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Investigating the Effect of Phenylbutyrate and Valproate Supplementation on Atherosclerotic Plaque Regression in a High Fat Diet Fed LDLR-/- Mouse Model

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Abstract

Objective: Evidence suggests that Endoplasmic Reticulum (ER) stress plays a causative role in the development of atherosclerosis. Our previous studies have shown that ER stress signals through glycogen synthase kinase (GSK)-3αβ to activate pro-atherogenic pathways. The purpose of this study is to determine if small molecule inhibitors of ER stress-GSK3αβ signaling can promote the regression of existing atherosclerotic lesions.

Methods: Four-week-old female low density lipoprotein receptor deficient (LDLR-/-) mice were fed a high fat diet for 16 weeks to establish atherosclerotic lesions. A subset of mice was sacrificed at this time to set the baseline for atherosclerotic progression. The remaining mice were switched to stand chow diet (control) or a standard chow diet supplemented with phenylbutyrate, a chemical chaperone that reduces ER stress, or valproate, a branch chain fatty acid that selectively inhibits GSK3αβ. These mice were harvested at 30 weeks of age and atherosclerotic lesions were quantified and characterized.

Results: Dietary supplementation with phenylbutyrate and valproate had no effect on body weight but did significantly reduce plasma cholesterol levels. Atherosclerotic lesion areas at the aortic sinus and total atherosclerotic volumes were significantly larger in the control group compared to the baseline group, indicating that the lesions continued to grow despite the switch from a high fat diet to chow diet. The supplemented mice had significantly smaller lesions than the control group, but not the baseline group. Both phenylbutyrate and valproate supplementation reduced lesional macrophage/foam cell content and necrotic core area, and increased smooth muscle cell content and collagen content, relative to control and baseline groups. These changes are indicative of more stable atherosclerotic lesions.

Conclusion: Small molecule inhibitors of ER stress-GSK3αβ signaling do attenuate the growth of existing atherosclerotic lesions and appear to increase lesion stability. It remains unclear whether these interventions can actually promote atherosclerotic regression.

Keywords: Atherosclerosis; Mouse model; Plaque regression; Dietary supplementation; ER stress

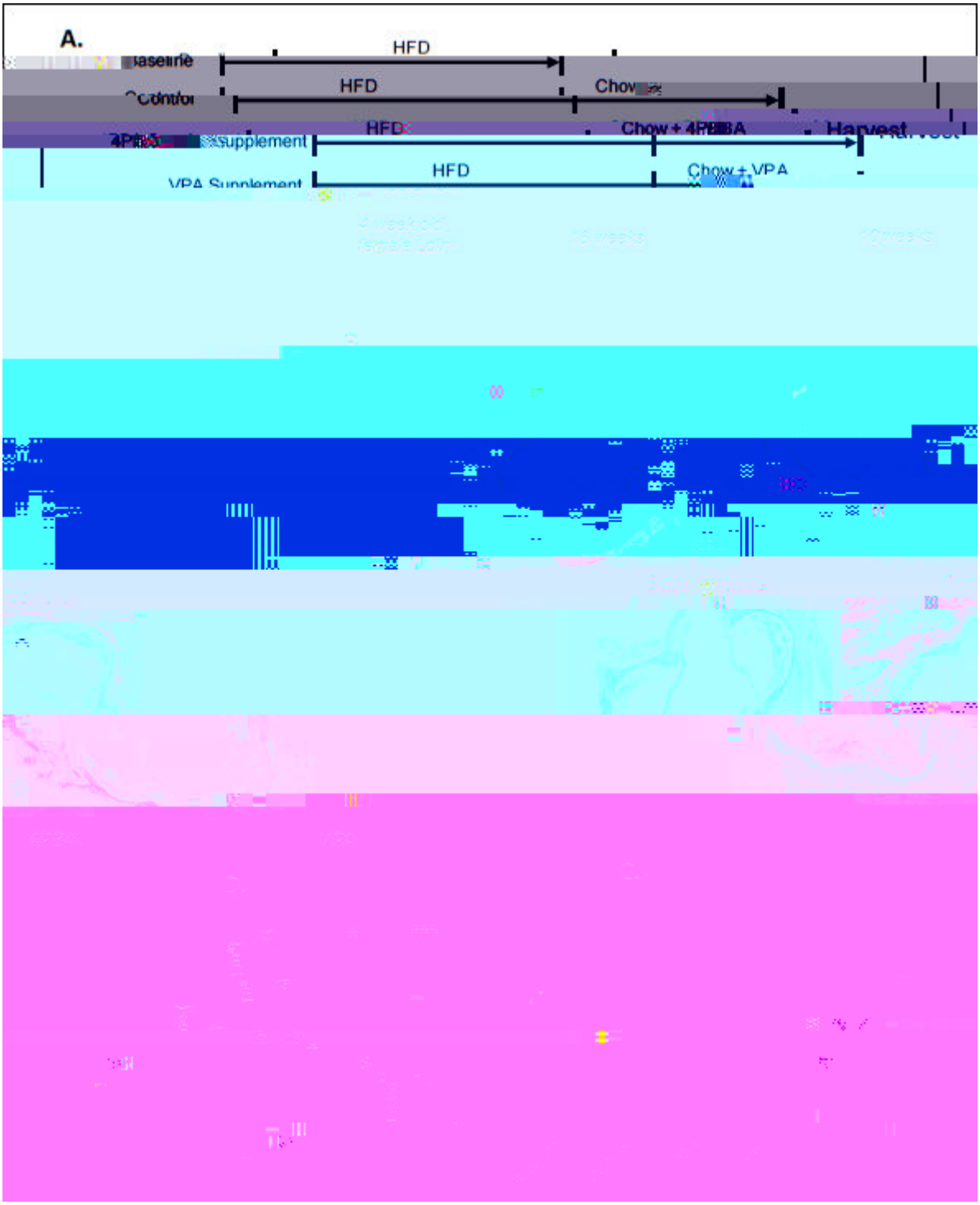
Introduction

Atherosclerosis is an inflammatory disease that involves the accumulation of lipid engorged macrophages in the walls of medium and large arteries [1]. Initially, atherosclerotic lesions grow asymptomatically, however more advanced lesions can significantly impede blood flow through the artery and cause unstable angina, stoke or myocardial infarction. These cardiovascular diseases are the leading cause of death in the world [2].

A great deal of research has been focused upon understanding the underlying molecular and cellular mechanisms of atherogenesis. It is now well established that atherosclerosis initiates at sites of endothelial injury, usually in regions of turbulent blood flow, including vascular bifurcations and inner curvatures [3]. Leukocytes and Low Density Lipoprotein (LDL) particles infiltrate the intima through gaps in the damaged endothelium. Intimal monocytes differentiate into macrophages which endocytose oxided-LDL and eferocytose apoptotic bodies thus becoming lipid laden foam cells. Macrophage/foam cells secrete cytokines that perpetuate the inflammatory response and promote vascular smooth muscle cell (VSMC) migration into the intima. VSMCs secrete collagen which contributes to the development of a fibrous cap over the growing lesion. If foam cell apoptosis exceeds macrophage eferocytosis, an acellular necrotic region of cellular debris (predominately cholesterol/lipids) accumulates within the lesion. This necrotic core destabilizes the lesion making it prone to rupture. If the lesion ruptures and the circulating blood contacts the lipid-rich contents of the lesion, a thrombus will rapidly form which may occlude blood flow through the vessel. Occlusion of a major artery will cause myocardial infarction or stroke [1,4].

Many interventions have been identified that can slow or block the progression of atherosclerosis, in pre-clinical model systems [5].

with phenylbutyrate (3.8 g/L in the drinking water), or control diet containing valproate (625 mg/kg). Control, phenylbutyrate-treated and valproate-treated groups were sacrifced after 10 additional weeks (30 weeks of age).



To investigate the efects of phenylbutyrate and valproate on atherosclerosis, the aortic sinus was removed and processed, as previously described [20]. Cross-sections of the aortic root were stained with Masson's trichrome stain (Figure 1B) and the atherosclerotic lesion areas (Figure 1C) and volumes were quantifed (Figure 1D) [20].

of supplementation on metabolic parameters

T ere were no signifcant diferences in fasting blood glucose level, body weight, liver weight, adipose weight between experimental groups (Table 1). Hepatic triglyceride and cholesterol levels were lower in the treatment groups compared to baseline (Figure 2A and 2B). Control, phenylbutyrate- and valproate-treated mice had signifcantly lower total plasma triglyceride and cholesterol levels compared to baseline (Figure 2C and 2D). T is diference is likely due to the switch from HFD to standard diet. Both phenylbutyrate- and valproate-treated mice had signifcantly lower total cholesterol levels compared to control.

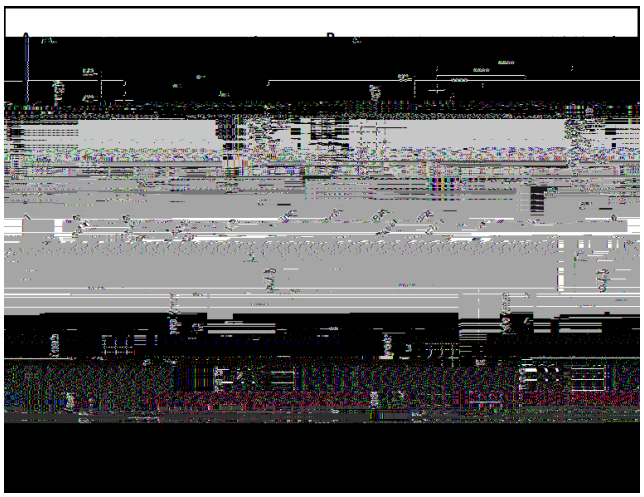


Figure 2: Hepatic and plasma lipid levels. Quantifcation of (A) Hepatic triglyceride, (B) Total cholesterol levels and fasting plasma (C) Triglyceride and (D) Total cholesterol levels from the indicated treatment groups. n=5-7 mice in each group; $^{*}p<0.05$, $^{**}p<0.01$, $^{****}p<0.0001$.

Characterization of atherosclerotic lesions

Mason's trichrome staining was used to assess necrotic core areas and collagen content within the atherosclerotic lesions. T ere were signifcantly larger acellular necrotic regions the atherosclerotic lesions from control mice compared to baseline and supplemented mice (Figure 3A and 3B). Collagen content, a marker for more stable atherosclerotic plaques, was signifcantly elevated in the phenylbutyrate and the valproate treated mice compared to both the baseline and the controls (Figure 3C and 3D).

Lesional vascular smooth muscle cell and macrophage/foam cell content was quantifed in each of the experimental groups using immunofuorescent staining with antibodies against smooth muscle α actin and CD107b (Mac-3), respectively. Both phenylbutyrate and valproate treatment resulted in signifcantly increased lesional VSMC content relative to baseline and control groups (Figure 4). T is is consistent with the observed increase in plaque collagen (Figure 3C).

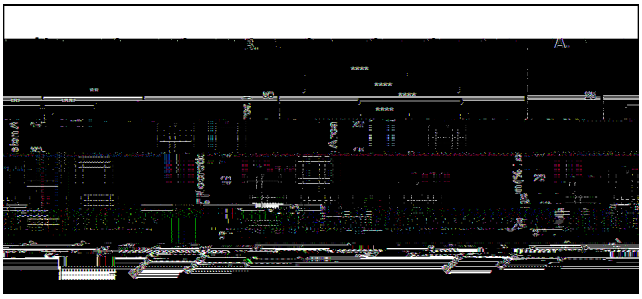


Figure 3: Necrotic core area in atherosclerotic plaques. (A) Quantifcation of necrotic core area in atherosclerotic plaques at the aortic sinus normalized to the lesion area. (B) Quantifcation of collagen staining area at the aortic sinus normalized to the lesion area. n=7-8 mice/group; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$.

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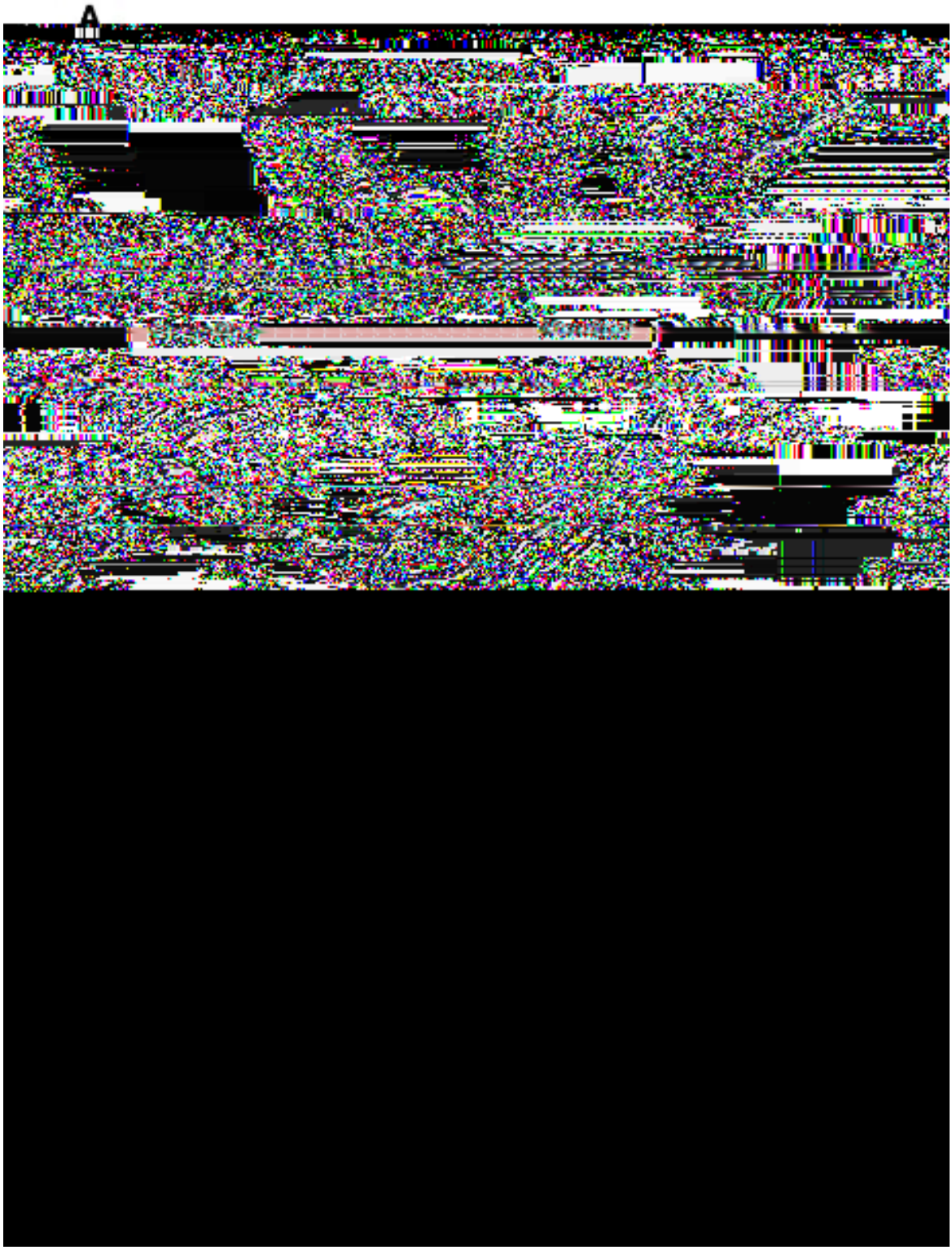

Figure 4: Vascular smooth muscle cell content in atherosclerotic plaques. (A) Representative sections of aortic sinus immunostained with an antibody against α-actin (green) and co-stained with DAPI (nuclei stain, blue). Atherosclerotic lesions are outlined (yellow). (B) Quantification of α-actin staining area normalized to the total lesion area. n=6-8 mice/group; $^{*}p<0.05$, $^{***}p<0.001$, $^{****}p<0.0001$.

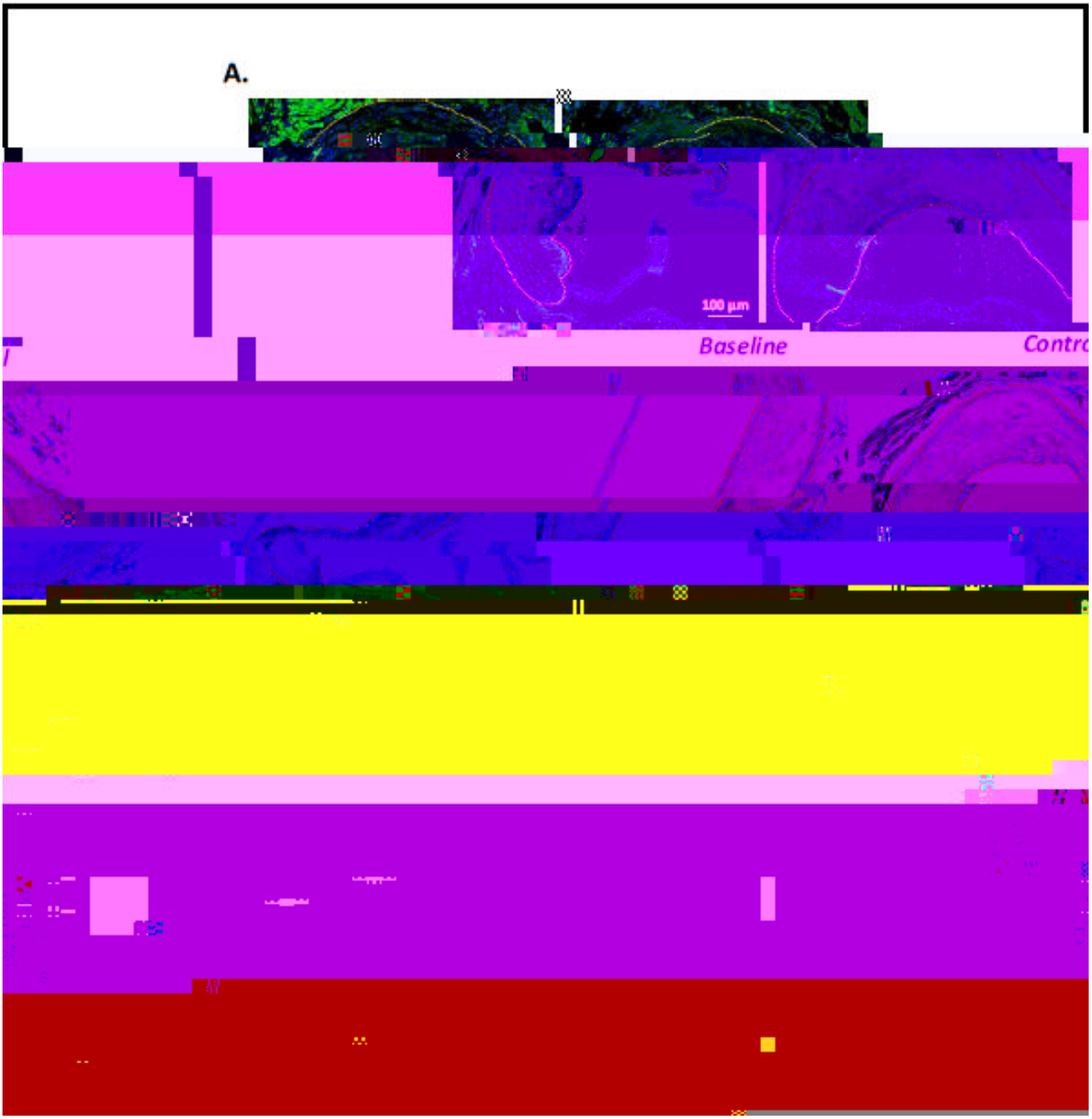

Figure 5: Macrophage content in atherosclerotic plaques. (A) Representative sections of aortic sinus immunostained with an antibody against CD107b/Mac3 (green) and co-stained with DAPI (nuclei stain, blue). Atherosclerotic lesions are outlined (yellow). (B) Quantification of CD107b/Mac3 staining area normalized to the total lesion area. n=6-8 mice/group; $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$.

Figure 6: Adaptive unfolded protein response markers in the atherosclerotic plaques. (A) Representative sections of aortic sinus stained with an anti-KDEL antibody (GRP78/94, green) and co-stained with DAPI (nuclei stain, blue). Atherosclerotic lesions are outlined (yellow). (B) Quantification of KDEL staining area normalized to the total lesion area. n=6-8 mice/group; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$.

Figure 7: Pro-apoptotic unfolded protein response markers in the atherosclerotic plaques. (A) Representative sections of aortic sinus immunostained with an antibody against CHOP (green) and co-stained merged with DAPI (nuclei stain, blue). Atherosclerotic lesions are outlined (yellow). (B) Quantification of CHOP staining area normalized to the total lesion area. n=6-8 mice/group; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$.

Discussion and Conclusion

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