12.25	<0.001	0.122	0.089	<0.001	0.975	0.178	0.023
Elastin (% plaque)	32.3 ± 11.14	36.33 ± 5.85	27.14 ± 3.44	17.85 ± 2.87	<0.001	0.672	0.301	0.002	0.051	<0.001	0.048
VSMC (%plaque)	15.83 ± 4.08	17 ± 4.76	14.14 ± 5.18	8.91 ± 2.76	0.115	0.984	0.705	0.130	0.668	0.177	0.423
Macrophages (% plaque)	9.4 ± 3.92	15 ± 2.45	19.78 ± 2.79	34.25 ± 4.99	<0.001	<0.001	<0.001	<0.001	0.038	<0.001	<0.001

P, one-way ANOVA; *P1*, RiEx vs R; *P2*, RiEx vs Ex; *P3*, RiEx vs Co; *P4*, Ri vs Ex; *P5*, Ri vs Co; *P6*, Ex vs Co; $p < 0.05$.

Table 3 Effects on the percentage of MMP-2, MMP3, MMP-9 and TIMP-1 content of the atherosclerotic plaques at the end (30 weeks) of the study in ApoE −/− mice.

Groups	Rimonabant + Exercise	Rimonabant	Exercise	Control	*P*	*P1*	*P2*	*P3*	*P4*	*P5*	*P6*
MMP-2 (% plaque)	6.43 ± 3.46	7.01 ± 1.21	4.85 ± 1.55	3.97 ± 0.46	0.108	0.701	0.231	0.178	0.798	0.124	0.665
MMP-3 (% plaque)	4.64 ± 0.18	5.62 ± 0.27	3.94 ± 1.04	9.53 ± 1.69	<0.001	0.349	0.505	<0.001	0.010	<0.001	<0.001
MMP-9 (% plaque)	7.82 ± 2.99	8.67 ± 3.33	11.15 ± 4.44	18.09 ± 4.47	<0.001	0.973	0.167	<0.001	0.565	0.001	0.005
TIMP-1 (% plaque)	11.23 ± 3.7	11 ± 5.35	14.54 ± 3.48	6.31 ± 1.46	0.001	0.999	0.116	0.042	0.332	0.197	<0.001

P, one-way ANOVA; *P1*, RiEx vs R; *P2*, RiEx vs Ex; *P3*, RiEx vs Co; *P4*, Ri vs Ex; *P5*, Ri vs Co; *P6*, Ex vs Co; $p < 0.05$.

study, the retardation and stabilization of plaques paralleled the suppression of MMP-3 and MMP-9 across groups. The amount of change in these MMPs was the same for exercise and rimonabant therapeutic modalities. Moreover, the combined treatment did not further decrease MMP levels relative to each intervention alone. We hypothesize that all treatment modalities exert equivalent MMP-related plaque-stabilizing actions. However, the proposed mechanisms require further investigation.

In contrast, both circulating and intraplaque concentration of MMP-2 were not affected by either rimonabant or exercise training. This finding may mirror the absence of any change in its cellular source, VSMCs. Although some reports have shown that exercise training[40] and endocannabinoids modulate the migration of VSMCs,[41] we did not detect any effect on their concentrations. Perhaps, improved laboratory techniques are necessary for the identification of VSMC functions within atherosclerotic plaques.

Another important finding was the considerable improvement in circulating MMP-3, MMP-9 and TIMP-1 levels over the 6-week treatment period in all active treatment groups, implicating an atheroprotective effect. The relative concentrations of MMP-3, MMP-9 and TIMP-1 within plaques almost paralleled the respective serum values across groups at the end of the study. It is worth mentioning that the wide-spectrum MMP inhibitor, TIMP-1, has been extensively reported to contribute to atherosclerotic plaque stability.[42] In our study, exercise therapy predominantly upregulated intraplaque TIMP-1 activity, while rimonabant treatment alone tended to increase TIMP-1. All treatment modalities significantly shifted the MMP-9/TIMP-1 equilibrium to be less proteolytically active, implicating an atheroprotective mechanism. Thus, from our results we could not infer superiority of any intervention. However, this is only a small part of the regulatory mechanisms in atherosclerosis and cannot describe the whole spectrum of plaque phenotypes. Perhaps, additional mechanisms are involved in plaque texture modification, which are beyond the scope of the present study.

There are several drawbacks to the present study. First, we used a valid animal model of atherosclerosis development in which spontaneous plaque rupture is rarely observed. Thus, we estimated plaque vulnerability indirectly via the quantification of ECM components and inflammatory cell infiltration. Second, the immunohistochemistry-based measurements do not directly express absolute protein activity, but instead provide a relative quantification of molecule concentrations. Third, TIMP-1 is a wide-spectrum MMP inhibitor that forms complexes with MMPs. These complexes inhibit the proteolytic activity of MMPs and concomitantly promote the activation of MMP pro-enzymes.

Thus, the immunohistochemical staining cannot distinguish the free-molecule content. Finally, several reports from medical societies about increased incidence of psychiatric side-effects, including depression and suicide, led American and European drug authorities to suspend rimonabant across the world. Despite the withdrawal of rimonabant, the study of the endocannabinoid system and its actions remains an intriguing field of scientific research and it may be of great interest to test an even lower dose, such as 1 mg/kg to 0.5 mg/kg to reduce side effects.[43]

In conclusion, rimonabant treatment and exercise training either alone or combined induce plaque regression and increase stability without any complementary effects of combining rimonabant and exercise training. Overall, the specific role of endocannabinoid signaling during atherosclerosis remains to be better elucidated.

Acknowledgements

This study was financed in part by Sanofi-Aventis. Nikolaos P.E. Kadoglou was awarded a grant by the Greek State Scholarship's Foundation.

References

1. Mancia G, Fagard R, Narkiewicz K, et al. 2013 ESH/ESC guidelines for the management of arterial hypertension: the Task Force for the Management of Arterial Hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). *Eur Heart J*. Jul 2013;34(28):2159—2219.
2. Libby P, Okamoto Y, Rocha VZ, Folco E. Inflammation in atherosclerosis: transition from theory to practice. *Circ J*. Feb 2010;74(2):213—220.
3. Kadoglou NP, Daskalopoulou SS, Perrea D, Liapis CD. Matrix metalloproteinases and diabetic vascular complications. *Angiology*. Mar-Apr 2005;56(2):173—189.
4. Scoditti E, Nestola A, Massaro M, et al. Hydroxytyrosol suppresses MMP-9 and COX-2 activity and expression in activated human monocytes via PKCalpha and PKCbeta1 inhibition. *Atherosclerosis*. Jan 2014;232(1):17—24.
5. Huxley RR, Lopez FL, MacLehose RF, et al. Novel association between plasma matrix metalloproteinase-9 and risk of incident atrial fibrillation in a case-cohort study: the Atherosclerosis Risk in Communities study. *PloS one*. 2013;8(3):e59052.
6. Brunner S, Kim JO, Methe H. Relation of matrix metalloproteinase-9/tissue inhibitor of metalloproteinase-1 ratio in peripheral circulating CD14+ monocytes to progression of coronary artery disease. *Am J Cardiol*. Feb 15 2010;105(4):429—434.
7. Basu P, Sen U, Tyagi N, Tyagi SC. Blood flow interplays with elastin: collagen and MMP: TIMP ratios to maintain healthy vascular structure and function. *Vasc Health Risk Manag*. 2010;6:215—228.
8. Kadoglou NP, Iliadis F, Liapis CD. Exercise and carotid atherosclerosis. *Eur J Vasc Endovasc Surg*. Mar 2008;35(3):264—272.
9. Palmefors H, DuttaRoy S, Rundqvist B, Borjesson M. The effect of physical activity or exercise on key biomarkers in atherosclerosis—a systematic review. *Atherosclerosis*. Jul 2014;235(1):150—161.
10. Agrotou S, Karatzi K, Papamichael C, et al. Effects of chronic anaerobic training on markers of sub-clinical atherosclerosis. *Hellenic J Cardiol*. May-Jun 2013;54(3):178—185.
11. Shon SM, Park JH, Nahrendorf M, et al. Exercise attenuates matrix metalloproteinase activity in preexisting atherosclerotic plaque. *Atherosclerosis*. May 2011;216(1):67—73.
12. Kadoglou NP, Kostomitsopoulos N, Kapelouzou A, et al. Effects of exercise training on the severity and composition of atherosclerotic plaque in apoE-deficient mice. *J Vasc Res*. 2011;48(4):347—356.
13. Kadoglou NP, Moustardas P, Kapelouzou A, et al. The anti-inflammatory effects of exercise training promote atherosclerotic plaque stabilization in apolipoprotein E knockout mice with diabetic atherosclerosis. *Eur J Histochem*. 2013;57(1):e3.
14. Rallidis LS. Are lower levels of LDL-cholesterol really better? Looking at the results of IMPROVE-IT: opinions of three experts - II. *Hellenic J Cardiol*. Jan-Feb 2015;56(1):4—6.
15. Singh M, Bedi US. Is atherosclerosis regression a realistic goal of statin therapy and what does that mean? *Curr Atheroscler Rep*. Jan 2013;15(1):294.
16. Zernecke A, Weber C. Improving the treatment of atherosclerosis by linking anti-inflammatory and lipid modulating strategies. *Heart (British Cardiac Society)*. Nov 2012;98(21):1600—1606.
17. Esch T. Endocannabinoid signaling in stress, medicine and wellness. *Med Sci Monit*. Aug 2005;11(8). ED3-5.
18. Steffens S, Mach F. Cannabinoid receptors in atherosclerosis. *Curr Opin Lipidol*. Oct 2006;17(5):519—526.
19. Sugamura K, Sugiyama S, Nozaki T, et al. Activated endocannabinoid system in coronary artery disease and antiinflammatory effects of cannabinoid 1 receptor blockade on macrophages. *Circulation*. Jan 6 2009;119(1):28—36.
20. Topol EJ, Bousser MG, Fox KA, et al. Rimonabant for prevention of cardiovascular events (CRESCENDO): a randomised, multicentre, placebo-controlled trial. *Lancet*. Aug 14 2010;376(9740):517—523.
21. Raichlen DA, Foster AD, Gerdeman GL, Seillier A, Giuffrida A. Wired to run: exercise-induced endocannabinoid signaling in humans and cursorial mammals with implications for the 'runner's high'. *J Exp Biol*. Apr 15 2012;215(Pt 8):1331—1336.
22. Raichlen DA, Foster AD, Seillier A, Giuffrida A, Gerdeman GL. Exercise-induced endocannabinoid signaling is modulated by intensity. *Eur J Appl Physiol*. Apr 2013;113(4):869—875.
23. Galdino G, Romero T, da Silva JF, et al. Acute Resistance Exercise Induces Antinociception by Activation of the Endocannabinoid System in Rats. *Anesth Analg*. Jun 26 2014.
24. Tiyerili V, Zimmer S, Jung S, et al. CB1 receptor inhibition leads to decreased vascular AT1 receptor expression, inhibition of oxidative stress and improved endothelial function. *Basic Res Cardiol*. Jul 2010;105(4):465—477.
25. Sugamura K, Sugiyama S, Fujiwara Y, et al. Cannabinoid 1 receptor blockade reduces atherosclerosis with enhances reverse cholesterol transport. *J Atheroscler Thromb*. Feb 26 2010;17(2):141—147.
26. Dol-Gleizes F, Paumelle R, Visentin V, et al. Rimonabant, a selective cannabinoid CB1 receptor antagonist, inhibits atherosclerosis in LDL receptor-deficient mice. *Arterioscler Thromb Vasc Biol*. Jan 2009;29(1):12—18.
27. O'Leary DH, Reuwer AQ, Nissen SE, et al. Effect of rimonabant on carotid intima-media thickness (CIMT) progression in patients with abdominal obesity and metabolic syndrome: the AUDITOR Trial. *Heart (British Cardiac Society)*. Jul 2011;97(14):1143—1150.
28. Mohapatra J, Sharma M, Acharya A, et al. Retinol-binding protein 4 : a possible role in cardiovascular complications. *Br J Pharmacol*. Dec 2011;164(8):1939—1948.
29. Netherland C, Thewke DP. Rimonabant is a dual inhibitor of acyl CoA:cholesterol acyltransferases 1 and 2. *Biochem Biophys Res Commun*. Aug 6 2010;398(4):671—676.
30. El-Remessy AB, Rajesh M, Mukhopadhyay P, et al. Cannabinoid 1 receptor activation contributes to vascular inflammation and

cell death in a mouse model of diabetic retinopathy and a human retinal cell line. *Diabetologia*. Jun 2011;54(6):1567—1578.
31. Goncalves I, Ares MP, Moberg A, et al. Elastin- and collagen-rich human carotid plaques have increased levels of the cysteine protease inhibitor cystatin C. *J Vasc Res*. 2008;45(5): 395—401.
32. Wang Y, Johnson JA, Fulp A, Sutton MA, Lessner SM. Adhesive strength of atherosclerotic plaque in a mouse model depends on local collagen content and elastin fragmentation. *J Biomech*. Feb 22 2013;46(4):716—722.
33. Pellegrin M, Alonso F, Aubert JF, et al. Swimming prevents vulnerable atherosclerotic plaque development in hypertensive 2-kidney, 1-clip mice by modulating angiotensin II type 1 receptor expression independently from hemodynamic changes. *Hypertension*. May 2009;53(5):782—789.
34. Prati P, Tosetto A, Casaroli M, et al. Carotid plaque morphology improves stroke risk prediction: usefulness of a new ultrasonographic score. *Cerebrovasc Dis*. 2011;31(3):300—304.
35. Chistiakov DA, Sobenin IA, Orekhov AN. Vascular extracellular matrix in atherosclerosis. *Cardiol Rev*. Nov-Dec 2013;21(6): 270—288.
36. Siasos G, Tousoulis D, Kioufis S, et al. Inflammatory mechanisms in atherosclerosis: the impact of matrix metalloproteinases. *Curr Top Med Chem*. 2012;12(10):1132—1148.
37. Ogita M, Miyauchi K, Morimoto T, et al. Association between circulating matrix metalloproteinase levels and coronary plaque regression after acute coronary syndrome—subanalysis of the JAPAN-ACS study. *Atherosclerosis*. Jan 2013;226(1): 275—280.
38. Lenglet S, Thomas A, Soehnlein O, et al. Fatty acid amide hydrolase deficiency enhances intraplaque neutrophil recruitment in atherosclerotic mice. *Arterioscler Thromb Vasc Biol*. Feb 2013;33(2):215—223.
39. Montecucco F, Di Marzo V, da Silva RF, et al. The activation of the cannabinoid receptor type 2 reduces neutrophilic protease-mediated vulnerability in atherosclerotic plaques. *Eur Heart J*. Apr 2012;33(7):846—856.
40. Michel-Monigadon D, Steffens S, Molica F, Mach F, Montecucco F. Update on the endocannabinoid-mediated regulation of gelatinase release in arterial wall physiology and atherosclerotic pathophysiology. *Expert Rev Cardiovasc Ther*. Dec 2012;10(12):1481—1486.
41. Rajesh M, Mukhopadhyay P, Hasko G, Pacher P. Cannabinoid CB1 receptor inhibition decreases vascular smooth muscle migration and proliferation. *Biochem Biophys Res Commun*. Dec 26 2008;377(4):1248—1252.
42. de Vries MR, Niessen HW, Lowik CW, Hamming JF, Jukema JW, Quax PH. Plaque rupture complications in murine atherosclerotic vein grafts can be prevented by TIMP-1 overexpression. *PloS one*. 2012;7(10):e47134.
43. Netherland CD, Pickle TG, Bales A, Thewke DP. Cannabinoid receptor type 2 (CB2) deficiency alters atherosclerotic lesion formation in hyperlipidemic Ldlr-null mice. *Atherosclerosis*. Nov 2010;213(1):102—108.