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Abstract: No Abstract

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Rupatadine Treatment is Associated to 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-piperidyliden]-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 [1].
Rupatadine is clinically effective in relieving symptomatic seasonal and perennial allergic rhinitis and it is well tolerated [2,3]. It displays a robust antagonistic activity towards histamine H\textsubscript{1} receptors at sub-nanomolar concentration \textit{in vitro}, and also prevents mast cell degranulation [1]. Additionally, it has also been shown to possess a PAF receptor antagonist activity, achieved at sub-micromolar concentration \textit{in vitro} [4]. Finally, rupatadine is able to reduce the recruitment of macrophages, eosinophils, basophils, neutrophils and inhibit platelet aggregation [5,6], 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 [7,8], rupatadine was administered with a high-fat diet (adjusted calories 42% from fat, 0.2% cholesterol) to 15 Apoe\textsuperscript{-/-} 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 to those in Apoe\textsuperscript{-/-} untreated mice (Supplementary Fig. S1).

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 liver, spleen, heart and kidney weight (Supplementary Fig. S2).

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).

To perform an all-round characterization of rupatadine treatment on the Apoe−/− model, we extensively reviewed a broad panel of histological features across several organs. The hepatic inflammatory status - parenchymal/perivascular hepatic infiltrates and sub-intimal hepatic macrophages - was overall comparable between groups. The glycogen deposition and steatosis degree in the liver were – as expected - likewise comparable between groups. In the kidney, glomerular lipidosis, a condition commonly found in Apoe−/− mice, characterized by the presence of large foamy macrophages within the glomerulus [7,8], was not influenced by rupatadine treatment. In lung, the presence of mast cells in the peribronchial and perivascular regions was comparable (Supplementary Fig. S3-5).

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 Fig. S3 and S4).

Atherosclerosis development was assessed in the aortic sinus and in the whole aorta [9,10]. Unexpectedly, the pharmacological treatment did result into an increased atherosclerotic plaque development at the aortic sinus (Figure 1A-C). The total aortic sinus area was comparable in the two groups (Figure 1D). Plaques were characterized by comparable necrotic core area (Figure 1E,F), extracellular matrix content (Figure 2A-D), area occupied by neutral lipids (Figure 2E-H) and macrophage amount (Figure 2I-L), in terms of both absolute values and percentage composition.
The increase in the aortic sinus plaque area by Rupatadine treatment of about 22% (0.675±0.129 in Rupatadine vs 0.551±0.089 mm² in Control; p=0.012), was partly confirmed in the aortic arch, the segment closer to the aortic sinus, where a similar - although non-significant - increase of about 12% was observed in Rupatadine mice (36.56±7.15 in Rupatadine vs 32.82±6.30% in Control; p=0.13; Figure 1G-I). Plaque development was instead comparable in the thoracic and abdominal segments between the two groups (Figure 1J,K).

Circulating IgE levels were not significantly modified by the treatment (Supplementary Fig. S6). 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). It has been shown that rupatadine inhibits mast cells degranulation [11], thus reducing the release of chemoattractant molecules including leukotriene B4 [12,13], 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 [14], administered at different doses to Apoe⁻/⁻ 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 lymphocyte [14]. In the present study a similar finding was obtained for macrophages; conversely, rupatadine-treated Apoe⁻/⁻ 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 (Figure 2M-P).

Several evidences from the literature indicate that T cells express H1, H2 and H4 histamine receptors [15,16]. We tested in vitro a possible direct effect of rupatadine on T lymphocytes (Supplementary Fig. S8-10). 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). Conversely, rupatadine promoted the polarization of CD4+ lymphocytes toward $T_h1$ and $T_h2$ subsets, suggesting an effect on T cell activation. $T_h1$ cells are known to play pro-atherogenic functions, whereas the impact of $T_h2$ in atherosclerosis is still controversial [17,18]. 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$^{-/-}$ mice fed with high-fat diet – a widespread, well characterized, but extreme atherosclerosis model. The impact of $H_1$ 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 $H_1$ antihistamine for the treatment of seasonal or chronic illnesses, especially if in the presence of predisposing conditions.

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Author Contributions

Marco Busnelli: Conceptualization, Investigation, Writing - Original Draft, Writing - Review & Editing; Stefano Manzini: Investigation, Formal analysis, Writing - Review & Editing, Visualization; Alice Colombo: Investigation; Fabrizia Bonacina: Investigation; Giuseppe D. Norata: Investigation, Resources; Investigation; Elsa Franchi: Investigation; Silvia Castiglioni: Investigation; Christos Andronis: Conceptualization; Eftychia Lekka: Conceptualization; Eugenio Scanziani: Investigation; Giulia Chiesa: Conceptualization, Resources, Writing - Review & Editing, Project administration, Funding acquisition

Competing Interests

Christos Andronis and Eftychia Lekka are senior investigators at Biovista, Athens, Greece. All other authors declare no competing interests.

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References


Figure 1. Atherosclerosis evaluation. Representative Hematoxylin&Eosin photomicrographs of aortic sinuses (n=15 per group, bar length = 500 μm) (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.

Figure 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+ 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 vs Control mice (M-P). * p = 0.048, § p = 0.022. Bar length = 250 μm. 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).
Supplementary Information

Rupatadine treatment is associated to atherosclerosis worsening and altered T lymphocyte recruitment

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**Experimental Design.** Apolipoprotein E-deficient female mice (Apoe-/-) were fed a Western-type diet, with (Rupatadine) or without (Control) 0.017% w/w rupatadine for 12 weeks.
Mice characterization. Mice weight gain (n=15 per group) (A) food (B) and water intake (C) were monitored (normalized to the number of animals in each cage). Body and organ weights at sacrifice are charted in D. Fasting total cholesterol and triglyceride concentration are plotted in E. Unpaired two-tailed Student’s T-test (D, E) or Mann-Whitney U test (B, C) were used to test for significant differences between the two groups, depending on data distribution. No statistical differences were found. Data are shown \( \pm \) SD (B-D), or as box plots.
Figure 3. Histological evaluation of liver, kidney, spleen, lymph node and lung. The occurrence of steatosis in the liver (A, B) and glomerular lipidosis in kidney (C, D) were comparable between Control and Rupatadine. No relevant histopathological findings were observed in spleen (E, F), lymph node (G, H) and lung (I, J) from both groups. Bar length: 100 μm.
### Histological features investigated in liver, kidney, lymph node, spleen and lung

Histological features were graded into 4 tiers and graphically represented with numerical values ranging from 0 to 3 in an heatmap.

#### Features were classified as follows:
- 0 (feature is absent)
- 1 (slight)
- 2 (mild)
- 3 (severe)

#### Liver
- **Infiltrate number**
  - Absent
  - Sub-intimal foamy macrophages
  - Glomerular lipidosis
  - Hyaline cast
  - Hydronephrosis
  - Tubular regeneration
  - Pelvic lymphocytic infiltrate
  - Follicular hyperplasia
  - Haemopoiesis
  - Periarteriolar inflammatory cells
  - Hypertrophy of arterial media
  - Foamy macrophages
  - Follicular hyperplasia
  - Cholesterol crystals
  - Sinus ectasia

#### Kidney
- **Infiltrate number**
  - Absent
  - Glomerular lipidosis
  - Hyaline cast
  - Hydronephrosis
  - Tubular regeneration
  - Pelvic lymphocytic infiltrate
  - Follicular hyperplasia
  - Haemopoiesis
  - Periarteriolar inflammatory cells
  - Hypertrophy of arterial media
  - Foamy macrophages
  - Follicular hyperplasia
  - Cholesterol crystals
  - Sinus ectasia

#### Lung
- **Infiltrate number**
  - Absent
  - Glomerular lipidosis
  - Hyaline cast
  - Hydronephrosis
  - Tubular regeneration
  - Pelvic lymphocytic infiltrate
  - Follicular hyperplasia
  - Haemopoiesis
  - Periarteriolar inflammatory cells
  - Hypertrophy of arterial media
  - Foamy macrophages
  - Follicular hyperplasia
  - Cholesterol crystals
  - Sinus ectasia

#### Lymph node
- **Infiltrate number**
  - Absent
  - Glomerular lipidosis
  - Hyaline cast
  - Hydronephrosis
  - Tubular regeneration
  - Pelvic lymphocytic infiltrate
  - Follicular hyperplasia
  - Haemopoiesis
  - Periarteriolar inflammatory cells
  - Hypertrophy of arterial media
  - Foamy macrophages
  - Follicular hyperplasia
  - Cholesterol crystals
  - Sinus ectasia

#### Summary
- **Group**
  - Rupatadine
  - Control

- **Features**
  - Glycogenosis
  - Steatosis
  - Glomerular lipidosis
  - Hyaline cast
  - Hydronephrosis
  - Tubular regeneration
  - Pelvic lymphocytic infiltrate
  - Follicular hyperplasia
  - Haemopoiesis
  - Periarteriolar inflammatory cells
  - Hypertrophy of arterial media
  - Foamy macrophages
  - Cholesterol crystals
  - Sinus ectasia

- **Infiltrate number**
  - Absent
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  - Hydronephrosis
  - Tubular regeneration
  - Pelvic lymphocytic infiltrate
  - Follicular hyperplasia
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  - Periarteriolar inflammatory cells
  - Hypertrophy of arterial media
  - Foamy macrophages
  - Follicular hyperplasia
  - Cholesterol crystals
  - Sinus ectasia

- **Infiltrate size**
  - Absent
  - Small (1: 1-10 cells)
  - Medium (2: 11-50 cells)
  - Large (3: more than 100 cells)

- **Supplementary Figure 4**
Histological detection of mast cells in lung. After toluidine blue staining (A,B), a comparable number of peribronchial (C) and perivascular (D) mast cells (red circles) was observed in the two groups. The upper and lower ends of the boxes indicate the 25th and 75th percentiles, respectively. Unpaired two-tailed Mann-Whitney’s U test was used to test for significant differences between the two groups. The length of the box shows the interquartile range within which 50% of the values are located. The solid grey lines denote the median. Bar length: 50 μm.
Quantification of plasma IgE. Plasma IgE levels at sacrifice were measured by enzyme-linked immunosorbent assay (ELISA). Significant differences were determined by unpaired Mann-Whitney’s U test, two-tailed. The upper and lower ends of the boxes indicate the 25th and 75th percentiles, respectively. The length of the box shows the interquartile range within which 50% of the values are located. The solid grey lines denote the median.
**Histological detection of mast cells in the heart.** No mast cells were observed in the atherosclerotic plaques (A, D). The number of mast cells was significantly reduced by the treatment with rupatadine in the adventitia immediately surrounding the aortic sinus (B, E). A comparable amount of mast cells was observed in the myocardial parenchyma of the two groups (C, F). Unpaired two-tailed Student’s T-test was used to test for significant differences between the two groups. The upper and lower ends of the boxes indicate the 25th and 75th percentiles, respectively. The length of the box shows the interquartile range within which 50% of the values are located. The solid grey lines denote the median. Arrows = mast cells. Bar length: 100 μm.
**Effect of Rupatadine on cell survival.** Percentage of live cells after 18 hours (A) and 4 days (B) of stimulation without (CTRL+) and with increasing concentration of Rupatadine (0.1, 1, 10, 25 and 50 µM). As negative control (CTRL-), not stimulated cells were concomitantly analyzed. Based on these results, 50 µM concentration was not considered in the analyses. Representative dot plots from flow cytometry analysis of cell granulosity (SSC-A) versus negative staining for live and dead (L/D) dye of CTRL-, CTRL- and cell incubated with Rupatadine (25 and 50 µM) are shown. Statistical analyses were performed with non-parametric One-way ANOVA with Multiple comparison. Data are presented as mean±SEM.

**p<0.01 vs CTRL-; § p<0.01 vs CTRL+**
**Effect of Rupatadine on CD4 T cell polarization.** Percentage of GATA3 (A) and Tbet (B) positive cells in CD4 T lymphocytes after 18 hours of stimulation without (CTRL+) and with increasing concentration of Rupatadine (0.1, 1, 10 and 25 µM). As negative control (CTRL-), not stimulated cells were concomitantly analysed. Representative dot plots from flow cytometry analysis of cell granularity (SSC-A) versus positive staining for transcription factors (GATA3 for Th2 and Tbet for Th1) of CTRL-, CTRL- and cell incubated with Rupatadine (25µM) are shown. Statistical analyses were performed with non-parametric One-way ANOVA with Multiple comparison. Data are presented as mean±SEM.

*p<0.05, ** p<0.01 vs CTRL-; § p<0.01 vs CTRL+
**Effect of Rupatadine on T cell proliferation.** Percentage of cells in proliferation in CD4 (A) and CD8 (B) T lymphocytes after 4 days of stimulation without (CTRL+) and with increasing concentration of Rupatadine (0.1, 1, 10 and 25 µM). As negative control (CTRL-), not stimulated cells were concomitantly analyzed. Representative dot plots from flow cytometry analysis of cell granularity (SSC-A) versus CFSE staining of CTRL-, CTRL- and cells incubated with Rupatadine (25µM) are shown. Statistical analyses were performed with non-parametric One-way ANOVA with Multiple comparison. Data are presented as mean±SEM.

** p<0.01 vs CTRL-
Immunohistochemical detection of CD4+ and CD8+ T lymphocytes in lymphoid organs. The ratio of CD4+ and CD8+ positive T lymphocytes was investigated via IHC in lymphoid follicles of the lymph node (A, B) and in white pulp and periarterial lymphatic sheaths of the spleen (C, D). The percentage of CD4+ and CD8+ T lymphocytes were comparable in the two group of mice, in both lymphoid organs. Unpaired two-tailed Student’s T-test (A, B) or Mann-Whitney U test (C, D) was used to test for significant differences between the two groups, depending on data distribution. Bar length: 100 μm.
A) Extracellular matrix
B) Extracellular matrix
C) Extracellular matrix area (mm²)
D) Extracellular matrix area (%)
E) Neutral lipids
F) Neutral lipids
G) Neutral lipids area (mm²)
H) Neutral lipids area (%)
I) Macrophages
J) Macrophages
K) Macrophage area (mm²)
L) Macrophage area (%)
M) T lymphocytes
N) T lymphocytes
O) T lymphocyte cells
P) T lymphocyte cells

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