Cysteinyl leukotriene receptor 1 antagonism prevents experimental abdominal aortic aneurysm

Antonio Di Gennaro, Ana Carolina Araújo, Albert Busch, Hong Jin, Dick Wägström, Emina Vorkapić, Kenneth Caidahl, Per Eriksson, Bengt Samuelsson, Lars Maegdefessel, and Jesper Z. Haeggeström

ABSTRACT

Cysteinyl-leukotrienes (cys-LTs) are 5-lipoxygenase-derived lipid mediators involved in the pathogenesis and progression of inflammatory disorders, in particular asthma. We have previously found evidence linking these mediators to increased levels of proteolytic enzymes in tissue specimens of human abdominal aortic aneurysm (AAA). Here we show that antagonism of the CysLT1 receptor by montelukast, an established antiasthma drug, protects against a strong aorta dilatation (−50% increase = aeurysm) in a mouse model of CaCl2-induced AAA at a dose comparable to human medical practice. Analysis of tissue extracts revealed that montelukast reduces the levels of matrix metalloproteinase-9 (MMP-9) and macrophage inflammatory protein-1α (MIP-1α) in the aortic wall. Furthermore, aeurysm progression was specifically mediated through CysLT1 signaling since a selective CysLT2 antagonist was without effect. A significantly reduced vessel dilatation is also observed when treatment with montelukast is started days after aeurysm induction, suggesting that the drug not only prevents but also stops and possibly reverts an already ongoing degenerative process. Moreover, montelukast reduced the incidence of aortic rupture and attenuated the AAA development in two additional independent models, i.e., angiotensin II- and porcine pancreatic elastase-induced AAA, respectively. Our results indicate that cys-LTs are involved in the pathogenesis of AAA and that antagonism of the CysLT1 receptor is a promising strategy for preventive and therapeutic treatment of this clinically silent and highly lethal disease.

Abdominal aortic aneurysm | inflammation | leukotriene | montelukast

A bdominal aortic aneurysm (AAA) is a clinically silent but life-threatening vascular disorder for which no medical prevention or treatment is currently available. It has a prevalence of about 5% in men and 1% in women over 60 y old and is associated with hypertension, atherosclerosis, and cigarette smoking (1). The disease process in the aortic wall is characterized by strong dilation of (thoracic and/or abdominal) aorta, infiltration of media, and adventitia layers by immune cells, such as macrophages, neutrophils, and T cells, which release inflammatory cytokines and other mediators, which in turn drive extracellular matrix degradation, eventually resulting in spontaneous aortic rupture. Several proteolytic enzymes have been described as biomarkers of degenerative AAA progression, and matrix metalloproteinases (MMPs) have been shown to play a pivotal role in the pathogenesis of AAA through direct vascular degeneration, control of inflammation, and induction of apoptosis (2–4).

The 5-lipoxygenase (5-LO) pathway of arachidonic acid metabolism leads to biosynthesis of leukotrienes (LTs), potent lipid mediators with proinflammatory biological actions. Among the LTs, cysteinyl-leukotrienes (cys-LTs) are well-known signaling molecules in human asthma, and drugs antagonizing the CysLT1 receptor have been established and important therapeutics for clinical management of asthma for more than a decade (5). However, more recently LTs have also been implicated in cardiovascular diseases such as atherosclerosis, myocardial infarction, and stroke (6–8). Less is known about the 5-LO pathway and leukotrienes in AAA disease. It has been proposed that leukotriene B4 (LTB4) plays a role in AAA as a chemotactic factor released from neutrophils within the intraluminal thrombus, and work in our laboratory identified cys-LTs as main 5-LO products in human AAA wall (9). We could also demonstrate that challenging the aneurysm wall with exogenous cys-LTs can induce the release of MMPs, and this action can be prevented by pretreatment of the tissue with the CysLT1 antagonist montelukast (9). Moreover, levels of local cys-LTs were enhanced in humans undergoing AAA surgery (10). However, the limited number of animal studies addressed to clarify the potential role of LTs in AAA has yielded different results in the angiotensin II (AngII)-induced model (11). On the other hand, targeting the LTB4 receptor 1 (BLT1) by genetic deletion or pharmacological antagonism afforded significant protection against AngII-induced AAA (12, 13). Recently, the inhibition of 5-LO by pharmacological or gene therapies has been described to attenuate aneurysm formation in two different AAA mouse models (14). In the present study, we used the in vivo AAA mouse model induced by periaortic application of CaCl2 to study the antiasthma drug montelukast as possible treatment strategy for preventive and therapeutic treatment of this clinically silent and highly lethal disease.

Significance

Cysteinyl-leukotrienes (cys-LTs) are lipid mediators involved in human inflammatory diseases, in particular asthma. We have previously identified cys-LTs in tissue specimens of human abdominal aortic aneurysm (AAA) and linked these mediators to increased metalloproteinase activity. Here we show in vivo that antagonism of the CysLT1 receptor by montelukast, an established antiasthma drug, protects against aneurysm in three mouse models of AAA at doses comparable to human medical practice. Together, these data support the role of cys-LTs in AAA and indicate a new potential therapeutic approach for treatment of this clinically silent and highly lethal disease.


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To whom correspondence may be addressed. Email: bengt.samuelsson@ki.se or jesper.haeggestrom@ki.se.

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to prevent aortic aneurysm. Additionally, we used two independent and well-characterized models of AAA, i.e., infusion of AngII into apolipoprotein E-deficient (ApoE<sup>−/−</sup>) mice and the porcine pancreatic elastase (PPE) infusion model in wild-type (WT) mice.

Our results indicate a pathophysiological role for cys-LTs in experimental AAA and suggest that pharmacological abrogation of CysLT1 signaling may be a clinical approach to protect against aortic wall degeneration and AAA development.

**Results**

Expression of 5-LO Pathway-Related Enzymes Is Up-Regulated in CaCl<sub>2</sub>-Induced AAA. Infrarenal aortas were harvested 21 d after the periaortic application of CaCl<sub>2</sub> and NaCl. Quantitative RT-PCR (qPCR) analysis revealed increased mRNA transcript levels for all 5-LO pathway enzymes (5-LO, FLAP, LTA<sub>4</sub> H, and LTC<sub>4</sub>S) with 5-LO (5.39 ± 1.86, P = 0.0181) and LTC<sub>4</sub>S (6.22 ± 2.82, P = 0.0342) folds increased in comparison with the control mice (Fig. 1A). The significant up-regulation of 5-LO and LTC<sub>4</sub>S

![Fig. 1.](image-url) The effect of montelukast on aorta dilatation in CaCl<sub>2</sub>-induced AAA. (A) 5-LO, FLAP, LTA<sub>4</sub> H, and LTC<sub>4</sub>S mRNA was determined by qPCR in aortic wall of C57Bl6/J mice 21 d after the treatment with CaCl<sub>2</sub> (n = 4) and compared with control mice (n = 4) treated with NaCl. (B) Bright field and representative immunofluorescence staining for 5-LO, FLAP, LTA<sub>4</sub> H, and LTC<sub>4</sub>S (Cy3, red), CD68 (FITC, green), and DNA (DAPI, blue) and merged picture. Dashed yellow lines indicate external elastic lamina. Dashed line squares represent selected area for high magnification. (Scale bars: 100 μm, inner square 10 μm.) (C) AAA was induced on mice by periaortic application of CaCl<sub>2</sub> (n = 25) or NaCl (n = 6). CaCl<sub>2</sub>-induced AAA mice were divided into four groups: group 1, no treatment (n = 10); group 2, mice treated with montelukast 0.1 mg/kg/d (n = 7); group 3, mice treated with montelukast 1 mg/kg/d (n = 8); and group 4, mice treated with CysLT2 antagonist 3 mg/kg/d (HAMI3379; n = 5). AAA was also induced on Alox5<sup>−/−</sup> mice by periaortic application of CaCl<sub>2</sub> (n = 7) or NaCl (n = 4) as control. After 21 d, aortas were harvested, stained, and circumferences were calculated. (D) AAA was induced by CaCl<sub>2</sub> application and mice were divided into three groups receiving montelukast 1 mg/kg/d for 21 (n = 8), 14 (n = 6), and 7 (n = 6) d after AAA induction. CaCl<sub>2</sub>-treated mice without montelukast (n = 10) and NaCl-treated mice (n = 6) were used as positive and negative control, respectively. At the end of the experiment, aortas were harvested, stained, and circumferences were calculated. (E) Homogenated aortas from separate C57Bl6/J mice treated with NaCl (n = 3), CaCl<sub>2</sub> (n = 3), CaCl<sub>2</sub> + montelukast 0.1 mg/kg/d (n = 3), CaCl<sub>2</sub> + montelukast 1 mg/kg/d (n = 4), and from Alox5<sup>−/−</sup> mice treated with NaCl (n = 3) and CaCl<sub>2</sub> (n = 3) were analyzed by zymography and bands were quantified. (F) Homogenated aortas from separate C57Bl6/J mice treated with NaCl (n = 3), CaCl<sub>2</sub> (n = 3), CaCl<sub>2</sub> + montelukast 0.1 mg/kg/d (n = 3), and CaCl<sub>2</sub> + montelukast 1 mg/kg/d (n = 3), and from Alox5<sup>−/−</sup> mice treated with NaCl (n = 3) and CaCl<sub>2</sub> (n = 3) were analyzed for the presence of MIP-1α by Bio-Plex mouse cytokine assay. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, L, lumen; n.s., nonsignificant.
mRNA indicates that cys-LTs are the main products of this pathway in the mouse AAA model, in agreement with what was observed in human AAA (9). We also checked a trend to increase the FLAP and LTC4H transcripts, indicating that other leukotriene signaling (LTA4H) might have a (small) role in AAA development.

In addition, immunohistochemistry was performed in CaCl2-treated mice. The results showed focal accumulation in media and adventitia layers of 5-LO, FLAP, and LTC4S positive cells that partially colocalized with positive cells for mouse macrophage marker CD68 (Fig. 1B). This result confirms previous reports, which indicated macrophages as important inflammatory cells (15, 16) and a source of LTs (11) during AAA progression.

CysLT1 Antagonism Reduces CaCl2-Induced AAA. The morphological analysis of sections stained by hematoxylin, 21 d after CaCl2 challenge confirmed a significant dilatation of the vascular wall (aorta circumference: 2.34 ± 0.57 mm) compared with NaCl-treated control (aorta circumference: 1.16 ± 0.102 mm, P = 0.0001), whereas the treatment of mice with montelukast 0.1 or 1 mg/kg/d reduced the CaCl2-induced AAA (aorta circumference: 1.76 ± 0.22 mm, P = 0.0479 and 1.62 ± 0.42 mm, P = 0.0022, respectively) (Fig. 1C). To ascertain that the protective effect of montelukast was leukotriene dependent we induced aneurysms in 5-LO−/− mice by CaCl2 (Fig. 2). Montelukast inhibited LTD4 production (1.84 ± 0.20 mm), while low dose montelukast did not show any effect (Fig. 1F). We also measured levels of LTD4 in Alox5−/− mice and found that this chemokine was not increased during AAA induction (Fig. 1F).

Montelukast Inhibits LTD4-Induced Expression of Cytokines and Chemokines in Human MonoMac6 Cells. MonoMac6 (MM6) cells were treated with either 1 μM montelukast (1 h) or vehicle, followed by challenge with 100 nM LTD4 (1 h). LTD4 alone increased mRNA levels of the cytokines TNFα (P < 0.0001) and IL-1β (P < 0.01) as well as chemokines MIP-1α (P < 0.0001) and MCP-1 (P < 0.0001) (Fig. 2A and Fig. S1A), while no effect was observed for the transcript levels of cytokines IL-10 and TGFβ1 (Fig. S1A). In addition, increased levels of TNFα (P < 0.05) and MIP-1α (P < 0.01) were detected in the supernatants of MM6 cells challenged with LTD4, while protein levels of the other mediators were not significantly increased (Fig. 2B and Fig. S1B). Pretreatment of cells with montelukast attenuated all LTD4-induced increases of cytokine/chemokine mRNA and protein to levels corresponding to the untreated control (Fig. 2B and Fig. S1B).

Montelukast as Treatment of Ongoing AAA Development. To test whether CysLT1 antagonism could not only prevent but also inhibit an already ongoing degenerative process in the aortic wall, we studied the effects of the drug at two time points after induction with CaCl2. Thus, in a separate set of experiments we started treatment with 1 mg/kg/d montelukast, 7 and 14 d after CaCl2 induction (Fig. 1D), and, interestingly, the drug significantly protected the aorta at both time points with a circumference of 1.76 ± 0.07 mm (P = 0.0075) and 1.57 ± 0.06 mm (P = 0.0017), respectively, compared to untreated mice.

Montelukast Prevents the Release of MMP-9 and MIP-1α. Cysteinyl-leukotrienes are powerful mediators of inflammation and have been implicated in the release of proteolytic enzymes as well as proinflammatory cytokines and chemokines from immune cells (18). Therefore, to detect the MMPs activity, we examined mouse aortas by zymography. We found a sixfold increase of MMP-9 in mice undergoing AAA induction by CaCl2 (5.84 ± 2.25, P = 0.0002 vs. NaCl) (Fig. 1E). Moreover, both doses of montelukast, 0.1 and 1 mg/kg/d, significantly attenuated this increase and maintained the protease almost at basal levels, corresponding to a twofold increase (1.84 ± 0.23, P = 0.0088 and 2.17 ± 0.26, P = 0.0040 vs. CaCl2, respectively), indicating that cys-LTs were the major, albeit not the sole, mediator of MMP-9 induction. Additionally, Alox5−/− mice showed no difference in MMP-9 activity between CaCl2- and NaCl-treated mice (P = 0.5 vs. NaCl; Fig. 1E). Altogether, these results show that montelukast prevents the release of MMP-9 in a leukotriene-dependent manner.

Next we measured cytokine/chemokine levels in the aortic wall of CaCl2-treated mice. Cytokines, i.e., interleukin (IL)-1β, IL-10, IL-13, tumor necrosis factor-α (TNFα), and chemokines, i.e., monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α) were analyzed in aorta homogenates. Out of these, only MIP-1α showed a significant increase in CaCl2-treated mice (17.04 ± 1.12 pg/mL) compared with basal levels (1.70 ± 0.54 pg/mL, P = 0.0001 vs. change in NaCl-treated mice). Treatment with montelukast (1 mg/kg/d) maintained MIP-1α near basal level (2.79 ± 2.42 pg/mL, P = 0.0008 vs. CaCl2) while low dose montelukast did not show any effect (Fig. 1F). We also measured levels of MIP-1α in Alox5−/− mice and found that this chemokine was not increased during AAA induction (Fig. 1F).
after 28 d of challenge with AngII (Fig. 3A). Additionally, immunostaining revealed colocalization of 5-LO, FLAP, and LTC₄S in macrophages mainly localized in the adventitia (Fig. 3B). A common complication associated with the infusion of AngII in ApoE⁻/⁻ mice is the aortic rupture. We observed that the rupture rate was significantly increased in the placebo group (45%) compared with montelukast-treated mice (15%) (*P = 0.03; Fig. 3C). Moreover, we observed that in the placebo group, mice died within the first week of AngII infusion, while in the group treated with montelukast, deadly ruptures were delayed until the last week of the experiment. We also examined MMP-9 expression within the suprarenal aortas of both groups and detected reduced levels of MMP-9 in the montelukast-treated group vs. placebo (*P = 0.03; Fig. 3D). Altogether, we can conclude that montelukast delays aortic rupture in AngII-infused mice.

Montelukast Decreases the Aortic Dilatation in the PPE-Infusion Model of AAA. In line with the results obtained in the previous two models, the induction of AAA by PPE infusion induced an up-regulation of transcript levels of 5-LO (3.05 ± 0.7 fold; *P = 0.04), FLAP (6.95 ± 1.0 fold; *P = 0.004), and LTA₄H (1.8 ± 0.2 fold; *P = 0.02) in comparison with the control group (Fig. 4A). We also found a trend to increase the levels of LTC₄S transcripts in this model. In addition, immunostaining of 5-LO

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**Fig. 3.** Effect of montelukast in AngII-infused ApoE⁻/⁻ mouse model. (A) 5-LO, FLAP, LTA₄H, and LTC₄S mRNA was determined by qPCR in aortic wall of AngII-infused ApoE⁻/⁻ (n = 4) and compared with ApoE⁻/⁻ mouse (n = 4). (B) Bright field and representative immunofluorescence staining for 5-LO, FLAP, LTA₄H, and LTC₄S (Cy3, red), CD68 (FITC, green), and DNA (DAPI, blue) and merged picture of suprarenal aorta in AngII-infused ApoE⁻/⁻. Dashed yellow lines indicate external elastic lamina. Dashed line squares represent selected area for high magnification. (Scale bars: 100 μm, inner square 10 μm.) (C) Aortic rupture rate was determined in AngII-infused ApoE⁻/⁻ treated with placebo (45%, 9 of 20) vs. montelukast group (15%, 3 of 20). (D) Homogenated infrarenal aortas from mice treated with placebo (n = 6) and montelukast (n = 5) were analyzed by zymography and bands were quantified. Data represent mean of experiments ±SEM. *P < 0.05, n.s., nonsignificant.

**Fig. 4.** Effect of montelukast in PPE infusion model. (A) 5-LO, FLAP, LTA₄H, and LTC₄S mRNA was determined by qPCR in aortic wall of PPE (n = 3) compared with sham unoperated animals (n = 3). (B) Bright field and representative immunofluorescence staining for 5-LO, FLAP, LTA₄H, and LTC₄S (Cy3, red), CD68 (FITC, green), and DNA (DAPI, blue) and merged picture of infrarenal aorta in PPE. Dashed yellow lines indicate external elastic lamina. Dashed line squares represent selected area for high magnification. (C) Representative ultrasound imaging of infrarenal aorta from mice treated with placebo and montelukast after 28 d of PPE infrarenal infusion. (D) Luminal diameter (relative dilatation from baseline) after 28 d of PPE infrarenal infusion in mice treated with placebo (n = 7) and montelukast (n = 5). (E) Homogenated infrarenal aortas from mice treated with placebo (n = 6) and montelukast (n = 5) were analyzed by zymography and bands were quantified. Data represent mean of experiments ±SEM. *P < 0.05, ***P < 0.001, L, lumen; n.s., nonsignificant.
pathway-related proteins (5-LO, FLAP, and LTC4S) were observed through colocalization with macrophages (Fig. 4B).

Ultrasound imaging revealed that montelukast treatment reduced significantly the increase of abdominal aortic diameter (AAD) at day 28 (48.4% ± 3.9 vs. 81.4 ± 10.6 placebo; P = 0.03) (Fig. 4 C and D). Moreover, treatment with montelukast for 4 wk revealed a reduced activity of MMP-9 (P = 0.03 vs. placebo; Fig. 4E).

Discussion

AAA is a common, slowly developing, and highly lethal vascular disorder in elderly people. The most dangerous clinical consequence of AAA is an acute rupture, which carries a mortality of 80%. The absence of clinical signs and biomarkers able to predict this disorder brings it to late diagnosis when the aneurysm is in an advanced stage, which may, or may not, be amenable to surgical intervention. Thus, a main medical goal is to find non-invasive pharmacological therapies, which can prevent, slow down, and possibly block the progression of the vascular wall degradation at an early stage of the disease process.

The AAA pathology is characterized by chronic inflammation and increased protease activity in the vessel wall leading to extracellular matrix degradation, aneurysm growth, and finally spontaneous rupture (19).

Previous work on human aneurysm tissue has suggested that leukotrienes may be involved in AAA disease (9, 20). However, studies with the AngII model have yielded variable results (11–13, 21). Thus, pharmacological inhibition or genetic ablation of 5-LO, the regulatory enzyme responsible for the biosynthesis of LTs, reduced vascular remodeling but did not afford significant protection against AAA (11, 22, 23), while positive results were achieved with interventions at the BLT1 receptor level (12, 13).

A possible reason for this discrepancy could be that blocking 5-LO inhibits not only the formation of proinflammatory LTs, but also biosynthesis of antiinflammatory, proresolving media tors such as lipoxins and resolvins, which have been shown to attenuate murine AAA (24, 25). More recently, a study from AstraZeneca demonstrated that pretreating mice with a potent 5-LO inhibitor before the induction of AAA by either AngII infusion in LDL−/− mice under high fat diet or PPE in WT mice reduced the aneurysm development (14).

Although these results are promising, high doses of the inhibitor were required and drugs targeting 5-LO are known to be associated with side effects, limiting the translational potential of these observations. Furthering our findings on increased 5-LO pathway enzymes, and at a dose of 1 mg/kg, the drug significantly reduced the rate of rupture in the AngII/ApoE−/− model (Fig. 3C) and attenuated aortic dilatation in the PPE model (Fig. 4D), apparently due to decreased MMP-9 activity (Figs. 3D and 4E).

Hence, we show in three independent in vivo AAA mouse models with specific characteristics (i.e., inflammatory homing: CaCl2 and PPE; and aortic dissection with thrombus: AngII) (26), the AngII model shows infiltration also of other leukocytes, i.e., B and T lymphocytes, and the aneurysm occurs in the suprarenal aorta (15, 16).

Both models exhibited increased levels of 5-LO pathway enzymes, and at a dose of 1 mg/kg, the drug significantly reduced the rate of rupture in the AngII/ApoE−/− model (Fig. 3C) and attenuated aortic dilatation in the PPE model (Fig. 4D), apparently due to decreased MMP-9 activity (Figs. 3D and 4E).

Although we believe that montelukast acts locally in the diseased artery, we cannot rule out that actions of the drug outside the vessel wall may also play a role in our AAA models. For instance, montelukast blocks CysLT1-dependent signaling in human Th2 cells that can migrate in response to cys-LTs and are believed to contribute to airway inflammation in asthma (34).

In the absence of noninvasive treatments for AAA (1), there is a continued strong medical need to develop preventive and therapeutic medications that can be used for patients with incipient AAA below the threshold for surgical repair. In recent years, several clinical trials have been carried out to test the protective ability of a variety of drugs such as β blockers, calcium channel blockers, mast cell inhibitors, ACE inhibitors, and the antibiotic doxycycline without any positive effect (36).

Although the reasons for these failures are currently under debate, there is a general consensus to continue the search for new drug targets for evaluation in human clinical trials. Since CysLT1 antagonists target both the inflammatory and proteolytic components of AAA pathogenesis, they would represent a drug candidate with a mode of action that has not yet been clinically tested. Furthermore, CysLT1 antagonists, typified by montelukast, are effective medications against human asthma, a disease which is experimentally and epidemiologically associated with AAA and rupture (37, 38). It has also been observed that AAA and asthma share several pathophysiological similarities (39, 40) and epidemiological data have revealed a positive correlation between use...
of montelukast and reduced cardiovascular risk in men (41). Moreover, CysLT1 antagonists are remarkably safe, allow a once-daily dose regimen, carry few side effects, and are available as generic drugs. Hence, we firmly believe that CysLT1 antagonists hold promise as anti-AAA agents and should soon be tested in a controlled clinical trial.

Materials and Methods

Animals. Wild-type and ApoE−/− male mice on the background C57BL6J were purchased from SCANDUR Sweden and Taconic, respectively. Alox5−/− mice on the C57BL6J background were a generous gift from Geraldine Canny (University of Lausanne, Lausanne, Switzerland). The housing and care of animals and all of the animal procedures used in this study were in accordance with national guidelines and approved by the Stockholm North Ethical Committee on Animal Experiments. Details of the mouse models of AAA are described in SI Materials and Methods.

Luminal Aortic Diameter Measurements by Ultrasound Imaging. Ultrasound was performed in the animals before the aneurysm induction by angiotensin (Ang) II and PPE infusion and then weekly until the end of week 4 as described (42).

Isolated Cells in Vitro. Cultivation and treatments of a monocyte/macrophage cell line followed a protocol described in ref. 11.

Immunohistochemistry and Measurements of mRNA, Protein, and Protease Activity. Histology and immunohistochemical analysis as well as measurements of mRNA and protein by qPCR and Bio-Plex analysis, respectively, are described in SI Materials and Methods. Gel zymography was used for assessment of metalloprotease activity, as described (9).

Statistical Analysis. Data were analyzed with one-way ANOVA followed by Tukey’s post hoc test. Real-time PCR and aortic dilatation in Alox5−/− mice data were analyzed by Student’s t test. In all cases, statistical significance was set to P < 0.05.

For further details of materials and methods, please see SI Materials and Methods.

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