



## Resolvin E1 attenuates atherosclerosis in absence of cholesterol-lowering effects and on top of atorvastatin



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### ABSTRACT

**Background and aims:** Besides LDL-cholesterol, local vascular inflammation plays a key role in atherogenesis. Efficient therapies to treat the inflammatory component of the disease have not been established. The discovery of specialized inflammation-resolving mediators, such as resolvins may provide new opportunities for treatment. This study examines whether the  $\omega$ -3 fatty acid eicosapentaenoic acid-derived resolvin E1 (RvE1), can reduce atherosclerosis, when administered alone or in combination with a cholesterol-lowering statin.

**Methods:** ApoE<sup>3</sup>Leiden mice were fed a hypercholesterolemic diet for 9 weeks and subsequently treated with RvE1-low (1 mg/kg/day), RvE1-high (5 mg/kg/day), atorvastatin (1.5 mg/kg/day) or the combination of atorvastatin and RvE1-low for the following 16 weeks.

**Results:** RvE1-low and RvE1-high reduced atherosclerotic lesion size to the same extent (−35%;  $p < 0.05$ ), attenuated the formation of severe lesions, also seen as a proportional increase in the presence of mild lesions, but did not alter plasma cholesterol levels. Cholesterol-lowering atorvastatin reduced atherosclerosis (−27%,  $p < 0.05$ ), and the combination of RvE1 and atorvastatin further attenuated lesion size (−51%,  $p < 0.01$ ) and increased the content of mild lesions. RvE1 did not affect plasma SAA, E-selectin, VCAM-1 or MCP-1 but did reduce plasma EPHX4 and down-regulated the local expression of pro-atherogenic genes in the aortae, (e.g. *Cd74*, *Cd44*, *Ccl2*, *Ccr5* and *Adam17*) and significantly inactivated IFN- $\gamma$  ( $p < 0.001$ ) and TNF- $\alpha$  ( $p < 0.001$ ) signalling pathways.

**Conclusions:** RvE1 attenuates atherogenesis both alone and on top of a statin. The local effects of RvE1 are demonstrated by the modulated aortic expression of genes involved in inflammatory and immune responses, without altering plasma cholesterol or circulating SAA.

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## 1. Introduction

Atherosclerotic plaque buildup is closely linked to an increased exposure to low-density lipoprotein (LDL) cholesterol, which has

been the rationale for treatment with LDL-lowering statins over the last several decades. While the outcome of statin treatment undeniably is a reduction in cardiovascular disease (CVD) mortality, this still remains a major cause of death worldwide [1]. However, elevated LDL levels may be more important in the induction of disease, while plaque progression and later potential rupture is caused by vascular inflammation rather than high LDL levels per se. Intimal retention of LDL and its subsequent modification and oxidation provide a chronic trigger of vascular inflammation. This initially involves the innate immune system, with monocyte recruitment and macrophage and dendritic cell activation, which initiates later adaptive immune responses [2,3]. Due to the

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complexity of these local inflammatory events in CVD development, it is challenging to single out one pathway as a therapeutic target, when considering an immune-based approach. Statins, in addition to their LDL-lowering effects, have specific anti-inflammatory properties in the vasculature [4] but may not offer sufficient control [5], nor have other attempts at treating atherosclerosis with anti-inflammatory drugs been successful [6]. A major contributing factor to plaque build-up and instability is thought to be compromised clearance mechanisms at the plaque level, possibly suggesting a local imbalance between pro-inflammatory events and counter-acting resolution mechanisms of the immune system [7] suggesting that resolution may be dysfunctional.

The discovery of specialized pro-resolving mediators (SPMs) may represent a therapeutic alternative to classic anti-inflammatory drugs by harnessing the body's own systems to regulate inflammation and promote homeostasis, including activation of endogenous clearance mechanisms [8]. Originally identified as oxygenation products of omega-3 fatty acids in resolving exudates of acute inflammation [9], these lipid-derived mediators with agonistic properties, such as resolvins, protectins, and maresins, are now established regulators of both acute and chronic inflammatory responses [10]. In a dual action these mediators dampen active pro-inflammatory pathways and concurrently activate pro-resolution functions such as phagocytic clearance and tissue repair mechanisms. Uniquely, they appear to do so without compromising host immune defense [10].

The ApoE\*3Leiden transgenic mouse has a humanized lipoprotein profile with elevated plasma cholesterol levels confined mainly to the VLDL/LDL-sized lipoprotein fractions [11]. The model is well-established to quantify the build-up of lesions and their potential amelioration by drug intervention, and is well-characterized for statins [12]. Using this model, we herein investigated the efficacy of resolvin E1 (RvE1), an endogenous SPM derived from eicosapentaenoic acid, in attenuating atherosclerotic lesion development. A putative anti-atherogenic effect of RvE1 was studied at two doses, and in combination with atorvastatin. A reference group was treated with atorvastatin alone.

## 2. Materials and methods

### 2.1. Ethics statement

Experiments were approved by an independent Ethical Committee on Animal Care and Experimentation (Zeist, the Netherlands; approval number DEC2680) and were performed in compliance with the European Commission Directive on the use of animals for scientific purposes.

### 2.2. Animals and treatments

Eighty female ApoE\*3Leiden transgenic mice were fed an atherogenic Western-type diet (HC) for a nine-week run-in period, starting at 12–16 weeks of age. This diet contains 40.5% sucrose, 20% acid casein, 15% cocoa butter, 10% corn starch, 5.45% cellulose, 5.1% mineral mixture, 1% choline chloride, 1% corn oil, 0.2% methionine and 0.4% cholesterol (all w/w) (AB-diets, Woerden, the Netherlands). After the run-in period, in which plasma cholesterol increased from  $2.8 \pm 0.4$  mM to  $17.5 \pm 2.5$  mM as expected for ApoE\*3Leiden transgenic mice, mice were divided into 5 treatment groups that were matched for total plasma cholesterol ( $n = 16$ /group) continuing on HC with 1) vehicle control, 2) 1.5 mg/kg/day atorvastatin, 3) 1 mg/kg/day RvE1 (RvE1-low), 4) 5 mg/kg/day RvE1 (RvE1-high), 5) or a combination of atorvastatin 1.5 mg/kg/day and RvE1 1 mg/kg/day (combination group), for the following 16 weeks. Atorvastatin (Lipitor, Pfizer, Capelle a/d IJssel, the Netherlands) was

supplemented to the diet (0.0015% w/w), and RvE1 and vehicle were administered daily by oral gavage between 9 and 10 a.m. Resolvin E1 (RvE1; RX-10001) was provided by Resolvyx Pharmaceuticals Inc. (Cambridge, MA, USA). The dose RvE1 used in this study was defined based on previous experiments in humans and mice. Briefly, pharmacokinetic analysis of a single dose, 10 mg,  $^{14}\text{C}$ -labeled RvE1 (approximately 0.15 mg/kg) in a Phase 1 clinical study revealed a half-life of about 7 h (Resolvyx unpublished; trial number NCT00941018). Furthermore, administration of repeated doses of 100 mg and higher in the same study were well-tolerated and without safety-related issues. In mice,  $^{14}\text{C}$ -labeled RvE1 at 1 mg/kg resulted in plasma concentrations greater than 3 nM, which is the  $\text{EC}_{50}$  defined in *in vitro* experiments. These levels ( $>3$  nM) were maintained for a period of 8 h post-administration. (Resolvyx unpublished).

All animals had free access to water and food. Body weight and food intake were monitored throughout the study. Animals were sacrificed by  $\text{CO}_2$  asphyxiation after 16 weeks of treatment (at 37–41 weeks of age) to collect hearts including the aortic roots which were fixed in formalin and embedded in paraffin for atherosclerosis analysis.

Prior to the main study described above, a pilot experiment was performed using the same experimental conditions but with 9 weeks of RvE1 intervention. This experiment served to investigate whether a dose of 1 mg/kg is efficacious in a setting of experimental diet-induced atherosclerosis and to establish a microarray gene expression dataset of aortae.

### 2.3. Biochemical, histological and microarray gene expression analyses

A detailed description of biochemical, histological and aortic microarray analyses is provided in Supplemental methods. In brief, plasma parameters (total cholesterol, serum amyloid A, E-selectin and alanine aminotransferase) were quantified using commercially available assays as described previously [13,14]. Atherosclerosis was scored blindly in 4 serial cross-sections (at 50  $\mu\text{m}$  intervals) of the aortic root. Morphometric analysis of lesion number and area was performed using cellD software (version 2.7; Olympus Soft Imaging Solutions, Hamburg, Germany), and lesion severity was scored according to the established classification of the American Heart Association [15,16]. Aortic genome-wide gene expression analyses were performed using Illumina microarray analysis followed by established normalisation and quality control protocols and pathway analysis as described [17,18].

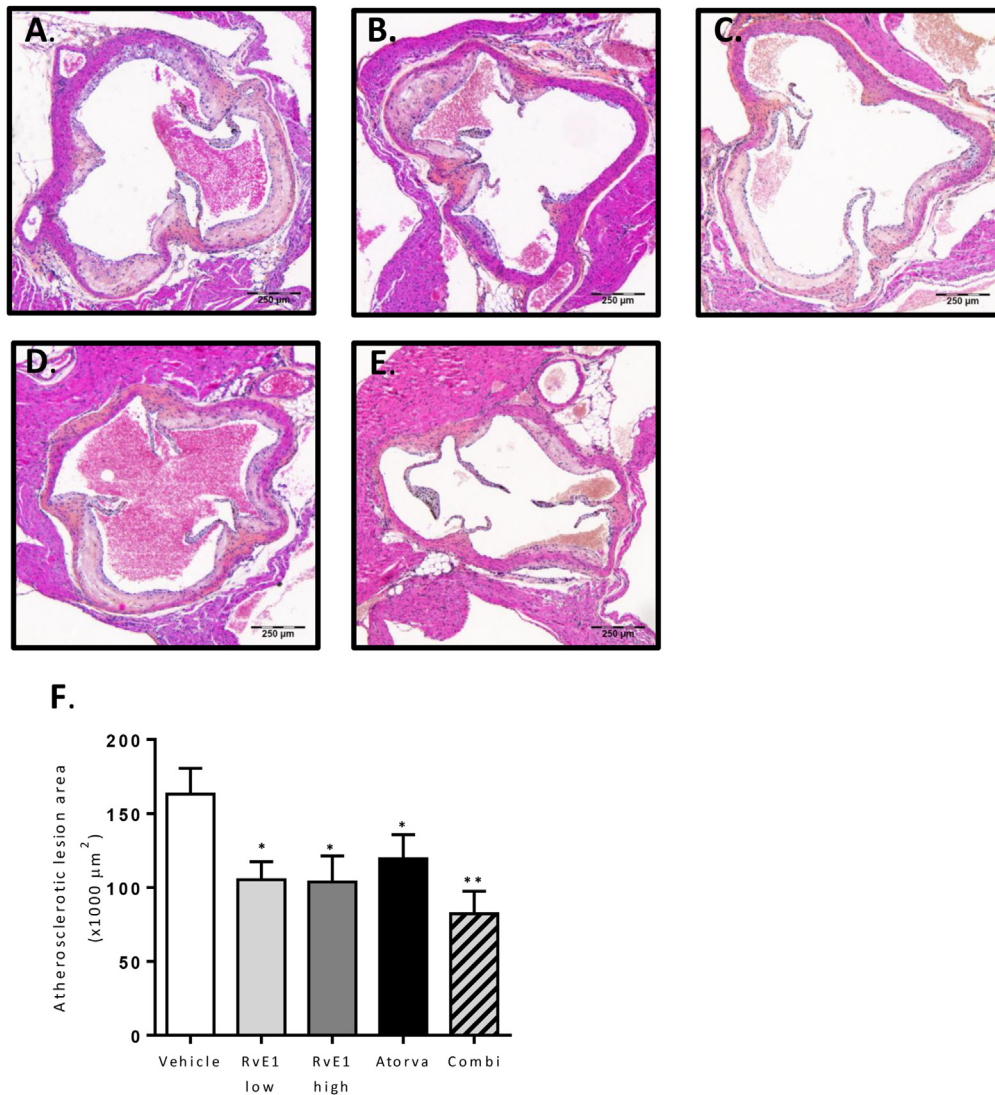
### 2.4. Statistical analysis

Significance of differences was tested using one-way ANOVA with LSD post-hoc test. Statistical tests were performed using SPSS software (version 20, IBM, Armonk USA). A  $p$ -value  $<0.05$  was considered statistically significant. All data are presented as mean  $\pm$  SEM.

## 3. Results

### 3.1. RvE1 treatment reduces atherosclerotic lesion area

ApoE\*3Leiden transgenic mice were fed the HC diet for 9 weeks to induce atherosclerotic lesions, after which treatment with RvE1 was started and continued for another 16 weeks until the end of the study. Analysis of atherosclerotic lesions in the valve area of the aortic root (Fig. 1A–E) revealed a clear treatment effect of RvE1 relative to vehicle in which the total lesion area was  $163,200 \pm 17,410 \mu\text{m}^2$ . Both RvE1-low ( $105,300 \pm 12,200 \mu\text{m}^2$ ) and



**Fig. 1.** Treatment with RvE1 reduces atherosclerotic lesion area. (A) Representative images of haematoxylin-phloxine-saffron stained cross-sections of the aortic root after 16 weeks of treatment with vehicle, (B) RvE1-low 1 mg/kg/day, (C) RvE1-high 5 mg/kg/day, (D) atorvastatin 1.5 mg/kg/day, (E) combination of atorvastatin 1.5 mg/kg/day and RvE1 1 mg/kg/day. (F) Quantification of total atherosclerotic lesion area in serial cross-sections of the aortic root after 16 weeks of treatment. Results are shown as mean  $\pm$  SEM (n = 15–16/group). \* $p < 0.05$ , \*\* $p < 0.01$  vs. the vehicle group.

RvE1-high ( $103,700 \pm 17,640 \mu\text{m}^2$ ) significantly attenuated lesion area by around 35% compared with vehicle control (both  $p < 0.05$  vs. vehicle) (Fig. 1F). The reference compound atorvastatin ( $119,400 \pm 16,410 \mu\text{m}^2$ ) reduced atherosclerosis to a comparable extent as RvE1 (27% reduction;  $p < 0.05$  vs. vehicle). Notably, the combination treatment had the strongest effect on atherosclerosis ( $82,300 \pm 1522 \mu\text{m}^2$ ) equaling a 51% reduction ( $p < 0.001$  vs. vehicle). The lesion area of this group tended to be 31% smaller than with atorvastatin ( $p = 0.11$  vs. atorvastatin).

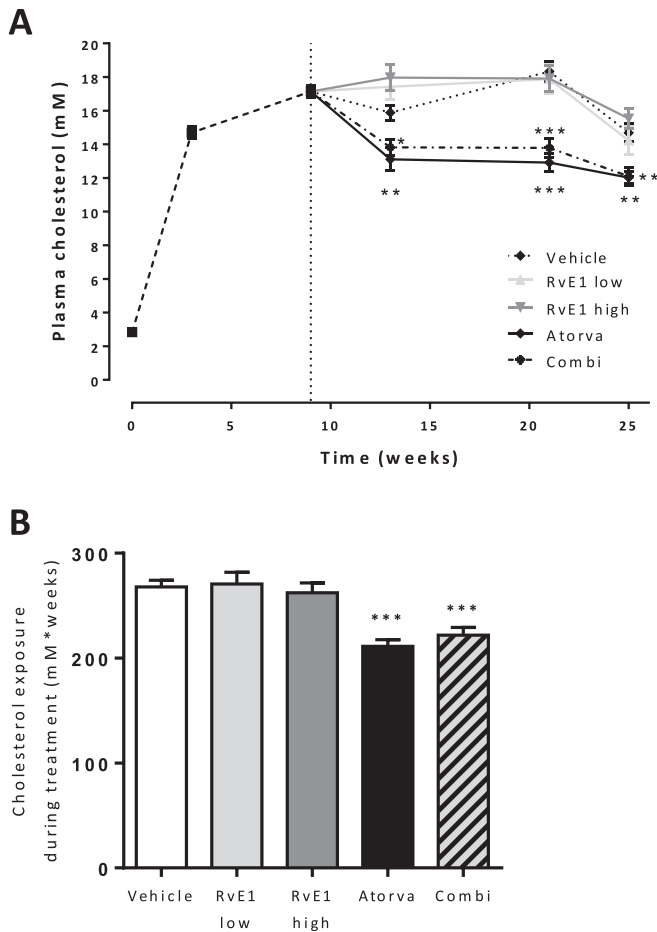
In ApoE\*3Leiden transgenic mice, the reduction in lesion area upon statin treatment is closely linked to the cholesterol-lowering effects of these drugs [11,12]. In the current study, atorvastatin reduced plasma cholesterol levels from  $17.5 \pm 0.3$  mM before the start of treatment to  $12.0 \pm 0.4$  mM at endpoint (18% reduction vs. vehicle;  $p < 0.001$ ) (Fig. 2A). RvE1 had no effect on plasma cholesterol levels at either dose, nor did RvE1 affect the lipoprotein profile or HDL cholesterol levels (data not shown). Combined treatment with RvE1 and atorvastatin reduced cholesterol levels to

the same degree as atorvastatin alone (18% reduction at endpoint;  $p < 0.01$  vs. vehicle) (Fig. 2A). Atorvastatin and the combination treatment reduced the total cholesterol exposure, calculated over the entire treatment period, by 21% and 17% (both  $p < 0.001$  vs. vehicle) respectively (Fig. 2B), while cholesterol exposure was not affected by RvE1.

### 3.2. RvE1 treatment improves lesion severity

To further elucidate how RvE1 interferes with the progression of atherosclerosis, aortic segments were analyzed for presence or absence of lesions and the number of lesion-free segments was determined (Fig. 3A). When lesions were present, each individual lesion was graded to determine its severity according to the classification system developed by the American Heart Association [15,16] (Fig. 3B).

In the vehicle group,  $8 \pm 3\%$  of all aortic segments were free of lesions (Fig. 3A). A higher content of lesion-free segments was

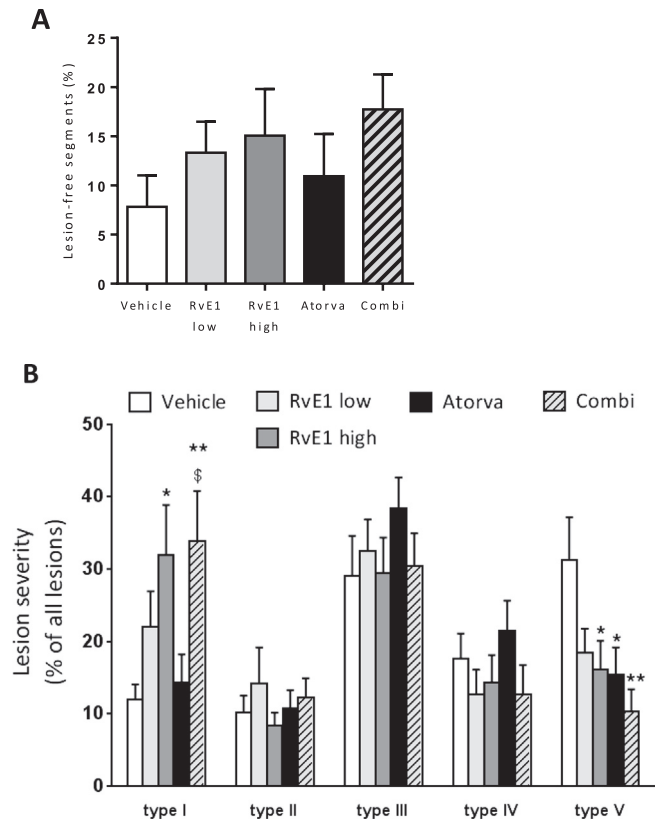


**Fig. 2. Treatment with RvE1 does not reduce plasma cholesterol.** (A) Plasma cholesterol levels over time in the different treatment groups from  $t = 0$  to  $t = 25$  weeks. (B) Cholesterol exposure during the 16-weeks treatment period. Results are shown as mean  $\pm$  SEM ( $n = 15$ – $16$ /group). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. the vehicle group.

observed with RvE1 (RvE1-low  $13 \pm 3\%$  and RvE1-high  $15 \pm 5\%$ ) and atorvastatin ( $11 \pm 4\%$ ) but these effects were not significant. The combination treatment had the highest content of lesion-free segments ( $18 \pm 4\%$ ,  $p = 0.07$  vs. vehicle).

A more refined analysis of lesion severity showed that all experimental groups exhibited lesions ranging from mild (I) to most severe (V) (Fig. 3B). However, there was a striking difference in distribution of severity scores in favor of milder lesions in the RvE1-treated groups. Most notably the content of mild type I lesions (early fatty streaks) was almost doubled in the low-dose RvE1 group, with  $22 \pm 5\%$  type I lesions, compared to  $12 \pm 2\%$  in the vehicle group ( $p = 0.17$ , Fig. 3B). The effect of RvE1 seemed to be dose-dependent as this percentage was further increased to  $32 \pm 7\%$  in the high-dose group ( $p < 0.01$  vs. vehicle). Although reference atorvastatin alone did not affect the content of type I lesions ( $14 \pm 4\%$ , n.s. vs. vehicle), the combination treatment resulted in significantly more of these mild lesions ( $34 \pm 7\%$ , combination vs. vehicle  $p < 0.01$ , combination vs. atorvastatin  $p < 0.05$ , Fig. 3B) indicating that the observed attenuation of lesion severity can be ascribed to RvE1.

In the vehicle control group  $31 \pm 6\%$  of the lesions were severe type V lesions. RvE1-treated groups contained less type V lesions (RvE1-low  $18 \pm 3\%$ , n.s. vs. vehicle and RvE1-high  $16 \pm 4\%$ ,  $p < 0.05$  vs. vehicle) which represents a reduction of 42% and 48%, respectively (Fig. 3B). The content of type V lesions was also reduced by



**Fig. 3. RvE1 treatment increases proportion of lesion-free segments and incidence of mild lesions.** (A) Percentage of lesion-free segments in the aortic root. (B) Lesion severity as a percentage of all lesions, ranging from type I (mild) to type V (severe) lesions, after 16 weeks of treatment. Results are shown as mean  $\pm$  SEM ( $n = 15$ – $16$ /group). \* $p < 0.05$  and \*\* $p < 0.01$  vs. the vehicle group. § $p < 0.05$  vs. the atorvastatin group.

atorvastatin ( $15 \pm 4\%$ ,  $p < 0.01$  vs. vehicle), and the combination treatment had the lowest content of type V lesions ( $10 \pm 3\%$ ,  $p < 0.01$  vs. vehicle) equaling a reduction of 67%.

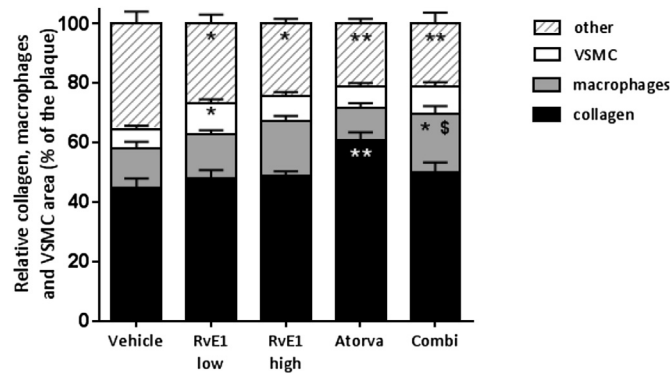
### 3.3. Effect of RvE1 treatment on the composition of aortic lesions

To investigate whether RvE1 treatment may affect the composition of aortic lesions, we analyzed the collagen area, the macrophage area, the vascular smooth muscle cell (VSMC) of the lesions. Data are shown as percentages of the plaque area (Fig. 4).

PicroSirius Red staining showed that the lesional collagen area was  $45 \pm 3\%$  in the vehicle control group (Fig. 4). This was not affected by RvE1 treatment ( $48 \pm 3\%$  in RvE1-low,  $49 \pm 1\%$  in RvE1-high). Atorvastatin treatment increased the collagen content ( $61 \pm 2\%$ ,  $p < 0.01$  vs. vehicle), an effect that was not observed in the combination treatment ( $50 \pm 3\%$ ).

The percentage of VSMC area (Fig. 4), as assessed by immunohistochemical staining for alpha smooth muscle actin, was  $6 \pm 1\%$  in vehicle control animals. RvE1-low treatment increased the VSMC content ( $11 \pm 1\%$ ,  $p < 0.05$ ). Neither RvE1-high ( $8 \pm 1\%$ ) nor atorvastatin treatment ( $7 \pm 1\%$ ) nor the combination treatment ( $9 \pm 1\%$ ) had a significant effect on the VSMC area of aortic lesions.

Immunohistochemical staining showed that MAC-3-positive cells (macrophages; Fig. 4) were present in aortic lesions of the vehicle control group (MAC-3-positive area  $13 \pm 2\%$ ). RvE1 treatment (RvE1-low dose  $15 \pm 1\%$ ; RvE1-high dose  $18 \pm 2\%$ ) and atorvastatin ( $11 \pm 2\%$ ) did not affect this area. The MAC-3 positive area was increased in the combination group ( $20 \pm 3\%$ ;  $p < 0.05$ ) when



**Fig. 4.** Effect of RvE1 treatment on the composition of aortic lesions. Percentages of collagen area (Picrosirius red staining), macrophage area (immunohistochemical staining of MAC-3) and vascular smooth muscle cell area (immunohistochemical staining of alpha smooth muscle actin) of atherosclerotic lesions after 16 weeks of treatment. The remaining lesion area (referred to as 'other') consists of e.g. necrotic core with cholesterol clefts and proteoglycans. Results are shown as mean  $\pm$  SEM ( $n = 13$ –15/group). \* $p < 0.05$  and \*\* $p < 0.01$  vs. vehicle.  $^{\$}p < 0.05$  vs. atorvastatin.

compared to vehicle control and to atorvastatin.

The remainder of the lesion area consisting of necrotic core with cholesterol clefts and proteoglycans was  $35 \pm 4\%$  in vehicle control animals (Fig. 4). RvE1 treatment significantly decreased the percentage of this area ( $27 \pm 3\%$  in RvE1-low and  $24 \pm 2\%$  in RvE1-high, both  $p < 0.05$ ). A significant decrease was also observed for atorvastatin treatment ( $21 \pm 2\%$ ,  $p < 0.01$ ) and the combination treatment ( $21 \pm 4\%$ ,  $p < 0.01$ ).

Targeted analysis of gene expression levels of chemokines and cell surface receptors commonly associated with pro-inflammatory macrophages (*Cxcl10*, *Cxcl11*, *Ccr7*) as well as receptors associated with macrophages contributing to resolution (*Cd163*, *Mrc1*) did not reveal an effect of RvE1 treatment (RT-PCR analysis; not shown). Atorvastatin did also not cause any change in the expression of these receptors.

#### 3.4. Effects of RvE1 on circulating markers of inflammation

To further characterize the anti-atherogenic effect of RvE1, we measured circulating markers of inflammation that are involved in atherogenesis. Serum amyloid A (SAA) is a liver-derived inflammation marker that can exert pro-atherogenic effects in the vasculature [19]. E-selectin and VCAM-1 are expressed on activated endothelial cells and involved in immune cell adhesion and recruitment [20]. In addition, we measured circulating levels of Monocyte Chemoattractant Protein-1 (MCP-1), a chemokine that is reported to play an important role in the pathogenesis of atherosclerosis [21]. SAA levels rose in the vehicle group from  $4.7 \pm 0.3 \mu\text{g/ml}$  at the beginning of the study (data not shown) to  $6.2 \pm 0.7 \mu\text{g/ml}$  at the end of the study. SAA levels were not affected by RvE1-low ( $6.4 \pm 0.5 \mu\text{g/ml}$ ), RvE1-high ( $6.9 \pm 0.4 \mu\text{g/ml}$ ), atorvastatin ( $5.4 \pm 0.3 \mu\text{g/ml}$ ), or the combination treatment ( $5.9 \pm 0.6 \mu\text{g/ml}$ ) (Table 1). E-selectin also increased over time in the vehicle control group, from  $51.8 \pm 1.4 \text{ ng/ml}$  at the start of the study (not shown) to  $68.1 \pm 2.8 \text{ ng/ml}$  at the end of the study. E-selectin levels were not affected by RvE1-low ( $65.7 \pm 3.0 \text{ ng/ml}$ ) or RvE1-high ( $67.5 \pm 1.5 \text{ ng/ml}$ ) (Table 1). Atorvastatin significantly reduced E-selectin ( $53.2 \pm 1.7 \text{ ng/ml}$ ;  $p < 0.001$  vs. vehicle), an effect that was not further enhanced by combination with RvE1 ( $51.1 \pm 2.4 \text{ ng/ml}$ ;  $p < 0.001$  vs. vehicle) (Table 1). Similarly, VCAM-1 levels increased over time in the vehicle control group from  $2.20 \pm 0.05 \mu\text{g/ml}$  at the start of the study (not shown), to  $3.56 \pm 0.22 \mu\text{g/ml}$  at the end of the study (Table 1). Comparable to the effects observed for E-selectin,

VCAM-1 levels were not affected by RvE1-low ( $3.25 \pm 0.09 \mu\text{g/ml}$ ) or RvE1-high ( $3.23 \pm 0.13 \mu\text{g/ml}$ ) while they were similarly reduced relative to vehicle control in atorvastatin-treated and combination-treated animals ( $2.68 \pm 0.20 \mu\text{g/ml}$  and  $2.79 \pm 0.08 \mu\text{g/ml}$  respectively; both  $p < 0.001$  vs. vehicle; Table 1). Circulating levels of the chemokine MCP-1 were not affected by RvE1 at either dose, nor were they affected by atorvastatin or the combination treatment of atorvastatin and RvE1 (Table 1). Since we observed no effect of RvE1 treatment on these conventional circulating markers of inflammation, we questioned whether it may affect a different type of inflammatory marker, namely epoxide hydrolase 4 (EPHX4), which is considered to be pro-atherogenic through its role in the degradation of the anti-inflammatory and atheroprotective epoxyeicosatrienoic acids (EETs) [22,23]. Plasma EPHX4 levels were  $30.9 \pm 2.0 \text{ ng/ml}$  in the vehicle control group at the end of the study (Table 1). Both RvE1-low and RvE1-high significantly reduced plasma EPHX4 ( $25.8 \pm 1.7 \text{ ng/ml}$ ,  $p < 0.05$  vs. vehicle in RvE1 low;  $23.5 \pm 1.5 \text{ ng/ml}$ ,  $p < 0.01$  vs. vehicle in RvE1 high). Atorvastatin treatment also reduced circulating EPHX4 ( $23.5 \pm 1.2 \text{ ng/ml}$ ,  $p < 0.01$  vs. vehicle), and the strongest reduction was observed in the combination-treated animals ( $20.7 \pm 1.1 \text{ ng/ml}$ ,  $p < 0.001$  vs. vehicle) (Table 1).

Alanine aminotransferase (ALAT), a liver integrity marker, increased during atherogenic diet feeding from  $53.3 \pm 2.1 \text{ U/L}$  at baseline, to  $139.4 \pm 24.8 \text{ U/L}$  at the end of the study in the vehicle control group. Treatment with RvE1 did not affect ALAT levels (RvE1-low  $116.3 \pm 13.2$ ; RvE1-high  $108.6 \pm 24.4$ ) (Table 1). Plasma ALAT levels were significantly lower in atorvastatin treated animals ( $76.5 \pm 12.6 \text{ U/L}$ ;  $p < 0.05$  vs. vehicle) and the combination treatment also resulted in attenuated ALAT levels ( $67.5 \pm 12.5 \text{ U/L}$ ,  $p < 0.05$  vs. vehicle, n.s. vs. atorvastatin) which were comparable to the atorvastatin group (Table 1).

#### 3.5. RvE1 down-regulates inflammatory genes and pathways in the vasculature

To evaluate potential early gene expression changes in aorta, aortae collected from ApoE\*3Leiden mice treated with RvE1 (1 mg/kg) or vehicle for a period of 9 weeks were used for a comprehensive gene expression analysis by microarray. RvE1 significantly affected the expression of 73 genes involved in the biological process "Inflammation" (see Supplementary Table S1) and 62 genes in "Immune cell trafficking" (see Supplementary Table S2). Among the genes down-regulated with RvE1 several are known to be involved in atherogenesis, e.g. *Ctss*, *Cd74*, *Cd44*, *Hpse*, *Ifr5*, *Ccl2*, *Csp1*, *Il20rb*, *Ccr5* and *Adam17*. Also, genes involved in antigen presentation and dendritic cell maturation, including *Lilrb3*, *Lair1*, *Casp1*, *Ctss*, *Irf5*, *Lat2*, *Tyrobp*, *Hla-dqb1*, *Hla-Dma*, *Cd74*, *Hla-Drb5* were down-regulated by RvE1. Subsequent pathway analysis revealed a significant inactivation of the pathways downstream of  $\text{IFN-}\gamma$  (Z-score  $-4.05$ ;  $p < 0.001$ ) and  $\text{TNF-}\alpha$  (Z-score  $-3.10$ ;  $p < 0.001$ ) in aortae of RvE1-treated mice. These results indicate pro-resolution of inflammation by RvE1 in atherogenesis.

## 4. Discussion

Herein we report that intervention with RvE1, an endogenous oxidation product of the  $\omega$ -3 PUFA eicosapentaenoic acid (EPA) with inflammation resolution properties [26], attenuated atherosclerotic lesion development when administered orally once daily over a period of 16 weeks in mice with established progressive atherosclerosis. RvE1 reduced total lesion area by 35%, and in animals treated with RvE1 the proportion of atherosclerotic lesions was significantly skewed towards mild (type I) lesions over more severe (type IV and V) lesions. RvE1 had no effect on plasma

**Table 1**  
Plasma parameters at the end of the study (t = 25 weeks).

	Vehicle	RvE1-low	RvE1-high	Atorva	Combi
SAA (µg/ml)	6.2 ± 0.7	6.4 ± 0.5	6.9 ± 0.4	5.4 ± 0.3	5.9 ± 0.6
E-selectin (ng/ml)	68.1 ± 2.8	65.7 ± 3.0	67.5 ± 1.5	53.2 ± 1.7***	51.1 ± 2.4***
VCAM-1 (µg/ml)	3.56 ± 0.22	3.25 ± 0.09	3.23 ± 0.13	2.68 ± 0.20***	2.79 ± 0.08***
MCP-1 (pg/ml)	42.4 ± 3.8	40.7 ± 3.3	46.1 ± 5.8	33.5 ± 2.8	42.3 ± 5.5
EPHX4 (ng/ml)	30.9 ± 2.0	25.8 ± 1.7*	23.5 ± 1.5**	23.5 ± 1.2**	20.7 ± 1.1***
ALAT (U/l)	139.4 ± 24.8	116.3 ± 13.2	108.6 ± 24.4	76.5 ± 12.6*	67.5 ± 12.5*

Results are shown as mean ± SEM (n = 12–16/group). \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 vs. the vehicle group.

cholesterol levels, which is in marked contrast to atorvastatin, the active comparator used. Atorvastatin attenuated atherosclerosis by 27% and reduced plasma cholesterol concentrations by 18%, but had no effect on lesion distribution towards milder lesions. Combination treatment with RvE1 and atorvastatin caused a more pronounced reduction of atherosclerotic lesion area (by 51%) with retained reduction in lesion severity, as observed with RvE1 alone. Gene expression analysis of aortae showed that RvE1 reduced activation of specific inflammatory pathways (IFN- $\gamma$  and TNF- $\alpha$ ) and diminished the expression of specific pro-atherogenic inflammatory factors.

In the current study we used atorvastatin as reference compound. The hypolipidemic effects of statins, among which atorvastatin, in the ApoE\*3Leiden transgenic mouse model have been described in many studies ([11,12] and references therein). The anti-atherosclerotic activities of statins in ApoE\*3Leiden transgenic mice are strongly related to their (V)LDL cholesterol-lowering effects [11], and high statin doses are needed to substantially quench inflammation [24,27,28]. The dose of atorvastatin employed in the present study was low (1.5 mg/kg/day) and plasma cholesterol was lowered by 18%. This hypolipidemic effect is in a clinically relevant range, i.e. as also observed in atorvastatin-treated patients ([12] and references therein). Under the experimental conditions employed, atorvastatin reduced atherosclerosis by 27%. Notably, the efficacy of RvE1 monotherapy to attenuate atherosclerotic lesion development was even more pronounced. Importantly, RvE1 significantly reduced lesion load and improved lesion severity in absence of an effect on plasma cholesterol which remained unaltered and high (~16 mM). RvE1 increased the content of mild type I lesions, an effect that was not observed in the case of atorvastatin. Furthermore, the content of severe type V lesions was decreased by RvE1. The composition of these severe lesions is considered to make them unstable and prone to rupture. Therefore, the observed shift towards a higher proportion of mild lesions and lower proportion of severe lesion in the RvE1 treated animals could indicate a beneficial effect of RvE1 on risk of plaque rupture. Defective immune-cell-mediated clearance in atherosclerotic lesions has been suggested as a major component in progressive disease, and is not restored by statin treatment [7]. In this respect, and in marked contrast to atorvastatin, RvE1 did not reduce the macrophage content in lesions. This is consistent with observations *in vivo* with another SPM, neuroprotectin D1, where resolution of inflammation-driven retinal neovascularization in a model of wet age-related macular degeneration (AMD) occurred in the absence of change in numbers of microglia but with clear conversion to a pro-resolution ramified shape [29]. The reduction in necrotic core in the atherosclerotic lesions of RvE1 treated animals may point to enhanced macrophage phagocytosis as previously reported [37]. We investigated a potential change in the gene expression of macrophage cell surface receptors that would allow differentiation between different subsets of macrophages, but we could not detect any difference between control or RvE1-treated animals. Importantly, many of the processes thought to be resolved by SPMs are

natural endogenous processes with a relatively short occurrence (a few weeks) during the entire process of atherogenesis (>20 weeks). For instance, the process of neutrophil infiltration into the vasculature takes place at around 10 weeks of atherogenic diet-feeding and lasts only for a few weeks, i.e. it cannot be studied at later time points [13,30]. Thus, it is likely that any histological analysis of immune cells in aortic lesions at endpoint does not allow identification of the inflammatory event during atherogenesis that was resolved by RvE1.

Furthermore, it is also increasingly recognized that macrophage cell surface molecule expression may not fully reflect the functional stage of a macrophage [31], and for technical reasons we were limited to a few markers. Thus, it is unlikely that immunohistochemical analysis of putative macrophage cell surface markers in aortic lesions at a 25-week endpoint would allow us to identify in full or even partially the macrophage effector profile contributing to lesion reduction in the presence of RvE1.

The observed reduction in EPHX4 by RvE1 may potentially contribute to inflammation resolution because low levels of EPHX4 may lead to increases in epoxyeicosatrienoic acids with known resolution properties [22,23]. Furthermore, inhibitors of EPHX4 have been shown to attenuate experimental atherosclerosis in *ApoE*<sup>-/-</sup> mice [40].

Microarray analysis of gene expression changes in aortae collected earlier in time (at 18 weeks of the study) revealed that RvE1-treated animals displayed reduced expression of genes related to inflammation and immune cell trafficking compared with the untreated controls. For instance, RvE1 down-regulated the expression of chemokines or chemokine receptors (e.g. *Ccl2*, *Ccr5*) suggesting a dampening effect on immune cell trafficking and innate immunity. Furthermore, RvE1 reduced the expression of MHC class II molecules suggesting a role for RvE1 in regulation of T-cell responses in atherosclerosis by reducing antigen presentation. Consistent with this, RvE1 was shown to regulate expression of costimulatory molecules CD-80 and CD-86 [32], and also dampen TH1 and TH17 T-cell responses, while upregulating IL-10 to promote corneal clearance in herpes simplex virus-induced ocular keratitis [33]. Novel findings in the present study were that several genes, whose products are known to be involved in atherogenesis, were beneficially affected by RvE1, including *Cd74*, *Casp1*, and *Cd44*. An integrated analysis of gene expression patterns across pathways showed that RvE1 significantly reduced activation of the inflammatory signaling routes triggered by TNF- $\alpha$  and IFN- $\gamma$ , both of which are known to control crucial pro-atherogenic pathways during lesion development [34,35]. The observed effects of RvE1 in downregulating pro-inflammatory mediators is concordant with previous studies on RvE1 that described attenuated secretion of pro-inflammatory cytokines, including IFN- $\gamma$ , IL-6, IL-1 $\beta$  and TNF- $\alpha$ , and the attenuation of neutrophil and macrophage infiltration at sites of inflammation [36,37]. Consistent with this and our findings, topical gingival treatment of periodontitis with RvE1 also reduced atherosclerosis in a rabbit model of disease, which was accompanied by a dampening effect on inflammation [38].

In the present study we studied for the first time the efficacy of resolvin E1 in a diet-inducible model of atherosclerosis. While we demonstrate the principle of atherosclerosis treatment with resolvin E1, the study was not designed to study or unravel the underlying mechanisms such as phenotypic changes of inflammatory cells. Future studies should include a refined characterization of circulating as well as tissue-resident inflammatory cells to provide insight into the mechanisms underlying these effects.

In the current study, *en face* Oil Red O lipid staining of the aorta, which is a commonly used method for quantification of atherosclerosis extension, was not performed. The method employed herein is an established method for analysis of atherosclerosis [11,13,24,25] that not only allows quantification of lesion load (with results comparable to those obtained from *en face* staining [25,39]), but also provides information on the severity of atherosclerotic lesions.

Taken together, our results demonstrate for the first time the efficacy of an SPM, RvE1, in an experimental model of diet-induced atherosclerosis, the ApoE\*3Leiden transgenic mouse, and further support the concept that defective resolution of inflammation may contribute to progression of atherosclerotic disease. While RvE1 showed comparable efficacy to that of an established statin in attenuating atherosclerosis, the more pronounced effect of their combined actions is indicative of a therapeutic potential of SPM in atherosclerosis, and the added benefit of activating pro-resolution pathways on top of existing first line treatment.

### Conflict of interest

PG and LW were employees of Resolvyx Pharmaceuticals Inc.

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### Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.atherosclerosis.2016.05.001>.

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