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The Clonal Hematopoiesis Mutation *Jak2*^{V617F} Aggravates Endothelial Injury and Thrombosis in Arteries with Erosion-like Intimas

Roberto Molinaro[1,8], **Rob S. Sellar**[2,3,4,8], **Amélie Vromman**[1,8], **Grasiele Sausen**[1], **Eduardo Folco**[1], **Galina K. Sukhova**[1], **Marie E. McConke**[3,4], **Claudia Corbo**[5,6], **Benjamin L. Ebert**[3,4,7], **Peter Libby**[1]

[1]Division of Cardiovascular Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts

[2]Department of Haematology, UCL Cancer Institute, London, UK

[3]Dana-Farber Cancer Institute, Boston, MA, USA

[4]Broad Institute of Harvard and MIT, Cambridge, MA, USA

[5]University of Milano-Bicocca, Department of Medicine and Surgery, NANOMIB Center, Monza 20900, Italy

[6]IRCCS Istituto Ortopedico Galeazzi, Milan 20161, Italy

[7]Howard Hughes Medical Institute, Boston, MA, USA

[8]These authors contributed equally.

Abstract

Background: Superficial plaque erosion causes many acute coronary syndromes. However, mechanisms of plaque erosion remain poorly understood, and we lack directed therapeutics for thrombotic complication. Human eroded plaques can harbor neutrophil extracellular traps (NETs) that propagate endothelial damage at experimental arterial lesions that recapitulate superficial erosion. Clonal Hematopoiesis of Indeterminate Potential (CHIP) denotes age-related clonal expansion of bone marrow-derived cells harboring somatic mutations in the absence of overt hematological disease. CHIP heightens the risk of cardiovascular disease, with the greatest increase seen in individuals with *JAK2*^{V617F}. Neutrophils from mice and humans with *JAK2*^{V617F} undergo NETosis more readily than *Jak2*^{WT} (wild-type) cells. We hypothesized that *JAK2*^{V617F}, by increasing propensity to NETosis, exacerbates aspects of superficial erosion.

Corresponding author: Peter Libby, Division of Cardiovascular Medicine, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115. plibby@bwh.harvard.edu.

CONFLICTS OF INTEREST

Dr. Libby's interests were reviewed and are managed by Brigham and Women's Hospital and Mass General Brigham in accordance with their conflict-of-interest policies.

Methods and results: We generated *Jak2*^{V617F} and *Jak2*^{WT} mice with heterozygous *Jak2*^{V617F} in myeloid cells. We induced areas of denuded endothelium that recapitulate features of superficial erosion and assessed endothelial integrity, cellular composition of the erosion, thrombosis rates, and response to ruxolitinib, a clinically available JAK1/2 inhibitor, in relation to genotype. Following experimental erosion, *Jak2*^{V617F} mice have greater impairment of endothelial barrier function and increased rates of arterial thrombosis. Neointimas in Jak2^{V617F} mice exhibit increased apoptosis, NETosis, and platelet recruitment. *Jak2*^{V617F} mice treated with ruxolitinib show increased endothelial continuity and reduced apoptosis in the neo-intima comparable to levels in *Jak2*^{WT}.

Conclusions: These observations provide new mechanistic insight into the pathophysiology of superficial erosion, the heightened risk for myocardial infarction in *JAK2*^{V617F} CHIP, and point the way to personalized therapeutics based on CHIP status.

Keywords

clonal hematopoiesis; superficial erosion; endothelial dysfunction; acute coronary syndromes; Janus kinase; neutrophil extracellular traps

INTRODUCTION

Recurrent event rates in acute coronary syndromes (ACS) remain unacceptably high despite contemporary therapies.[1] Current clinical and pathological data suggest an ongoing shift in the mechanisms of the thrombotic complications of human atherosclerosis.[2–5]. ST segment elevation myocardial infarction (STEMI) is waning as non-STEMI rises as a proportion of ACS. In the era of intense LDL lowering, human atherosclerotic plaques contain less lipid and fewer macrophages. We have marshalled evidence that rupture of lipid-rich, thin-capped fibroatheroma, so-called “vulnerable plaques,” now cause fewer ACS, while superficial erosion has risen as a proportion of ACS (25–30%).[6 7] Post-mortem analysis of human lesions that have provoked erosion, in contrast with those that rupture, revealed prominent accumulation of proteoglycan and glycosaminoglycans (GAGs) rather than a paucity of interstitial collagen and thin fibrous caps,[8, 9] suggesting a fundamental difference in the pathophysiology of ACS provoked by superficial erosion. Decades of study have illuminated the cellular and molecular mechanisms of genesis of the thin-capped, lipid-rich atheroma. Yet few studies have probed the fundamental mechanisms of superficial erosion.

Our previous data, coupled with observations on human atheromata, suggest a role for neutrophils and neutrophil extracellular traps (NETs) in superficial erosion and subsequent thrombosis.[10–14] When stimulated, neutrophils, components of the innate immune system, can expel extracellular strands of decondensed DNA in complex with histones and neutrophil granular proteins to produce NETs. These structures may ensnare microorganisms, but also recruit tissue factor procoagulant [15, 16], entrap platelets and fibrin, and thus participate in the pathogenesis of thrombosis. [17, 18]

We recently developed and validated a procedure that produces experimentally in mice some of the characteristics of human lesions that provoke superficial erosion. Studies in this preparation demonstrated that peptidyl arginine deiminase 4 (PAD4), an enzyme that

is critical for DNA decondensation and NET formation, participates causally in acute thrombotic complications in these intimal lesions that recapitulate features of superficial erosion.[12] We further demonstrated that local delivery via targeted nanoparticles of a PAD4 inhibitor could limit NET formation and preserve endothelial structural and functional integrity in regions prone to experimental erosion.[19] NETs can propagate and amplify endothelial injury and thus heighten the risk of thrombosis.[20] Moreover, we previously demonstrated the presence of NETs in human eroded lesions, supporting their participation in the pathogenesis of human atherosclerosis.[5, 10–12]

Recent research has conclusively linked certain somatic mutations in bone marrow-derived cells that cause clonal hematopoiesis of indeterminate potential (CHIP) with cardiovascular disease.[21] CHIP denotes the age-related clonal expansion of leukocytes with these acquired mutations[22, 23] found in hematological malignancies in the absence of overt hematological disease. Among the CHIP mutations, the V617F gain-of-function mutation in Janus kinase 2 (*JAK2*^{V617F}) confers a several-fold increased risk for cardiovascular events even in those without an altered blood count (i.e. without a myeloproliferative neoplasm). In patients with a myeloproliferative neoplasm due to *JAK2*^{V617F}, arterial and venous thrombotic events lead as causes of mortality.[24, 25] Moreover, individuals with *JAK2*^{V617F} CHIP also have an increase in major thrombotic events.[26] In humans and mice *JAK2*^{V617F} primes neutrophils to undergo NETosis.

The findings that NETosis contributes to the pathogenesis of ACS due to superficial erosion and the relationship between *JAK2*^{V617F} and increased NETosis prompted the hypothesis that *JAK2*^{V617F} exacerbates aspects of superficial erosion. We tested here whether mice bearing *Jak2*^{V617F} in myeloid cells show accentuated NETosis, endothelial injury, and thrombosis at sites of flow disturbance in arteries with erosion-like intimas. We also tested the hypothesis that the clinically used JAK1/2 inhibitor ruxolitinib alleviates such effects of *Jak2*^{V617F}, thus identifying a potential adjunctive therapeutic intervention for selected patients with ACS due to superficial erosion.

METHODS

Animals

This study was approved by Brigham and Women's Hospital (BWH) Institutional Animal Care and Use Committee (IACUC) and conducted following the NIH Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training. The mice were certified free of common pathogens by the suppliers and maintained at Harvard Institutes of Medicine, BWH Center of Comparative Medicine (CCM) animal facility, with standard Pico5058 diet and water ad libitum with 12 hours light-dark cycle.

Generation of *Jak2*^{V617F} and *Jak2*^{WT} chimeric mice

Previously described *Jak2*^{V617F} mice[27] or *Jak2*^{WT} mice[26] were crossed with Lyz2-Cre mice. The marrow from these CD45.2 animals was harvested, processed and transplanted into lethally irradiated recipient *Ldlr*$^{-/-}$ C57B6 (CD45.1) mice (6–8-weeks-old) as previously described.[26]

Ruxolitinib treatment

Both *Jak2*^{WT} and *Jak2*^{V617F} mice were treated with 90 mg/kg ruxolitinib (in 5% dimethylacetamide (DMAC, Sigma-Aldrich) by oral gavage, twice daily for 72 hours before cuff placement for 24 hours as previously described.[26] Mice were randomized to treatment groups and analysis of tissue sections was performed by a researcher blinded to genotype and treatment group.

Arterial Injury

All animal experiments were performed in accordance with the guidelines of the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals approved by the IACUC of the Harvard Medical Area Standing Committee on Animals (Protocol #: 2016N000293; PI: Libby, Peter). The arterial injury procedure was conducted as previously described.[14] Summarily, after the mice were anesthetized with 90–200 mg/kg of ketamine (KetaVed Vedco) and 10 mg/kg of xylazine (AnaSed) intraperitoneally, an incision was made to give access to the left common carotid artery (LCCA). The electrical injury was executed using a bipolar microcoagulator (Erbe ICC 200, USA) with current pulse of 4 watts for 4 seconds. After recovery from anesthesia, the animals were fed a standard diet and water ad libitum. Post-operative analgesia was administered using 0.05–0.1 mg/kg of buprenorphine (first dose prior to animal recovering and second dose at 8–12 h from the first dose for the first day; once the second day). Post-surgical animals were evaluated daily for a minimum period of 4 days as required by the BWH CCM institutional policy.

Arterial Flow perturbation

The *in vivo* flow perturbation induction was performed as previously published by Franck at al.[11] Four weeks after the arterial injury completion, a non-constricted polyethylene cuff (NCC; internal diameter of 500 μm; Proto Labs, USA) or a constricted polyethylene cuff (CC; internal diameter of 250 μm; Proto Labs, USA) were placed at the mice left common carotid artery (LCCA) for a period of 6 or 24 hours.

Immunohistochemistry

The mice were euthanized by CO2 inhalation accompanied by blood withdrawal via cardiac puncture and perfused with cold PBS followed by 4% paraformaldehyde. The LCCA was harvested and embedded in an optimal cutting temperature medium (OCT). The specimens were cut in sections of 8 μm.

Immunohistochemical staining was performed using rat anti-CD31 [1:50] (BD Pharmingen, USA) for endothelial cells, rat anti-Ly6G [1:350] (BioXcell, USA) for neutrophils, rat anti-Mac2 [1:50] (Cederline) for macrophages, rabbit anti-citrH3 [1:150] (Abcam, USA), for citrullinated histone H3, rat anti-CD41 [1:1000] (Biolegend, USA) for thrombosis, rat anti-CD8-A [1:20] for DC8+ T cells, and rat anti-CD4 [1:20] (BD Pharmigen, USA) for CD4+ T cells. The apoptosis staining was performed using the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Millipore, USA) following the manufacturer's instructions. The images were acquired using a Nikon Optiphot-2 microscope equipped with Olympus DP27 camera. Histological quantification was performed blindly by computer-assisted immunostaining (Image-Pro Plus, Media Cybernetics).

Statistical analysis

Statistical analysis was performed using Graphpad Prism (version 9), with comparisons between groups using unpaired t-test, Mann-Whitney U test, or Fisher's exact test as indicated in the figure legends.

RESULTS

We generated *Jak2*^{V617F} and *Jak2*^{WT} mouse cohorts using the previously described mouse with heterozygous *Jak2*^{V617F} driven from the endogenous promoter crossed with mice with Lyz2-Cre.[26] To limit mutant *Jak2* to bone marrow-derived cells we constructed chimeric mice by bone marrow transplantation as described in the Methods section. Assessment of chimerism at 4 weeks post transplantation of donor cells confirmed successful engraftment (Supplementary figure S1).

To evaluate the role of the *Jak2*^{V617F} mutation on the pathogenesis of eroded arterial lesions, we performed a dual-step surgery on mouse carotids to create an arterial intimal lesion recapitulating features of superficial erosion as previously reported.[11, 12] *Ldlr*$^{-/-}$ mice transplanted with *Jak2*^{V617F} bone marrow cells (abbreviated *Jak2*^{V617F} mice) showed an increased number of blood monocytes and lymphocytes, red blood cells, and increased hemoglobin and hematocrit compared to *Ldlr*$^{-/-}$ mice transplanted with WT bone marrow cells (abbreviated *Jak2*^{WT} mice). However, *Jak2*^{V617F} mice showed similar neutrophil and platelet counts compared to *Jak2*^{WT} mice (Supplementary figure S1).

Next, we evaluated whether the *Jak*^{V617F} mutation altered the endothelial barrier function and thrombosis after introducing flow disturbance in a carotid arterial segment previously injured to resemble the substrate of a human eroded plaque. Constrictive cuff (CC) placement and subsequent analysis at 6 h after induction of flow disturbance significantly impaired endothelial barrier function (as assessed by Evans blue) in the group of mice bearing the *Jak2*^{V617F} mutation compared to control mice (P=0.004, Figure 1A, B). At 24h after CC placement, we observed an increased incidence of thrombosis in the group of mice bearing the *Jak2*^{V617F} mutation compared to *Jak2*^{WT}mice (Figure 2A), with thrombosis seen in 7 out of 8 (87.5%) vs. 2 out of 8 (25%) respectively (P=0.04) (Figure 2B). At this time point we also analyzed intimal endothelial continuity, neutrophil recruitment, NET accumulation, and apoptosis (Figure 3A). Immunohistochemical evaluation of the artery revealed reduced endothelial continuity (Figure 3B) and an associated increase in NET content (Figure 3C), assessed by CD31 and H3 citrulline staining respectively, in *Jak2*^{V617F} mice compared to *Jak2*^{WT} animals. In contrast, neutrophil recruitment (LyG6+ cells) did not differ significantly between the groups (Figure 3D), suggesting a qualitative rather than quantitative difference in neutrophils between groups. Furthermore, the labeling of the 3'-OH ends of fragmented DNA during apoptotic cell detection through the ApopTag® Terminal deoxynucleotidyl Transferase revealed an increased apoptotic signal in the neo-intima in *Jak2*^{V617F} mice (Figure 3E). The quantitation of the neo-intima area between *Jak2*^{WT} and *Jak2*^{V617F} mice affirmed a reproducible surgical procedure, as this measurement did not differ significantly in the carotid arteries from the two genotypes (Figure 3F).

To test our hypothesis that the *Jak2*^{V617F} variant increases thrombosis in eroded arteries, we stained carotids for CD41 (integrin alpha 2b, primarily expressed on megakaryocytes and platelets) to probe the contribution of CD41^{+} platelets to thrombus formation in mice as in humans.[28] Immunohistochemical analysis revealed a statistically significant increase in CD41 signal in the regions subjected to disturbed flow in *Jak2*^{V617F} compared to the *Jak2*^{WT} arteries, corroborating our findings obtained using the Evans blue assay (Figure 4A, B). In aggregate, these findings support a close relationship between *Jak2*^{V617F} and NET formation, endothelial damage, and thrombotic events observed at the intimal level in lesions associated to superficial erosion.

Our previous studies have demonstrated both the participation of NET formation in the mechanisms of superficial erosion as well as the ability of the JAK-1/2-STAT inhibitor ruxolitinib to abrogate NET formation in human and mouse neutrophils, and in experimental venous thrombosis in mice.[26] Ruxolitinib is approved for the treatment of myelofibrosis, polycythemia vera and steroid-refractory acute graft-versus-host disease [29, 30]. We therefore sought to investigate whether treatment with ruxolitinib could ameliorate the aggravated arterial superficial erosion seen in *Jak2*^{V617F} mice. We pre-treated either *Jak2*^{WT} or *Jak2*^{V617F} mice for 72h, twice a day, with 90 mg/kg ruxolitinib before electrical injury followed by CC placement for 24 hours. *Jak2*^{V617F}mice treated with ruxolitinib showed greater preservation of endothelial continuity when compared to the untreated *Jak2*^{V617F} controls (Figure 5A,B). In addition, ruxolitinib-treated *Jak2*^{V617} mice showed endothelial integrity similar to *Jak2*^{WT} mice after the introduction of flow disturbance, suggesting that ruxolitinib can indeed reverse the adverse effects of the *Jak2*^{V617F} mutation. Ruxolitinib-treated *Jak2*^{V617F} mice had fewer intimal apoptotic cells than controls (Supplementary Figure 2), whilst neutrophil accumulation and CD41 staining did not differ significantly after ruxolitinib treatment (Supplementary Figure 3). NET formation in the neo-intima of *Jak2*$^{V617F.}$ mice fell significantly following treatment with ruxolitinib (Figure 5C) such that there was no significant difference between genotypes (Figure 5D). Following treatment with ruxolitinib, no mice in either *Jak2*^{WT} or *Jak2*^{V617F} developed thrombosis (Figure 5E).

DISCUSSION

Superficial erosion now accounts for up to a third of ACS. CHIP mutations have gained increasing recognition as a newly identified risk factor for the development of ACS, with the greatest risk seen in individuals with *JAK2*^{V617F}. Having previously demonstrated the key participation of NETosis in the pathophysiology of superficial erosion, as well as the propensity for neutrophils harboring *JAK2*^{V617F} to undergo NETosis, we hypothesized that *JAK2*^{V617F} exacerbates the pathological features of superficial erosion.

In mice studied under conditions that recapitulate features of human superficial erosion, we demonstrate that those with *Jak2*^{V617} bone marrow have aggravated superficial erosion including impaired endothelial integrity, increased apoptosis and NETosis in neo-intimas, augmented platelet recruitment, and increased rates of thrombosis. These adverse consequences occurred despite similar levels of neutrophil recruitment, a finding that suggests a qualitative difference in behavior of these neutrophils between genotypes.

Treatment with the JAK-STAT inhibitor ruxolitinib ameliorated these functions related to increased intimal injury and thrombosis in *Jak2*^{V617F} mice.

The indication that a clinically available therapeutic intervention might reduce the severity of atherothrombotic events in patients harboring the *JAK2*^{V617F} mutation raises several translational perspectives. First, these findings should spur the quest for specific inhibitors of the mutant JAK2 to minimize the unwanted effects.[31] Second, these findings point the way toward personalization of therapy. For decades we have triaged ACS patients based on the scalar electrocardiogram – an over century-old technology.[32] The development of point of care assessment of ACS due to erosion vs. plaque rupture could help tailor therapy. A combination of biomarkers reflecting NET formation such as citrullinated histones, circulating DNA, and myeloperoxidase merit consideration in this regard. Furthermore, seeking *JAK2*^{V617F} in peripheral blood could inform genetically-allocated therapy with a selective JAK2 inhibitor. Such targeting of therapy based on genotype has revolutionized cancer therapy, but application to this approach has lagged in atherothrombotic disease. The present findings provide a pathway to address this opportunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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R.M., R.S.S, A.V., and P.L. These authors take responsibility for all aspects of the reliabiliy and freedom from bias of the data presented and their discussed intepretation. The contribution of other authors is given in the acknowldegements.

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Highlights

- Mechanisms of plaque erosion remain poorly understood
- Clonal hematopoiesis due to mutant JAK2 predisposes to myocardial infarction
- Mice bearing mutant myeloid Jak2 show increased liability to endothelial damage
- A JAK kinase inhibitor can mitigate superficial intimal damage due to Jak2 mutation

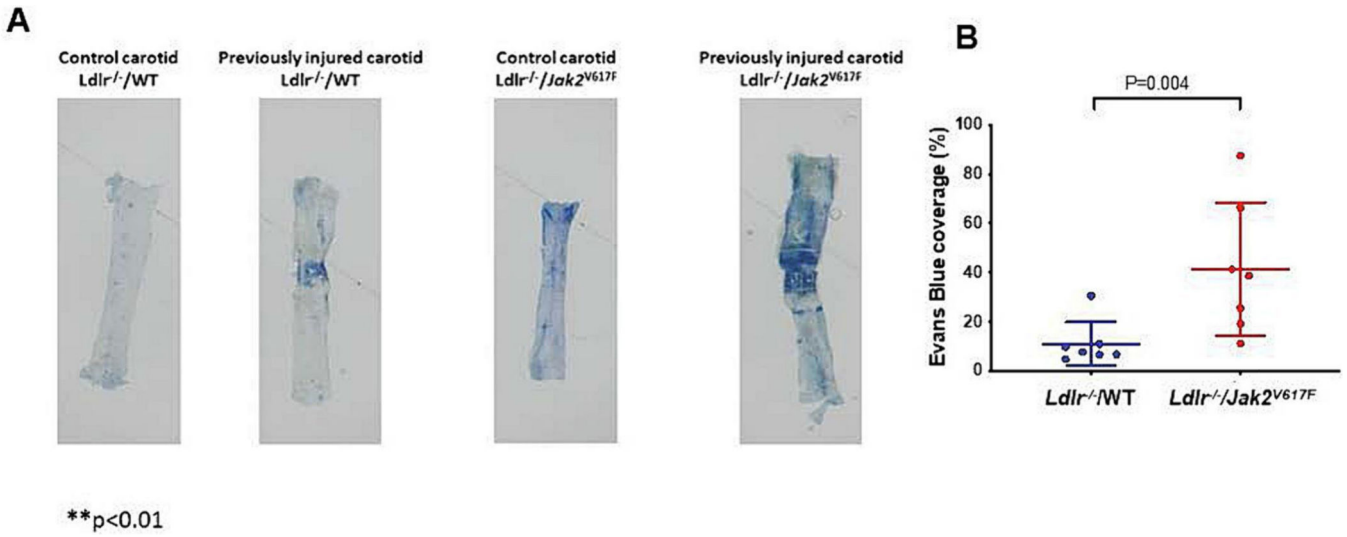

Figure 1: Mice with *Jak2*^{V617F} bone marrow have impaired endothelial barrier function compared to *Jak2*^{WT} mice following electrical injury and flow disturbance.

A. Representative images of Evans blue exclusion analysis of control (non-injured) and previously injured (electric injury plus flow disturbance induced by constrictive cuff placement for 6h) carotids from *Ldlr*$^{-/-}$/WT *Lyz2cre*$^{+/-}$ (***Ldlr*$^{-/-}$*/WT***) and *Ldlr*$^{-/-}$*/V617F Jak2/WT Lyz2cre*$^{+/-}$ ***(Ldlr*$^{-/-}$*/Jak2*^{V617F}*)*** mice (n=7 per group). **B.** Combined data of Evans blue exclusion analysis of previously injured arteries of *Ldlr*$^{-/-}$*/Jak2*^{V617F} mice compared to *Ldlr*$^{-/-}$*/WT* mice (P=0.004) using unpaired t-test.

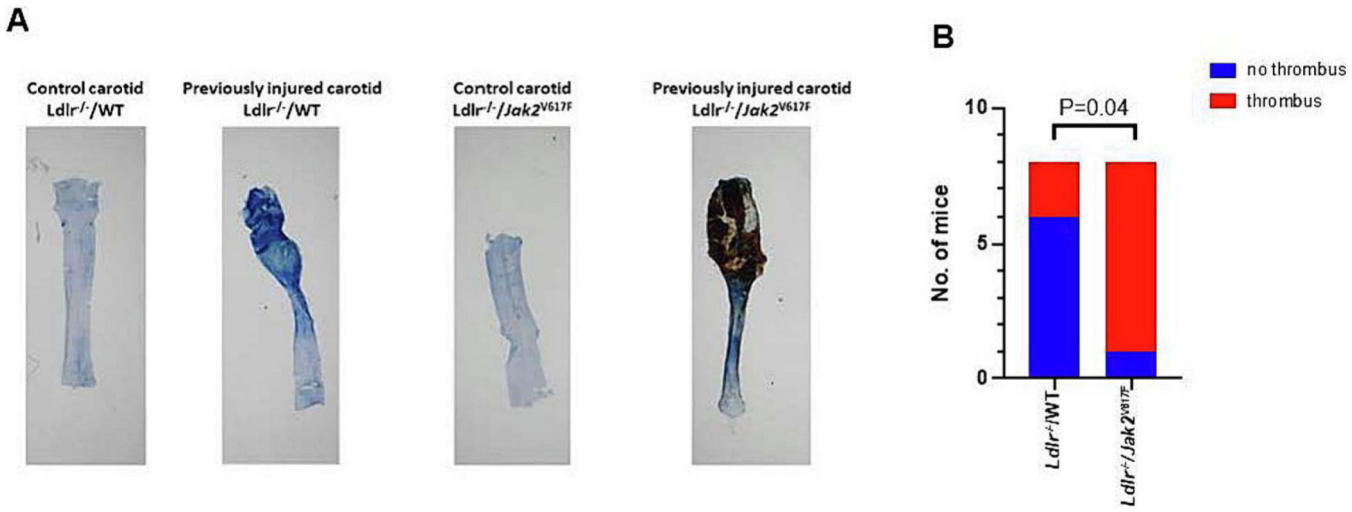

Figure 2. Mice with *Jak2*^{V617F} bone marrow have increased thrombosis rates following electrical injury and flow disturbance.

A. Representative images of Evans blue exclusion analysis of control (non-injured) and previously injured (electric injury plus flow disturbance induced by constrictive cuff placement for 24 h) carotids from *Ldlr*$^{-/-}$*/WT Lyz2cre*$^{+/-}$ ***(Ldlr*$^{-/-}$***/WT)*** and *Ldlr*$^{-/-}$*/V617F Jak2/WT Lyz2cre*$^{+/-}$ ***(Ldlr*$^{-/-}$***/Jak2*^{V617F}***)*** mice (n=8 per group). **B.** Comparison of thrombosis incidence in previously injured *Ldlr*$^{-/-}$*/ Jak2*^{V617F} */WT Lyz2cre*$^{+/-}$ mice (P=0.04, Fisher's exact test).

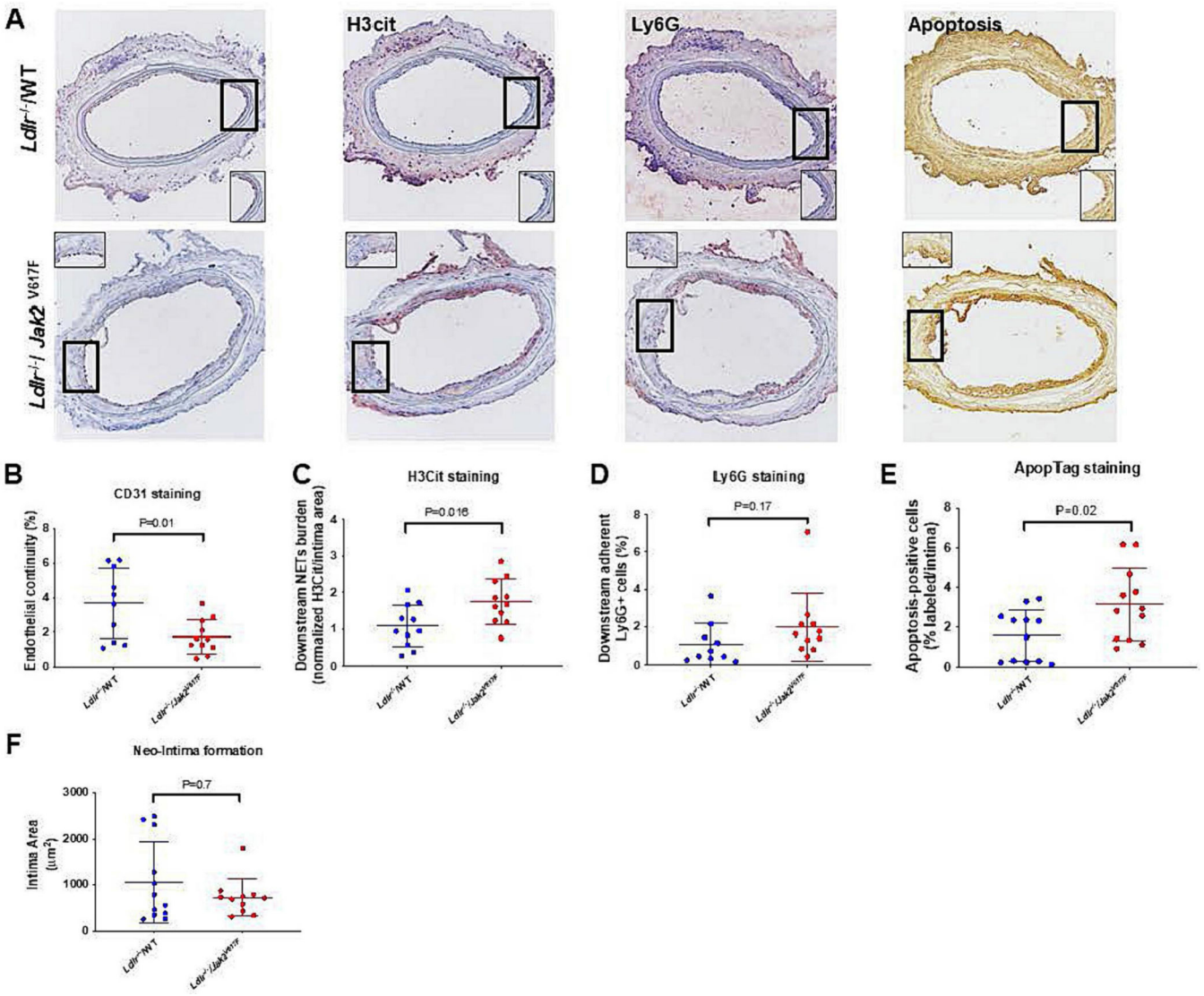

Figure 3. Neo-intimas in *Jak2* ^{V617F} mice have increased NETosis and apoptosis.

A. Representative histological sections showing endothelial cells (CD31), neutrophils (Ly6G), citrullinated histone (H3Cit) and apoptosis immunohistochemical staining of carotid arteries from *Ldlr*$^{-/-}$*/WT Lyz2cre*$^{+/-}$ ***(Ldlr***$^{-/-}$***/WT)*** or *Ldlr*$^{-/-}$*/V617F Jak2/WT Lyz2cre*$^{+/-}$ ***(Ldlr***$^{-/-}$***/Jak2***^{V617F}***)*** mice. Histological sections are shown at x20 magnification with inserts shown at X60 magnification. **B-F**. Combined results of the quantification of staining in histological sections, stained as indicated. Results are expressed as mean ± SD of n=10–12 mice per group compared using unpaired t-test

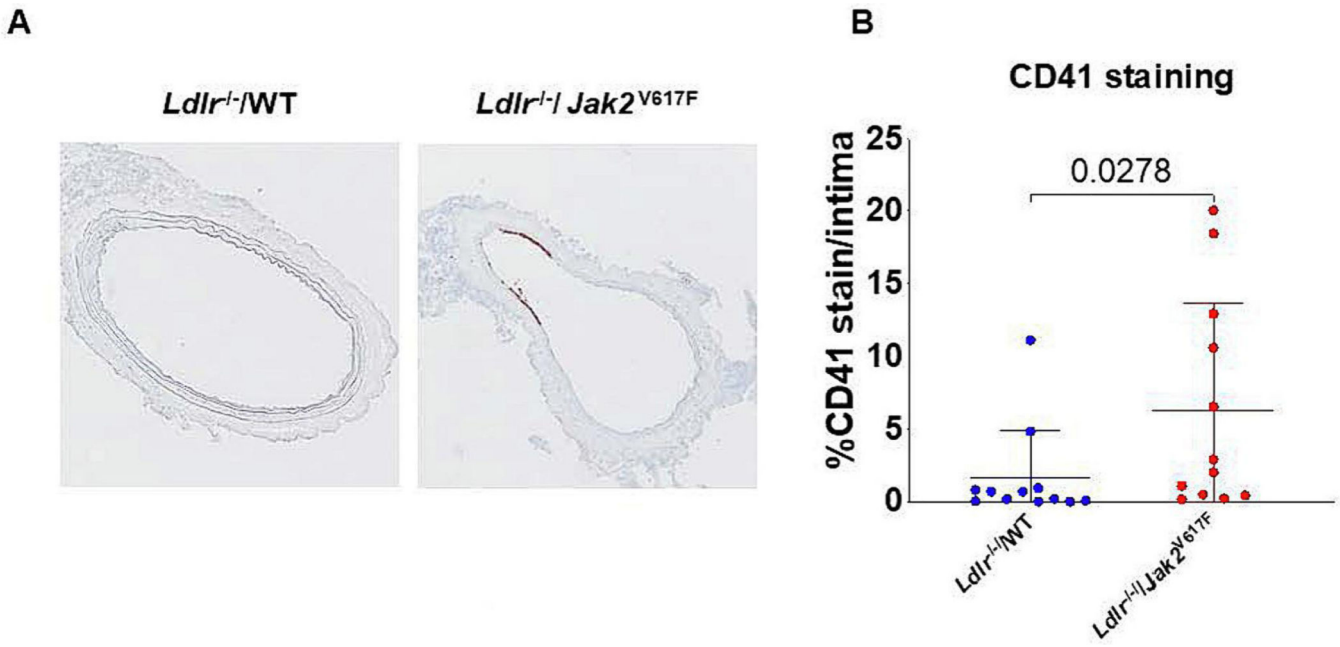

Figure 4. Regions subjected to disturbed flow in mice with *Jak2*^{V617} bone marrow have increased numbers of CD41^{+} platelets.

A. Representative images of CD41 immunohistochemical staining of carotid arteries from *Ldlr*$^{-/-}$*/WT Lyz2cre*$^{+/-}$ ***(Ldlr*$^{-/-}$*/WT)*** or *Ldlr*$^{-/-}$*/V617F Jak2/WT Lyz2cre*$^{+/-}$ ***(Ldlr*$^{-/-}$*/Jak2*^{V617F}*)*** mice, x20, **B.** Combined results of the quantification of staining in histological sections. Results are expressed as mean ± SD of n=12 mice per group compared using Mann-Whitney test.

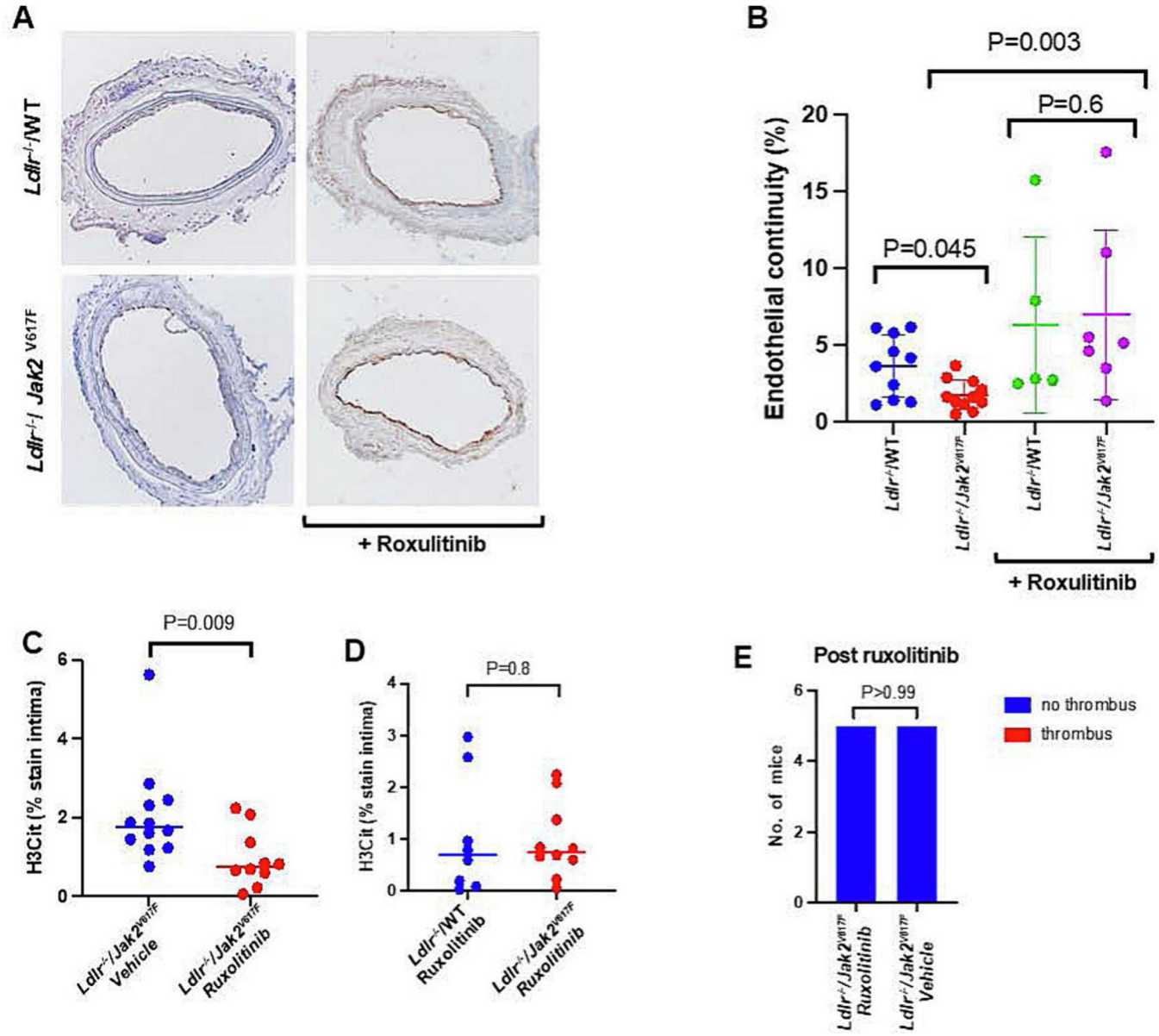

Figure 5: Ruxolitinib treatment increases endothelial continuity in *Jak2*^{V617F} mice.

A. Representative images (left image) of CD31 immunohistochemical staining of carotid arteries from *Ldlr*$^{-/-}$*/WT Lyz2cre*$^{+/-}$ ***(Ldlr*$^{-/-}$*/WT)*** or *Ldlr*$^{-/-}$*/V617F Jak2/WT Lyz2cre*$^{+/-}$ ***(Ldlr*$^{-/-}$*/Jak2*^{V617F}*)*** mice, x20, **B.** Combined results of the quantification of staining in histological sections. Results are expressed as mean ± SD of n=5–12 mice per group compared using Mann-Whitney U test. **C-D.** Comparison of H3cit staining following treatment with ruxolitinib between **C,** untreated and treated *Jak2*^{V617F} mice and **D,** treated *Jak2*^{WT} versus *Jak2*^{V617F} mice. **E.** Thrombosis rates in treated *Jak2*^{WT} versus *Jak2*^{V617F} mice.