



# Rupatadine Treatment Is Associated with Atherosclerosis Worsening and Altered T Lymphocyte Recruitment

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Rupatadine, an *N*-alkyl pyridine derivative (8-chloro-11-[1-[(5-methyl-3-pyridyl)methyl]-4-piperidylidene]-6,11-dihydro-5H-benzo[5,6]cyclohepta[1,2b]pyridine), is a non-sedating, second-generation antihistamine and platelet-activating factor (PAF) antagonist currently employed for the treatment of allergies.<sup>1</sup>

Rupatadine is clinically effective in relieving symptomatic seasonal and perennial allergic rhinitis and it is well tolerated.<sup>2,3</sup> It displays a robust antagonistic activity toward histamine H<sub>1</sub> receptors at subnanomolar concentration *in vitro*, and also prevents mast cell degranulation.<sup>1</sup> Additionally, it has also been shown to possess a PAF receptor antagonist activity, achieved at submicromolar concentration *in vitro*.<sup>4</sup> Finally, rupatadine is able to reduce the recruitment of macrophages, eosinophils, basophils, and neutrophils, and to inhibit platelet aggregation,<sup>5,6</sup> all features that could be favorably exploited against atherosclerosis development.

Given the pleiotropic anti-inflammatory effects that rupatadine exerts, in this study we assessed the potential beneficial effect of chronic rupatadine treatment on atherosclerosis development. To this aim, similarly to what we did in analogous studies,<sup>7,8</sup> rupatadine was administered with a high-fat diet (adjusted calories 42% from fat, 0.2% cholesterol) to 15 *Apoe*<sup>-/-</sup> female mice at the highest tolerated dose

(170 mg/kg diet). The treatment lasted for 12 weeks. Atherosclerosis development, as well as possible systemic effects and impact on the lipid profile were evaluated and compared with those in *Apoe*<sup>-/-</sup>-untreated mice (► **Supplementary Fig. S1**, available in the online version).

The treatment did not significantly affect daily food intake (control: 0.106 ± 0.008 g/g body weight; rupatadine: 0.105 ± 0.007 g/g body weight, *p* = 0.87), water intake (control: 0.147 ± 0.01 mL/g body weight; rupatadine: 0.142 ± 0.02 mL/g body weight, *p* = 0.28), or body weight (control: 25.23 ± 0.85 g; rupatadine: 25.81 ± 1.77 g, *p* = 0.28). No significant differences between groups were also found in the liver, spleen, heart, and kidney weight (► **Supplementary Fig. S2**, available in the online version).

Moreover, plasma total cholesterol (control: 880.6 ± 124.7 mg/dL; rupatadine: 838.7 ± 142.1 mg/dL, *p* = 0.40) and triglyceride (control: 160.6 ± 29.7 mg/dL; rupatadine: 181.0 ± 53.1 mg/dL, *p* = 0.21) levels were comparable between groups (► **Supplementary Fig. S2**, available in the online version).

To perform an all-round characterization of rupatadine treatment on the *Apoe*<sup>-/-</sup> model, we extensively reviewed a broad panel of histological features across several organs. The hepatic inflammatory status—parenchymal/perivascular hepatic infiltrates and subintimal hepatic macrophages—was overall comparable between groups. The glycogen deposition and steatosis degree in the liver were—as expected—

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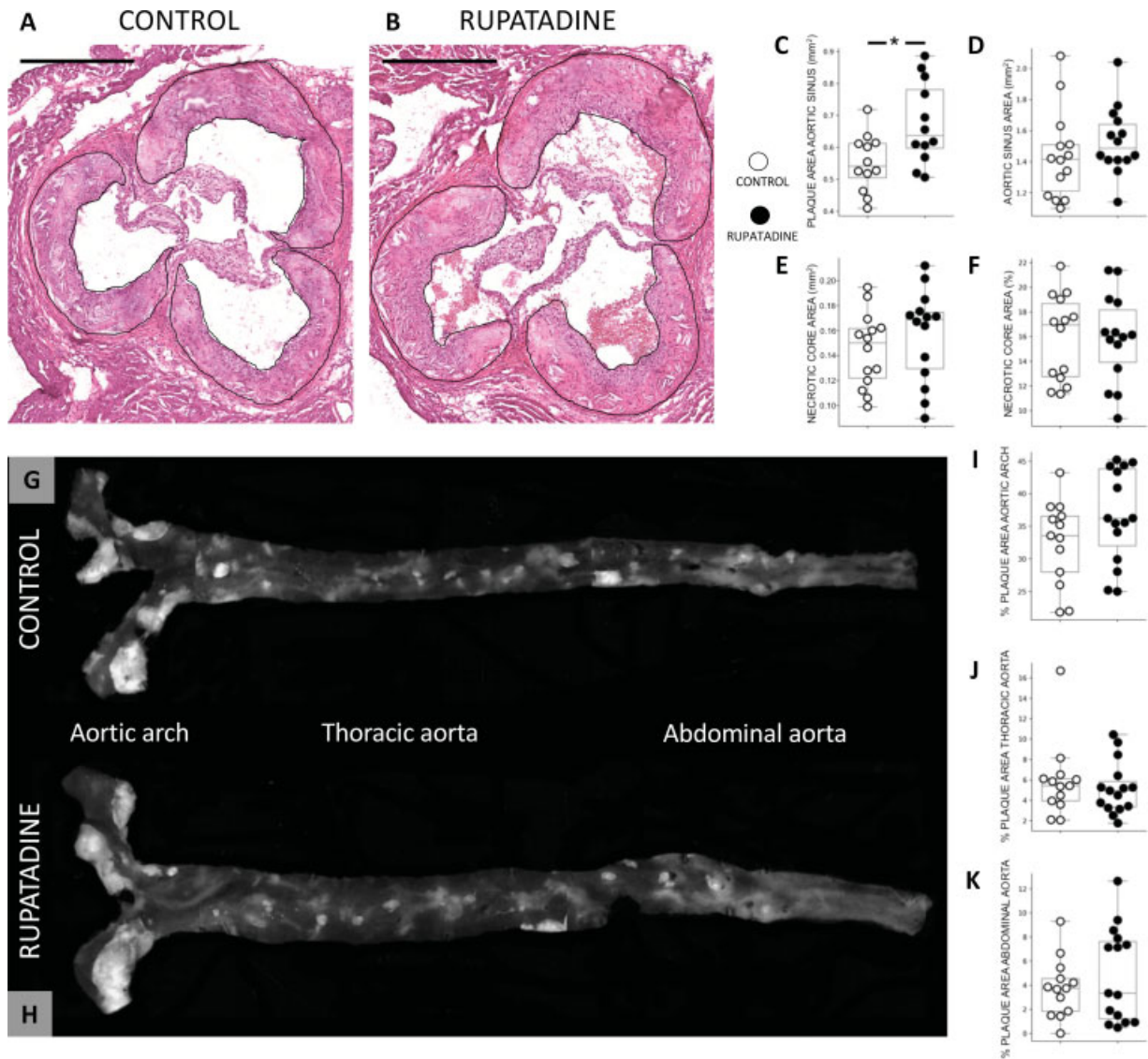
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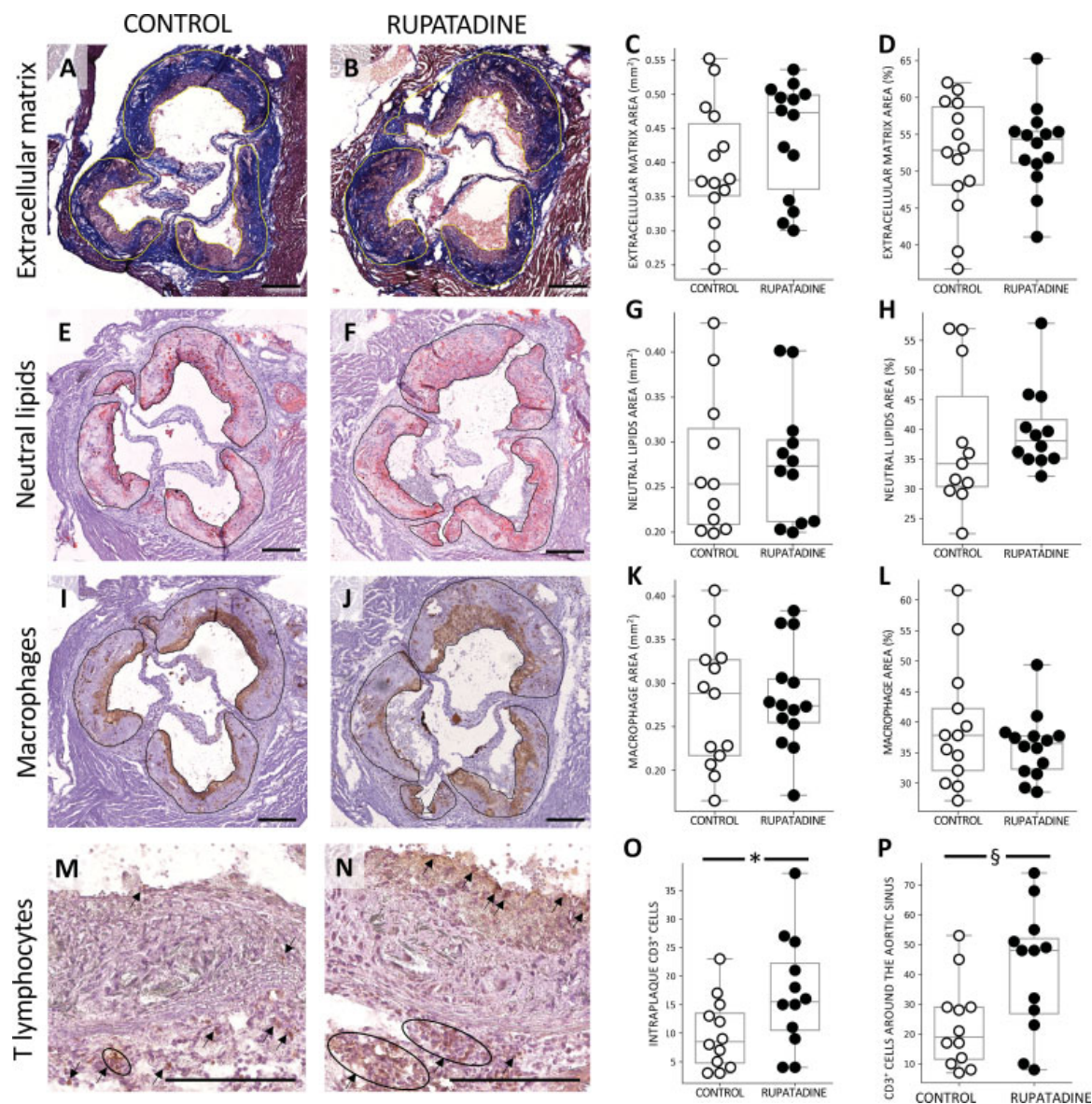
**Fig. 1** Atherosclerosis evaluation. Representative hematoxylin & eosin photomicrographs of aortic sinuses ( $n = 15$  per group, bar length = 500  $\mu\text{m}$ ) (A, B). Box plot of aortic sinus values showing a significantly increased plaque development in rupatadine-treated mice (C). Comparable area of the aortic sinus (D) and necrotic core extension (E, F) in plaques from both groups. Representative image of whole aortas ( $n = 15$  per group), prepared with the en face technique (G, H). Box plots of plaque extent (percentage over entire area) in the aortic arch (I), thoracic (J), and abdominal aorta (K). Only in the aortic arch of rupatadine mice, a tendency toward an increase in plaque size was observed. Statistically significant differences were determined by unpaired two-tailed Student's  $t$ -test.  $*p = 0.011$ .

likewise comparable between groups. In the kidney, glomerular lipidosis, a condition commonly found in *Apoe*<sup>-/-</sup> mice, characterized by the presence of large foamy macrophages within the glomerulus,<sup>7,8</sup> was not influenced by rupatadine treatment. In lung, the presence of mast cells in the peribronchial and perivascular regions was comparable (**Supplementary Figs. S3–S5**, available in the online version).

Spleens were comparable in terms of hemopoiesis and follicular hyperplasia, almost absent or present to a very slight degree in a small number of samples. In the lymph node, the accumulation of foamy macrophages was similar in both groups. Follicular hyperplasia, the presence of cholesterol crystals and sinus ectasia were almost absent in all samples of both groups (**Supplementary Figs. S3 and S4**, available in the online version).

Atherosclerosis development was assessed in the aortic sinus and in the whole aorta.<sup>9,10</sup> Unexpectedly, the pharmacological treatment did result in an increased atherosclerotic plaque development at the aortic sinus (**Fig. 1A–C**). The total aortic sinus area was comparable in the two groups (**Fig. 1D**). Plaques were characterized by comparable necrotic core area (**Fig. 1E, F**), extracellular matrix content (**Fig. 2A–D**), area occupied by neutral lipids (**Fig. 2E–H**) and macrophage amount (**Fig. 2I–L**), in terms of both absolute values and percentage composition.

The increase in the aortic sinus plaque area by rupatadine treatment of approximately 22% ( $0.675 \pm 0.129$  in rupatadine vs.  $0.551 \pm 0.089$  mm<sup>2</sup> in control;  $p = 0.012$ ) was partly confirmed in the aortic arch, the segment closer to the aortic sinus, where a similar—although nonsignificant—increase of approximately 12% was observed in rupatadine mice ( $36.56 \pm 7.15$  in



**Fig. 2** Histological and immunohistochemical characterization of atherosclerotic plaques at the aortic sinus. Representative photomicrographs of sections stained with Masson's trichrome to highlight extracellular matrix (A–D). Deposition of neutral lipids is revealed by O.R.O. staining (E–H). Plaque macrophage content, assayed with a macrophage-specific immunohistochemistry (anti-Mac2 antibody; I–L). An increased count of CD3<sup>+</sup> T lymphocytes, both as single cells (*arrows*) and clusters (*circles*), was observed in the atherosclerotic plaque as well as in the myocardium immediately surrounding the aortic sinus of rupatadine versus control mice (M–P). \* $p = 0.048$ , § $p = 0.022$ . Bar length = 250  $\mu\text{m}$ . Statistically significant differences were determined by unpaired Student's *t*-test (C, D, K, L, O, P) or by unpaired Mann–Whitney's U-test (G, H) based on the data distribution (assayed on residuals by Shapiro–Wilk normality test).

rupatadine vs.  $32.82 \pm 6.30\%$  in control;  $p = 0.13$ ; ► **Fig. 1G–I**). Plaque development was instead comparable in the thoracic and abdominal segments between the two groups (► **Fig. 1J, K**).

Circulating immunoglobulin E levels were not significantly modified by the treatment (► **Supplementary Fig. S6**, available in the online version). No mast cells were detected in plaques and a comparable number of mast cells was observed in the myocardial parenchyma of the two groups. Interestingly, in rupatadine-treated mice, a reduced amount of mast cells was detected in the adventitia immediately surrounding the aortic sinus (► **Supplementary Fig. S7**, available in the online version). It has been shown that rupatadine

inhibits mast cell degranulation,<sup>11</sup> thus reducing the release of chemoattractant molecules including leukotriene B<sub>4</sub>,<sup>12,13</sup> able to recruit more mast cell progenitors to the site of inflammation. We hypothesize that, in the group of treated mice, rupatadine reduced the enrollment of mast cells around the aortic sinus through this mechanism.

Albeit unexpected, the results on atherosclerosis seem to fall partly in line with a previous report on two other antihistamine molecules, cetirizine and fexofenadine,<sup>14</sup> administered at different doses to *ApoE*<sup>-/-</sup> mice. At a lower concentration, both compounds significantly augmented plaque deposition in the aortic sinus and aorta of mice, an



effect that was completely abolished at a higher dosage. Both treatments had no effect on the number of macrophages and T lymphocytes.<sup>14</sup> In the present study, a similar finding was obtained for macrophages; conversely, rupatadine-treated *ApoE*<sup>-/-</sup> mice showed a 70% increase in the amount of T lymphocytes infiltrating the plaque and a 80% increase in the amount of T lymphocytes within the myocardial parenchyma around the aortic sinus (► **Fig. 2M–P**).

Several evidences from the literature indicate that T cells express H<sub>1</sub>, H<sub>2</sub>, and H<sub>4</sub> histamine receptors.<sup>15,16</sup> We tested in vitro a possible direct effect of rupatadine on T lymphocytes (► **Supplementary Figs. S8–S10**, available in the online version). Rupatadine did not affect cell proliferation, nor CD4/CD8 polarization, a finding also supported by the specific staining performed on lymphoid organs (► **Supplementary Fig. S11**, available in the online version). Conversely, rupatadine promoted the polarization of CD4<sup>+</sup> lymphocytes toward T<sub>H</sub>1 and T<sub>H</sub>2 subsets, suggesting an effect on T cell activation. T<sub>H</sub>1 cells are known to play proatherogenic functions, whereas the impact of T<sub>H</sub>2 in atherosclerosis is still controversial.<sup>17,18</sup> Altogether these results can partly explain the observed effect of rupatadine on atherosclerosis development.

In conclusion, rupatadine was shown to affect plaque progression in *ApoE*<sup>-/-</sup> mice fed with high-fat diet—a widespread, well characterized, but extreme atherosclerosis model. The impact of H<sub>1</sub> antihistamine on cardiovascular disease in humans has been scarcely explored. Although we cannot directly translate our results to a clinical condition of established atherosclerotic disease, taken together our data suggest a cautionary survey of patients regularly taking H<sub>1</sub> antihistamine for the treatment of seasonal or chronic illnesses, especially if in the presence of predisposing conditions.

#### Author Contributions

M.B.: conceptualization, investigation, writing: original draft, writing: review and editing; Stefano Manzini: investigation, formal analysis, writing: review and editing, visualization; Alice Colombo: investigation; F.B.: investigation; G.D.N.: investigation, resources; E.F.: investigation; Silvia Castiglioni: investigation; C.A.: conceptualization; E.L.: conceptualization; E.S.: investigation; Giulia Chiesa: conceptualization, resources, writing: review and editing, project administration, funding acquisition.

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#### Conflict of Interest

C.A. and E.L. are senior investigators at Biovista, Athens, Greece. All other authors declare no competing interests.

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