Chronic Intake of the Selective Serotonin Reuptake Inhibitor Fluoxetine Enhances Atherosclerosis

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Objective—Cardiovascular diseases and depression are the leading causes of disability in Western countries. Clinical data on potential cardiovascular effects of serotonin reuptake inhibitors (SSRIs), the most commonly used antidepressant drugs, are controversial. In addition to blocking serotonin reuptake transporter in the brain, SSRIs deplete the major peripheral serotonin (5-hydroxytryptamine [5-HT]) storage by inhibiting serotonin reuptake transporter–mediated uptake in platelets. In this study, we aimed to investigate the effect of chronic SSRI intake on the development of atherosclerosis.

Approach and Results—Treatment of apolipoprotein E–deficient mice with the SSRI fluoxetine for 2, 4, or 16 weeks increased atherosclerotic lesion formation, with most pronounced effect during early plaque development. Intravital microscopy of carotid arteries revealed enhanced myeloid cell adhesion on fluoxetine treatment. Mechanistically, we found that fluoxetine augmented vascular permeability and increased chemokine-induced integrin-binding activity of circulating leukocytes. In vitro stimulation of murine blood demonstrated that fluoxetine, but not 5-HT, could directly promote β1 and β2 integrin activation provided C-C motif chemokine ligand 5 was also present. Similar effects were observed with the SSRI escitalopram. Enhanced C-C motif chemokine ligand 5–induced integrin activation by fluoxetine was also confirmed in a human neutrophil-like cell line. In contrast to the proatherogenic properties of fluoxetine, pharmacological inhibition of the peripheral 5-HT synthesizing enzyme tryptophan hydroxylase 1 did not promote atherosclerosis, suggesting that the proatherogenic effect of fluoxetine occurs independent of peripheral 5-HT depletion.

Conclusions—SSRI intake may promote atherosclerosis and therefore potentially increase the risk for acute cardiovascular events by a mechanism that is independent of 5-HT depletion.

Visual Overview—An online visual overview is available for this article. **(*Arterioscler Thromb Vasc Biol*. 2018;38: 1007-1019. DOI: 10.1161/ATVBAHA.117.310536.)**

Key Words: antidepressive agents ■ atherosclerosis ■ fluoxetine ■ integrins ■ serotonin

Serotonin or 5-hydroxytryptamine (5-HT) is best known for its role as a neurotransmitter in the central nervous system, where it regulates various functions, such as mood, behavior, and appetite. Impaired 5-HT signaling is associated with psychiatric disorders, such as depression. However, the vast majority (≈95%) of 5-HT can be found in the periphery where it is produced by tryptophan hydroxylase 1 (TPH1) mainly in enterochromaffin cells in the gastrointestinal tract.[1–3] On synthesis, 5-HT is secreted into the circulation, where it is directly taken up by platelets via the serotonin reuptake transporter (SERT) and stored in their dense granules. After activation, platelets release their granule content, including 5-HT.[4] It has been shown that peripheral 5-HT is involved in a variety of different physiological processes, including regulation of vascular tone,[5] vascular permeability,[6] intestinal motility,[7] platelet aggregation, and degranulation.[8] The 5-HT signaling is mediated by various receptors (5-HTR1 to 5-HTR7) and terminated on the part of the receptor ligand 5-HT by reuptake via SERT into platelets.[4] SERT is also expressed on neurons where it represents the primary target of the first-line antidepressants called selective serotonin reuptake inhibitors (SSRIs).[1] Because platelets are not able to synthesize 5-HT themselves, chronic intake of SSRIs results in a depletion of platelet 5-HT and thereby loss of its main peripheral storage.[9] According to the World Health Organization, >300 million people worldwide are experiencing depression.[10]

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Nonstandard Abbreviations and Acronyms

5-HT	5-hydroxytryptamine
5-HTR	serotonin receptor
ApoE	apolipoprotein E
CCL5	C-C motif chemokine ligand 5
HFD	high-fat diet
ICAM-1	intercellular adhesion molecule 1
RT	room temperature
SERT	serotonin reuptake transporter
SSRI	selective serotonin reuptake inhibitor
TPH1	tryptophan hydroxylase 1
VCAM-1	vascular cell adhesion molecule 1

Consequently, SSRIs, such as fluoxetine , are among the most common drugs being prescribed.[11,12] Clinical studies investigating potential cardiovascular effects of SSRIs and the risk for developing an acute cardiovascular event are controversial. On the one hand, there are published data showing no increased or even lower cardiovascular risk for patients taking SSRIs.[13–16] On the other hand, different studies report opposing findings, suggesting a link between SSRI intake and a higher risk for developing cardiovascular events.[17–19] Interpreting these conflicting results becomes even more complex when considering the fact that depression per se is a well-established cardiovascular risk factor.[20] Because results from existing clinical studies are inconclusive to clarify a possible association between SSRI intake and cardiovascular diseases, other than observational studies are needed. We, therefore, aimed to shed light on this topic by investigating the effect of chronic SSRI intake on atherosclerosis onset and progression in a mouse model.

Materials and Methods

Please see the Major Resources Table in the online-only Data Supplement for additional experimental details.

Mice

Male apolipoprotein E–deficient (ApoE$^{-/-}$) mice on C57Bl/6 background aged 6 to 8 weeks were either fed a high-fat diet (HFD) only (Western diet; ssniff) for 2, 4, or 16 weeks, respectively (control group), or treated in parallel with fluoxetine (160 mg/L; Prozac, Lilly) via the drinking water.[6] In a selective experimental condition, fluoxetine treatment was initiated 2 weeks before starting HFD feeding and then continued for another 2 weeks in parallel to HFD. Wild-type C57Bl/6 mice were treated with fluoxetine for 2 weeks. In other experiments, ApoE$^{-/-}$ mice received daily intraperitoneal injections of TPH1 inhibitor LP533401 (25 mg/kg, Dalton Pharma Services) dissolved in dimethyl sulfoxide and diluted in aqua ad injectabilia or equal volume of vehicle in parallel to 2 or 4 weeks HFD feeding.[21] At the end point, mice were anesthetized with ketamine/xylazine, and blood was obtained via cardiac puncture unless otherwise noted. Heart, aorta, spleen, and femurs were harvested after PBS perfusion. All animal experiments were approved by the local Ethics committee (District Government of Upper Bavaria; License Number: 55.2-1-54-2532-111-13) and conducted in accordance with the institutional and national guidelines.

Intravital Microscopy

Intravital microscopy was used to analyze leukocyte–endothelial interactions along the carotid artery of ApoE$^{-/-}$ mice after 4 weeks HFD±fluoxetine treatment as described previously.[22] In brief, antibodies against Ly6G-phycoerythrin (PE) and CD11b-650NC were injected to label myeloid cells and allowed to circulate for 10 minutes to label peripheral myeloid cells. Intravital microscopy of the surgically exposed left external carotid artery was performed using a BX51 (Olympus) microscope. For image acquisition and analysis, Olympus Excellence software was used. Rolling flux was measured as the number of cells passing a reference line perpendicular to blood flow within 30 seconds. When no rolling was observed for at least 30 seconds, cells were defined to be adherent.

Quantification of 5-HT and Chemokines in Serum and Plasma

Murine blood was collected retro-orbitally. For serum, blood was collected in Serum Gel Z tubes and allowed to clot for 30 minutes at room temperature (RT). Plasma was obtained from EDTA-anticoagulated whole blood. To avoid platelet activation, 1 µmol/L prostaglandin E1 (Cayman) was added. The 5-HT was measured with Serotonin Fast Track ELISA (Labordiagnostika Nord). CXC motif chemokine ligand 4 and chemokines C-C motif chemokine ligand 5 (CCL5) were analyzed in serum and in supernatant of activated platelets using DuoSet ELISA kits (R&D systems).

Histology and Immunohistochemistry

Hearts were isolated after perfusion with PBS and frozen in Tissuetek (Sakura Finetek). Aortic roots were cut in 5-µm thick serial cryosections. For the quantification of lesion size, sections were stained with Oil-Red O. Plaque macrophage content was quantified using an antibody against Mac-2 visualized with the alkaline phosphatase enzyme detection system using the VECTASTAIN ABC-AP staining kit (Vector Laboratories). Total collagen was quantified via Sirius red staining. For analysis, the Leica Application Suite LAS V4.3 software was used.

Cholesterol Measurements

Total plasma cholesterol concentrations were quantified using a colorimetric assay (CHOD-PAP, Roche) according to the manufacturer's protocol. The neutral lipid content in blood leukocytes was determined with Amplex Red Cholesterol Assay Kit (Invitrogen)[23] and presented as ratio of esterified to total cholesterol. After erythrocyte lysis in ammonium chloride potassium lysis buffer, cells were resuspended in cold 1× buffer and incubated on ice for 1 hour. Samples were heated at 60°C for 30 minutes to destroy endogenous esterases, followed by shaking for 30 minutes at RT. To measure total and esterified cholesterol, the assay was performed according to the manufacturer's protocol with and without esterase.

Assessment of Blood Cell Counts

Blood leukocyte, platelet counts, and mean platelet volume were measured from EDTA-anticoagulated whole blood with a hematology analyzer (scil Vet ABC Hematology Analyzer).

Flow Cytometry

To obtain single cell suspensions, spleens were meshed through a 70-µm cell strainer, and femurs were centrifuged for 2 minutes at 9000*g*. Erythrocytes were lysed using ammonium chloride potassium buffer. After Fc blocking with CD16/CD32 antibody for 5 minutes on RT, cells were stained for 30 minutes in the dark with an antibody mix for neutrophils and monocytes (CD45.2-fluorescein isothiocyanate, CD11b-PerCP, Ly6G-PE, CD115-allophycocyanin [APC], Ly6C-PE/Cy7). Neutrophils were identified as CD45^{+}CD11b^{+}Ly6G^{+}, classical monocytes as CD45^{+}CD11b^{+}CD115^{+}Ly6C^{high}. For staining of aortic endothelial cells, aortas were flushed with PBS, excised from aortic arch to iliac bifurcation, and digested with collagenase IV and DNase I at 37°C at 750 rpm for 40 minutes as previously described.[24] After filtering and Fc blocking, cells were stained with antibodies against intercellular adhesion molecule 1 (ICAM-1)-APC, vascular cell adhesion molecule 1 (VCAM-1)-PerCP/Cy5.5, CD45-APCeFluor780, CD31-PE/Cy7, and CD107a-BV421. Aortic endothelial cells were

determined as $CD45^{-}CD31^{+}CD107a^{+}$. For staining of leukocyte adhesion molecules, blood was incubated with antibodies against CD11b-PerCP, CD11a-PE/Cy7, CD18-fluorescein isothiocyanate, CD49d-PE, PSGL-1-PerCP, CD62L-fluorescein isothiocyanate, and CD31-PE/Cy7 at 4°C for 30 minutes. Lipid transporters were stained with antibodies against CD36-PE, SRI/anti-rabbit BV421, ABCA1-Alexa Fluor 405, and ABCG1/anti-rabbit AF488. Data were acquired on a BD FACSCanto II flow cytometer (BD Biosciences) and analyzed with FlowJo v10.2 software (Tree Star, Inc).

Flow Cytometric Analysis of Platelet–Leukocyte Aggregates

Blood was carefully obtained via cardiac puncture using a 26G needle and a syringe containing 100 µL acid citrate dextrose buffer. A total of 150 µL blood was incubated with 1 mL red blood cell Lysis/Fixation Solution (BioLegend) for 15 minutes at RT. After washing and Fc blocking, cells were stained (CD45.2-fluorescein isothiocyanate, CD11b-PerCP, Ly6G-ACP/Cy7, CD115-PE, CD41-APC) for 20 minutes at RT. Platelet–leukocyte aggregates were measured by flow cytometry with a low flow rate and identified as leukocytes, which were also positive for platelet marker CD41.

In Vivo Permeability Assay

$ApoE^{-/-}$ mice fed an HFD for 2 weeks±fluoxetine were injected intravenously with 0.5% Evans blue solution in 0.9% saline (40 mg/kg, Sigma Aldrich). After 30 minutes, mice were anesthetized and perfused with PBS. Spleen, heart, and kidney were collected, air dried, and weighted. To extract Evans blue, organs were incubated with 450 µL formamide at 56°C for 24 hours.[25] The optical density of the extracted dye was measured at 620 nm. Vascular leakage was determined as ng of Evans blue extravasated per mg tissue using a standard curve. Endothelial permeability in aortic arches was assessed by confocal laser scanning microscopy. Aortic arches were dissected, fixed in 4% paraformaldehyde solution, mounted on microscopy slides after imaging with a Leica TCSII SP8 3X (Leica Microsystems) equipped with a ×20 multi-immersion objective (numeric aperture, 0.75), and a motorized stage. The whole sample was acquired as a tilescan of 775×775×120 to 200 µm xyz stacks. Evans blue was excited with a continuous white-light laser tuned at 580 nm, and the emitted fluorescence was collected in the 660 to 720 nm range with a hybrid diode detector. Tilescan volumes were reconstructed and processed using Imaris 8.4 (Bitplane, Switzerland). Z sectioning allowed to remove the external adventitial layer, strongly autofluorescent, and to expose the underneath Evans blue–positive signal of the endothelium, which was quantified as a volume after application of an ad hoc mask.

Murine Integrin Activation Assay

Murine blood was drawn from wild-type or $ApoE^{-/-}$ mice as indicated in the respective experiment. Erythrocytes were lysed, and leukocytes were resuspended in Hanks' balanced salt solution with Ca^{2+}/Mg^{2+} and 0.5% BSA. In the presence of recombinant ICAM-1/Fc chimera or VCAM-1/Fc chimera (2.5 µg/mL; R&D Systems), which were prelabeled with anti-human IgG Fc-PE, cells were incubated with 5-HT (1 µmol/L), fluoxetine (1 µmol/L), or escitalopram (0.1 µmol/L, Sigma Aldrich) for 15 minutes at 37°C after stimulation with CCL5 (5 µg/mL, Peprotech) for 5 minutes.[26] Cells were stained, and neutrophils were identified as $CD45^{+}CD11b^{+}Ly6G^{+}Gr1^{+}$ and classical monocytes as $CD45^{+}CD11b^{+}Ly6G^{-}Gr1^{high}$. ICAM-1 or VCAM-1 binding was determined via geometric mean fluorescence intensity of PE-positive cells.

Assessment of High-Affinity β2 Integrin Conformation on HL 60 Cells

The human promyelocytic leukemia cell line HL 60 was cultured in RPMI (Roswell Park Memorial Institute)-1640 medium, containing 10% fetal calf serum and 1% penicillin/streptomycin. For differentiation into neutrophil-like cells, 1×10^6 cells were cultured in 10 mL RPMI-1640 (10% fetal calf serum, 1% penicillin/streptomycin) medium supplemented with 1.3% dimethylsulfoxyd for 6 days.[27,28] To analyze the high-affinity conformation of β2 integrins on HL 60 cells, an anti-CD11/CD18 antibody (mAb24)[29] and the secondary Alexa Fluor 488–conjugated (Fab')2 antibody were used as previously described.[30] HL 60 cells were resuspended in adhesion medium (1.2 mmol/L Ca^{2+}, 1 mmol/L Mg^{2+}, 0.25% [wt/vol] BSA, 0.1% [v/v] glucose, 20 mmol/L HEPES, pH 7.4 in Hanks' balanced salt solution) containing mAb24 (5 µg/mL) and fluoxetine (1 µmol/L) or 5-HT (1 µmol/L) and stimulated with CCL5 (5 µg/mL) or left unstimulated for 20 minutes at 37°C. Cells were fixed and analyzed using a fluorescence-activated cell sorter BD LSRFortessa 5 L (BD Bioscience). High-affinity β2 integrin conformation was measured by mean fluorescence intensity.

Isolation and In Vitro Stimulation of Platelets

Blood was taken by heart puncture using a 26G needle and a syringe containing 100 µL acid citrate dextrose buffer into a citrate tube. Platelet-rich plasma was obtained by centrifugation at 100*g* for 10 minutes at RT without break. After washing, platelets were obtained via centrifugation at 2600*g* for 5 minutes at RT and resuspended in 300 µL prewarmed tyrode buffer. The 2×10^8 platelets were activated by stimulation with 0.5 U/mL thrombin for 20 minutes at 37°C. After pelleting, supernatants were collected for ELISA. Platelet activation was determined by CD62P-PE staining after flow cytometry analysis of geometric mean fluorescence intensity.

In Vitro Stimulation of Murine Endothelial Cells

Murine endothelial cells SVEC4-10 were grown to confluence in RPMI-1640 (10% fetal calf serum, 1% penicillin/streptomycin). Cells were pretreated with fluoxetine or 5-HT (1 µmol/L) in serum-free medium for 15 minutes, after stimulation with tumor necrosis factor-α (10 ng/mL; BioLegend) for 6 hours. After washing, cells were stained with antibodies against ICAM-1-APC and VCAM-1-PerCP/Cy5.5. Protein expression levels were measured by flow cytometry and calculated via mean fluorescence intensity.

Isolation of Monocytes and Neutrophils From Bone Marrow

Bone marrow cells were obtained from femurs by centrifugation. Cells of 3 mice were pooled, and neutrophils or monocytes were isolated by negative selection using Neutrophil or Monocyte Isolation Kit (Miltenyi Biotec). The obtained purity of neutrophils and monocytes was 98% and 85%, respectively.

Quantitative Real-Time Polymerase Chain Reaction

Aortas were homogenized and lysed using the TissueLyser LT (Qiagen) for extraction of total RNA (peqGold Trifast and Total RNA kit, Peqlab). After reverse transcription (PrimeScript RT reagent kit, Clontech), real-time polymerase chain reaction was performed with the 7900HT Sequence Detection System (Applied Biosystems) using the KAPA PROBE FAST Universal qPCR kit (Peqlab). Primers and probes were purchased from Life Technologies or self-designed. Target gene expression was normalized to HPRT (hypoxanthine-guanine phosphoribosyltransferase) and presented as relative transcript level ($2^{-\Delta\Delta Ct}$). For comparison of relative 5-HTR and SERT expression between murine monocytes and neutrophils, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as additional housekeeping control for normalization.

Generation of 5-HTR2a-Overexpressing HEK293 Cells

The plasmid carrying the human coding sequence for the 5-HT receptor 2a (5-HTR2a) flanked by *Bam*HI (5′) and *Xho*I (3′) restriction sites was purchased from cDNA Resource Center. The coding region was cloned into the pcDNA5/FRT/TO expression vector (Invitrogen), N-terminally fused to the coding sequence for yellow fluorescent protein. For stable and inducible expression of the construct, Flp-In

T-Rex-293 (HEK [human embryonic kidney] 293) cells, cultured in DMEM (10% fetal calf serum, 1% penicillin/streptomycin), were transfected using EcoTransfect reagent (OZ Biosciences), and clones were selected with hygromycin (0.25 mg/mL; Invivogen). Receptor expression in isolated clones was induced by tetracycline (0.5 µg/mL; Sigma Aldrich) 2 days before the experiment.

Calcium Assay

Intracellular calcium concentrations were measured using FLPR Calcium 5 Assay Kit (Molecular Devices). Briefly, stably transfected cells were seeded with medium containing tetracycline in 96 wells, pretreated with 0.01% poly-D-lysine (Sigma Aldrich). After incubation for 48 hours, cells were loaded with FLIPR Calcium 5 dye for 1 hour at 37°C. Fluorescence of the calcium sensitive dye (excitation: 485 nm, emission 525 nm) was measured at 37°C before and after addition of fluoxetine or 5-HT with an Infinite F200 PRO microplate reader (Tecan Trading AG). Relative light units were normalized to basal levels and presented as x-fold over basal level.

Statistical Analysis

All data are shown as mean±SEM. Statistical analyses were performed using GraphPad Prism 7 software. To test for Gaussian distribution, D'Agostino Pearson omnibus or Shapiro–Wilk normality test was applied. After comparing variances via F test, Student *t* test was used for normally distributed data with equal variances. If variances were significantly different, Welch *t* test was applied. Mann–Whitney *U* test was performed if normality test failed. For highly screwed data sets, log transformation was applied before statistical calculations (only applied for data shown in Figure IIIB in the online-only Data Supplement). For multiple comparisons, after testing for normal distribution, either 1-way ANOVA followed by Bonferroni post hoc test or Kruskal–Wallis test followed by Dunn post hoc test was applied. For 2 independent factors, 2-way ANOVA followed by Bonferroni post hoc test was performed. Outliers were determined by Tukey method. A $P<0.05$ was considered statistical significant.

Results

Fluoxetine Treatment Depletes Platelet 5-HT Without Affecting Free Circulating Plasma Levels

To validate the efficiency of chronic fluoxetine treatment on peripheral 5-HT depletion in ApoE$^{-/-}$ mice, we first performed a time course experiment. It has been reported that chronic oral intake of 18 mg/kg per day fluoxetine in mice resulted in fluoxetine plasma concentrations equivalent to those measured in patients receiving 20 to 80 mg/d fluoxetine.[31] Therefore, we decided to administrate the dose of 160 mg/L fluoxetine via the drinking water,[6] which approximately corresponds to 18 mg/kg per days. Plasma levels of 5-HT reflecting free circulating 5-HT not stored by platelets were generally low and not significantly changed by fluoxetine (Figure 1A). As expected, fluoxetine induced a significant decrease of serum 5-HT, which was already detectable after 7 days of treatment (Figure 1B). This indicates that SERT inhibition specifically inhibited 5-HT storage in platelets. After 14 days, a depletion efficiency of 88% was achieved (Figure 1C). Furthermore, we observed that HFD feeding progressively decreased 5-HT serum levels in ApoE$^{-/-}$ mice, possibly because of enhanced platelet activation stage and consequently reduced storage (Figure 1D).

Fluoxetine Treatment Enhances Atherosclerotic Plaque Formation

To investigate the influence of fluoxetine on the onset and progression of atherosclerosis, ApoE$^{-/-}$ mice received fluoxetine treatment in parallel to HFD feeding for 2, 4, or 16 weeks, respectively. We did not observe any effect of fluoxetine on body weight or plasma total cholesterol levels at any of the investigated time points (Tables I and II in the online-only Data Supplement). In addition, fluoxetine did not affect the neutral lipid content in circulating leukocytes as evidenced by similar ratios of esterified to total cholesterol levels in treated and control mice (Figure IA in the online-only Data Supplement). Moreover, no changes in protein levels of cholesterol transporters were detectable on blood nonclassical monocytes on fluoxetine treatment (Figure IB in the online-only Data Supplement).

Atherosclerotic lesion size in aortic roots was increased at all stages of atherosclerosis compared with untreated controls (Figure 2). Acute fluoxetine treatment was previously shown to transiently elevate plasma 5-HT concentrations, as a consequence of blocked 5-HT uptake by platelets.[32] To exclude the possibility that the proatherogenic effects might be solely caused by a short-term peak of 5-HT plasma levels during onset of fluoxetine treatment, we also performed a pretreatment experiment, starting fluoxetine administration before HFD onset. However, the resulting plaque phenotype was similar to the one observed in our initial experimental setup (Figure II in the online-only Data Supplement).

Because the proatherogenic effect was most pronounced in the early phase of atherogenesis and this stage of plaque formation mainly involves myeloid cell recruitment, we assessed macrophage plaque content by immunohistochemistry after 4 weeks HFD. We found significantly higher amounts of Mac-2–positive cells in aortic roots of fluoxetine-treated mice, indicating enhanced neointimal macrophage content (Figure III in the online-only Data Supplement). However, the relative plaque macrophage content was not changed. At a more advanced stage (16 weeks HFD), we did no longer observe differences in the amount of lesional macrophages (Figure IVA and IVB in the online-only Data Supplement). Similarly, fluoxetine treatment did not affect relative plaque collagen content at advanced stage (Figure IVC and IVD in the online-only Data Supplement).

Fluoxetine Treatment Transiently Reduces Circulating Leukocyte and Platelet Counts Without Affecting Myelopoiesis

During atherogenesis, monocytes and neutrophils are mobilized from the bone marrow into the blood and recruited into the arterial wall. We asked whether higher intimal macrophage content might be a consequence of enhanced monocyte infiltration, possibly because of changes in endothelial activation or myelopoiesis and mobilization from the bone marrow. Surprisingly, blood count analysis during the initial time course experiment revealed a marked decrease in circulating granulocyte counts after 14 days of fluoxetine treatment (Figure VA in the online-only Data Supplement). More precisely, the blood count data of mice treated with fluoxetine in parallel to 2 weeks HFD revealed an overall reduction of circulation leukocyte numbers, mainly granulocytes and monocytes. Furthermore, platelet numbers were significantly lower in fluoxetine-treated mice (Figure VB in the online-only Data Supplement). These differences were no longer detectable

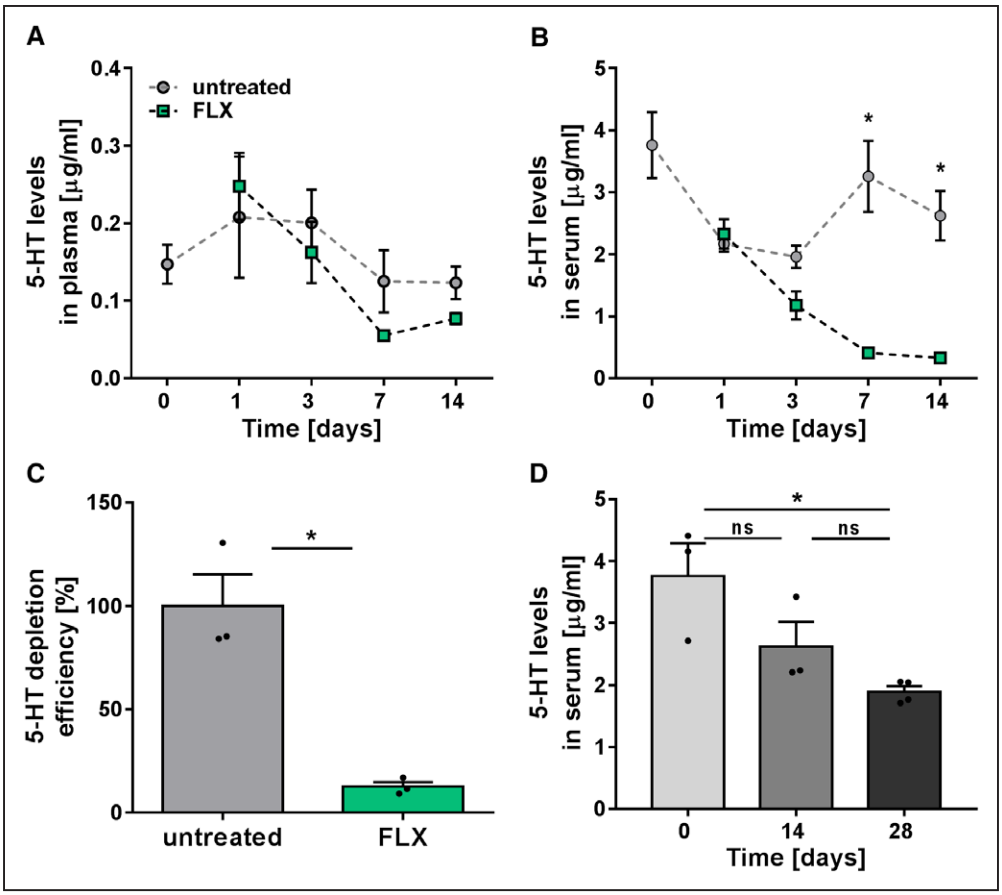

Figure 1. Influence of fluoxetine (FLX) treatment and high-fat diet (HFD) on serotonin (5-hydroxytryptamine [5-HT]) levels. Quantification of 5-HT levels in plasma (**A**) and serum (**B**) of apolipoprotein E–deficient (ApoE$^{-/-}$) mice after 0, 1, 3, 7, and 14 d of HFD±FLX. Data show mean±SEM, n=3, 2-way ANOVA followed by Bonferroni multiple comparison test: *P<0.05. **C**, Quantification of 5-HT depletion efficiency after 14 d of FLX treatment in percent. Data show mean±SEM, n=3, Welch t test: *P<0.05. **D**, Quantification of 5-HT levels in serum of ApoE$^{-/-}$ mice after 0, 14, and 28 d of HFD. Data show mean±SEM, n=3 to 4, Kruskal–Wallis test followed by Dunn multiple comparison test: *P<0.05, ns indicates not significant.

after 4 and 16 weeks of fluoxetine treatment (Figure VC and VD in the online-only Data Supplement) and did not occur in wild-type mice without atherogenic background (Figure VE in the online-only Data Supplement). Moreover, we did not find any differences in neutrophil and monocyte counts in bone marrow or spleen, indicating that fluoxetine did not affect myelopoiesis (Figure VI in the online-only Data Supplement). The transient reduction of circulating myeloid cells might be a consequence of their enhanced recruitment to the arterial wall, in particular during early lesion formation.

Fluoxetine Treatment Enhances In Vivo Adhesion of Myeloid Cells to Carotid Arteries

To validate this hypothesis, we performed in vivo imaging of myeloid cell recruitment to the carotid artery bifurcation using intravital fluorescence microscopy. After 4 weeks of HFD and fluoxetine treatment, we administered fluorescently labeled antibodies to identify circulating myeloid cells (CD11b^{+}) and neutrophils (Ly6G^{+}). Although rolling on the endothelium was not affected, the number of adhering myeloid cells to the vessel wall was 2-fold higher in fluoxetine-treated mice compared with untreated control mice (Figure 3). The effect was even more striking when selectively quantifying neutrophil adhesion, which was 3.5-fold higher. Because selectin-dependent leukocyte–endothelium interactions were not altered, we speculated that fluoxetine treatment might affect integrin-binding affinity, which is relevant for leukocyte adhesion.

Fluoxetine Treatment Does Not Affect Platelet Characteristics

Platelets were previously described to aggravate atherosclerosis by forming platelet–leukocyte aggregates and thereby releasing the platelet-derived CCL5 and CXC motif chemokine ligand 4.[33] We speculated that the depletion of 5-HT storage might affect these proatherogenic platelet properties. However, no differences in circulating platelet–leukocyte aggregates between fluoxetine-treated and control mice were detectable (Figure VII in the online-only Data Supplement). Similarly, fluoxetine treatment did not affect mean platelet volume, an indicator of platelet reactivity (Figure VIIIA in the online-only Data Supplement). Moreover, CCL5 and CXC motif chemokine ligand 4 serum levels of fluoxetine-treated and untreated control mice were comparable (Figure VIIIB in the online-only Data Supplement). In vitro activation of isolated platelets after 2 weeks HFD and fluoxetine treatment also did not reveal any differences in CXC motif chemokine ligand

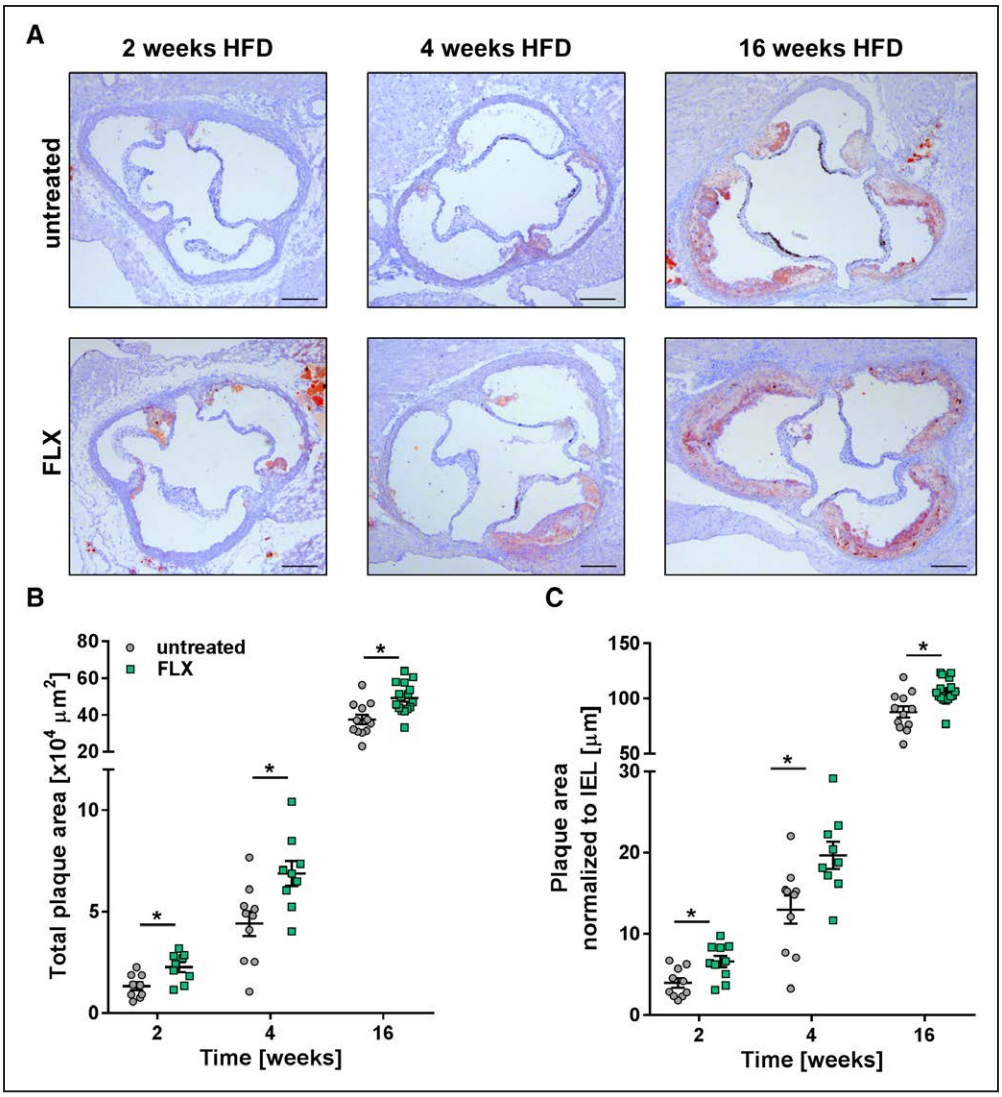

Figure 2. Fluoxetine (FLX) treatment enhances atherosclerosis. Untreated and FLX-treated apolipoprotein E–deficient mice were fed a high-fat diet (HFD) for 2, 4, and 16 wk, and plaque formation was quantified via Oil Red O (ORO) staining of frozen sections of aortic roots. **A**, Representative images after 2, 4, or 16 wk HFD±FLX. Scale bars indicate 200 µm. Quantitative analysis of ORO staining as total plaque area per cross-section (**B**) or ratio between total plaque area and internal elastic lamina (IEL; **C**) after 2 (n=9), 4 (n=9–10), and 16 (n=12–16) wk HFD. Data show mean±SEM. Student *t* test: **P*<0.05.

4 release compared with the control group (Figure VIIIC in the online-only Data Supplement). Furthermore, the expression of CD62P, a surface marker for platelet activity, was not altered (Figure VIIID in the online-only Data Supplement).

Fluoxetine Treatment Increases Vascular Permeability

In atherosclerosis, the barrier function of the endothelium is disrupted leading to increased vascular permeability. This facilitates transendothelial migration of leukocytes to the arterial intima.[34] Because platelet 5-HT was reported to be involved in the regulation of vascular integrity,[6] we investigated the effect of fluoxetine on vessel leakage using an in vivo permeability assay. Fluoxetine treatment for 2 weeks in parallel to HFD enhanced vascular permeability compared with mice fed a HFD only, as evidenced by an increased amount of Evans blue captured per milligram of tissue in spleen, kidney, and heart (Figure IXA in the online-only Data Supplement). The confocal imaging of aortic arches also suggested that Evans blue–positive areas tended to be larger (*P*=0.09; Figure IXB through IXC in the online-only Data Supplement).

Fluoxetine Treatment Does Not Increase Endothelial Adhesion Molecule Expression

Enhanced myeloid cell adhesion on endothelium might be promoted by increased expression of adhesion molecules on arterial endothelium or circulating leukocytes. To clarify the potential effects on endothelial cells and aortic inflammatory cytokines, we measured aortic mRNA expression of adhesion molecules, chemokines, as well as chemokine receptors after 4 weeks of fluoxetine treatment and HFD (Figure X in the online-only Data Supplement). We did not detect any changes that would support augmented leukocyte recruitment. Given that differences in surface expression levels might be because

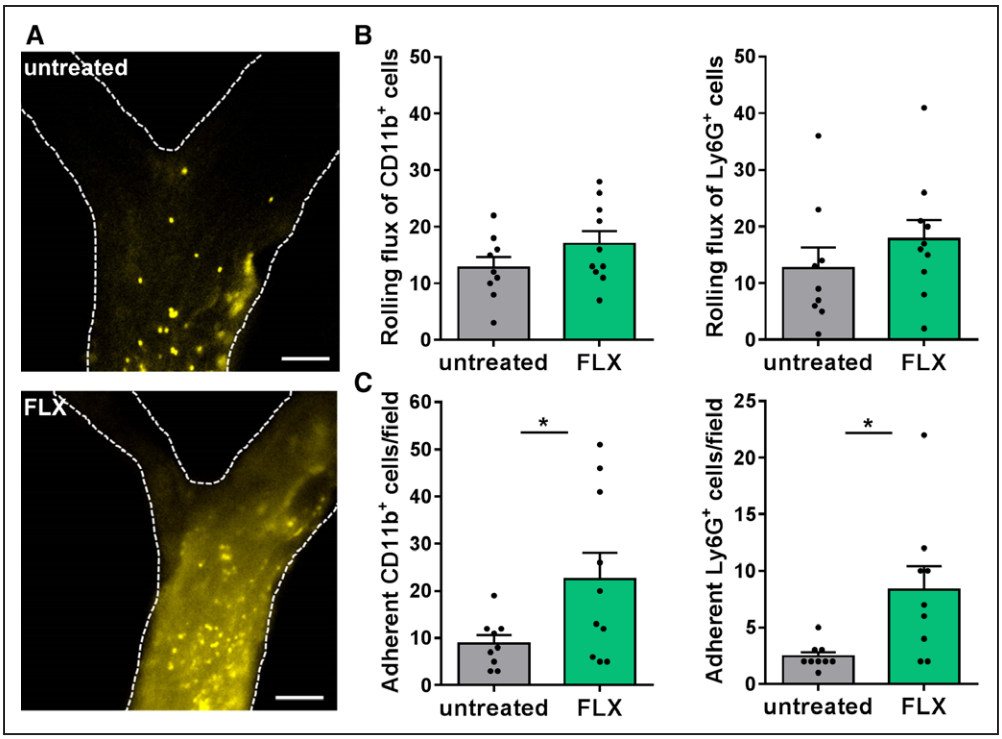

Figure 3. Fluoxetine (FLX) treatment promotes adhesion to carotid arteries. Untreated and FLX-treated apolipoprotein E–deficient mice were fed a high-fat diet for 4 wk, and intravital microscopy of the left carotid artery was performed. Myeloid cells or neutrophils were identified by intravenous injection of antibodies against CD11b or Ly6G 10 min before measurement. **A**, Representative images of adherent CD11b⁺ cells. Scale bars indicate 100 μm. **B**, Quantitative analysis of the number of rolling CD11b⁺ and Ly6G⁺ cells. **C**, Quantitative analysis of counted adherent CD11b⁺ and Ly6G⁺ cells. Data show mean±SEM, n=9 to 10, **P*<0.05, Welch *t* test or Mann–Whitney *U* test.

of post-transcriptional changes, we further analyzed surface protein levels on aortic endothelial cells using flow cytometric analysis. However, no increase of endothelial ICAM-1 and VCAM-1 surface expression in fluoxetine-treated mice was detectable (Figure 4A). Moreover, in vitro fluoxetine or 5-HT treatment of tumor necrosis factor-α–stimulated murine endothelial cells SVEC4-10 did not affect surface expression levels of ICAM-1 or VCAM-1 (Figure XI in the online-only Data Supplement).

Fluoxetine Treatment Enhances CCL5-Induced Integrin Activation

We subsequently focused on circulating leukocytes. Measurement of adhesion molecules on blood neutrophils, more precisely selectins and integrins, did not reveal differences between fluoxetine-treated and control mice (Figure 4B). Because integrin-mediated binding depends on conformational changes rather than regulation of surface expression, we analyzed β1 and β2 integrin-binding activity to their natural ligands ICAM-1 and VCAM-1 in response to leukocyte stimulation with CCL5 (Figure 4C and 4D). After 2 weeks of in vivo fluoxetine treatment, circulating neutrophils and classical monocytes exhibited an increased CCL5-induced binding affinity to ICAM-1 and VCAM-1. To better clarify whether enhanced integrin activation in mice chronically treated with fluoxetine is a consequence of platelet 5-HT depletion or possibly a direct effect of fluoxetine on blood leukocytes, we performed additional in vitro experiments. In vitro stimulation of whole blood obtained from wild-type mice revealed increased CCL5-induced integrin activation after fluoxetine but not 5-HT treatment (Figure 5A). This finding suggests a direct effect of fluoxetine on leukocytes independent of peripheral 5-HT depletion. Of note, enhanced integrin-mediated binding was only observed on chemokine costimulation, whereas fluoxetine alone did not promote binding to VCAM-1 or ICAM-1 (Figure 5B–5C). In line with these findings measuring integrin, high-affinity conformation with the mAb24 reporter antibody revealed upregulation of β2 integrin activation in the presence of fluoxetine on CCL5-stimulated human HL 60 cells compared with CCL5 treatment alone. Similar to murine leukocytes, treatment with 5-HT did not affect CCL5-induced integrin activation and fluoxetine stimulation in the absence of CCL5 did not induce β2 integrin activation in the human system (Figure 5D). This implies that the fluoxetine-mediated and 5-HT–independent effect on integrin activation is also relevant for human cells and CCL5 dependent. To examine whether the increase of integrin activity is solely mediated by fluoxetine or possibly also triggered by other SSRIs, we performed additional integrin activity assays using escitalopram. Escitalopram is the newest clinically available SSRI, showing advantages in terms of efficacy and tolerability compared with other SSRIs. Compared with other SSRIs, which block 5-HT transport of SERT only by orthosterically binding in the central cavity, escitalopram additionally binds to an allosteric site.[35,36] Remarkably, escitalopram also strongly increased VCAM-1 binding on monocytes and neutrophils in the presence of CCL5 while

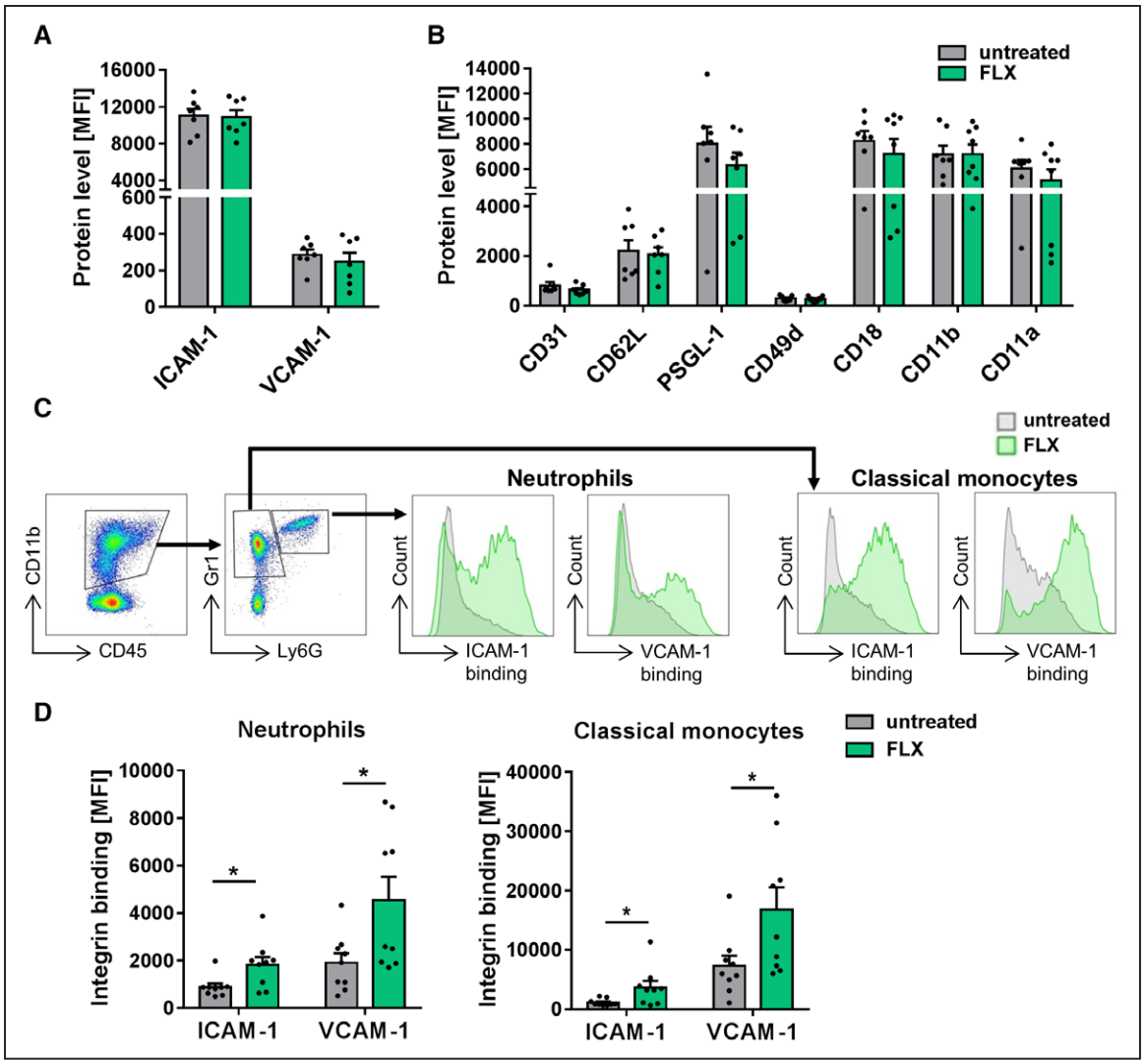

Figure 4. Fluoxetine (FLX) treatment augments integrin activation. Apolipoprotein E–deficient mice were fed with a high-fat diet (HFD) for 2 wk and treated in parallel±FLX. Expression of adhesion molecules on aortic endothelial cells (**A**) and circulating neutrophils (**B**) was measured by flow cytometry and expressed as geometric mean fluorescence intensity (MFI). Data show mean±SEM, n=7, Student *t* test: **P*<0.05. **C** and **D**, Blood leukocytes of control and FLX-treated mice were stimulated with C-C motif chemokine ligand 5 and the binding of neutrophils ($CD45^+CD11b^+Gr1^+Ly6G^+$) and classical monocytes ($CD45^+CD11b^+Gr1^{high}Ly6G^-$) to recombinant intercellular adhesion molecule 1 (ICAM-1)/Fc chimera or vascular cell adhesion molecule 1 (VCAM-1)/Fc chimera was measured by flow cytometry via geometric MFI. Data show mean±SEM, n=8 to 9, Welch *t* test: **P*<0.05.

the effect on ICAM-1 binding was less pronounced (Figure XII in the online-only Data Supplement).

According to previously published data, fluoxetine may not only bind to SERT but also to several 5-HTRs.[37] We measured mRNA levels of SERT and several 5-HTRs in isolated murine monocytes and neutrophils (Figure XIII in the online-only Data Supplement). In addition to SERT, we were able to detect 5-HTR1b and 5-HTR2a mRNA expression in monocytes and to a lower extend in neutrophils. Affinity assays reported binding of fluoxetine to 5-HTR2a with much higher affinity than to 5-HTR1b.[37] Because 5-HTR2a is a G protein–coupled receptor coupled to G_q[38] and intracellular Ca^{2+} is essential for integrin activation,[39–41] we tested the possibility that fluoxetine might generate an increase of Ca^{2+} flux via 5-HTR2a binding. However, fluoxetine did not induce a Ca^{2+} response in stably 5-HTR2a expressing HEK293 cells, whereas 5-HT stimulation resulted in a strong intracellular Ca^{2+} increase (Figure XIV in the online-only Data Supplement).

Pharmacological TPH1 Inhibition Does Not Promote Atherogenesis

To strengthen our hypothesis that the proatherogenic effect of fluoxetine treatment was not a consequence of peripheral 5-HT depletion, we used an additional approach for in vivo 5-HT depletion using a pharmacological inhibitor of the peripheral 5-HT-synthesizing enzyme TPH1. Daily administration of the TPH1 inhibitor LP-533401 for 2 weeks in parallel to HFD resulted in efficient depletion of peripheral 5-HT (Figure 6A). Of note, TPH1 inhibition did not influence circulating leukocyte or platelet counts (Figure 6B). Interestingly, chronic treatment with TPH1 inhibitor had an opposing effect on early plaque formation compared with

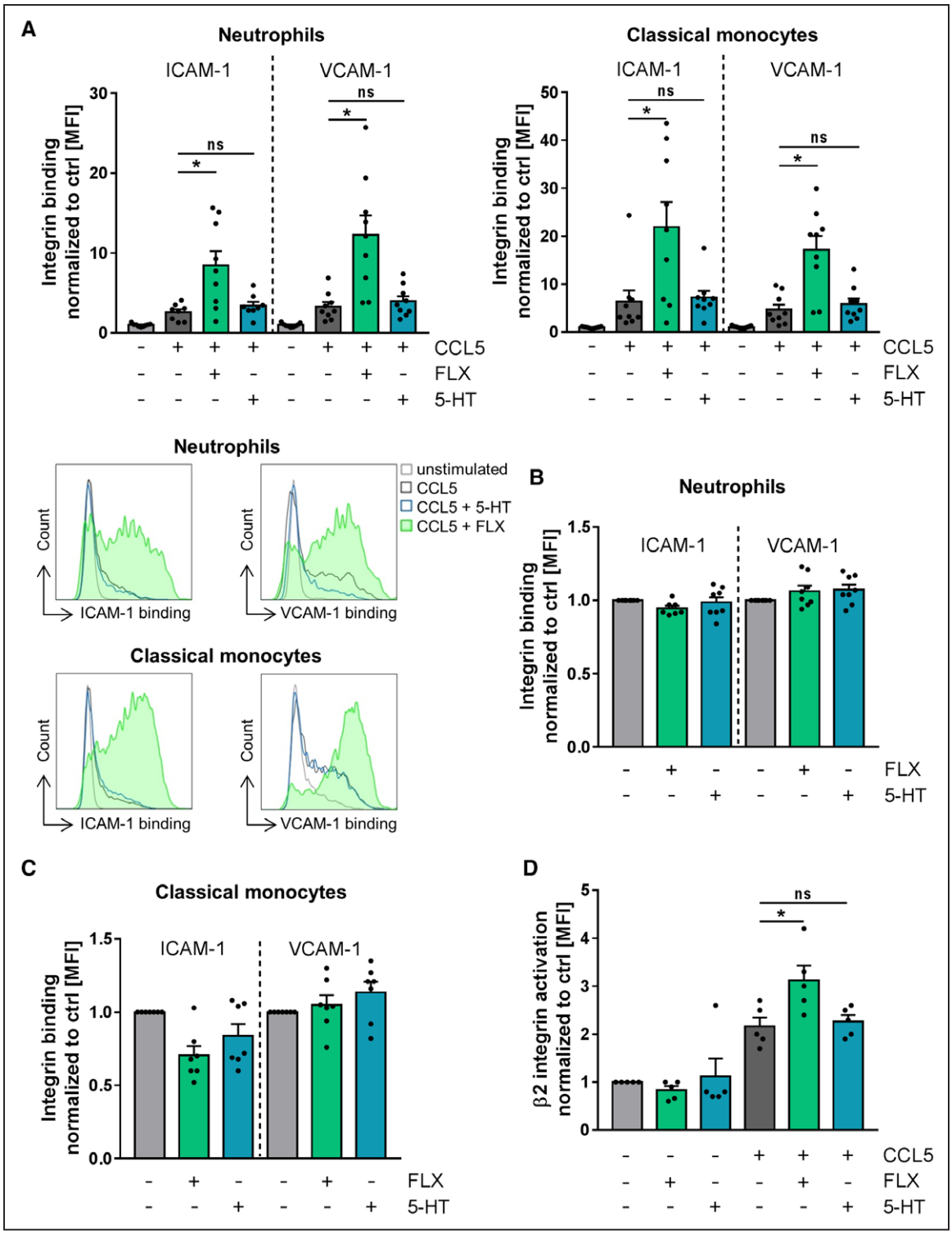

Figure 5. Fluoxetine (FLX) increases C-C motif chemokine ligand 5 (CCL5)–induced integrin activity after in vitro stimulation of murine blood leukocytes and human neutrophil-like cells. Blood of C57Bl/6J mice was stimulated in vitro with (**A**) or without (**B** and **C**) CCL5 in the presence of FLX or serotonin (5-hydroxytryptamine [5-HT]), and integrin activity was assessed via leukocyte binding to recombinant intercellular adhesion molecule 1 (ICAM-1)/Fc chimera or vascular cell adhesion molecule 1 (VCAM-1)/Fc chimera measured by flow cytometry via geometric mean fluorescence intensity (MFI). **D**, Human HL 60 cells, which were differentiated into neutrophil-like cells, were stimulated with CCL5 in the presence of FLX or 5-HT. CCL5-mediated β2 integrin activation was quantified via binding to mAb24. Data show mean±SEM, n=7 to 9 (**A**–**C**), n=5 (**D**), 1-way ANOVA followed by Bonferroni multiple comparison test: **P*<0.05, ns indicates not significant.

fluoxetine, evidenced by significantly smaller lesions compared with the vehicle group (Figure 6C–6E) after 2 weeks treatment, without affecting body weight or cholesterol levels (Table III in the online-only Data Supplement). However, this antiatherogenic effect was no longer observed after 4 weeks of TPH1 inhibitor treatment (Figure 6C–6E),

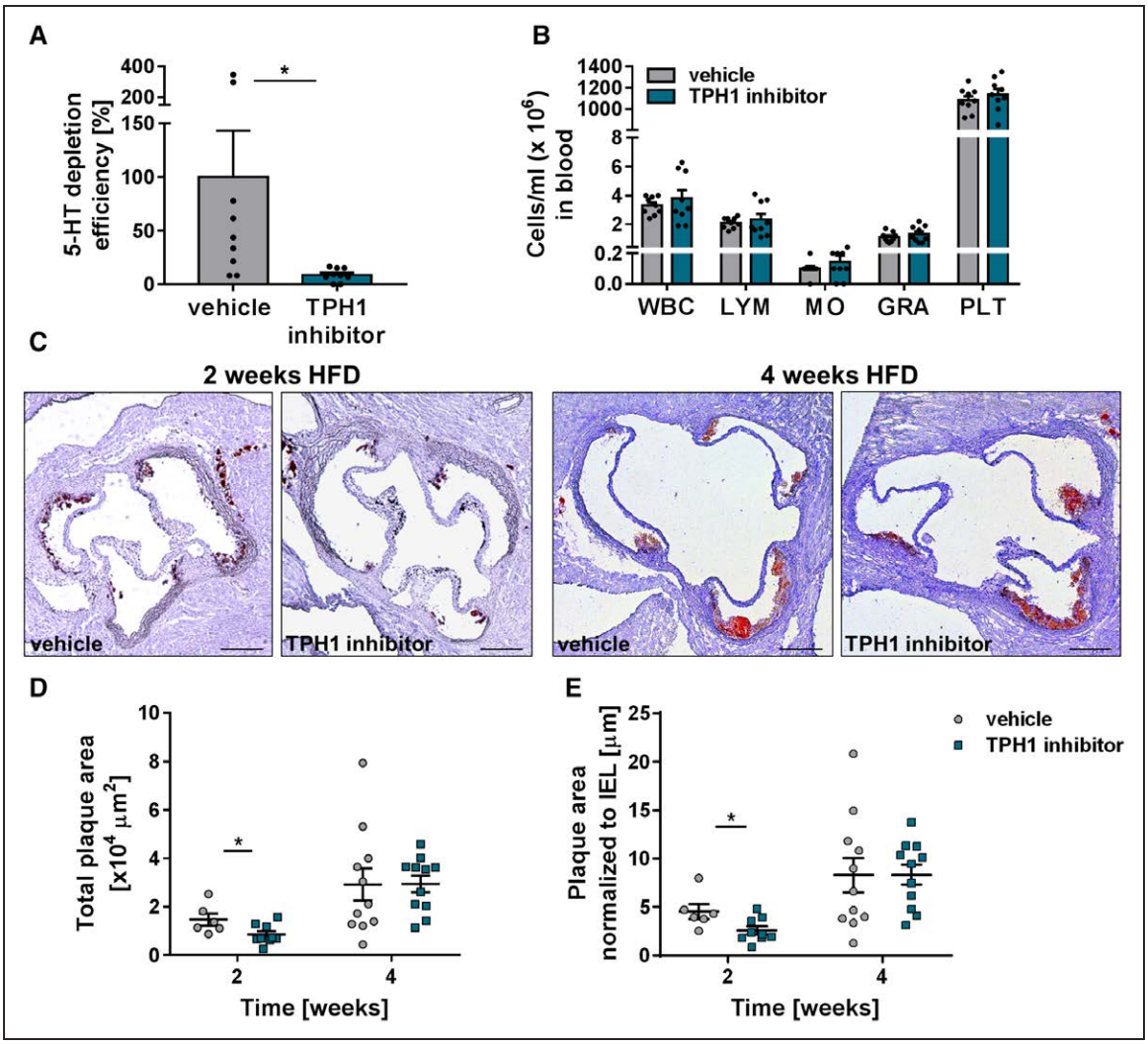

Figure 6. Depletion of peripheral serotonin by tryptophan hydroxylase 1 (TPH1) inhibition does not aggravate atherosclerosis. Apolipoprotein E–deficient mice were fed a high-fat diet (HFD) for 2 wk and received daily injections of TPH1 inhibitor LP533401 (IP 25 mg/kg) or vehicle (dimethyl sulfoxide). **A**, TPH1 inhibitor efficiency was calculated as reduction of serum serotonin (5-hydroxytryptamine [5-HT]) levels determined by ELISA. **B**, Blood counts of white blood cells (WBC), lymphocytes (LYM), monocytes (MO), granulocytes (GRA), and platelets (PLT) were measured with a hematology analyzer. Data show mean±SEM, n=9, Student *t* test or Mann–Whitney *U* test: **P*<0.05. **C**, Representative images of Oil Red O–stained sections of aortic roots of mice treated for 2 and 4 wk. Scale bars indicate 200 µm. Quantitative analysis of atherogenesis as total plaque size (**D**) or normalized to internal elastic lamina (IEL; **E**) after 2 (n=6–9) and 4 (n=11) wk treatment. Data show mean±SEM, Student *t* test: **P*<0.05.

resulting in a similar lesion size within aortic roots of vehicle and fluoxetine-treated mice.

Discussion

Cardiovascular disease and depression are the most important health burdens in high-income countries, and there is increasing evidence for a causal relationship between both diseases.[42] There is a higher frequency of depression among patients with cardiovascular diseases,[42] and depression might also represent a cardiovascular risk factor.[43,44] Although antidepressants, such as SSRIs, are the first-line therapy for depression, clinical data on potential cardiovascular effects of SSRI treatment are inconclusive.[13–19] Here, we provide evidence that chronic intake of the SSRI fluoxetine promotes atherosclerotic plaque formation in a mouse model of atherosclerosis by enhancing integrin activity on circulating leukocytes.

Platelet 5-HT is involved in inflammatory diseases, such as obesity,[45] colitis,[46] or asthma.[47] Moreover, it was shown that 5-HT triggers immune cell activation leading to proinflammatory cytokine production.[46] Thus, the proatherogenic phenotype after fluoxetine treatment was surprising because we expected a more anti-inflammatory phenotype after platelet 5-HT depletion.

Arterial inflammation is caused by endothelial cell activation that promotes leukocyte recruitment, which is an initial step in atherogenesis. We found that fluoxetine increased vascular permeability and enhanced leukocyte recruitment by amplifying chemokine-induced activation of integrins on circulating monocytes and neutrophils. Conversely, blocking 5-HT synthesis with a pharmacological TPH1 inhibitor attenuated plaque formation in the initial lesion formation, an effect that was no longer present at the later stage of

atherogenesis. Thus, fluoxetine mediates proatherogenic effects despite peripheral 5-HT depletion. We may speculate that after chronic fluoxetine intake, enhanced leukocyte recruitment because of integrin activation might override anti-inflammatory effects caused by depletion of peripheral 5-HT. In fact, although displaying enhanced in vivo leukocyte adhesion and plaque formation, fluoxetine-treated mice had significantly reduced CCL5 mRNA levels in aortas. Because we did not observe any modulation of endothelial adhesion molecules VCAM-1 and ICAM-1 by fluoxetine, we assume that the augmented vascular permeability could be a secondary consequence of fluoxetine-mediated leukocyte activation and arterial infiltration. However, we cannot exclude potential effects of fluoxetine on endothelial cell–cell junctions.

The transient decrease in circulating leukocytes (mainly granulocytes) and enhanced arterial adhesion are somewhat conflicting with previously published data. Duerschmied et al[9] reported that absence of peripheral 5-HT by genetic TPH1 deficiency or pharmacological depletion with fluoxetine in wild-type mice causes mild neutrophilia and a diminished neutrophil adhesion to postcapillary venules. In a subsequent study, the same group demonstrated that acute fluoxetine treatment transiently increases plasma 5-HT concentrations because of impaired uptake by platelets, thereby promoting leukocyte–endothelial interactions.[32] However, we showed that fluoxetine pretreatment caused a plaque phenotype similar to the one observed in our standard experimental setup, arguing against the possibility that transiently elevated 5-HT plasma levels provoke atherosclerosis outcome.

More likely, the opposing results might be explained by differences in the vascular bed (micro- versus macrovasculature) and the atherogenic background in our mouse model. Duerschmied et al[9] measured venular leukocyte recruitment in the microcirculation while we performed intravital microscopy of atherosclerosis-prone carotid arteries. Previous studies already highlighted dissimilarities in neutrophil adhesion between micro- and microvasculature.[22,48] Site-specific differences in the mechanisms underlying leukocyte recruitment between the different vascular beds may account for this phenomenon. Moreover, the underlying hypercholesterolemia because of ApoE deficiency in combination with HFD leads to endothelial dysfunction in our atherosclerosis mouse model.[49] The relevance of the inflammatory setting is supported by the finding that wild-type mice treated with fluoxetine did not have the transient reductions in blood cell counts as observed in ApoE$^{-/-}$ mice.

At inflammatory sites, leukocytes extravasate into the vessel wall through a series of events called leukocyte adhesion cascade. This process, consisting of leukocyte rolling on the endothelium, followed by adhesion and finally transendothelial migration, is predominately mediated by selectins and integrins.[50] Several studies showed that integrin activation and clustering are crucial in atherogenesis.[22,48] Although we did not observe increased expression of adhesion molecules on circulating neutrophils, we found an enhanced CCL5-induced β1 and β2 integrin-binding activity on blood neutrophils as well as classical monocytes in fluoxetine-treated mice. Our data suggest that the effect of fluoxetine on integrin activation is only detectable in the presence of enhanced chemokine levels because it is the case during an inflammatory setting like chronic hypercholesterolemia. Solely fluoxetine but not 5-HT increased integrin activation on cells (murine and human), supporting the notion that the proatherogenic effects of fluoxetine are independent of its role in platelet 5-HT depletion. Our conclusion is further strengthened by the observation that pharmacological inhibition of the 5-HT synthesizing enzyme TPH1 did not aggravate atherosclerosis. Interestingly, TPH1 inhibition with LP533401 mitigated lesion formation after 2 weeks HFD, an effect no longer observed after 4 weeks. In fact, a previous study reported that platelet-specific JAM-A (junctional adhesion molecule A) deficiency only affected early lesion formation in aortic roots evidenced after 2 weeks of HFD but not at later stages.[51] We speculate that platelet reactivity, which might be particularly relevant at the onset of atherogenesis, is altered by TPH1 inhibition. LP533401 was previously described to reduce obesity by promoting brown adipose tissue thermogenesis.[21] Whether pharmacological blocking of TPH1 and thus reducing peripheral 5-HT may not only be effective in treating obesity and other related clinical disorders, such as type 2 diabetes mellitus, but also atherogenesis or atherothrombotic events deserves further investigations.

A limitation of our study is that we could not ultimately clarify how fluoxetine enhances integrin activation. In agreement with the literature, we detected 5-HTRs (ie, 5-HTR1b, 5-HTR2a) and SERT mRNA expression in murine monocytes and neutrophils.[52] Fluoxetine might affect integrin activity through signaling via binding either to SERT or to one of the 5-HTRs. In fact, a direct interaction between αIIbβ3 integrin and SERT in platelets has been reported to influence SERT activity.[53] The other explanation might be that fluoxetine interacts with one of the 5-HTRs expressed by myeloid cells and thereby affects integrin activation. In support of this possibility, affinity assays revealed binding of fluoxetine to different 5-HTRs.[37] Here, we were able to detect 5-HTR1b and 5-HTR2a mRNA expression in murine monocytes and neutrophils. The exact mechanism of integrin inside-out signaling is still incompletely understood. It is believed that G protein–coupled receptor stimulation, via phospholipase C and downstream activation of the small GTPase Rap1, mediates integrin conversion to the high-affinity conformation and thereby ICAM-1 and VCAM-1 binding.[41,54,55] However, we excluded the possibility that fluoxetine interacts with 5-HTR2a to stimulate an intracellular Ca^{2+} response. Because 5-HT induced a strong intracellular Ca^{2+} response via 5-HTR2a, the question arises why fluoxetine but not 5-HT enhanced integrin activity in vitro. Considering that the 2 detected 5-HTRs are coupled to different G proteins (5-HTR1b to G_i and 5-HTR2a to G_q),[38] it is likely that fluoxetine- and 5-HT–mediated 5-HTR signaling responses might be different depending on which receptors are preferentially activated. In fact, fluoxetine binds to 5-HTR2a with a much higher affinity than to 5-HTR1b.[37]

Because enhanced integrin-mediated binding was only observed on CCL5 costimulation, whereas fluoxetine alone did not promote integrin binding to VCAM-1 or ICAM-1, another possible explanation might be that fluoxetine directly acts on CCL5 chemokine receptors CCR1 and CCR5. It is also conceivable that fluoxetine prevents the inactivation of integrins, thereby prolonging integrin activity. Thus, the exact

mechanism by which fluoxetine amplifies integrin activity remains to be clarified.

A crucial question that arises is whether the proatherogenic effect of fluoxetine is unique or possibly a common side effect of SSRIs in general. In support of the latter possibility, the effect of escitalopram, the newest approved SSRI, was comparable to fluoxetine-induced binding to VCAM-1. The effect on β2 integrins was less prominent. In support of a broader implication, a study with adult female cynomolgus macaques treated with the SSRI sertraline showed extended coronary artery atherosclerosis in treated primates.[56] Additional studies testing various SSRIs in common experimental models of atherosclerosis are needed to further address the proatherogenic effects of these drugs and the underlying mechanisms.

In conclusion, we provide the first experimental proof that the SSRI fluoxetine may promote atherosclerosis by amplifying CCL5-mediated integrin activation in a 5-HT–independent manner and therefore potentially enhances the risk for acute cardiovascular events, such as myocardial infarction. Given the increasing use of antidepressant drugs, potential proatherogenic effects of SSRIs in cardiovascular risk patients and incidence of acute cardiovascular events should be carefully monitored.

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Disclosures

None.

References

1. Baganz NL, Blakely RD. A dialogue between the immune system and brain, spoken in the language of serotonin. *ACS Chem Neurosci*. 2013;4:48–63. doi: 10.1021/cn300186b.
2. Walther DJ, Peter JU, Bashammakh S, Hörtnagl H, Voits M, Fink H, Bader M. Synthesis of serotonin by a second tryptophan hydroxylase isoform. *Science*. 2003;299:76. doi: 10.1126/science.1078197.
3. Berger M, Gray JA, Roth BL. The expanded biology of serotonin. *Annu Rev Med*. 2009;60:355–366. doi: 10.1146/annurev.med.60.042307.110802.
4. El-Merahbi R, Löffler M, Mayer A, Sumara G. The roles of peripheral serotonin in metabolic homeostasis. *FEBS Lett*. 2015;589:1728–1734. doi: 10.1016/j.febslet.2015.05.054.
5. Van Nueten JM, Janssens WJ, Vanhoutte PM. Serotonin and vascular reactivity. *Pharmacol Res Commun*. 1985;17:585–608.
6. Cloutier N, Paré A, Farndale RW, Schumacher HR, Nigrovic PA, Lacroix S, Boilard E. Platelets can enhance vascular permeability. *Blood*. 2012;120:1334–1343. doi: 10.1182/blood-2012-02-413047.
7. Hendrix TR, Atkinson M, Clifton JA, Ingelfinger FJ. The effect of 5-hydroxytryptamine on intestinal motor function in man. *Am J Med*. 1957;23:886–893.
8. Lopez-Vilchez I, Diaz-Ricart M, White JG, Escolar G, Galan AM. Serotonin enhances platelet procoagulant properties and their activation induced during platelet tissue factor uptake. *Cardiovasc Res*. 2009;84:309–316. doi: 10.1093/cvr/cvp205.
9. Duerschmied D, Suidan GL, Demers M, Herr N, Carbo C, Brill A, Cifuni SM, Mauler M, Cicko S, Bader M, Idzko M, Bode C, Wagner DD. Platelet serotonin promotes the recruitment of neutrophils to sites of acute inflammation in mice. *Blood*. 2013;121:1008–1015. doi: 10.1182/blood-2012-06-437392.
10. WHO. Depression. http://www.who.int. Accessed April 21, 2017.
11. National Center for Health Statistics. Health, United States, 2015: With Special Feature on Racial and Ethnic Health Disparities. https://www.cdc.gov/nchs/data/hus/hus15.pdf. Accessed June 9, 2017.
12. Wong DT, Perry KW, Bymaster FP. Case history: the discovery of fluoxetine hydrochloride (Prozac). *Nat Rev Drug Discov*. 2005;4:764–774. doi: 10.1038/nrd1821.
13. Coupland C, Hill T, Morriss R, Moore M, Arthur A, Hippisley-Cox J. Antidepressant use and risk of cardiovascular outcomes in people aged 20 to 64: cohort study using primary care database. *BMJ*. 2016;352:i1350.
14. Pizzi C, Rutjes AW, Costa GM, Fontana F, Mezzetti A, Manzoli L. Meta-analysis of selective serotonin reuptake inhibitors in patients with depression and coronary heart disease. *Am J Cardiol*. 2011;107:972–979. doi: 10.1016/j.amjcard.2010.11.017.
15. Stewart JC, Perkins AJ, Callahan CM. Effect of collaborative care for depression on risk of cardiovascular events: data from the IMPACT randomized controlled trial. *Psychosom Med*. 2014;76:29–37. doi: 10.1097/PSY.0000000000000022.
16. Kimmel SE, Schelleman H, Berlin JA, Oslin DW, Weinstein RB, Kinman JL, Sauer WH, Lewis JD. The effect of selective serotonin re-uptake inhibitors on the risk of myocardial infarction in a cohort of patients with depression. *Br J Clin Pharmacol*. 2011;72:514–517. doi: 10.1111/j.1365-2125.2011.04008.x.
17. Biffi A, Scotti L, Corrao G. Use of antidepressants and the risk of cardiovascular and cerebrovascular disease: a meta-analysis of observational studies. *Eur J Clin Pharmacol*. 2017;73:487–497. doi: 10.1007/s00228-016-2187-x.
18. Coupland C, Dhiman P, Morriss R, Arthur A, Barton G, Hippisley-Cox J. Antidepressant use and risk of adverse outcomes in older people: population based cohort study. *BMJ*. 2011;343:d4551.
19. Rieckmann N, Kronish IM, Shapiro PA, Whang W, Davidson KW. Serotonin reuptake inhibitor use, depression, and long-term outcomes after an acute coronary syndrome: a prospective cohort study. *JAMA Intern Med*. 2013;173:1150–1151. doi: 10.1001/jamainternmed.2013.910.
20. Lett HS, Blumenthal JA, Babyak MA, Sherwood A, Strauman T, Robins C, Newman MF. Depression as a risk factor for coronary artery disease: evidence, mechanisms, and treatment. *Psychosom Med*. 2004;66:305–315.
21. Crane JD, Rengasamy P, Mottillo EP, et al. Inhibiting peripheral serotonin synthesis reduces obesity and metabolic dysfunction by promoting brown adipose tissue thermogenesis. *Nat Med*. 2015;21:166–172.
22. Ortega-Gomez A, Salvermoser M, Rossaint J, et al. Cathepsin G controls arterial but not venular myeloid cell recruitmentclinical perspective. *Circulation*. 2016;134:1176–1188.
23. Corcoran MP, Lichtenstein AH, Meydani M, Dillard A, Schaefer EJ, Lamon-Fava S. The effect of 17β-estradiol on cholesterol content in human macrophages is influenced by the lipoprotein milieu. *J Mol Endocrinol*. 2011;47:109–117.
24. Sager HB, Dutta P, Dahlman JE, et al. RNAi targeting multiple cell adhesion molecules reduces immune cell recruitment and vascular inflammation after myocardial infarction. *Sci Transl Med*. 2016;8:342ra80. doi: 10.1126/scitranslmed.aaf1435.
25. Radu M, Chernoff J. An in vivo assay to test blood vessel permeability. *J Vis Exp*. 2013;73:e50062. doi: 10.3791/50062.
26. Drechsler M, de Jong R, Rossaint J, Viola JR, Leoni G, Wang JM, Grommes J, Hinkel R, Kupatt C, Weber C, Döring Y, Zarbock A, Soehnlein O. Annexin A1 counteracts chemokine-induced arterial myeloid cell recruitment. *Circ Res*. 2015;116:827–835. doi: 10.1161/CIRCRESAHA.116.305825.
27. Schymeinsky J, Then C, Walzog B. The non-receptor tyrosine kinase Syk regulates lamellipodium formation and site-directed migration of human leukocytes. *J Cell Physiol*. 2005;204:614–622. doi: 10.1002/jcp.20323.
28. Srinivasan S, Wang F, Glavas S, Ott A, Hofmann F, Aktories K, Kalman D, Bourne HR. Rac and Cdc42 play distinct roles in regulating PI(3,4,5)

P3 and polarity during neutrophil chemotaxis. *J Cell Biol*. 2003;160:375–385. doi: 10.1083/jcb.200208179.
29. Evans R, Lellouch AC, Svensson L, McDowall A, Hogg N. The integrin LFA-1 signals through ZAP-70 to regulate expression of high-affinity LFA-1 on T lymphocytes. *Blood*. 2011;117:3331–3342. doi: 10.1182/blood-2010-06-289140.
30. Pick R, Begandt D, Stocker TJ, et al. Coronin 1A, a novel player in integrin biology, controls neutrophil trafficking in innate immunity. *Blood*. 2017;130:847–858. doi: 10.1182/blood-2016-11-749622.
31. Dulawa SC, Holick KA, Gundersen B, Hen R. Effects of chronic fluoxetine in animal models of anxiety and depression. *Neuropsychopharmacology*. 2004;29:1321–1330. doi: 10.1038/sj.npp.1300433.
32. Herr N, Mauler M, Witsch T, Stallmann D, Schmitt S, Mezger J, Bode C, Duerschmied D. Acute fluoxetine treatment induces slow rolling of leukocytes on endothelium in mice. *PLoS One*. 2014;9:e88316. doi: 10.1371/journal.pone.0088316.
33. Huo Y, Schober A, Forlow SB, Smith DF, Hyman MC, Jung S, Littman DR, Weber C, Ley K. Circulating activated platelets exacerbate atherosclerosis in mice deficient in apolipoprotein E. *Nat Med*. 2003;9:61–67. doi: 10.1038/nm810.
34. Patrick L, Lehoux S, Gros R, Bobryshev Y V, Chistiakov DA, Orekhov AN. Endothelial barrier and its abnormalities in cardiovascular disease. *Front Physiol*. 2015;6:365.
35. Coleman JA, Green EM, Gouaux E. X-ray structures and mechanism of the human serotonin transporter. *Nature*. 2016;532:334–339. doi: 10.1038/nature17629.
36. Sanchez C, Reines EH, Montgomery SA. A comparative review of escitalopram, paroxetine, and sertraline: are they all alike? *Int Clin Psychopharmacol*. 2014;29:185–196. doi: 10.1097/YIC.0000000000000023.
37. Koch S, Perry KW, Nelson DL, Conway RG, Threlkeld PG, Bymaster FP. R-fluoxetine increases extracellular DA, NE, as well as 5-HT in rat prefrontal cortex and hypothalamus: an *in vivo* microdialysis and receptor binding study. *Neuropsychopharmacology*. 2002;27:949–959. doi: 10.1016/S0893-133X(02)00377-9.
38. Millan MJ, Marin P, Bockaert J, Mannoury la Cour C. Signaling at G-protein-coupled serotonin receptors: recent advances and future research directions. *Trends Pharmacol Sci*. 2008;29:454–464. doi: 10.1016/j.tips.2008.06.007.
39. Schaff UY, Yamayoshi I, Tse T, Griffin D, Kibathi L, Simon SI. Calcium flux in neutrophils synchronizes beta2 integrin adhesive and signaling events that guide inflammatory recruitment. *Ann Biomed Eng*. 2008;36:632–646. doi: 10.1007/s10439-008-9453-8.
40. Rowin ME, Whatley RE, Yednock T, Bohnsack JF. Intracellular calcium requirements for beta1 integrin activation. *J Cell Physiol*. 1998;175:193–202. doi: 10.1002/(SICI)1097-4652(199805)175:2<193::AID-JCP9>3.0.CO;2-J.
41. Herter J, Zarbock A. Integrin regulation during leukocyte recruitment. *J Immunol*. 2013;190:4451–4457. doi: 10.4049/jimmunol.1203179.
42. Hare DL, Toukhsati SR, Johansson P, Jaarsma T. Depression and cardiovascular disease: a clinical review. *Eur Heart J*. 2014;35:1365–1372. doi: 10.1093/eurheartj/eht462.
43. Ladwig KH, Baumert J, Marten-Mittag B, Lukaschek K, Johar H, Fang X, Ronel J, Meisinger C, Peters A; KORA Investigators. Room for depressed and exhausted mood as a risk predictor for all-cause and cardiovascular mortality beyond the contribution of the classical somatic risk factors in men. *Atherosclerosis*. 2017;257:224–231. doi: 10.1016/j.atherosclerosis.2016.12.003.
44. Catalina-Romero C, Calvo-Bonacho E. Depression and cardiovascular disease: time for clinical trials. *Atherosclerosis*. 2017;257:250–252. doi: 10.1016/j.atherosclerosis.2017.01.004.
45. Kim HJ, Kim JH, Noh S, Hur HJ, Sung MJ, Hwang JT, Park JH, Yang HJ, Kim MS, Kwon DY, Yoon SH. Metabolomic analysis of livers and serum from high-fat diet induced obese mice. *J Proteome Res*. 2011;10:722–731. doi: 10.1021/pr100892r.
46. Ghia JE, Li N, Wang H, Collins M, Deng Y, El-Sharkawy RT, Côté F, Mallet J, Khan WI. Serotonin has a key role in pathogenesis of experimental colitis. *Gastroenterology*. 2009;137:1649–1660. doi: 10.1053/j.gastro.2009.08.041.
47. Lechin F, van der Dijs B, Orozco B, Lechin M, Lechin AE. Increased levels of free serotonin in plasma of symptomatic asthmatic patients. *Ann Allergy Asthma Immunol*. 1996;77:245–253. doi: 10.1016/S1081-1206(10)63263-2.
48. Drechsler M, Megens RT, van Zandvoort M, Weber C, Soehnlein O. Hyperlipidemia-triggered neutrophilia promotes early atherosclerosis. *Circulation*. 2010;122:1837–1845. doi: 10.1161/CIRCULATIONAHA.110.961714.
49. d'Uscio LV, Baker TA, Mantilla CB, Smith L, Weiler D, Sieck GC, Katusic ZS. Mechanism of endothelial dysfunction in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol*. 2001;21:1017–1022.
50. Nourshargh S, Alon R. Leukocyte migration into inflamed tissues. *Immunity*. 2014;41:694–707. doi: 10.1016/j.immuni.2014.10.008.
51. Karshovska E, Zhao Z, Blanchet X, et al. Hyperreactivity of junctional adhesion molecule A-deficient platelets accelerates atherosclerosis in hyperlipidemic mice. *Circ Res*. 2015;116:587–599. doi: 10.1161/CIRCRESAHA.116.304035.
52. Dürk T, Panther E, Müller T, Sorichter S, Ferrari D, Pizzirani C, Di Virgilio F, Myrtek D, Norgauer J, Idzko M. 5-Hydroxytryptamine modulates cytokine and chemokine production in LPS-primed human monocytes via stimulation of different 5-HTR subtypes. *Int Immunol*. 2005;17:599–606. doi: 10.1093/intimm/dxh242.
53. Carneiro AM, Cook EH, Murphy DL, Blakely RD. Interactions between integrin alphaIIbbeta3 and the serotonin transporter regulate serotonin transport and platelet aggregation in mice and humans. *J Clin Invest*. 2008;118:1544–1552. doi: 10.1172/JCI33374.
54. Ghandour H, Cullere X, Alvarez A, Luscinskas FW, Mayadas TN. Essential role for Rap1 GTPase and its guanine exchange factor CalDAG-GEFI in LFA-1 but not VLA-4 integrin mediated human T-cell adhesion. *Blood*. 2007;110:3682–3690. doi: 10.1182/blood-2007-03-077628.
55. Han J, Lim CJ, Watanabe N, Soriani A, Ratnikov B, Calderwood DA, Puzon-McLaughlin W, Lafuente EM, Boussiotis VA, Shattil SJ, Ginsberg MH. Reconstructing and deconstructing agonist-induced activation of integrin alphaIIbbeta3. *Curr Biol*. 2006;16:1796–1806. doi: 10.1016/j.cub.2006.08.035.
56. Shively CA, Register TC, Appt SE, Clarkson TB. Effects of long-term sertraline treatment and depression on coronary artery atherosclerosis in premenopausal female primates. *Psychosom Med*. 2015;77:267–278. doi: 10.1097/PSY.0000000000000163.

Highlights

- Chronic selective serotonin reuptake inhibitor treatment leads to larger atherosclerotic lesions in apolipoprotein E–deficient mice because of enhanced leukocyte adhesion to the arterial wall.
- The proatherogenic effect of fluoxetine is independent of peripheral 5-hydroxytryptamine depletion.
- Selective serotonin reuptake inhibitors enhance chemokine-induced integrin-binding activity of murine leukocytes and human neutrophil-like HL 60 cells.