Dietary alpha-linolenic acid increases the platelet count in ApoE<sup>−/−</sup> mice by reducing clearance

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Running title: Dietary α-linolenic acid and platelet clearance

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Key points

1. Dietary alpha-linolenic acid decreases platelet clearance in a mouse model of atherosclerosis;

2. Alpha-linolenic acid reduces platelet activation and TACE-dependent GPIb cleavage;

Abstract

We reported previously that dietary intake of alpha-linolenic acid (ALA) reduces atherogenesis and inhibits arterial thrombosis. Here, we analyze the substantial increase in the platelet count induced by ALA and the mechanisms of reduced platelet clearance. 8-week-old male apolipoprotein E knockout (ApoE−/−) mice were fed a 0.21g% cholesterol diet complemented by either a high (7.3g%) or low ALA (0.03g%) content. Platelet counts doubled after 16 weeks ALA feeding, whereas the bleeding time remained similar. Plasma glycocalcin and glycocalcin index were reduced, while reticulated platelets, thrombopoietin and bone marrow megakaryocyte colony forming units remained unchanged. Platelet content of liver and spleen were substantially reduced, without affecting macrophage function and number. GPIb shedding, exposure of P-selectin and activated integrin αIIbβ3 upon activation with thrombin were reduced. Dietary ALA increased the platelet count by reducing their clearance in the RES. The latter appears to be mediated by reduced cleavage of GPIb by TACE, and reduced platelet activation/expression of procoagulant signaling. Ex vivo, there was less adhesion of human platelets to von Willebrand factor under high shear conditions after ALA treatment. Thus, ALA may be a promising tool in transfusion medicine and in high turnover/high activation platelet disorders.
List of abbreviations

ALA alpha-linolenic acid
TPO thrombopoietin
LCn3-FA long-chain omega-3 fatty acids
EPA eicosapentaenoic acid
DHA docosahexaenoic acid
EDTA ethylenediaminetetraacetic acid
BSA bovine serum albumine
HRP horseradish peroxidase
FITC fluorescein isothiocyanate
vWF von Willebrand factor
TACE TNF-alpha converting enzyme
PI3K phosphatidylinositol 3 kinase
PKC protein kinase C
PS/PE phosphatidylserine/phosphatidylethanolamine
Introduction
The platelet count is regulated by a complex interplay of thrombopoietin (TPO), pro- and anti-apoptotic molecules Bak/Bcl, platelet activation, the expression of procoagulant and proapoptotic factors including phosphatidylserine/phosphatidylethanolamine and the caspase system, clustering and/or loss of specifically glycosylated receptors such as GPIb and, finally, clearance by the reticuloendothelial system from the circulation1–6.

Long chain-Omega-3 fatty acids (LCn3-FA) exert beneficial cardiovascular effects on atherosclerosis and inflammation, and they have been demonstrated to induce antithrombotic and antiplatelet effects and to reduce mortality7–13. Although the Alpha Omega trial did not show a significantly different event rate among patients receiving LCn3-FA on top of state-of-the-art therapy for myocardial infarction14, previous studies and subgroup analyses demonstrated a beneficial effect of omega-3 FA11,15. An inexpensive and abundantly available alternative to marine-derived omega-3 is the plant derived alpha-linolenic acid (ALA), which by itself or by chain elongation to LCn3-FA EPA, DPA and DHA, has a number of beneficial effects as well, although they are somewhat less well characterized than the LCn3-FAs. Furthermore, some landmark studies investigating nutritional interventions (mediterranean diet and nuts -walnuts in particular- as food additives) have been performed, where ALA is likely to contribute to the main beneficial effects16–19.

We have recently reported on the mechanisms of ALA and its anti-inflammatory, antiatherosclerotic and anti-platelet effects20,21. Dietary ALA was incorporated into various adipose tissues in a mouse model of atherosclerosis and switched the balance towards the anti-inflammatory class of the thromboxane/prostacycline mediators20. Interestingly, we have shown that the nutritional intake of ALA resulted in prolonged time to occlusion in a mouse model of carotid thrombosis21.
Given the reduction in platelet activation and based on the above-mentioned observations, we hypothesized that platelets could circulate longer in the blood stream due to reduced clearance signaling thus increasing the net platelet counts. Preliminary observations of increased platelet counts in pilot experiments encouraged us to extend our studies to further analyze this hypothesis and its potential mechanisms with eventual translational applications of this finding.

Methods

Animals and diet

8-week-old male C57/BL6 ApoE−/− mice were fed a 0.21g% cholesterol diet for 16 weeks containing either high ALA (7.3g%, D06080702, Research Diets, New Brunswick, NJ, U.S.A.; n=20) or low ALA (0.03g%, D06080701 Research Diets; n=20). ALA was given as flaxseed oil and substituted for with cocoa butter in the control group. At 24 weeks of age, animals were euthanized and tissues were harvested. All animal experiments were approved by the local ethic committee for animal protection.

Blood analyses

Mice were fasted over night before blood was drawn. Plasma and red cells were stored at -80°C. Total blood cell count was performed on a ScilVet ABCplus (Horiba, Kyoto, Japan) using EDTA-anticoagulated blood.

Bleeding times

Measurements of bleeding times was performed according to Jansen et al. Mice were anesthetized by intraperitoneal injection of sodium pentobarbital and then the last 5 mm of the tail was cut with a razor. Tails were placed in saline at 37°C and the time to bleeding cessation was determined with a stopwatch.
**Reticulated platelets**

Newly synthesized platelets (reticulated platelets) were determined by Thiazole Orange staining. Briefly, 5 μl whole blood were mixed with 50 μl Thiazole Orange solution (Sigma Aldrich, USA, 0.1 μg/ml in methanol) and incubated 15 min in the dark. After staining, platelets were fixed by addition of 1 ml 1% PFA in PBS and placed on ice. Samples were immediately analyzed by flow cytometry on a FacsCanto (BD Bioscience, Heidelberg, Germany) and the percentage of positive platelets calculated with the FacsDiva software.

**Plasma Glycocalicin and Glycocalicin Index (GI)**

Plasma glycocalicin was assayed by ELISA. Briefly, 96-well plates (Costar, USA) were coated with the antibody 7A9 (directed against the extracellular part of GpIb, the kind gift of Bernard Nieswandt, Wurzburg, Germany) at 5 μg/ml in 50 mM NaHCO3, and incubated at 4°C overnight. The next day plates were washed 3 times with PBS/Tween 20 0.1% and then blocked with 5% BSA-5% milk in PBS, for 2 hours at 37°C. After washing again 3 times with PBST plates were incubated with standards and samples diluted in 1% BSA-1% milk in PBS, and incubated for 1 hour at 37°C. Plates were washed again 3 times and incubated with the HRP-conjugated detection antibody 15E3 for 30 min at 37°C. Plates were finally washed 4 times with PBST and incubated with TMB substrate solution (BD Bioscience, Heidelberg, Germany), for 5 minutes. The reaction was stopped with 0.5 M sulphuric acid and absorbances read at 450 nm with a Spectramax plate reader (Molecular Probes, USA). To determine the glycocalicin index, absolute plasma concentrations were multiplied for the platelet count normalized to 250,000/μl24.

**Plasma Thrombopoietin, IL-6 and TXB2**

Plasma concentrations of thrombopoietin, IL-6 and thromboxane B2 were determined on samples stored at -80°C by Multiplex-assay (Cytolab, Dallikon, Switzerland).
**Platelet activation**

For platelet activation studies, washed platelets were obtained from citrate-anticoagulated blood after mice euthanasia as described elsewhere\(^25\) and resuspended in platelet buffer (10 mM HEPES, 140 mM NaCl, 3 mM KCl, 0.5 mM MgCl\(_2\) hexahydrate, 0.5 mM NaHCO\(_3\), 10 mM glucose, pH=7.4). Platelets were activated with thrombin (0.05 and 0.1 U/ml) or collagen (final concentration 5 μg/ml) in flow cytometry tubes and simultaneously incubated with specific anti-P-selectin and anti-activated integrin α\(_{\text{IIb}}\)β\(_3\) antibodies (Emfret analytics, Eibelstadt, Germany), for 15 minutes in the dark. Reaction was stopped by addition of 200 μl PBS and samples were analyzed immediately on a FacsCanto (BD Bioscience, Heidelberg, Germany).

For detection of phosphatidylserine, washed platelets were incubated with thrombin and Annexin V-FITC (Roche, Basel, Switzerland) for 15 min, the reaction stopped by addition of PBS and samples analyzed by flow cytometry.

**Platelet survival**

Platelet survival was determined by *in vivo* platelet labeling. Briefly, mice were injected in the tail vein with X488 (an IgG derivative directed against the β subunit of the complex GPIb, Emfret Analytics, Eibelstadt, Germany), 0.15 μg/gram body weight. A small blood sample (5 μl) was taken at various time points (2, 24, 48, 72 and 96 hours) and mixed with buffer A (10 mM HEPES, 140 mM NaCl, 3 mM KCl, 0.5 mM MgCl\(_2\) hexahydrate, 0.5 mM NaHCO\(_3\), 10 mM Glucose, pH 7.4) containing Aster Jandl citric-based anticoagulant (85 mM sodium citrate dihydrate, 69 mM citric acid, 20 mg/ml glucose, pH 4.6). Diluted platelet rich plasma was isolated after centrifugation for 8 min at 125 g and analysed immediately on a FacsCanto (BD Bioscience, Heidelberg, Germany). Positive platelets at 2h were set as 100% and all subsequent time points were calculated accordingly.
Tissue processing of liver and spleen

Spleens and livers from 3 mice/group were stored in OCT medium at -80°C. Frozen sections (5 μm) were obtained and fixed in ice cold acetone for 30 min. Slides were blocked with 10% goat serum in PBS for 1 hour at room temperature, then incubated with the rat anti-mouse α2b (CD41) ab and the rabbit anti-mouse CD68 ab (both Abcam, UK), diluted in 1% BSA in PBS, overnight at 4°C. After washing in PBS, sections were incubated with a goat anti-rat ab conjugated with Alexa 647 and a goat anti-rabbit conjugated with Alexa 488, for 1 hour at room temperature. Slides were washed with PBS and coverslips were mounted with DAPI-mounting medium (Dako, Glostrup, Denmark). Slides were stored at -20°C until analysis. Single images were taken using an Olympus BX51 microscope equipped with an Olympus DP70 camera (Olympus, Tokyo, Japan) and overlayed with Image J software (NIH, USA).

GPIbα shedding by TACE

Cleavage of the GPIbα extracellular domain after platelet activation by thrombin was monitored by flow cytometry. Washed platelets from mice were activated with thrombin (0.5 or 1 U/ml). Samples were stained for the platelet marker α2b (CD41) and for GPIb with fluorophore-conjugated antibodies (Emfret Analytics, Eibelstadt, Germany) and then analysed on a FACSCanto (BD Bioscience, Heidelberg, Germany). GPIb cleavage was expressed as % fluorescence versus the resting state on CD41 positive cells. In the experiments using inhibitors, platelets were incubated simultaneously with thrombin and either the broad spectrum metalloprotease inhibitor GM-6001 or the specific inhibitor for TACE (TAPI-1), p38 (SB203580), PI3K (wortmannin and LY294002) or PKC (calphostin C) as well as the FITC-conjugated GPIb antibody, then analysed as above.
**Megakaryocyte colony-forming-unit assay**

Femura from euthanized mice were harvested and cells from bone marrow flushed with 3 ml Iscove’s MDM (Lonza, Basel, Switzerland) with 2% FBS with a syringe attached to a 21 G needle. Cells were counted and resuspended at a concentration of $2.2 \times 10^6$ cells/ml. 100 μl of the cell suspension was added to 2 ml MegaCult medium containing cytokine (Stemcell, Vancouver, Canada), then 1.2 ml of cold collagen were added and cells vortexed and plated in duplicate into a chamber slide. Cells were grown for 7 days, then the chamber slides were fixed in ice-cold acetone and MK-CFU stained for acetylcholinesterase activity following the Mega Cult protocol (Stemcell, Canada). Slides were scored under a light microscope (Leica, Wetzlar, Germany).

**Platelet adhesion to vWF**

Platelet adhesion to vWF was assessed with the Bioflux system (Fluxion, San Francisco, USA). Blood from healthy donors was incubated with ALA (Cayman chemicals, Denver, USA) at the concentrations of 7.5, 15 and 30 μM or vehicle (ethanol) for 1 hour at room temperature and platelets stained with calcein (Enzo Life Science, Lausen, Switzerland). Bioflux plates were coated with human vWF at 100 μg/ml (Haematological Technologies, Essex Junction, USA), then blocked with 5% BSA in PBS for 10 min. Plates were placed on an inverted microscope and 1 ml blood applied to the inlet well. A shear force of 200 dynes/cm² was applied and the platelets allowed to adhere to vWF for 10 min. Images were taken at the end of the experiment and the platelet-covered area was measured with the Bioflux software.

**Statistics**

Values are expressed as mean ± SEM. Statistical analysis was performed using an unpaired two-tailed Student’s t-test or a one-way Anova with Bonferroni post-hoc test. Significance was accepted at $P < 0.05$. Values are expressed as mean ± SEM.
Results

Dietary alpha-linolenic acid (ALA) increases the platelet counts without affecting the bleeding times

To assess the effects of dietary ALA on platelet number and function, ApoE-/- mice were given a high-fat diet supplemented with low or high ALA. After 16 weeks of diet, the platelet count in the mice fed high ALA had increased to 1591±119 plt/μl compared to 1052±60 plt/μl (fig. 1A). Despite increased platelet count, bleeding times did not differ significantly (fig. 1B).

TPO and bone marrow macrophages are unchanged after a high ALA diet

In order to further analyze the mechanisms for the difference in platelet count in the high ALA mice, bone marrow megakaryocytes were evaluated. In a colony-forming unit assay, megakaryocytes isolated from femura showed no difference in the number of CFU between low or high-ALA mice (fig. 1C). Staining of bone marrow sections for the megakaryocyte marker CD41 also showed no difference in the number of these cells in the two groups (fig. 1D). When plasma levels of TPO were assayed, no difference was found between the two groups of mice (fig. 1E). Both these results ruled out the possibility that ALA could increase platelet production by increasing MK number in the bone marrow or by inducing TPO production. Accordingly, reticulated platelets, an independent measure of platelet production, were numerically lower in the high ALA group but the difference was not significant (fig. 1F). Interestingly, there was a decrease of plasma TXB2 and PGE2 in the high ALA fed animals but it didn’t reach statistical significance due to an important interindividual-variability in the samples (data not shown).

ALA reduces the platelet turnover as demonstrated by the glycocalicin and the glycocalicin index

Plasma glycocalicin was used to determine platelet turnover, as it has been shown to correlate with platelet production/clearance\textsuperscript{24,26}. It was significantly lower in the mice fed the high ALA
diet (25±4 μg/ml compared to 53±27 μg/ml, fig 2A). The glycocalcin index (GCI, plasma glycocalcin normalized to the platelet count) was also significantly lower in the high ALA group (6.6±3 compared to 15±5, fig 2B).

**ALA reduces the TACE mediated cleavage of glycocalcin and preserves GPIb expression**

To further elucidate potential mechanisms, we analyzed the TNF-α converting enzyme (TACE)-mediated cleavage of the extracellular portion of GPIbα upon platelet activation. This is an important negative feedback mechanism by which platelets are rendered progressively less capable to adhere to vWF and to aggregate, thus avoiding excessive platelet activation\(^2^7,^2^8\). Since we found a reduction of plasma glycocalcin following a high ALA diet (fig. 2A), we hypothesized that GPIbα cleavage by TACE following platelet stimulation was reduced. The results were in line with our previous finding: while GPIbα levels on resting platelets were not different in the two groups (data not shown), GPIbα shedding was significantly reduced after platelet activation with both thrombin concentrations used (fig. 2C). When the specific TACE inhibitor TAPI-1 was added, GPIb cleavage was completely abrogated, showing that receptor cleavage upon thrombin stimulation is TACE-dependent (fig. 2C). The use of a broad spectrum metalloprotease inhibitor (GM-6001) abrogated GPIb shedding as well (fig. 2C). In order to further elucidate possible mechanisms of TACE inhibition by ALA, the same experiment was performed in the presence of specific inhibitors of p38, PI3K and PKC (supplemental fig 1B). Only the p38 inhibitor SB203580 was able to reduce TACE-dependent GPIb cleavage, blunting the difference between the low and high-ALA group (fig. 2D). This demonstrates a role for p38 in mediating TACE inhibition by ALA.
Platelet half-life is increased whereas platelet clearance in the liver and spleen are reduced by ALA

Platelet survival was determined by in vivo platelet labeling, which allows their clearance to be analysed over 5 days. Platelet lifespan was not increased upon ALA treatment, but platelet half-life was prolonged in the high ALA group, even though it did not reach statistical significance (fig. 3A). Since it has been shown that platelet lifespan is regulated by the pro- and antiapoptotic molecules Bax and BclXL, we also analyzed the expression of these molecules by Western blotting but did not find a difference in the platelets from the two groups of mice, in agreement with our result (data not shown). Analysis of activation of caspase-3, a mediator of apoptosis, also showed no difference between the two groups (supplemental fig. 1A).

Senescent platelets are removed from the circulation by the reticuloendothelial system of the spleen and liver by recognition of specific residues in glycosylated proteins\(^3,4\). In order to analyze platelet clearance in these two organs, cryosections were stained for the platelet marker $\alpha_{2b}$. In both cases, the CD41-positive area was decreased in the high ALA group, being in the spleen 94,72±8,38 μm\(^2\) compared to 143,48±14,42 μm\(^2\), and in the liver 33,22±1,80 μm\(^2\) compared to 54,09±9,34 μm\(^2\) (fig. 3C-D). Liver sections were also co-stained for the macrophage marker CD68 and the overlay of the two fluorescences revealed co-localization of the signal, showing indeed platelets removal by macrophages (fig. 3B, arrows). In order to exclude that this phenomenon was due to an inhibition of macrophage function, CD68 positive area was quantified and found not to be different in the two groups (fig. 3E). Macrophage function was also assessed by injection of fluorescently-labeled platelets isolated from untreated animals into ALA-treated mice, followed by harvesting of livers and spleens after 2 hours and analysis of cryosections (supplemental fig. 2). This experiment showed no difference in platelet uptake as well,
demonstrating that macrophage function was not altered by the ALA treatment and neither was responsible for the reduced platelet clearance observed.

**ALA reduces the exposure of P-selectin and activated integrin α_{IIb}β_{3}**

We analyzed exposure of phosphatidylserine/phosphatidylethanolamine (PS/PE), P-selectin and active α_{IIb}β_{3} integrin on washed platelets from the two groups of mice after stimulation with thrombin (0.05 or 0.1 U/ml) or collagen (5 μg/ml). P-selectin exposure and integrin activation were both significantly reduced after stimulation with 0.1 U/ml thrombin, but interestingly after collagen stimulation the reduction did not reach statistical significance (fig. 4A-B). Analysis of the platelets which were positive for both P-selectin and activated integrin also showed a significant reduction in the high ALA group after stimulation with either thrombin or collagen (fig. 4C). This indicates an effective inhibition of the intracellular pathways leading to granule release and calcium mobilization. Binding of Annexin V was also reduced but the difference did not reach statistical significance (fig. 4D).

**ALA dose-dependently reduces platelet adhesion and aggregation to vWF under high-shear flow**

To examine the effect of ALA on vWF-GPIb binding we performed experiments of platelet adhesion to vWF under high shear flow. We used human blood from healthy donors in order to translate our results from the animal model into a clinically significant scenario. Incubation of whole blood with ALA in increasing concentrations demonstrated a dose-dependent inhibition of platelet adhesion to vWF and platelet aggregation after 10 minutes of flow compared to vehicle (fig 5, supplemental video 1 and 2).
Discussion

In the last decade, a number of clinical trials have been performed on the efficacy of omega-3 fatty acids as cardioprotective agents, with discordant results\textsuperscript{11,29,30}. A recent meta-analysis found ALA to be associated with a lower risk of cardiovascular disease\textsuperscript{31,32}.

Here we show that dietary ALA consistently increases the platelet count to a biologically relevant degree (i.e. 51\%) in the same mouse model of atherosclerosis (ApoE-/- mice) in which we previously have documented a reduction in atherosclerosis by this dietary intervention\textsuperscript{20}. The increased number of platelets did not translate into a shortened bleeding time; on the contrary, bleeding time tended to be prolonged in mice fed a high ALA diet. Thus, the increased platelet count seems to compensate for the reduced aggregation response under these dietary conditions.

We further elucidated the mechanisms involved. The reduction in plasma glycocalicin and glycocalicin index as well as the slightly reduced number of reticulated platelets supports the hypothesis of unchanged, stable platelet production and reduced platelet clearance by the reticuloendothelial system. Immunofluorescence staining of liver and spleen sections from animals on a high ALA diet confirms a striking reduction of CD41 fluorescence, consistent with our hypothesis of a reduced clearance induced by dietary ALA. In agreement with this observation plasma TPO concentration in the two groups were comparable, and the number of megakaryocyte colony forming units in the bone marrow exactly identical. In addition, ALA did not alter the homoeostasis of the apoptotic pathway nor did it increase caspase-3 activity. Since macrophages could also be affected by ALA leading to a reduced platelet uptake, we tested liver macrophage number in ALA-treated animals and found them to be not different in the two groups. Injection of fluorescently-labeled platelets from untreated mice into ALA-treated mice also showed no difference in platelet uptake in liver and spleen, confirming that macrophage function was not altered. This result is in agreement with our previous work showing that
macrophage recruitment into atherosclerotic plaque was not affected by ALA\textsuperscript{20}, and confirms our hypothesis that the reduced platelet activation is the basis for the reduced clearance. The reduced expression of the active conformation of integrin $\alpha_{2b}\beta_3$ (platelet CD41) and of P-selectin (CD62-P) upon activation with thrombin or collagen may also lead to reduced clearance from the circulation.

We also provide evidence that platelet activation by thrombin or collagen resulted in a reduced cleavage of GPIb by the enzyme TACE, thus reducing the phagocytosis signal in the RES of the liver and spleen. To our knowledge, this is the first time that an omega-3 FA is reported to inhibit the TACE activity. Since TACE has been reported to be directly activated by p38\textsuperscript{33}, and previous work form our group has demonstrated an inhibition of p38 phosphorylation by ALA in platelets\textsuperscript{21}, we hypothesized that ALA could inhibit TACE by reducing p38 activation in response to thrombin stimulation. The use of a specific p38 inhibitor was able to reduce thrombin-induced GPIb cleavage and to blunt the difference observed between the low and the high ALA mice. Our results collectively demonstrate that thrombin-induced GPIb shedding is specifically mediated by TACE and that ALA inhibits TACE activity via p38, although the involvement of other pathways cannot be excluded.

To translate our results into a functional assay and into a potentially clinically relevant setting, we analyzed platelet adhesion and aggregation under flow onto vWF in human blood preincubated with ALA; after an incubation as short as 1 hour ALA reduced platelet adhesion and aggregation on von Willebrand factor, suggesting a reduced platelet activatability under a number of conditions (thrombin or collagen-activation, high shear flow).

Taken together, our data indicate an increased platelet count due to reduced clearance but also reduced activatability of the platelets; however no evidence of an effect on the apoptosis pathway could be observed. The finding is novel and to our knowledge, this is the first time this
observation has been made. The timing of the platelet count elevation appears of interest; in a preliminary series of experiments we have found that no increase can be observed until 6 weeks after the nutritional intervention. We purposely chose the ApoE-/- model because it mimics the clinical situation of atherosclerosis with increased platelet activation. Whether the ApoE-/- model represents a low grade inflammatory model combined with a Western type diet that is improved by ALA is not clearly understood. Preliminary data indicate that wild-type mice seem to increase their platelet counts more modestly. However, a number of data indicate a consistent reaction in the human model, among them the data on human platelet adhesion under flow.

In conclusion, we show that dietary ALA reduces platelet clearance by reducing platelet activation and turnover, and that ALA inhibits TACE-mediated GPIb cleavage via inhibition of p38. The findings could be of interest in transfusion medicine where they may be applicable in platelet preservation, and in diseases with conditions of high platelet turnover and/or pathological platelet activation.

**Acknowledgement**

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**Authorship contributions**

SS performed the experiments and wrote the manuscript; MR performed the experiments; CL performed the experiments; TFL and CMM revised the manuscript; JHB designed the project and wrote the manuscript.
Disclosure of conflicts of interest

The authors have no conflict of interest to disclose.

References


Figure Legends

Fig. 1 Dietary ALA increases the platelet count but not their production. Platelet count in ApoE-/– mice was significantly increased after 16 weeks of high ALA diet (A; n=30; p= 0.0002). In contrast, tail bleeding time was prolonged but not significantly (B; n=5, p>0.05). Megakaryocytes from bone marrow were harvested and assayed for colony forming units, revealing no difference between low and high-ALA mice (C; n=3; p>0.05). Bone marrow sections were stained for the megakaryocyte marker CD41, 3 images per section were taken at random and CD41+ cells scored (D; n=3, p>0.05, magnification 20X). Plasma TPO levels were not significantly different in the high ALA group (E; n=13, p>0.05). Reticulated platelets representing newly synthesized platelets were not different between the groups (F, n=15, p>0.05).

Fig. 2 ALA reduces platelet turnover and TACE-mediated GPIb shedding. Plasma glycocalcin (as marker for platelet turnover) was significantly decreased in mice fed with the high ALA diet, as well as the glycocalcin index (A and B, n=17; p<0.001). GPIb cleavage after platelet activation with 0.5 and 1 U/ml thrombin was monitored by flow cytometry of washed platelets. Results are expressed as percentage of fluorescence versus resting state (C, n=10, * p= 0.027, ** p= 0.003). In presence of the specific TACE-inhibitor TAPI-1 or the broad metalloprotease inhibitor GM6001, cleavage was completely abrogated (C, n=5). Addition of the p38 specific inhibitor SB203580 (at concentration of 20 and 40 μM) blunted the difference between low and high-ALA platelets, indicating that the TACE inhibition by ALA is p38-dependent (D; n=8; * p<0.05).

Fig. 3 Platelet clearance in spleen and liver is reduced after a high-ALA diet. Platelet half-life was increased in the high ALA compared with the low ALA group (A; n=6, p=0.07). Liver cryosections were stained for the platelet marker CD41 (red) and the macrophage marker CD68.
(green) and images were taken with an Olympus microscope (B, magnification 20X, scale bar 100 μm). Colocalization is shown by a merge of the two fluorescent signals (yellow, arrows). Spleen and liver cryosections were also analyzed for the platelet marker CD41 (green), the positive area quantified with the software AnalySIS 3.1 (Soft Image Solutions) and found to be significantly reduced in the high ALA mice in both organs (C and D, n=3, *p=0.03, **p=0.005). Macrophage number in the spleen and liver was not affected by the ALA treatment as shown by quantification of the CD68-positive area (E, n=3, p>0.05).

**Fig. 4 Expression of P-selectin and integrin α2β3 activation are reduced after a high ALA diet.** Washed mouse platelets were activated with thrombin (0.05 and 0.1 U/ml) or collagen (5 μg/ml) and stained with specific fluorophore-conjugated antibodies for P-selectin (A) and the activated form of the integrin α2β3 (B). Samples were analyzed by flow cytometry. In both cases there was a reduced positivity in platelets from high ALA mice (n=8; *p=0.045; **p=0.01). The percentage of double positive platelets was also found to be significantly lower after a high ALA diet (C, n=8, *p=0.03; **p=0.004). Binding of Annexin V to phosphatidylserine/phosphatidylethanolamine was decreased overall in high ALA mice but did not reach statistical significance (D, n=5, p>0.05).

**Fig. 5 ALA reduces platelet adhesion and aggregation to vWF under flow.** Blood from human healthy donors was incubated with 7.5, 15 and 30 μM ALA or vehicle (ethanol) for 1 hour, then adhesion to vWF was quantified at a shear rate of 2500 s⁻¹ for 10 minutes with the Bioflux software. There was a dose-dependent reduction of platelet adhesion and aggregation which was significant for the highest ALA concentration used (D, n=6, *p=0.02).
Fig. 5

![Image of two panels showing platelet aggregation with bar chart indicating aggregation to vWF.](image)

- Low ALA
- High ALA

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<th>Group</th>
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