List of publications

1. **Malladi N**, Alam MJ, Maulik SK, Banerjee SK. The role of platelets in non-alcoholic fatty liver disease: From pathophysiology to therapeutics. Prostaglandins Other Lipid Mediators. 2023 Jul 20;169:106766. doi: 10.1016/j.prostaglandins.2023.106766. Epub ahead of print. **IF: 4.3**
2. **Malladi N**, Johny E, Uppulapu SK, Tiwari V, Alam MJ, Adela R, Banerjee SK. Understanding the Activation of Platelets in Diabetes and Its Modulation by Allyl Methyl Sulfide, an Active Metabolite of Garlic. Journal of Diabetes Research. 2021 Oct 19;2021:6404438. doi: 10.1155/2021/6404438. **IF: 2.9**
3. **Malladi N**, Devidas L, Balaji SS, Alam MJ, Banerjee SK. Paricalcitol attenuates oxidative stress and inflammatory response in the liver of NAFLD rats. Cellular and Molecular Life Sciences.2023. (Under communication)
4. Ubaid T, Soumalya S, **Malladi N**, Roshan K, Bugga P, Praloy C, Banerjee SK. Knockdown of SCN5A alters metabolic-associated genes and aggravates hypertrophy in the cardiomyoblast. Cell biology international, 2023. (under communication)

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Research Article

Understanding the Activation of Platelets in Diabetes and Its Modulation by Allyl Methyl Sulfide, an Active Metabolite of Garlic

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Background. Diabetes mellitus (DM) is a chronic metabolic disorder associated with higher risk of having cardiovascular disease. Platelets play a promising role in the pathogenesis of cardiovascular complications in diabetes. Since last several decades, garlic and its bioactive components are extensively studied in diabetes and its complications. Our aim was to explore the antiplatelet property of allyl methyl sulfide (AMS) focusing on ameliorating platelet activation in diabetes. *Method.* We used streptozotocin- (STZ-) induced diabetic rats as model for type 1 diabetes. We have evaluated the effect of allyl methyl sulfide on platelet activation by administrating AMS to diabetic rats for 10 weeks. Flow cytometry-based analysis was used to evaluate the platelet activation, platelet aggregation, platelet macrophage interaction, and endogenous ROS generation in the platelets obtained from control, diabetes, and AMS- and aspirin-treated diabetic rats. *Results.* AMS treatment for 10 weeks effectively reduced the blood glucose levels in diabetic rats. Three weeks of AMS (50 mg/kg/day) treatment did not reduce the activation of platelets but a significant ($p < 0.05$) decrease was observed after 10 weeks of treatment. Oral administration of AMS significantly ($p < 0.05$) reduced the baseline and also reduced ADP-induced aggregation of platelets after 3 and 10 weeks of treatment. Furthermore, 10 weeks of AMS treatment in diabetic rats attenuated the endogenous ROS content ($p < 0.05$) of platelets and platelet macrophage interactions. The inhibition of platelet activation in diabetic rats after AMS treatment was comparable with aspirin treatment (30 mg/kg/day). *Conclusion.* We observed an inhibitory effect of allyl methyl sulfide on platelet aggregation, platelet activation, platelet macrophage interaction, and increased ROS levels in type 1 diabetes. Our data suggests that AMS can be useful to control cardiovascular complication in diabetes via inhibition of platelet activation.

1. Introduction

Diabetes mellitus (DM) is one of the chronic and complex disorders among all metabolic diseases. "Diabetes mellitus" is characterized by persistent hyperglycemia with disturbances of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, insulin action, or both. Changes in lifestyle and rapid urbanization have increased the incidence of diabetes and its prevalence day by day. According to the International Diabetes Federation (IDF), 463 million adults (20–79-year age) are diabetic in the year 2019 with a record of 4.2 million deaths. Further, IDF interprets that there could be still more diabetic patients by the years 2030 and 2045 with an estimation of 578.4 and 700.2 million cases, respectively [1].

Type 1 diabetes is a condition that resulted from insufficient production of insulin by the pancreas while type 2 is due to improper utilization of produced insulin and ultimately leads to higher glucose levels in the circulatory system [2]. Both type 1 and type 2 diabetes require careful monitoring and control of blood glucose levels, if not this uncontrolled condition over time may root to several complications including cardiovascular disease (CVD). Cardiovascular complications in diabetes occurring as the disease progresses and leads to premature mortality [3–5]. IDF has suggested that diabetic patients are having 2-3 times more

probability of getting CVDs than nondiabetic patients [6]. The central mechanism for these cardiovascular complications in diabetes includes an imbalance in the systems which maintain the homeostasis of blood coagulation and fibrinolysis [7]. This imbalance results in diabetic thrombocytopathy [8], a condition that majorly affects platelet function and ultimately results in heart attacks or stroke in diabetes [9–12]. Scientific research has shown that antiplatelet therapy can reduce cardiovascular complications in diabetes and premature death [13, 14].

Despite having many drugs and therapies for the management of diabetes, it is still under the category of life-threatening diseases because of its complications. Reducing these cardiovascular complications in diabetes has become more challenging than controlling the disease itself. This has made an immense impact on scientific researchers for exploring new strategies for diabetic treatment. Dietary therapy in diabetes is one among them showing a tremendous effect on preventing as well as controlling diabetes [15, 16].

Among large numbers of nutritional diets, garlic has been showing a very promising effect on diabetic as well as diabetic complications [17–21]. An antiplatelet property has been observed in raw garlic, preventing cardiovascular complications in diabetes [22–24]. Scientific literature strongly supports that garlic is showing these beneficial effects mainly due to its sulfur-containing compounds [25–27]. Allyl methyl sulfide (AMS), an active metabolite observed inside the body after oral administration of raw garlic, has been shown to effect diabetes by reducing glucose levels, increasing insulin levels, and reducing hepatic oxidative stress caused by glucotoxicity in diabetes [28, 29]. However, the effect of AMS on diabetic complications in thrombosis especially on platelet activation and aggregation is yet to be reported.

Therefore, in this present study, we aim to understand the various altered parameters of platelets isolated from diabetic rats and the effect of garlic metabolite, allyl methyl sulfide, on altered parameters of diabetic platelet.

2. Materials and Methods

2.1. Reagents. Allyl methyl sulfide (cat no. A34201-25G) and phorbol 12-myristate 13-acetate (PMA) (cat no. P8139-1MG) were obtained from Sigma (St. Louis, Missouri, USA). Flow cytometry antibodies such as CD61 FITC antibody (cat no. 104305) and APC anti-mouse/rat CD62P (P-selectin) antibody (cat no. 148303) are from Bio Legend (San Diego, California, USA), and CD14 PE antibody (cat.no: 561707) is from BD Bioscience (Franklin Lakes, NJ, USA). Adenosine diphosphate (ADP) was obtained from Hi-Media (cat no. RM437-1G). H_2DCFDA dye (cat no. C6827) was from Invitrogen. Cell culture reagents such as RPMI-1640 (cat no. 31800-014), antibiotic-antimycotic (100X) (cat no. 15240062), and fetal bovine serum (cat no.10270106) are from Gibco.

2.2. Animals and Study Design. Wistar rats 200-250 g were used to develop diabetes and evaluate the effect of AMS on altered platelet function in diabetes. Animals were procured from Jeeva Life Sciences (Hyderabad, India). The study was approved by the Institutional Animal Ethics Committee (IAEC) of National Institute of Pharmaceutical Education and Research (NIPER), Guwahati, India (NIPER/BT/2020/37). The animals were housed in individually ventilated cages (IVC) at an animal house facility of NIPER, Guwahati, under standard conditions (temperature 23 ± 1 °C, 50 ± 15% relative humidity, and 12 h light/dark cycle). Wistar rats were allowed free access to food and water ad libitum during the study. Post seven days of acclimatization, animals were randomly allocated into four groups ($n = 6$): group 1: control; group 2: diabetes (STZ 35 mg/kg); group 3: diabetes+AMS (50 mg/kg); and group 4: diabetes+aspirin (30 mg/kg). All these rats were maintained for 10 weeks. Every week, the body weight of all animals was recorded to understand the body weight gain or loss during the experimental period and glucose levels were monitored by using a glucometer (Accu Chek Active, Roche). The doses of AMS and aspirin were chosen from our previous work and scientific literature [30, 31].

2.3. Induction of Diabetes. Diabetes was chemically induced in Wistar rats (weight 200-250 g) with streptozotocin. After 6 hours of fasting, adult Wistar rats were administered with a single intraperitoneal (I.P) injection of a freshly prepared solution of streptozotocin (STZ) in ice-cold citrate buffer (0.01 M, pH 4.5) at a dose of 35 mg/kg body weight. Animals were then monitored for the next seven days for their blood glucose levels by using a glucometer (Accu Chek Active, Roche). The induction of diabetes was confirmed by monitoring fasting blood glucose. The rats with >250 mg/dl of blood glucose levels were considered diabetic.

2.4. Oral Dosing. Allyl methyl sulfide was administered orally at a dose of 50 mg/kg in corn seed oil for 10 weeks in diabetes Wister rats and referred to as the 'diabetes +AMS' group. Aspirin was given at a dose of 30 mg/kg in 0.5% carboxy methyl cellulose (CMC) orally to another group of diabetes Wistar rats and referred to as the 'diabetes+aspirin' group. The control group was referred to the rats feed with corn seed oil.

2.5. Cell Culture. Human macrophages (THP-1 cells; an acute monocytic leukemia cell line) were a kind gift from Translational Health Science and Technology Institute (THSTI, Faridabad, India). The cells were cultured in the RPMI 1640 medium enriched with fetal bovine serum (10% *v*/*v*) and antimicrobial agents (antibiotic and antimycotic) (100 U/ml) and cultured under standard conditions that are 37°C and 5% CO_2 incubator (eppendorf CellXpert C170).

2.6. Blood Collection and Isolation of Platelets. The platelets were isolated from blood by dual centrifugation. It has been carried out at room temperature. Preventive measures have been taken to avoid platelet activation during the process. Initially, the animals were anesthetized with the help of isoflurane. Through retroorbital plexus using capillary tube, blood was collected into a tube containing 3.8% sodium citrate (9 : 1 ratio) and centrifuged at 500 rpm for 15 minutes at

20°C temperature. The centrifugation results in isolation of platelet-rich plasma (PRP) as the upper layer in a tube. Almost 3/4th of this layer was taken into a fresh tube using a wide-bore pipette tip. Further, platelets were pelleted by centrifuging the PRP at 400 g for 10 minutes at 20°C. The platelets pellet was washed, resuspended in HEPES-Tyrode buffer.

2.7. Measuring the Number of Activated Platelets by Flow Cytometer. The *in vivo* activated status of isolated platelets was measured by flow cytometry using fluorochrome-tagged antibodies. All these works were carried out within 2 hours of blood collection and took all measures to avoid platelet activation during handling of the sample. 7 μl of PRP was taken in a tube and diluted by adding 50 μl of HEPES-Tyrode's buffer having 1% BSA. Samples were added with antibody mix having FITC anti-mouse/rat CD61 antibody (platelet surface marker) and APC anti-mouse/rat CD62P antibody (platelet activation marker) diluted with HEPES-Tyrode's buffer (with 1% BSA). Stained platelets were incubated in dark for 30 minutes at room temperature. Samples were immediately analyzed by using Attune™ NxT Flow Cytometer, where analysis was done by taking 50,000 events for each sample. The compensation was done using individual antibody-stained cells and unstained cells in order to avoid spillover from one channel to other. Platelets were gated on forward light scatter (FSC) vs. side light scatter (SSC) plot, and the percentage of CD62P positive cells is enumerated among platelets positive for CD61 cells which give % activated platelets.

2.8. Measuring Platelet Aggregation in Presence of ADP, a Platelet Agonist, by Flow cytometry. With a few modifications to previously mentioned procedure, the hypersensitivity of platelets in presence of platelet-aggregating agents (ADP) has been observed by measuring the percentage aggregation of platelets by flow cytometry [32]. 50 μl of freshly isolated platelet-rich plasma (PRP) was resuspended in 450 μl of HEPES-Tyrode buffer. Platelets were activated by adding 20 μM ADP as a final concentration and incubated for 10 minutes at 37°C under shaking conditions at 1000 rpm using a thermoshaker. Diluted PRP without agonist was used as a negative control. Cells were analyzed by flow cytometry for the presence of platelet aggregation. Platelets were differentiated from platelet aggregated by gating cells on forward light scatter (FSC) versus side light scatter (SSC) plot where the platelet aggregates are having increased size and density.

2.9. Analysis of Platelet and Macrophage Interaction by flow cytometry. The heterogeneous interactions of platelets with macrophage were analyzed by flow cytometry method using phorbol 12-myristate 13-acetate- (PMA-) differentiated THP-1 macrophages [33, 34].

For monocyte differentiation to macrophage, THP-1 monocytes (1.5×10^6 cells) were seeded in 6-well plate having 1 ml of Roswell Park Memorial Institute medium (RPMI-1640) which was supplemented with 100 μg/ml streptomycin, 100 U/ml penicillin, and 10% fetal bovine serum. The monocytes were differentiated to macrophages by adding 50 ng phorbol 12-myristate 13-acetate (PMA) and incubated for 48 hours in an incubator maintaining 5% CO_2 at 37°C under humidified conditions. After 48 hours of incubation, media was changed to fresh RPMI-1640 complete media without the addition of PMA and incubated for 48 hours in an incubator.

For macrophage and platelet coculture study, isolated PRP of 7 μl was added in a culture plate having macrophages derived from THP1 and incubated for 30 minutes in a CO_2 incubator. After successive coculturing of platelets, each well has been washed with PBS to remove floating platelets. Cells were trypsinized by adding 0.25% trypsin EDTA and incubated in a CO_2 incubator for 2 minutes. The detached cells were collected in an individual tube and centrifuged at 400 g for 10 minutes at 20°C. The supernatant was discarded; the pellet was washed with PBS and fixed by incubating with 4% paraformaldehyde for 10 minutes. The cells further were washed and resuspended in PBS for labelling with fluorescently conjugated antibodies of macrophage surface marker (CD14 PE) and platelet-specific marker (CD61 FITC). The cells were incubated in dark for 30 minutes at room temperature and analyzed by Attune™ NxT Flow Cytometer. Analysis was done by gating the macrophage population under FSC vs. SSC plot. To compensate for the overlapping spectra single-stained cells were used. Finally, we measured the mean fluorescence intensity of CD61-FITC in macrophage (CD14+) population inorder to quantify platelet macrophage interaction.

2.10. Measurement of Endogenous ROS in Platelets. Intracellular ROS was measured by using 2′,7′-dihydrodichlorofluorescein diacetate, H_2DCFDA, dye as per the procedures mentioned in articles [35, 36]. The freshly isolated PRP (7.0 μl) was diluted in 100 μl of HEPES-Tyrode's buffer and incubated with 10 μM of H_2DCFDA dye under dark conditions for 30 min at 37°C. Later, samples were further diluted with 200 μl HEPES-Tyrode's buffer and mixed gently with a pipette. The tubes were centrifuged at 400 g for 10 minutes at 20°C for pelleting the cells and discarding the supernatant. Further, the pellet was resuspended by adding 100 μl of HEPES-Tyrode's buffer. The unstained samples were used as a negative control. All of these samples were analyzed using Attune™ NxT Flow Cytometer by taking 10,000 events where the mean fluorescent intensity of DCFDA in platelet population was determined for each sample.

2.11. Statistical Analysis. All the statistical analysis was performed using GraphPad Prism version 8.0.2(263) (GraphPad Software, San Diego, CA, USA), and its comparisons were made utilizing ANOVA test followed by Tukey's test post hoc analysis. Results were expressed as mean ± standard error, and a p value < 0.05 was considered significant.

3. Results

3.1. Body Weight and Blood Glucose Level Changes in Rats. We monitored body weights and blood glucose levels of

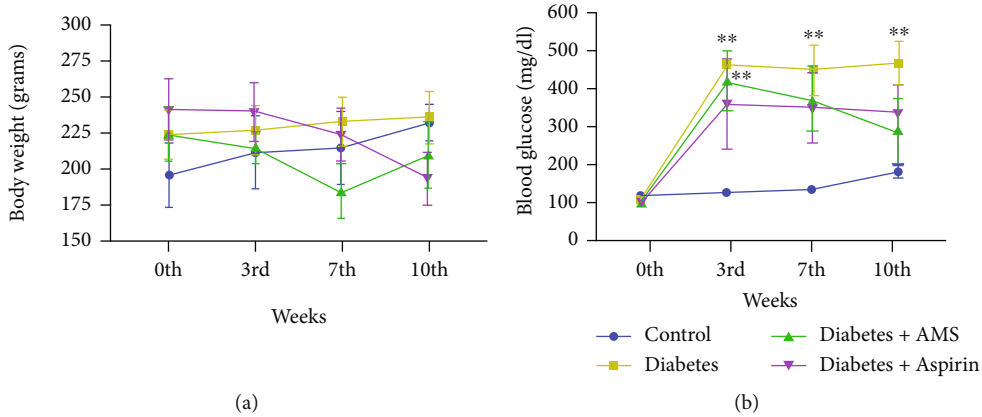

FIGURE 1: (a) Body weights and (b) blood glucose levels of the control, diabetes, AMS-treated, and aspirin-treated rats at the end of the 0^{th}, 3^{rd}, 7^{th}, and 10^{th} weeks. All values are represented as mean ± SEM ($N = 4$-6). $^{**}p < 0.01$ vs. the control.

experimental rats until 10 weeks. The mean values of body weight in grams of all study groups were compared in 0^{th}, 3^{rd}, 7^{th}, and 10^{th} week and plotted as a graph. As shown in Figure 1(a), we did not observe any significant body weight changes among all four groups. The blood glucose levels of the diabetes group significantly increased at all (3^{rd}, 7^{th}, 10^{th} weeks) the time points when compared to the control. However, AMS treatment decreased the glucose levels at 7 and 10 weeks when compared to the 0^{th} and 3^{rd} week of treatment (Figure 1(b)).

3.2. Effect of AMS on Platelet Activation in Diabetes. The *in vivo* activated status of platelets in the control, diabetes, and treatment groups after 3^{rd} and 10^{th} weeks of study duration has been analyzed by flow cytometry. The graph represents percent CD61 positive on the X-axis and CD62P-positive platelets on the Y-axis (Figure 2(a)). The dual positive cells are considered activated platelets, and percent of the activated platelets were represented as bar graphs. At the end of 3^{rd} week, the percentage of activated platelets was significantly increased in the diabetes group (~32.4%) when compared to the control (~19.11%). We did not observe any significant changes in the AMS and aspirin-treated groups (Figure 2(b)). Similarly, at the end of 10^{th} week, the percentage of activated platelets was increased significantly in the diabetes group (~47.19%) when compared to the control group (~35.24%). Moreover, both aspirin and AMS treatments significantly decreased this activation of platelets (~30.15 and 25.22%, respectively) induced in diabetes (Figure 2(c)).

3.3. Effect of AMS on ADP-Induced Platelet Aggregation. To assess the effect of AMS on platelet aggregation, flow cytometry analysis was performed on platelets derived from all the experiment groups in the absence or presence of ADP at the 3^{rd} week and 10^{th} week. Figure 3(a) represents FSC vs. SSC density plots showing the 3^{rd} week platelet aggregation with and without ADP where aggregates are gated as R2. In the absence of ADP, we did not observe any significant change in the percentage of platelet aggregation in the diabetes group compared to the control. However, a significant decrease (3-fold) in percent aggregation was observed in the AMS- and aspirin-treated diabetes groups compared to diabetes (Figures 3(a) and 3(b)). Similarly, as shown in Figures 3(a) and 3(c), we observed a significant induction (~1.6-fold) in platelet aggregation by ADP in the diabetes group when compared to the control. With AMS and aspirin treatment, we observed a significant decrease (~3.8- and 4.2-fold, respectively) in platelet aggregation when compared to the diabetes group.

Similar flow cytometry analysis in the presence and absence of ADP was performed at the end of the 10^{th} week, and results were represented as FSC vs. SSC density plots (Figure 4(a)). In the absence of ADP, we observed that the percentage of platelet aggregation increased 1.2-fold in diabetic rats when compared to the control (Figures 4(a) and 4(b)). A significant ($p < 0.05$) decrease in the percentage of platelet aggregation was observed after AMS and aspirin treatment (~2.3- and 3.3-fold, respectively) when compared to diabetes. Similarly, ADP-induced aggregation was increased by 1.1-fold in diabetes while it was decreased by 1.1- and 1.5-fold in the AMS and aspirin treatment groups, respectively (Figures 4(a) and 4(c)).

3.4. Effect of AMS on Diabetes-Induced Reactive Oxygen Species Generation in Platelets. After platelet activation and aggregation study, we measured the reactive oxygen species (ROS) levels in the platelets isolated from the control, diabetic, and treated diabetic rats at 10^{th} week of the study duration. ROS levels in platelets were measured by using 2′,7′-dihydrodichlorofluorescein diacetate (H_2DCFDA) dye and analyzed it by flow cytometry. H_2DCFDA dye is a direct measure of amount of ROS present in cells, and the results are expressed as the mean fluorescent intensity (MFI) of DCFDA (Figure 5). Platelet ROS levels was significantly ($p < 0.05$) increased in the diabetic group when compared to the control. However, the ROS levels was decreased significantly ($p < 0.05$) in platelets isolated from the AMS-treated diabetic rats when compared to diabetes rats. We did not observe any significant change in ROS levels in platelets isolated from the aspirin-treated diabetic rats.

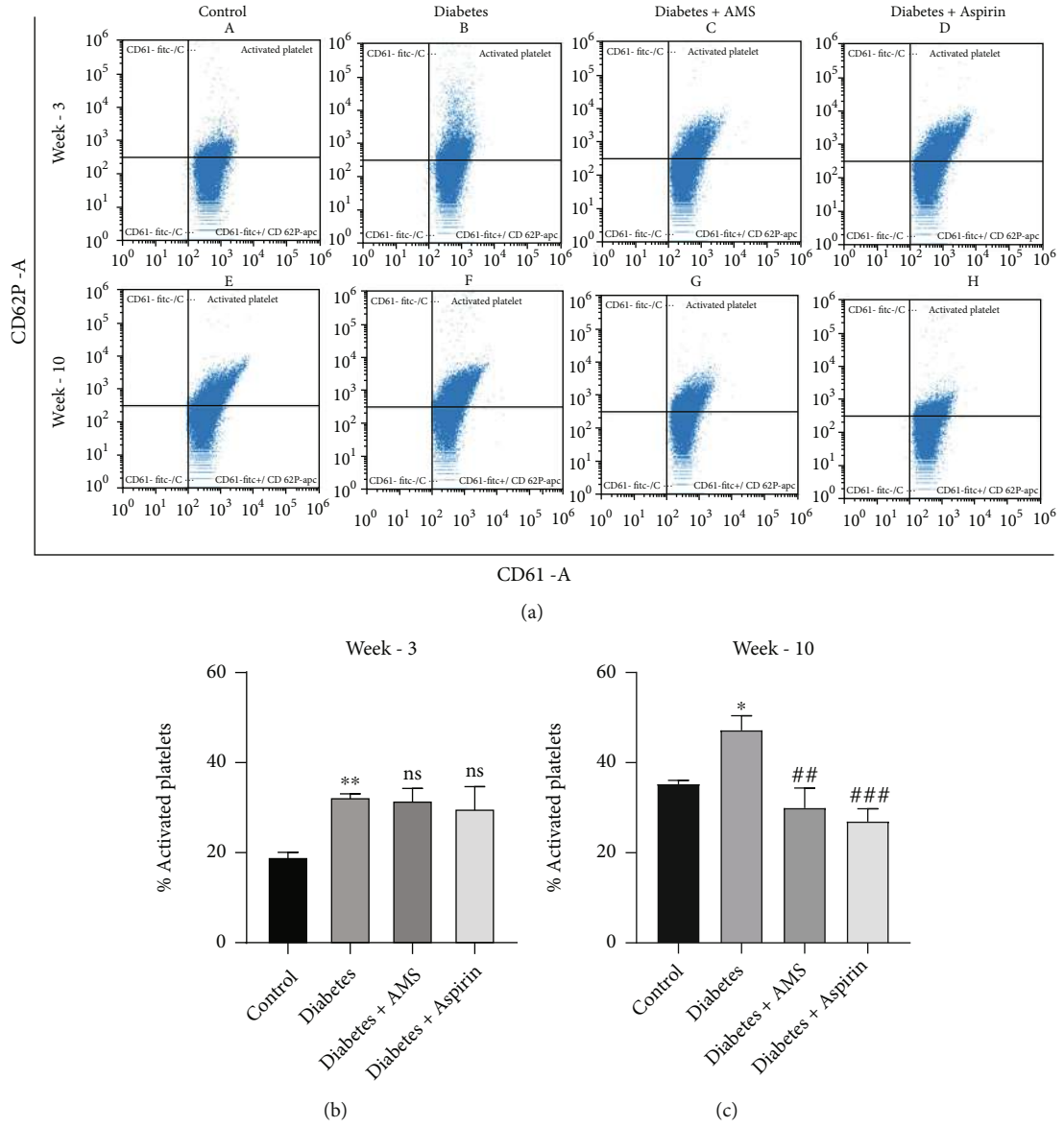

Figure 2: Flow cytometry analysis of platelet activation showing percentage dual positive cells (CD61+, CD62P+). (a) Representation of the scattered plots of percentage platelet activation in the control (A, E), diabetes (B, F), AMS-treated diabetic (C, G), and aspirin-treated diabetic (D, H) groups after 3 and 10 weeks of study. Bar graph showing percentage platelet activation at the end of the 3rd week (b) and 10th week (c). All values are represented as mean ± SEM ($N = 4$-5). $^{*}p < 0.05$ vs. the control; $^{**}p < 0.01$ vs. the control; $^{\#\#}p < 0.01$ vs. diabetes; and $^{\#\#\#}p < 0.001$ vs diabetes; ns: nonsignificant.

3.5. Effect of AMS on Diabetes-Induced Macrophage and Platelet Interaction. Platelet macrophage interaction has been considered an important phenomenon for platelet activation in diabetes [37, 38]. To look at this interaction, macrophage and platelet were incubated together and FACS analysis was performed. THP 1, a well-known monocyte cell, were differentiated with phorbol 12-myristate 13-acetate (PMA) and later cocultured with platelets collected from the control, diabetes, and AMS-/aspirin-treated rats at 10^{th} week of study duration. The results are shown in mean fluorescent intensity of CD61-FITC (marker of platelets) among CD14-positive THP1 macrophages. The data was represented as a bar graph (Figure 6). The increased mean fluorescent intensity was considered an aggregate cell of macrophages and platelets. In our data, the significant ($p < 0.05$) increase (~2.4-fold) in mean florescent intensity of CD61-FITC was observed in macrophages cocultured with diabetes platelets when compared to the control. Although we observed a decrease in macrophage platelet interactions in the AMS and aspirin treatment groups (~1.2- and 1.5-fold) when compared to the diabetic group, these changes were not significant.

4. Discussion

Diabetes mellitus is not a single clinical entity but a spectrum of diseases with various diabetic complications [39–41]. Several reports have shown a strong relation

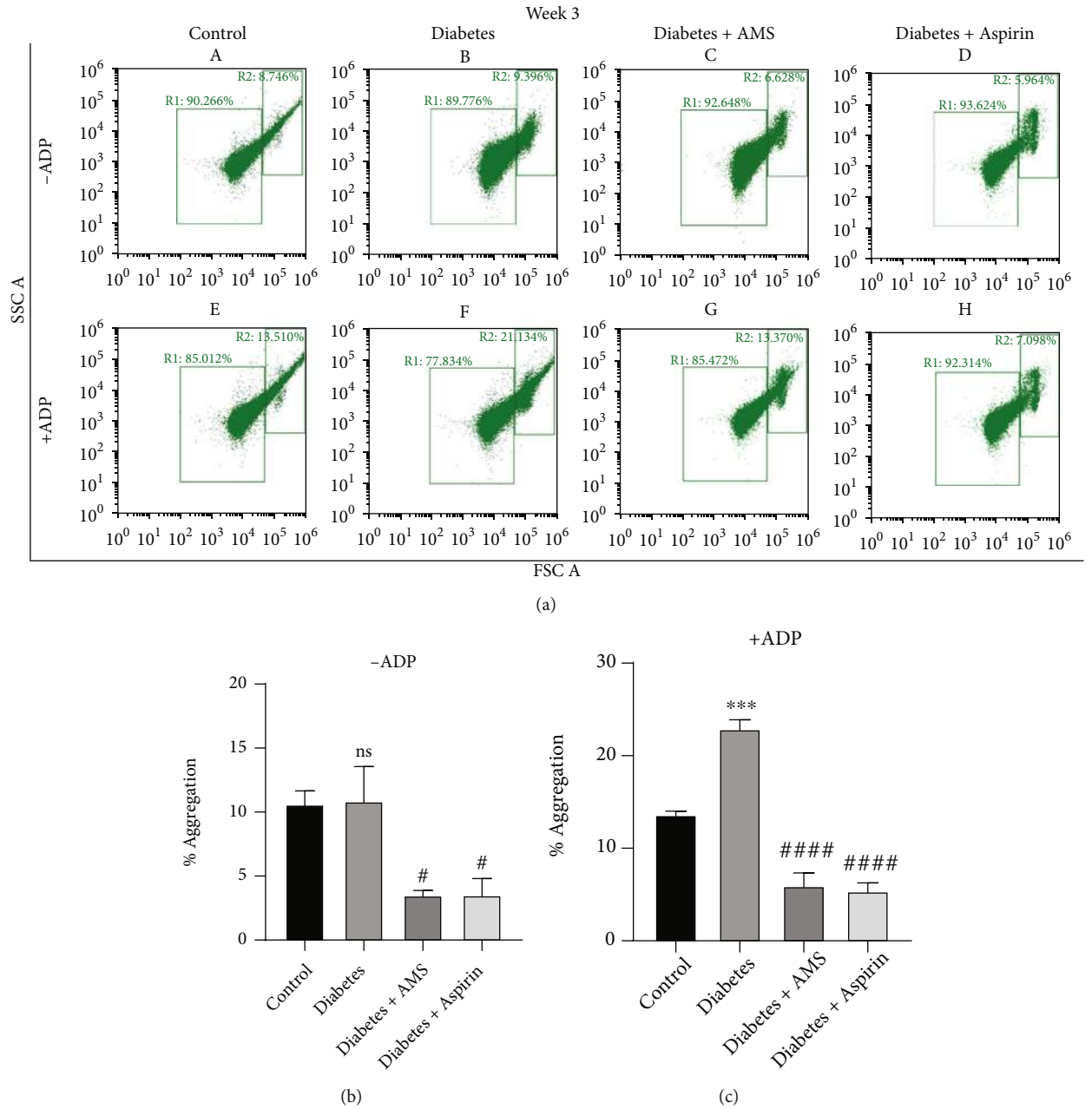

Figure 3: Flow cytometry analysis of platelet aggregation after 3-week treatment. (a) Scatter plot of forward vs. side scatter of platelets from the control (A, E), diabetic (B, F), AMS-treated diabetic (C, G), and aspirin-treated diabetic (D, H) groups, representing percentage of platelet aggregation in the absence and presence of ADP. Bar graph represents percentage of platelet aggregation observed in absence (b) and presence (c) of ADP. All values are represented as mean ± SEM ($N = 3$). $^{***}p < 0.001$ vs. the control; $^{\#}p < 0.05$, $^{\#\#\#\#}p < 0.0001$ vs. diabetes; ns: nonsignificant.

between diabetes mellitus and premature cardiovascular events [4, 5]. The underlining mechanisms of cardiovascular complication in diabetes include several physiological and pathological changes in the heart, blood vessels, blood cells, and kidneys [42]. Previous scientific literature identified numerous risk factors i.e., hyperglycemia [43–45], dyslipidemia [46, 47], inflammation [48, 49], endothelial dysfunction, and oxidative stress [50, 51], which together can induce several complexities including cardiovascular complication in diabetes. Studies have also identified that alteration of normal platelet function as one of the major risk factors of diabetic complication and characterized by increased thromboxane synthesis [52], reduced membrane fluidity [53], and increased expression of activation-dependent adhesion molecules (e.g., GpIIb-IIIa and P-selectin) [54]. All of these changes make platelets more reactive and create a prothrombic environment in a diabetes patient [55, 56]. Nevertheless, the studies focusing on platelet dysfunction in type 1 diabetes (T1DM) and its modulation by pharmacological agents are limited. Recent literature says that among all the popular natural remedies, organosulfur compounds from garlic have shown a potential antidiabetic

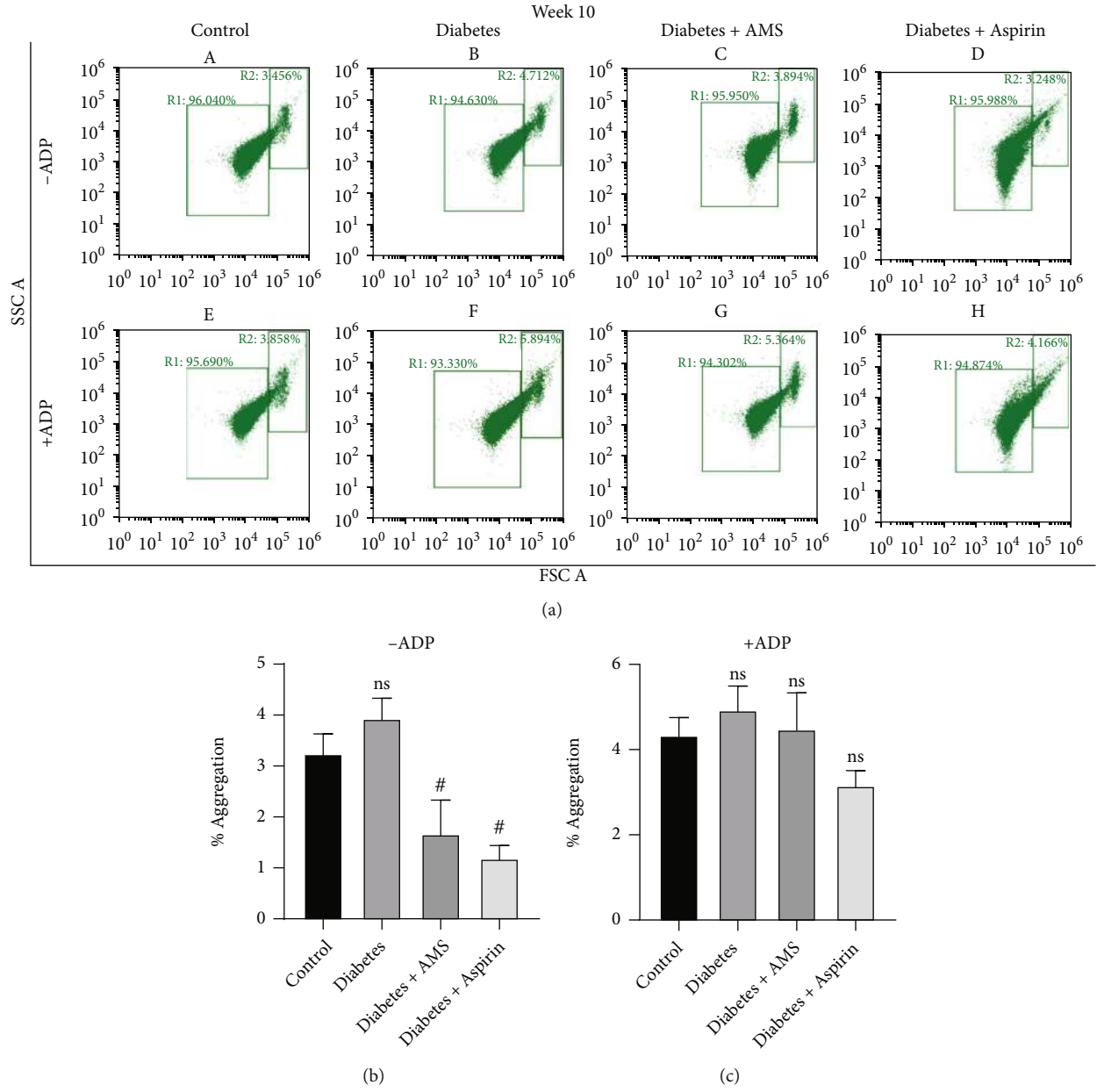

Figure 4: Flow cytometry analysis of platelet aggregation after 10-week treatment. (a) Scatter plot of forward vs. side scatter of platelets from the control (A, E), diabetic (B, F), AMS-treated diabetic (C, G), and aspirin-treated diabetic (D, H) groups, representing percentage of platelet aggregation in the absence and presence of ADP. Bar graph represents percentage of platelet aggregation observed in absence (b) and presence (c) of ADP. All values are represented as mean ± SEM ($N = 3$). $^{\#}p < 0.05$ vs. diabetes; ns: nonsignificant.

and as well as antithrombotic effect in diabetic individual [17, 18, 22, 23, 57]. Previous research work also supported the role of garlic to attenuate cardiovascular complications in diabetes [18]. Allyl methyl sulfide (AMS) is one of the important sulfur compounds obtained from garlic, and studies showed that AMS is a major metabolite that is detected in the human breath and plasma [28]. Our previous study on AMS suggested that chronic administration of AMS is safe in control rats, where the body weight, food, and water intake along with the histopathology of major organs and serum biomarkers remained normal [30]. Further, the same study showed a beneficial effect of AMS on isoproterenol-induced cardiac fibrosis and dysregulated extracellular matrix (ECM) deposition in the myocardium [30]. Also, our recent finding showed a cardioprotective effect of AMS in pressure overload-induced cardiac hypertrophy and heart failure by ameliorating endogenous antioxidants and mitochondrial function [31]. Further research identified the therapeutic role of AMS on type 1 diabetes where different parameters like blood glucose, HbA1c, oxidative stress, inflammation, and insulinotropic activity were normalised after AMS treatment. All the above parameters remained normal in control rats after AMS administration [29, 58]. However, there is no study to find the effect of AMS on platelet activation. Therefore, in the current study, we determined the major platelet alterations in STZ-induced diabetic

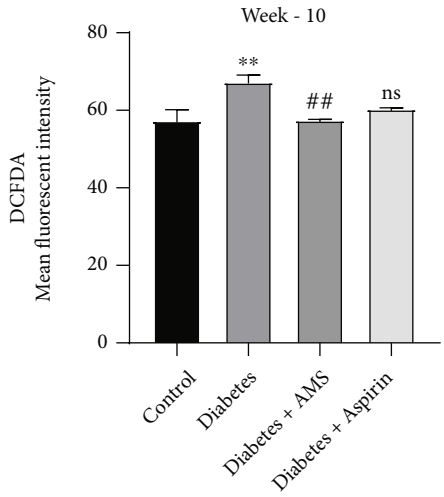

Figure 5: Comparison of ROS levels among the control, diabetes, and treatment groups. All values are represented as mean ± SEM ($N = 3$-4). $^{**}p < 0.01$ vs. the control; $^{\#\#}p < 0.01$ vs. diabetes; ns: nonsignificant.

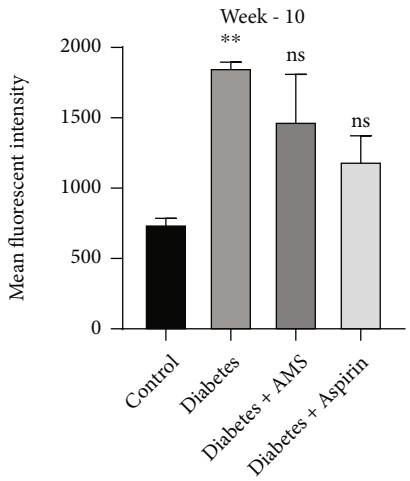

Figure 6: Macrophage platelet interaction in the control, diabetes, and treatment groups. All values are represented as mean ± SEM ($N = 3$-5). $^{**}p < 0.01$ vs. the control; ns: nonsignificant.

rats and explored the antithrombotic effect of AMS, a bioactive derivative of garlic, mainly focusing on platelet activation.

In diabetes, controlling hyperglycemia is the primary goal to reduce complications of diabetes. Poor glycemic control affects platelet activation and vascular dysfunction in diabetes. A previous study reported that a low dose of aspirin reduces blood glucose levels in diabetic rats [59]. In our present study, AMS administration to rats reduced blood glucose levels. The result showed that AMS is more effective to reduce blood glucose level in diabetic rats than aspirin. Our data is supporting the recent studies on AMS, where AMS administered to STZ-induced diabetic rats showed a significant decrease in blood glucose level [58, 60].

Hyperglycemia along with other factors helps to aggregate the platelets in the presence of a small stimulus. Such platelets are referred to as hyperactivated platelets [61]. This hyperactivated platelet has distinct morphology and expresses P-selectin (CD69P) and GP IIb/IIIa receptor on the surface [62–64]. Supporting the previous data, our present study also observed an increase in platelet activation (CD62P levels) in type 1 diabetic rats when compared to nondiabetic rats. This activation of platelets was higher at a later stage (10 weeks) when compared to the initial stage (3 weeks) of diabetes. Moreover, we also observed the effect of AMS on platelet activation. Interestingly, we found a little reduction in platelet activation after initial treatment for 3 weeks with AMS but a significant decrease was found after 10 weeks of treatment. Here, our data suggest that long-term administration of AMS has a superior effect on reduction of platelet activation in diabetes.

Increased platelet activation results in increased platelet aggregation and has been detected in diabetes mellitus [37, 38]. This increased platelet aggregation is a result of increased systemic production of TxA 2 by platelet [65], increased sensitivity of platelets agonist like ADP [66], and impaired production of platelet aggregation inhibitors PGI 2 and NO [67, 68]. The previous finding has shown that the platelet of the diabetes patient was found to be 1.6-fold more sensitive to the ADP-induced aggregation than that of nondiabetes persons [69]. In the present study, we observed the aggregation of platelets (basal level) and its sensitivity to ADP stimuli in both the early (3^{rd} week) and late (10^{th} week) stages of diabetes. At early stages of the disease, there was no difference in the baseline aggregation property of platelets between the diabetes and control groups. However, in the presence of ADP, the diabetic platelets showed an increase in aggregation than the control group. The data suggests that the platelets from diabetic rats were highly sensitive and prone to thrombus formation. A decrease in baseline aggregation was observed in diabetic rats after AMS and aspirin treatment. The percentage of aggregation was also reduced when the platelets were activated by the addition of ADP in treatment groups. The study suggests that AMS has a beneficial effect in reducing the platelet sensitivity and aggregatory properties. Similar to platelet aggregation data in the 3^{rd} week, we observed a decrease in baseline platelet aggregation in the 10^{th} week after AMS and aspirin treatment. Interestingly, in the 10^{th} week of diabetes, we did not observe any significant increase in ADP-induced platelet aggregation when compared to the control. Although we cannot explain the reason for not showing the sensitivity of platelets after ADP addition at week 10, it may be due to desensitization of P2Y1 and P2Y12 ADP receptors of platelets after the long-term interaction with endogenous ADP in chronic diabetes [70]. Further, the control group at 10^{th} week showed higher basal platelet aggregation than the control group at 3^{rd} week. This can be explained by the fact that age itself may enhance the platelet aggregation in the absence of diabetes [71]. After treatment with AMS for 10 weeks, we observed a decrease in ADP induced aggregation. The data overall indicated that inhibition was less at later stages of diabetes and may be due to the alteration of platelet structure and expression of major protein levels that resist the AMS's beneficial effect.

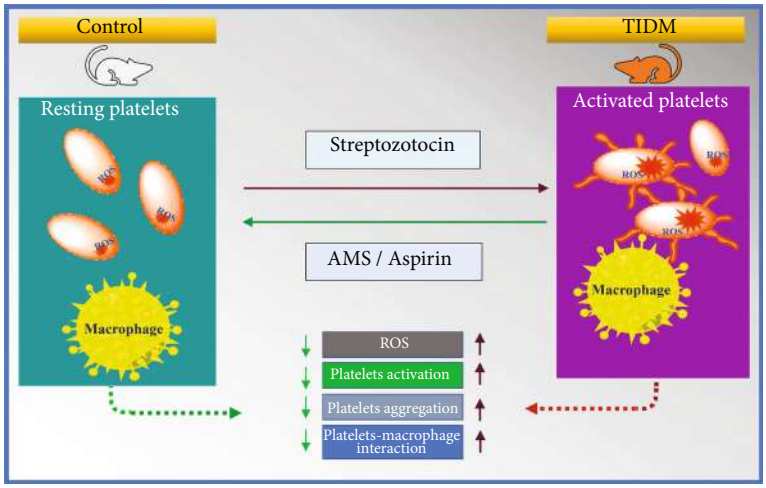

Figure 7: Attenuation of the platelet activation and platelet-macrophage interaction in type 1 diabetes by AMS. AMS: allyl methyl sulfide; ROS: reactive oxygen species; T1DM: type1 diabetes mellitus.

Next, we correlated the platelet aggregation phenotype with intracellular ROS production. Increased ROS has been observed in activated platelets and activates the PKC pathway which led to platelet hyperactivity and aggregation [72]. Supporting the previous literature, our study also found increased ROS content in diabetic platelet when compared to the control. A recent study indicates that AMS administration improved the oxidative stress in STZ-induced hyperglycemic rats [58]. Based on their observation, we evaluated the endogenous ROS generation in platelets after AMS treatment and found an inhibitory effect of AMS on ROS production in platelets.

Increased ROS production can activate the platelets and help to participate in the signaling event of atherosclerosis in diabetes by forming aggregates with monocytes through P-selectin–PSGL-1 interactions [73]. Therefore, platelets play an important role in promoting inflammation in diabetes. The inflammatory condition created by PSGL-1-mediated monocyte activation leads to the synthesis and release of various chemokines, cytokines, and reactive oxygen species. Monocyte platelet interactions also have a role in the coagulation system by a surface expression of phosphatidylserine [74]. Previous literature suggested that increased platelet-monocyte aggregates are the indicator of *in vivo* platelet activation. These aggregates are responsible for the prothrombic stage and play a major role in the development of atherosclerosis in type 1 diabetes [75, 76]. Additionally, macrophage accumulation also plays a vital role in causing diabetic complications [77]. It was reported that a similar interaction between macrophages and platelets causes releases of chemokines and phagocytosis of platelets which further involve in atherothrombosis formation [78, 79].

In the present study, we observed platelet macrophage interactions to evaluate the platelet activation status as well as proinflammatory condition in type 1 diabetes. Here, in the present study, we observed an increase in macrophage and platelet interaction at late stages of diabetes compared to control, whereas after AMS treatment, diabetic rats showed less macrophage and platelet interactions compared to diabetes. The study suggests that AMS can reduce the macrophage and platelet interaction and can further inhibit pro-inflammatory condition which leads to vascular complication in diabetes.

5. Conclusion

In this study, we found that allyl methyl sulfide inhibits platelet activation and their aggregation in type 1 diabetes. It has also shown an inhibitory effect on increased platelet-macrophage interaction in type 1 diabetes. The increased platelet activation was associated with elevated levels of ROS. Further, our results showed a decrease in ROS levels in platelets after AMS treatment. The summary of the present study has been represented in Figure 7. Overall, this study provides a piece of strong evidence that treatment with AMS could prevent the phenotypic platelet changes seen in type 1 diabetes and may act as a potential therapeutic molecule for cardiovascular complications especially thrombosis in diabetes.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Ethical Approval

Institutional Animal Ethics Committee (IAEC) Approval (NIPER/BT/2020/37) from National Institute of Pharmaceutical Education and Research (NIPER)-Guwahati to conduct the animal experiment was obtained.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

S.K.B., R.A, and N.M have designed the study. Animal handling and dosing were done by N.M. and S.K.U. Blood collection, platelet isolation, and sample preparation for flow analysis were performed by N.M., S.K.U., and V.T. Flow cytometry experiment design and analysis of platelet samples were done by both N.M and E.J. Statistical analysis and writing of the manuscript were done by S.K.B., R.A, M.J.A, and N.M.

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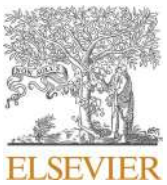

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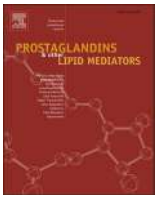

The role of platelets in non-alcoholic fatty liver disease: From pathophysiology to therapeutics

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ABSTRACT

Platelets are one of the key mediators in thrombosis as well as in the progression of many diseases. An increase in platelet activation and a decrease in platelet count is associated with a plethora of liver diseases. In non-alcoholic fatty liver disease (NAFLD), platelets are highly activated and participate in the disease progression by enhancing the pro-thrombotic and pro-inflammatory state. Some altered platelet parameters such as mean platelet volume, plateletcrits, and platelet distribution width, aspartate transaminase to platelet ratio index, liver stiffness to platelet ratio and red cell distribution width to platelet ratio were found to be associated with NAFLD disease. Further, platelet contributes to the progression of cardiovascular complications in NAFLD is gaining the researcher's attention. An elevated mean platelet volume is known to enhance the risk of stroke, atherosclerosis, thrombosis, and myocardial infarction in NAFLD. Evidence also suggested that modulation in platelet function using aspirin, ticlopidine, and cilostazol help in controlling the NAFLD progression. Future research should focus on antiplatelet therapy as a treatment strategy that can control platelet activation in NAFLD as well as its cardiovascular risk. In the present review, we have detailed the role of platelets in NAFLD and its cardiovascular complications. We further aimed to highlight the growing need for antiplatelet therapy in NAFLD.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD), is one of the rapidly growing global burden of chronic liver diseases [1]. Non-alcoholic fatty liver (NAFL) and Non-alcoholic steatohepatitis (NASH) are two different stages of NAFLD. NAFL is a condition developed by an enhanced fat in more than 5% of hepatocytes but has slight or no inflammation in the liver. Further NAFL typically does not advances to cause liver injury or complications. Nevertheless, NASH, on the other hand, is an inflammatory process that damages the liver cells and is defined by hepatocyte ballooning, lobular inflammation and fibrosis [2]. Fibrosis can later progress to cirrhosis and hepatocellular carcinoma (HCC), which are life-threatening complications of NAFLD [3]. The global prevalence of NAFLD is 29.8% by the year 2019 [4]. Furthermore, the incidence of NAFLD increases with obesity, type 2 diabetes mellitus (T2DM) and dyslipidemia [5,6]. Western diet, changes in the microbiota, predisposing genetic variations, and obesity combine in a complicated way to cause NAFLD. This consequences in an imbalance of lipid homeostasis by an abnormal accumulation of triglycerides (TG) and other lipids in liver cells [7]. Recently, platelets have been known to participate in various liver-associated pathologies apart from their physiological role in hemostasis [8]. Platelets are known to cause liver inflammation by enhancing the sinusoidal endothelium recruitment of leukocytes and can further activate effector cells, thus amplifying liver damage [9]. Various long-term studies have shown that platelets are responsible for the contribution and progression of NAFLD primarily by creating a pro-inflammatory and profibrotic environment in the liver [10]. Metabolic dysfunction is also associated with raising the risk of cardiovascular diseases (CVDs) such as hypertension, coronary heart disease, cardiomyopathy and cardiac arrhythmias in NAFLD [11,12]. Furthermore, an increased marker of platelet activation, such as mean platelet volume (MPV), is one of the mechanisms mediating cardiovascular complications associated with NAFLD/NASH [13,14]. In NAFLD, platelets are found to be highly activated and accumulated in hepatic sinusoids [15]. Although several drugs are applied in humans to treat NAFLD, very few approaches are there to reduce platelet activation and

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hepatic fibrosis. The treatment of NAFLD remains unsuccessful due to its complexity in the wide-ranging NAFLD stages and coexisting illnesses [16].

2. NAFLD epidemiology

In the past two decades, NAFLD pervasiveness increased worldwide with economic prosperity and a global rise in obesity [17]. The global prevalence of NAFLD was given as 25.2% and 29.8% by a metanalysis in the year 2016 and by a comprehensive review in 2019 respectively [4,18]. Further, the analysis represented the prevalence of NASH in NAFLD patients, which was shown as 59.10%. Moreover, it was also mentioned the prevalence of metabolic disorders such as obesity, T2DM, hyperlipidemia, hypertension, and metabolic syndrome with 51.34%, 22.51%, 69.16%, 39.34% and 42.54%, respectively in NAFLD [18]. Additionally, a recent systemic study of 72 publications highlighted that the prevalence of NAFLD was higher for men (39.7%) than for women (25.6%) [19]. As estimated by the United States, the future burden of NAFLD will rise 21% which is from 83.1 million cases to 100.6 million cases in between the years 2015–2030. Similarly, the NASH cases will rise from 16.52 million cases to 27.00 million, which is 63% in between 2015 and 2030. Also, it was estimated the rising prevalence of NAFLD-related diseases such as decomposed cirrhosis was shown to increase by 168% and HCC by 137%, with 78,300 deaths (178%) by 2030 [20]. The prevalence of NAFLD is two-fold higher in the T2DM population when compared to the general population. Also, a meta-analysis among 49,419 T2DM patients has showed the global prevalence of NAFLD, NASH and advanced-stage fibrosis among patients with T2DM accounts for 55%, 37.3% and 17%, respectively [21]. Another study showed that the prevalence of hepatic steatosis and NAFLD was high in the aged (61–76 years) population with T2DM [22]. The data from the Third National Health and Nutrition Examination Survey conducted among the population of the United States showed that the prevalence of NAFLD was 40.3% among the 60–74 age group while 39.2% among more than 74 years old [23]. A systemic review considered twenty studies between 2000 and 2019 with 3901 NAFLD patients showed that a pooled prevalence of NAFLD in type 1 diabetes was 19.3% and which was increased to 22.0% specifically among the adult population [24]. The prevalence of NAFLD is also higher in obese patients compared to the general population (50–90% vs 15–30%) [25]. Further, a study showed that 40% of NAFLD patients globally are non-obese subjects with a fifth of them being lean [26]. Although 1 in 4 adults is affected by NAFLD globally, it is highly unforeseeable because of the lack of global and national health awareness programs [27].

3. Risk factors for NAFLD

3.1. Genetic factors

Various studies have shown that genetic factors and polymorphism regulate NAFLD incidence, intensity, and prognosis [28]. Patatin-like phospholipase domain-containing protein 3 (PNPLA3) is associated with phospholipid and triglyceride remodeling in lipid droplets of the liver [29]. The association of PNPLA3 polymorphism with NAFLD was identified in 2008 [30]. Later it has become a research hotspot in the pathogenesis of NAFLD [31]. Among Asian, Hispanic, and Caucasian populations, the G allele at the PNPLA3 gene was identified as a risk factor for NAFLD and NASH [31]. Another study suggested that the mechanism of fibrosis development in individuals with NAFLD can be explained by polymorphism of the PNPLA3 rs738409 G allele which occurs by directly stimulating the specific fibrogenic pathways and indirectly through portal inflammation [32]. Further, this PNPLA3 re738409 gene polymorphism on NAFLD is not restricted to race and age [31].

Transmembrane 6 superfamily member 2 (TM6SF2), another gene associated with NAFLD risk, was known to regulate the fat metabolism in the liver by altering the release of TG-rich lipoproteins and hepatic lipid droplet content [33–35]. A study on the Chinese Han population showed an increased risk of NAFLD in the population with TM6SF2 rs58542926 T-allele polymorphism [36]. Another study on NAFLD showed that TM6SF2 rs58542926 T-allele increased liver fibrosis through the deposition of triglyceride and cholesterol in the liver of NAFLD patients [37]. Furthermore, evidence also suggests that there is an additive effect of the coexistence of PNPLA3 and TM6SF2 variants on NAFLD risk [38]. The co-presence of TM6SF2, PNPLA and membrane-bound O-acyltransferase Domain containing 7 (MBOAT7) variants affect the NAFLD development by modulating the lipid droplets accumulation and mitochondrial functionality followed by metabolic reprogramming toward HCC [39]. A study on Asian paediatric patients proved that the four risk variants (PNPLA3 rs738409, TM6SF2 rs58542926, Sorting, and assembly machinery component 50 (SAMM50) rs2073080 and rs3761472) as independent risk factors for NAFLD and have a synergistic effect on NAFLD progression [40]. Similarly, another cohort study on 515 patients revealed that PNPLA3 and TM6SF2 polymorphisms are associated with increased liver injury in NAFLD by a different mechanism where MBOAT7 polymorphism is linked to fibrosis, TM6SF2 polymorphism is linked to hepatic fat accumulations and PNPLA3 polymorphism causes both higher steatosis and fibrosis [41].

3.2. Diabetes

Many studies have confirmed a bidirectional association between T2DM and NAFLD [42,43]. A study conducted on 629 T2DM patients showed that higher body weight, heightened diastolic blood pressure, and increased gamma-glutamyl transferase (GGT) levels were associated with liver steatosis in T2DM [44]. A study conducted on 308,095 women in Korea showed that NAFLD could be an independent risk factor for gestational diabetes [45]. A community-based cohort study showed that T2DM patients diagnosed with NAFLD have 2.2-fold higher mortality than those without NAFLD [46]. Although studies identified T2DM as a risk factor for NAFLD, few studies showed an association between NASH and T2DM. In a large cohort consisting of biopsy-proven NAFLD and diabetes patients, 67% of the study population was predicted to develop NASH and 77% to develop advanced fibrosis [47].

Further, patients with NAFLD and T2DM are in increased danger of developing cardiovascular complications [48]. Severe glucose-lipid metabolism disorder, insulin resistance, high BMI, increased fasting plasma glucose, total cholesterol, low-density lipoprotein (LDL) cholesterol, fasting insulin, HOMA-IR and chemerin are major risk factors for NAFLD in T2DM [49]. NAFLD has drawn special attention among individuals with T2DM because of the presence of NAFLD with altered metabolic status and organ damage in patients with T2DM [50].

3.3. Dyslipidaemia

Dyslipidaemia is a condition of an individual with elevated serum triglyceride and LDL-cholesterol levels along with declined high-density lipoprotein (HDL) cholesterol levels [51]. A case-control cohort study of the Southern community showed hypercholesterolemia and body mass index but not fat and folate intake were significantly associated with an elevated risk of NAFLD [52]. A retrospective cross-sectional study on the Mexican population having 215 subjects, showed a significant association between liver fibrosis and increased triglyceride and cholesterol levels. This study suggests dyslipidemia as a vital risk factor in fibrosis and cirrhosis in NAFLD [53]. Among all dyslipidemia factors, hypertriglyceridemia is associated greater with NAFLD than hyper-LDL cholesterolemia and hypo-HDL cholesterolemia [54]. Furthermore, a study showed that hypertriglyceridemia, a lipid metabolism disorder, is an essential risk factor for NAFLD [55]. Moreover, a population-based study has proven that high triglyceride levels are associated with an increased risk of NAFLD among obese individuals [56]. Similarly, a

cross-sectional study showed that the triglyceride to HDL-cholesterol ratio was independently connected with NAFLD in healthy populations. It also suggests that triglyceride to HDL-cholesterol ratio can be considered a surrogate for NAFLD [57].

3.4. Obesity

Obesity is an independent risk factor for developing NAFLD. A meta-analysis suggested that obesity could predict the prognosis of NAFLD [58]. A systemic review of twenty-one cohort studies, including 381,655 individuals showed a 3.5-fold increase in acquiring NAFLD among obese individuals [59]. Furthermore, one study analyzed a linear relationship between BMI and future prediction of NAFLD using Humedica and Health Improvement Network (THIN), two large electronic health record (EHR) databases. This association study inferred that obese individuals (BMI: 30–32.5 kg/m2) have a 5–10-fold increased risk of NAFLD/NASH while patients with morbid obesity (BMI: 37.5- to 40-kg/m2) have a 10–14-fold increased risk [60]. Genetically directed T2DM, obesity, and central obesity are also associated with an increased risk of NAFLD [61]. Moreover, compared to central obesity caused by a sedentary lifestyle, general obesity caused by overeating may create a greater risk of NAFLD, although both are independent risk factors for NAFLD [62]. Further, nationwide research conducted in Sweden has proven that the offspring of obese mothers are at a higher risk of developing NAFLD [63].

3.5. Hypertension

Several studies including system biology analysis, systematic review, meta-analysis of observational studies and cross-sectional studies showed that hypertension is not only a risk factor for NAFLD but also there is always a two-way relation between hypertension and NAFLD. For example, a systematic biological investigation revealed that hypertension might be independently associated with NAFLD development [64]. Additionally, a study showed there is a 1.55-fold increased risk of developing hypertension in NAFLD and a 1.63-fold increased risk of developing NAFLD in hypertension [65]. A cross-sectional analysis including ten studies with 42,711 participants showed hypertension as a risk factor for NAFLD with an odd ratio of 1.43 [66]. Further research showed that increased systolic and diastolic blood pressure was positively linked to an increased NAFLD risk [67,68].

3.6. Age, gender and ethnicity

A retrospective study showed that people above 50 years with high serum uric acid are at higher risk of NAFLD. People less than 50 years with increased diastolic blood pressure and LDL are at high risk of NAFLD development [69]. Furthermore, a systemic review identified that among the general population, men are at higher risk of NAFLD whereas women are at 19% lower risk than men [70]. There are racial variations in the prevalence, severity, genetic risk, and prognosis of NAFLD [71]. Post-menopausal women aged 56–60 are more affected by NAFLD than men of similar age [72]. A study conducted by the United States found that NAFLD and NASH are more prevalent in the Hispanic population. Further African Americans have a lower risk of hypertriglyceridemia, suggesting a lower prevalence of NAFLD. They showed that the African-Americans population had lower hepatic inflammation and fibrosis levels and were similar to the Caucasian and Latinos populations [73].

4. NAFLD pathophysiology

Various theories have developed to understand the fundamental mechanism underlying the initiation and development of NAFLD. The "first hit" theory consists of the accumulation of hepatic fat in the liver, which sensitizes the liver to the “second hit" which leads to hepatocyte injury, inflammation and fibrosis [74,75]. Although the "two-hit" theory is widely used to describe NAFLD pathogenesis, it cannot explain the various metabolic and molecular variations that occur in NAFLD [76]. NAFLD pathophysiology can be better explained by the "multi-hit" hypothesis (Fig. 1), in which NAFLD pathogenesis is caused by a multifaceted communication between, obesity, environmental factors, predisposing genetic variants, and changes in the microbiota which disrupt the liver homeostasis of lipids with an excess accumulation of triglycerides and other lipids in hepatocytes [77,78]. This excess accumulation of fats is caused due to increased transport of free fatty acids, inadequate ß oxidation of fatty acid, and augmented *de novo* lipogenesis in the liver [79]. This excess accumulated fat further causes lipotoxicity and leads to organelle dysfunction mainly mitochondrial dysfunction and endoplasmic reticulum stress [80,81]. Insulin resistance is one of the active players in developing NAFLD [82]. Insulin resistance may increase the hepatic fat accumulation in the form of triglycerides together with increased circulating free fatty acids associated with the development of steatosis in NAFLD [83]. Further ER stress activates various signalling pathways, including C/EBP homologous protein (CHOP) and Jun N-terminal kinase (JNK) dependent upregulation of proapoptotic BH3-only proteins which leads to BCL2-associated X protein (Bax) activation, lysosomal permeabilization, mitochondrial dysfunction, caspase activation, and subsequent lipo-apoptosis. Lipo-apoptosis plays a significant role in advancing steatosis into steatohepatitis [84]. Further, an imbalance between the generation of reactive oxygen species (ROS) and antioxidants may cause a malfunction in mitochondria [85]. An alteration in mitochondrial redox balance contributes to mitochondrial dysfunction and oxidative stress in NAFLD [86]. Mitochondrial dysfunction may further increase fatty acid oxidation, reactive oxygen species (ROS) generation and oxidative stress, which lead to hepatocyte damage in NAFLD patients [87]. Moreover, this mitochondrial dysfunction may cause steatosis by down regulating various mitochondrial proteins such as G protein subunit alpha 12 (Gα12), sirtuin, ubiquitin-specific peptidase 22 (USP22) and hypoxia-inducible factor 1-alpha [88]. Increased oxidative stress and altered molecular factors mainly chemokines, adipokines such as adiponectin, leptin, gherlin, resistin and visfatin and pro-inflammatory cytokines such as interleukin 6 (IL 6), interleukin 1β (IL 1β), tumour necrotic factor 1α (TNF 1α) and interleukin 18 (IL18) are also critical players in the advancement of NAFLD to NASH [89,90]. NAFLD is linked to increased hepatic expression of numerous genes implicated in *de novo* lipogenesis (DNL) in humans [91]. Insulin and glucose regulate hepatic DNL independently through the activation of sterol regulatory element binding protein-1c (SREBP-1c) and carbohydrate-responsive element-binding protein (chREBP), which transcriptionally activate virtually all DNL-related genes [91]. The up-regulation of these lipogenic transcription factors like SREBP1c, chREBP, and peroxisome proliferator-activated receptor gamma (PPAR- γ) results in the upregulation of steatosis [92–94]. Furthermore, upstream activation of an inhibitor of nuclear factor kappa B kinase (IKK) causes enhanced signalling of the transcription factor NF-kB in steatosis [95].

5. The function and importance of platelets

Gulio Bizzozero first described platelets as ’spherules piastrine’ means little plates, and he is the first to show that platelets are anucleate cells [96]. These anucleate cells originate from precursor cells megakaryocytes and are released into the bloodstream after detaching from precursor cell. The process of platelet formation from megakaryocytes is known as thrombopoiesis [97]. Blood platelet plays the main role in maintaining the hemostasis in the body [98]. The primary hemostasis is performed as a series of acts like platelet activation, adhesion, and aggregation at the site of injury [99,100]. In physiological conditions, platelets are inhibited from activation by endothelial cells that release nitric oxide (NO) and prostaglandin I2 (PGI2) [101,102]. If damage occurs to the blood vessels, platelet activation and essential platelet

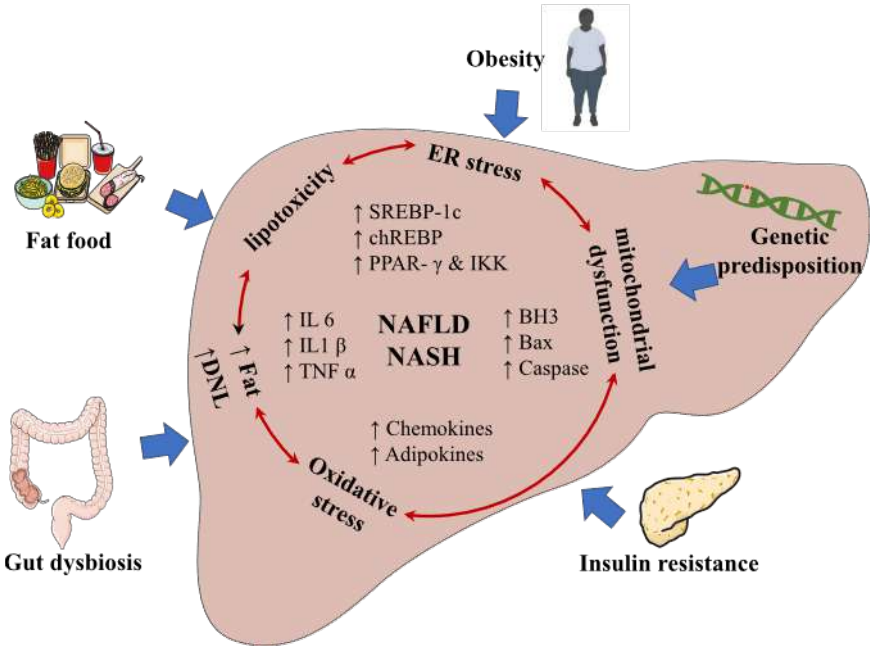

Fig. 1. Multi-hit hypothesis of NAFLD. NAFLD is caused by a complex interaction of various factors, including western diet, obesity, insulin resistance, gut dysbiosis, and genetic predisposition. These interactions increase fat accumulation, de novo lipogenesis (DNL), oxidative stress, endoplasmic reticulum stress (ER stress), lipotoxicity and mitochondrial dysfunction which upregulate various genes such as Interleukin 6 (IL 6), interleukin 1β (IL 1β) and tumour necrotic factor 1α (TNF 1α); adipokines; chemokines; Bcl-2 homology domain 3 (BH3); BCL2-associated X protein (Bax) and caspase activation; lipogenic transcription factors like sterol regulatory binding protein-1c (SREBP-1c), carbohydrate-responsive element-binding protein (chREBP) and peroxisome proliferator-activated receptor gamma (PPAR- γ).

mediators initiate the coagulation process [99]. In primary hemostasis, platelet adhesion to the exposed extracellular matrix via von Willebrand factor (vWF), an adhesive glycoprotein presents in the blood. The vWF forms a bridge between a glycoprotein GP Ib-IX-V receptor complex present on the platelet surface and collagen in the injured vascular wall. Another platelet receptors, GP Ia/IIa, and GP VI mediate adhesion of platelet to collagen [103]. Further, the platelet aggregation was mediated by the GP IIb-IIIa, an integrin receptor. GP IIb-IIIa normally presents on the membrane of platelet in an inactive form. Upon platelet activation, conformational changes expose the GP IIb-IIIa ligand binding sites and activates the receptor which is causing fibrinogen, its primary ligand, get bind to activated GP IIb-IIIa and initiates the aggregation process through "outside-in" signalling of the receptor [98,104,105]. Upon activation, platelet may release their dense granular content, including procoagulant mediators such as adenosine diphosphate (ADP), calcium, and prostaglandins, which further activate platelets and involve in the coagulation process [106,107]. A similar mechanism is seen in thrombosis and vascular blockage, resulting in heart attack and stroke after burst atherosclerotic lesions [108]. Additionally, a recent research study showed that platelets participates in preserving vascular homeostasis even in the absence of injury or inflammation [109]. Platelets also have essential role in regulating immunity and inflammation and also participate in the progression of disease [110,111]. Platelets are drawn to inflamed vessels in the early stages of inflammatory reactions, where they increase vascular permeability by releasing pro-permeability substances such as vascular endothelial growth factor A (VEGF-A), serotonin, and platelet-activating factor (PAF) [112]. These inflammatory mediators are stored in α-granules of platelet; upon platelet activation, α-granules release their content. Furthermore, P-selectin (CD62P), a surface receptor of platelet, helps in the interaction between platelet, leukocytes and endothelium. Platelet factor 4, a CXC chemokine, promotes the release of leukocyte pro-inflammatory cytokine, chemotaxis, phagocytosis and generation of ROS [111]. Through direct and indirect processes, platelets participate in the innate immune response and perform a major role in the fight against infection [113]. Through their receptors, mostly by expressing toll-like receptors (TLRs) on their surface and internal compartments, platelets detect invasive infections, which causes activation of platelets and antimicrobial proteins release [114,115]. Antimicrobial proteins specifically attack the bacteria to stop the infection from spreading [113, 116]. After an initial immune trigger, platelets get activated first by interacting directly with T cells and through the release of a RANTES (Regulated on Activation, Normal T Expressed and Secreted) chemokine. Further, activation of platelet occurs by recruiting more T cells which generate amplification of immune reactivity [117]. Platelets also help in the differentiation of $CD4^+$ T cells into regulatory T cells and T helper, which promote macrophage polarization toward the pro-inflammatory phenotype [118,119].

6. Role of platelets in liver pathophysiology

Recently, numerous studies have demonstrated that the platelets participate in liver homeostasis and also play an important role in liver diseases progression (Fig. 2) [120,121]. Under physiological shear stress, resting platelets can bind to human hepatic endothelial cells through the integrins αIIbβ3 and αVβ3. This binding triggers platelet to release CXCL8 and CCL2, which further chemoattract immune cells, a possible mechanism causing liver injury [122]. Platelets also play a role in sepsis-induced liver dysfunction [123]. Sepsis is accompanied by increased platelet activation, which triggers neutrophil extracellular traps (NETs) formation [124,125]. NET is formed through binding of platelet P-selectin and neutrophil PSGL-1 followed by a few secondary adhesion interactions, including those mediated by platelet GPIb and leukocyte Mac-1 (M2), stabilise the interactions between activated platelets and neutrophils. Furthermore, NET along with intravasation platelets, damages the endothelium, which results in extravasated platelet aggregation in the liver. These extravasated platelets release various growth factors such as thromboxane A2 (TXA2), 5-hydroxytryptamine (5-HT), plasminogen activator inhibitor type 1 (PAI-1) and transforming growth factor beta 1 (TGF-ß), which may promote liver fibrosis, portal hypertension and immunoparalysis [124]. Also it has been demonstrated that a low platelet count was one of the hallmark after partial hepatectomy or liver transplantation and potent predictor of postoperative liver dysfunction and mortality[126]. Platelets are considered to have complex behaviour in maintain the liver homeostasis [127]. Platelet also well known to participate in liver regeneration where the activated platelet released vascular endothelial growth factor (VEGF) promote hepatocyte proliferation by stimulating the endothelial cells to produce the hepatocyte growth factor (HGF). Additionally, the direct contact between platelets and endothelial cells encourages the

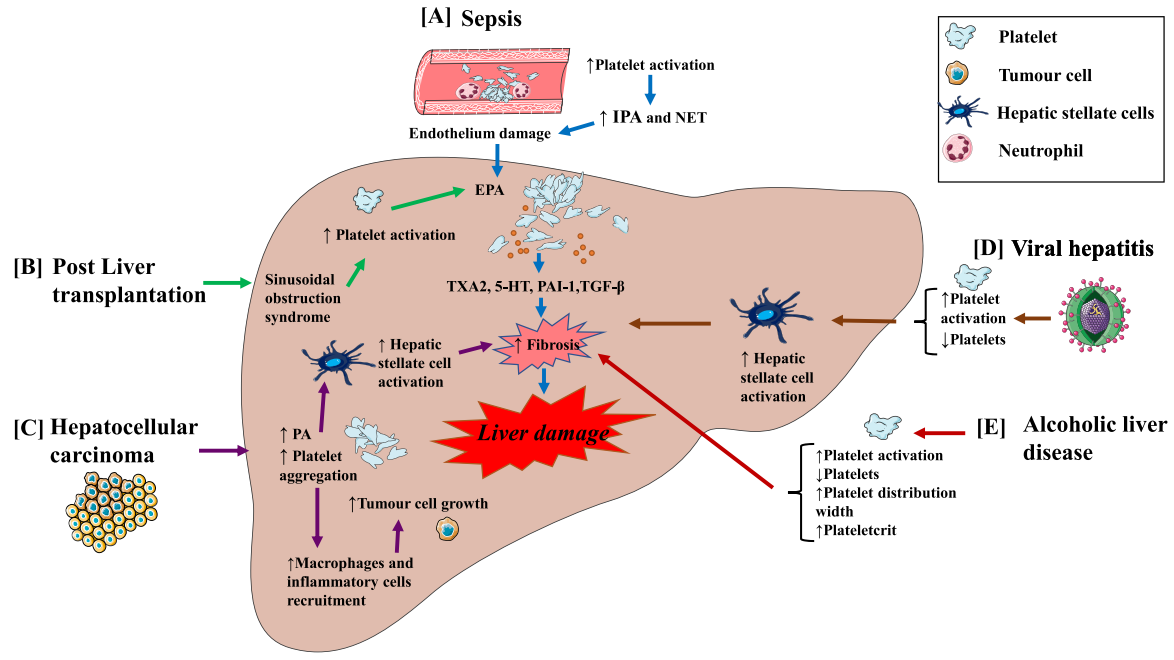

Fig. 2. Role of platelets in various liver pathologies. [A] There is an increased platelet activation in sepsis which increases the intravasation platelet aggregation (IPA) along with Neutrophil extracellular traps (NET) formation. Furthermore, NET and IPA promote extravasated platelet aggregation (EPA) and release of various chemokines (thromboxane A2 (TXA2), 5-hydroxytryptamine (5-HT), Plasminogen activator inhibitor type 1 (PAI-1), Transforming growth factor beta 1 (TGF-ß), leading to increased fibrosis and liver damage. [B] An increased platelet activation is associated with the sinusoidal obstructive syndrome as a post-liver transplantation complication, further leading to EPA formation in the liver and ultimately increasing liver fibrosis. [C] Hepatocellular carcinoma is also accompanied by an increased platelet activation (PA) and aggregation, leading to increased macrophage, and inflammatory cell accumulation ultimately increases tumour cell growth. [D] An increased platelet activation and decreased platelets are present in viral hepatitis, which further activates hepatic stellate cells and increases fibrosis. [E] An increase in platelet activation, platelet distribution width and plateletcrit, along with a decrease in platelets, is associated with increased fibrosis in alcoholic fatty liver disease.

release of VEGF and interleukin 6 that support liver regeneration [128]. Platelets vigorously participate in the pathogenesis of sinusoidal obstruction syndrome (SOS), a rare condition caused by post liver transplantation [129]. In SOS, the damaged sinusoidal endothelial cell due to toxicity of corticosteroid pulse (an immunosuppressant therapy) and the negative regulators released by activated platelets cause extravasated platelet aggregation in spaces of Disse, which may participate in liver damage [130]. Moreover, extravasated platelet aggregation in the space of Disse and platelet activation initiated by the damage of liver sinusoidal endothelial cell (LSECs) during hepatic ischemic reperfusion or immunosuppressive treatment were found to be the primary cause of liver damage in post-liver transplantation [131]. In chronic liver disease and cirrhosis, decreased platelet count was found to be a marked characteristic [132]. Now we discuss the role of platelets in liver diseases, including viral hepatitis, alcoholic liver disease, liver inflammation and regeneration, and cancer.

6.1. *Viral hepatitis*

The activation of platelets and viral infections frequently coexist. As a result of viral infection, the host defence mechanism activates platelets by releasing platelet activation mediators [133]. In chronic hepatitis C, a decreased platelet count and impaired platelet aggregation were found with advanced fibrosis [134]. Another study on chronic hepatitis C showed a fast improvement of decreased platelet count with the administration of direct-acting antiviral agents in hepatitis C patients [135]. The development of thrombocytopenia in chronic viral hepatitis is primarily influenced by altered thrombopoietin (TPO) production, portal hypertension, and splenomegaly due to advanced liver fibrosis [136]. Further study suggested that platelet count can be used to diagnose cirrhosis in viral hepatitis [137]. Most of the platelets in the cirrhotic liver were found in the sinusoidal space of the inflamed periportal region. Moreover, this platelet build-up in the liver is associated with chronic hepatitis may contribute to thrombocytopenia and liver fibrosis by stimulation of hepatic stellate cells (HSCs) [138].

6.2. *Hepatocellular carcinoma*

Hepatocellular carcinoma (HCC) is primary liver cancer that typically appears in association with cirrhosis or chronic hepatitis [139]. Patients with HCC are characterized by increased PAC-1 and CD62p-positive platelets, which indicates more activated platelets present in HCC [140]. In HCC, activated platelets were observed to participate in the progression of HCC by enhancing tumour cell growth and modifying the hepatic microenvironment towards tumorigenesis by facilitating the accumulation of macrophages in the liver and altering the phenotype of the inflammatory cell population. Additionally, pro-fibrotic signalling and the activation of hepatic stellate cells are caused by activated platelets [141]. Moreover, another research demonstrated that HCC is linked to higher levels of platelet aggregation and elevated platelet adhesive glycoprotein von Willebrand factor levels in cirrhotic patients independent of the severity of the cirrhosis or the level of thrombocytopenia [142]. Furthermore, the platelet-induced tumour cell proliferation is mediated by the inhibition of Kruppel-like factor 6 (KLF6) by platelet-released TGF-ß action on HCC cells [143]. In poorly differentiated HCC, higher plasma levels of ADP, platelet activation and increased platelet tumour binding suggest the role of platelets in regulating HCC differentiation [144].

6.3. *Alcoholic fatty liver disease*

Research showed that decreased platelet count is significantly accompanied by alcoholic fatty liver disease. Furthermore, the study showed that an increase in platelet microparticles (a marker of platelet

activation) was associated with alcoholic fatty liver (Al-FL), hepatitis-C liver cirrhosis (C-LC) and alcoholic liver cirrhosis (Al-LC) [145]. In contrast, a study showed that some patients with alcoholic liver cirrhosis had reduced platelet activation and aggregation [146]. Altered levels of serum oxylipin profile, mainly elevated 20- Hydroxyeicosatetraenoic acid (20-HETE), correlated with liver fibrosis in patients with alcohol use disorder and alcoholic hepatitis. Various oxylipins derived from ω-6 fatty acid arachidonic acid have increased the platelet aggregations. Further altered oxylipin correlated with platelet count which suggested a strong link between inflammation and platelet aggregation in alcohol use disorder and alcoholic hepatitis [147]. Platelet indices, namely mean platelet volume (MPV), plateletcrit (PCT) and platelet distribution width (PDW) found to be significantly altered in alcoholic hepatitis [148]. A study showed an increase in MPV, PDW, and decreased platelet count observed in alcoholic liver cirrhosis [149]. The platelet parameter namely plateletcrit can be used as a diagnostic marker for alcoholic liver disease [150].

Table 1
A list of altered blood platelet parameters in NAFLD.

Blood Platelet parameter	Participants	Result (s) driven	Inference	Reference
Platelet count	1048 patients with liver-biopsy-proven NAFLD.	A gradual decrease in platelet count with increasing liver fibrosis severity.	In NAFLD patients, platelet count may be an excellent diagnostic marker to identify severe fibrosis.	[173]
Platelet count	148 patients with biopsy-proven NAFLD.	Platelet count was identified as an independent predictor of cirrhosis with 100% sensitivity and 95% specificity.	Platelet count can determine cirrhosis in NAFLD patients	[174]
Platelet count	527 NAFLD patients.	Five years follow up study has shown a reduced platelet count among NAFLD patients.	NAFLD patients has increased risk of platelet count reduction compare to non NAFLD.	[175]
Platelet count	1350 patients with ultrasonography proven NAFLD.	Decreased platelet count was found in the mild fatty liver compared to moderate and severe fatty liver.	Platelet count can be used to know the severity of NAFLD disease.	[176]
Platelet count	587 NAFLD patients.	No association identified between NAFLD and platelet count	Thrombocytopenia may not correlate with NAFLD	[177]
Platelet count	1969 participants.	Increased WBC count and platelet count was observed	WBC and platelet count are associated with liver steatosis severity.	[178]
Platelet count, MPV and PDW	3592 NAFLD patients.	Decreased platelet count with increased MPV and PDW was observed	Platelet count, MPV and PDW are associated with severity of NAFLD, further MVP associated with lipid metabolic disorders in NAFLD.	[179]
Platelet count and MPV	829 NAFLD patients with 584 males and 245 females.	Decreased platelet count was observed in both males and females as age increased. MPV increased in females with age but no change in males.	Gender dimorphism exists in NAFLD for the platelet characteristics.	[180]
MPV	842 NAFLD patients.	Increased MPV was observed in NAFLD with a standardised mean difference of 0.45 and 0.67 using fixed and random effect models respectively.	Increased MPV indicates an increase activity of platelets in NAFLD patients.	[181]
MPV	628 obese patients.	Increased MPV was found among nonalcoholic hepatosteatosis individuals.	MPV is significantly associated with nonalcoholic hepatosteatosis.	[182]
MPV	128 obese adults with and without NAFLD.	Increased MPV was observed with a positive association with insulin resistance in NAFLD patients.	MPV can be used as a marker in NAFLD for predicting atherosclerosis.	[183]
MPV and neutrophil to lymphocyte ratio	873 patients with biopsy-proven NAFLD.	Increased MPV and the N/L ratio were observed in NASH and advanced fibrosis patients.	In NAFLD, platelet volume and neutrophil to lymphocyte ratio can be used as a non-invasive marker for identifying NASH.	[184]
RDW and MPV	53 NAFLD patients.	Increased RDW and MPV in NAFLD patients with hepatosteatosis.	RDW and MPV can be used as predictors of hepatosteatosis.	[185]
RDW and MPV	65 NAFLD patients.	A 21 months case control study showed increased MPV and RDW observed in NAFLD.	MPV and RDW can be used as marker for assessing onset and severity of NAFLD.	[186]
PDW and Neutrophil/ Lymphocyte (N/L) and Monocyte/ Lymphocyte (M/L)	99 NAFLD patients.	Elevated PDW, N/L, and M/L Ratios are significantly linked with hepatic steatosis.	PDW, N/L, and M/L can be diagnostic criteria for hepatic steatosis in NAFLD.	[187]
APRI	757 obese children and adolescents with ultrasonography-proven NAFLD.	Increased APRI in males compared to females.	APRI can be a positive predictor of NAFLD in males and a negative predictor in females.	[188]
RPR	388 NAFLD patients.	Increased RPR was observed in advanced fibrosis patients than in no fibrosis.	Significant associations exist between NAFLD and RPR.	[189]
PCT	225 biopsy-proven NAFLD patients.	Increased PCT was found in NAFLD patients.	PCT associated with hepatosteatosis in NAFLD.	[190]
APRI	100 NAFLD patients.	Increased AST and decreased platelets were found in NAFLD, suggesting increased APRI in NAFLD.	In urban slum-dwelling patients with NAFLD, APRI is more accurate than the fibrosis-4 (FIB-4) and FIB-5 index for predicting fibrosis.	[191]
LPR index	173 biopsy-proven NAFLD patients.	Higher areas under the receiver operating characteristic curve were observed for LPR index for detecting fibrosis compared to other parameters in NAFLD.	The LPR index could be the finest identifier of fibrosis stages in NAFLD.	[192]
PDW	129 NAFLD patients.	The highest area under the receiver operative characteristic curve was identified for PDW and TG/HDL ratio in NAFLD patients.	TG/HDL ratio and PDW can be used to identify the advancement of NAFLD.	[193]

APRI; Aspartate transaminase to platelet ratio index, LPR; Liver stiffness to platelet ratio, MPV; Mean Platelet Volume, PDW; Platelet Distribution Width, PCT; Plateletcrit, RDW; Red cell distribution width, RPR; Red cell distribution width to platelet ratio.

7. Platelets in NAFLD

7.1. Altered blood platelets in NAFLD

Various research has shown that altered platelet parameters is associated with NAFLD and NASH development (Table 1). Like other liver diseases, NAFLD was also found to have a low platelet count [151, 152]. Further this decreased platelet count relate to the degree of fat infiltration in liver tissue of NAFLD [153]. A study suggested that along with platelet count, albumin and type IV collagen 7 S level can be used in combination to predict the prognosis of NAFLD and to categorize individuals with NAFLD at a higher risk of all-cause mortality [154]. Moreover, a study showed that the thrombocytopenia is less among NAFLD patients without cirrhosis when compared to NAFLD with cirrhosis patients [155]. From the past researches the only well-known causes of thrombocytopenia in liver illness are portal hypertension and severe cirrhosis, which resulted from enhanced platelet pooling and accelerated breakdown of the platelets in an enlarged spleen [156]. Supporting to this, a study showed thrombocytopeneia and granulocytopeneia was due to the hypersplenism in NAFLD [157]. Platelet production was known to be regulated by thrombopoietin, primarily produced by the liver, which was identified to be decreased in seriously damaged liver tissue [158]. In contrast, a study showed that thrombopoietin level was significantly elevated in NAFLD [159]. Furthermore, a recent study found no correlation between platelet count and thrombopoietin level [160]. Further, extensive research is warranted to fully understand the mechanistic link underlying the decreased platelet count in NAFLD. Another platelet parameter which widely altered in well-known liver diseases namely chronic hepatitis C and HCC is MPV [161,162]. MPV defines the size of platelet which directly reflects the platelet activation and function. It was understood that a rise in MPV served as a risk indicator for a rise in platelet activation [163]. MPV is well known unique surrogate indicator for the early identification of NAFLD [13]. In NAFLD, a significantly higher MPV was observed when compared to healthy individuals [160,164]. Further this MPV, indicating the size of platelet, correlates with the activity of platelets in NAFLD [165]. Moreover, a study showed that the MPV can be used as marker for monitoring the patients with NAFLD [166]. In patients with central obesity, elevated MPV and IL-6 readings may be utilized as a diagnostic for the presence of NAFLD [167]. Further, MPV was observed to be higher in non-obese NAFLD than obese NAFLD [168]. NAFLD patients are also characterized by presence of an increased PCT and PDW [169]. PCT measures the volume of platelet occupied in blood which widely used to detect the platelet quantitative disorders [170]. Platelet distribution width (PDW) defines the volume variability in platelet size and platelet anisocytosis [171]. An increased PDW is a marker of platelet activation which has been reported previously in diabetes, cardio-cerebrovascular diseases and cancer [172]. Also increase in parameters such as aspartate transaminase to platelet ratio index (APRI), liver stiffness to platelet ratio (LPR) and red cell distribution width to platelet ratio (RPR) are positively correlates with fibrosis in NAFLD (table-1). Further, platelet indices may use as a diagnostic tool to evaluate the liver fibrosis and steatosis in liver diseases alternative to the traditional use of ultrasonography and histology [169].

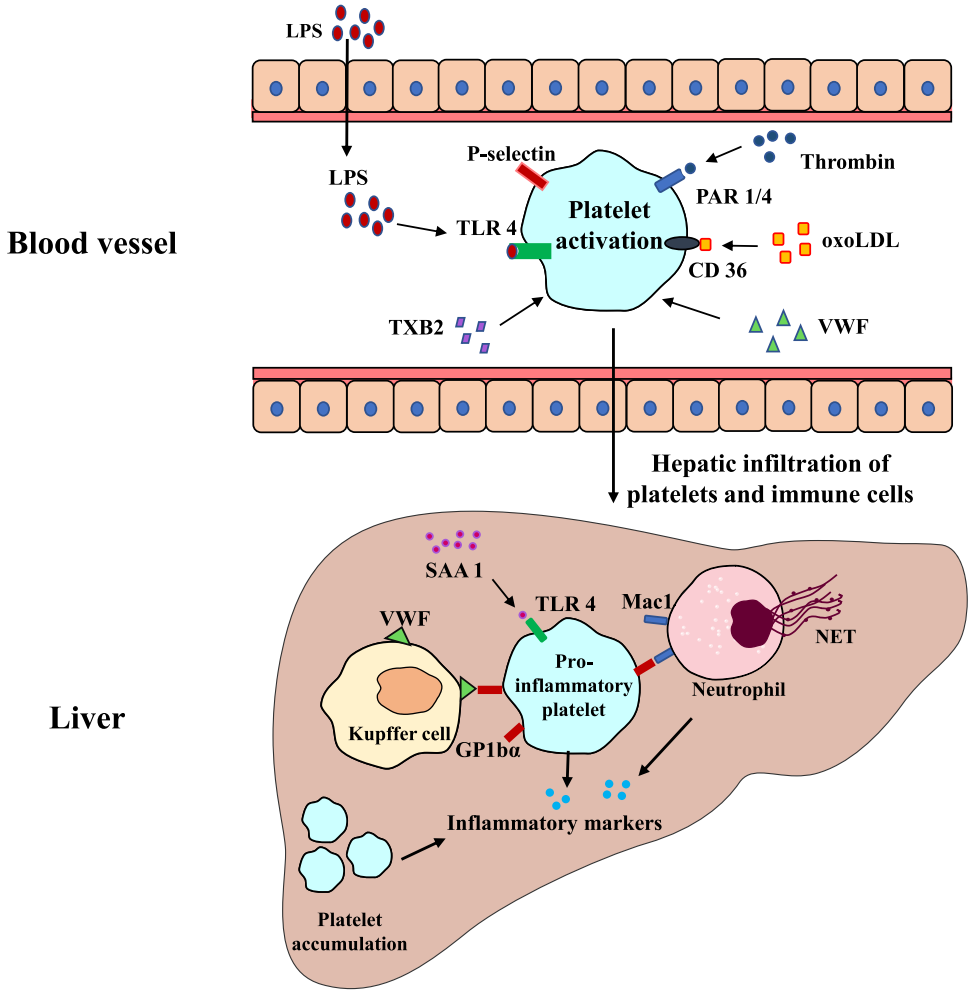

Fig. 3. Platelets in NAFLD. Various platelet activators increased in systemic circulation in NAFLD. In which thrombin bind to PAR1/4 receptor, and oxoLDL bind to CD36 on platelets and thereby activates platelets. Thromboxane B2 (TxB2) and Von Willibrand Factor (vWF) also act on platelets. The elevated LPS act on Toll like receptor 4 (TLR 4) of platelet. All these activators activate platelets. Activated platelets along with leukocytes infiltrate and accumulate in hepatocytes. The accumulated platelet participates in inflammation by interacting with Kupffer cells via GP1bα-vWF and with neutrophils via GP1bα-Mac1 interactions. These interactions lead to release of pro-inflammatory markers from activated platelets hence called pro-inflammatory platelets. Serum amyloid A protein (SAA 1) activates the platelets via interacting with Toll like receptor 4 (TLR 4).

7.2. Platelet activation and aggregation in NAFLD

The blood coagulation cascade was recognised to be profoundly activated in NAFLD, which caused the disease to worsen (Fig. 3). Thrombin, a serine protease is one of the coagulation indicators, which was found to be increased in serum of HFD-fed mice. Additionally, thrombin is recognised to be a key factor in liver damage and inflammation in HFD-fed mice [194]. Thrombin is well known platelet activator which activate platelets by cleaving PAR 1 and PAR 4 which present on human platelets [195]. Recent research has shown that thrombin helps in maintaining inflammation by stimulating the synthesis of IL-1α on platelets and macrophages [196]. Increases in fibrinogen, factor VIII, and vWF factor as well as a decrease in antithrombin were observed in NAFLD [197]. Fibrinogen-like protein 2 (FGL2) is capable of directly cleaving prothrombin into thrombin which was found to be elevated in severe NAFLD [198]. Further, fibrinogen are also known to promote platelet aggregation [199]. Moreover, there is a significantly greater levels of CD40 ligand, oxidised LDL and endogenous thrombin potential found in NAFLD patients which can be considered as the evidence of altered platelet/coagulative cascade [200]. This oxLDL can interact with platelet CD36 and platelet scavenger receptor a1 (SR-A1) and activate the platelets [201].

In a study, increased urinary 11-dehydro-TxB2 and serum TxB2, marker for COX1 activation and platelet activator were detected and correlates with increased sP-selectin indicating the existence of in vivo platelet activation in NAFLD. This increased platelet activation and cyclooxygenase I activation further correlates with the up-regulation of serum lipopolysaccharide (LPS) in NASH patients indicating LPS is one of the triggering factors for platelet activation NAFLD [202]. A study showed that *Escherichia coli* LPS may increase liver damage in NAFLD by inducing macrophage and platelet activation through the TLR4 pathway-mediated over-production of eicosanoids [203,204]. Moreover, another study shown a positive relation between TxB2 and liver enzyme ALT [205]. Additionally, TxB2 is associated with Pro-C3, a fibrosis marker, and Aspartate transaminase to platelet ratio index (APRI), confirming the platelet have an important function in liver damage among NAFLD [206].

The general haemostatic profile of non-cirrhotic NAFLD patients and healthy subject was similar. Additionally, obesity is probably a contributing factor to the pro-thrombotic characteristics such as a pro-thrombotic structure of fibrin clot and hypo fibrinolysis in patients with NAFLD [207]. Obesity-related pathophysiological alterations may influence the mega-karyopoiesis cellular microenvironment, releasing certain platelet transcriptome patterns. These modifications may have an impact on overall health and function of the liver. A transcriptome study on platelets showed that the platelet IL-6 signalling via Suppressor of cytokine signalling 3 (SOCS3) pathway may mediate the inflammatory response in obesity resulting in the progression of NAFLD [208]. Further platelet RNA sequencing in obese patients has shown a greater expression of platelet transcripts in obese patients than control where the upstream targets were proposed to be transglutaminase 2, interferon gamma (IFNγ), and CCAAT/enhancer-binding protein alpha (CEBPα) which known to involve in NAFLD disease progression [209]. Following surgical weight loss, platelet-specific alterations in gene expression have been seen in obese patients. These changes suggest potential paths for decreased cardio metabolic risk following weight loss in disease like NAFLD [208].

In the animal models of NAFLD, suppression of the PAF receptor gene decreased the inflammatory response, enhanced glucose homeostasis, and delayed the onset of NAFLD [210]. An increase in spontaneous aggregation of platelets is also seen in NAFLD patients, allowing for the inclusion of NAFLD as one of the risk factors for thrombophilia alterations in primary haemostasis. Further the spontaneous aggregation was found to be increased with hypertension in NAFLD patients [165]. Additionally, a study showed the degree of spontaneous aggregation was significantly higher in patients with hypercholesterolemia. A decrease in the activity of spontaneous aggregation, coagulation, and an increase in the anticoagulant potential of the blood are observed with statin therapy in patients with hypertension and NAFLD [211].

In fatty liver, there was an increased expression of the SAA1 protein, a serum amyloid A (SAA) family of proteins, which was identified to promote platelet activation and aggregation via TLR2 signalling. Furthermore, inhibiting SAA1 expression in vivo reduced intrahepatic immune cell infiltration as well as aggregation of platelets in the hepatic tissues of NAFLD mice [212]. In NAFLD and NASH, platelets developed more inflammatory transcripts, namely TLR4, ICAM1, MPO, IL1R1, and CXCR1than those in leukocytes, indicating that platelets are part of the inflammatory network of NASH/NAFLD [213]. In addition to the pro-inflammatory phenotype of platelet that promotes inflammation and platelet accumulation, NAFLD also showed NET deposition in the liver sinusoids, which exacerbated liver inflammation and damage [214]. Recent studies have also shown that lysosomal acid lipase (LAL), an enzyme necessary for intracellular cholesterol transport, plays a role in the pathophysiology of NAFLD [215,216]. From basic steatosis to non-alcoholic steatohepatitis and cryptogenic cirrhosis, patients with NAFLD have a gradual decline in LAL activity [217]. Further study showed this reduced blood LAL correlates with platelet count in NAFLD [218]. LAL gradually decreases with the disease stage in both blood and platelets, indicating that low LAL is harmful to the onset of NAFLD [219]. In individuals with well-compensated cirrhosis, soluble Glycoprotein VI (GPVI) also known as CD36, was considerably higher, suggesting early platelet activation [220]. Further patients with NAFLD have abnormally high levels of the soluble form of CD36 (sCD36), and this level is strongly correlated with the histological degree of hepatic steatosis [221]. Activated Kupffer cells participate in the prognosis of NAFLD by raising the inflammatory cytokines, causing necrotic inflammation in hepatocytes, changing the expression of oxidative damage-related genes, and increasing expression of fibrosis-associated genes [222]. A recent study showed that Kupffer cells also mediated intrahepatic platelet accumulation at different stages of NAFLD. In advanced NASH, platelet-expressed GPIb plays a major role in platelet-Kupffer cell communication and NASH maintenance via binding with VWF expressed on Kupffer cells. Furthermore, interacting platelets also attract immune cells through the GPIbα-activation-dependent release of α-granules containing proinflammatory mediators [15]. According to a prior study, lowering plasma ADAMTS13, a gene for production of enzymes required in coagulation, causes an accumulation of ultra large von Willibrand factor (ULVWF) multimers, which activates platelets. A non-alcoholic steatohepatitis (NASH) patient who was obese and had a severe ADAMTS13 activity deficit was also discovered to have lethal recurrent thrombotic thrombocytopenic purpura [223].

8. Platelets link to cardiovascular complications in NAFLD

The altered haemostasis in NAFLD leading to the increased thrombotic events in patients and causing increased cardiovascular risk in NAFLD [197,224]. A complex relationship exists between the liver and the cardiometabolic risk factors in NAFLD, which are responsible for heart disease, arrhythmias, and CVD [225]. Studies showed a promising role of platelets in the pathogenesis of various metabolic diseases [226]. A study proven that an altered platelet transcriptome and increased platelet aggregation may be connected with a higher cardiovascular risk in severely obese patients [209]. Further, a study demonstrated that the surgical weight loss in obese patients, platelet-specific alterations in gene expression have seen. These altered platelet transcripts are related to potential paths involve in decreasing cardiometabolic risk following weight loss in NAFLD [208].

An increased MPV was observed to be correlated with increased cardiovascular complications [227]. Various diseases such as stroke, thrombosis, and myocardial infarction are all linked to MPV [181]. In NAFLD, elevated MPV has been accompanying to an enhanced risk of atherosclerosis and may serve as a marker for cardiovascular events in

NAFLD [14,160,228]. According to a study, people with an MPV of more than 10.05 and a NAFLD CV (cardiovascular) score of more than 3.98 are at higher risk for CVD, acute coronary syndrome, stroke, and mini-stroke deaths [229]. Furthermore, in NAFLD with no metabolic risk factors such as obesity, hypertension and diabetes mellitus, MPV cannot be considered a cardiovascular risk predictor in NAFLD [230]. The existence of systemic inflammation also leads to cardiovascular events in NAFLD patients. In addition to the pro-inflammatory phenotype of systemic platelets, raised circulatory levels of vWF in NAFLD patients may partially explain the association between low-grade systemic inflammation and increased cardiovascular risk in NAFLD patients [213,214]. In obese adolescents with NAFLD, a strong correlation existed between the APRI and carotid intima-media thickness test, suggesting higher APRI score may be able to predict a worsening cardiovascular risk [231]. An increased expression of S100A9 in platelets is a novel CVD risk factor associated with obesity [209].

9. Antiplatelet therapy in NAFLD

Although there are no licensed drugs available for treatment of NAFLD, there are few proposed mechanisms, which are being targeted to manage the NAFLD. Further CVD risk can be minimised by treating the coexisting features of metabolic syndrome in NAFLD. The proposed treatment strategies include lifestyle modifications, antidiabetic drugs, lipid lowering drugs, fibrates, omega-3 polyunsaturated fatty acids, vitamin E, angiotensin receptor blockers, sodium-glucose transporter 2 inhibitors [232].

According to a meta-analysis, antiplatelet therapy such as aspirin and P2Y12 inhibitors has shown a protection against advanced liver fibrosis in NAFLD patients [233,234]. Another human study showed a link between the reduced prevalence of NAFLD among patients regularly receiving aspirin [235]. A study on high-fat, high-calorie (HF/HC) diet or choline-deficient, L-amino acid-defined (CDAA) diet induced NAFLD rat model, the antiplatelet therapy, namely aspirin, ticlopidine, and cilostazol decreased liver steatosis, inflammation, and fibrosis. Among these, cilostazol was found to exert its pharmacological effect by attenuating mitogen-activated protein kinase (MAPK) activation, which is stimulated by oxidative stress and platelet-derived growth factor via inhibiting signal transduction from Akt to c-Raf [236]. Liver fibrosis in the rat model, treated with enoxaparin and low and high-dose aspirin, showed a significant improvement in fibrosis grade [237]. Similarly aspirin and enoxaparin also reduced fibrosis in cirrhosis rats where aspirin also found to have liver regenerative action in cirrhosis rats [238]. A study on the US population showed a significant correlation between the use of aspirin with the decreased sign of liver fibrosis among chronic liver disease [239]. Daily aspirin use decreased the worsening histologic characteristics of NAFLD and NASH patients as well as the likelihood of progression to advanced fibrosis over time, in a prospective analysis of 151 biopsy-proven NAFLD patients [240]. Furthermore, aspirin use in T2DM showed that it could systemically and concurrently alleviate atherosclerosis as well as NAFLD by reducing the cholesterol build-up in the aorta, liver and reducing inflammation by diminishing nuclear factor kappa B (NFk-B) and TNF α, both of which are mediated by PPAR δ - AMP-activated protein kinase (AMPK) - Peroxisome proliferator-activated receptor-gamma coactivator (PGC)−1 α signalling pathway. Aspirin also inhibits CCR2 and causes macrophages to exhibit an anti-inflammatory phenotype [241]. A low-dose aspirin supplementation in biliary liver fibrosis mice model showed a reduction in the progression of liver fibrosis by reducing the circulating serum and hepatic levels of platelet-derived growth factor-β [242]. A study demonstrated that aspirin, clopidogrel, and ticagrelor preventing the progression of NASH-triggered HCC [15]. Anticoagulant therapy namely thrombin inhibitor dabigatran has shown a beneficial effect against HFD induced fatty liver disease in mice [194].

10. Conclusion

Platelets are one of the mediators in the progression of NAFLD. An altered platelet parameter in blood, along with an increase in activation and accumulation of platelets in the liver was identified in NAFLD. Furthermore, these activated platelets are termed proinflammatory phenotypes that interact with immune cells and initiate or amplify inflammation and fibrosis in NAFLD. These altered platelet parameters in NAFLD are strongly associated with increased cardiovascular risk. Controlling platelet activation and inhibiting the hepatic accumulation of platelets may help in suppressing the progression of the disease. Although there are evidence of platelets, participating in NAFLD progression by accumulating in the hepatic environment but the most of the literature confines to animal studies. Further studies in humans are required on role of platelets inside the liver apart from altered blood platelets in NAFLD. Very few studies showed the role of antiplatelet drugs such as aspirin and clopidogrel in treating NAFLD, it is still limited. Hence, well-designed future clinical research should focus on understanding the role of platelets in NAFLD and its therapeutic strategies to reduce NAFLD progression via inhibiting platelet activation.

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Data availability

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Conflict of interest

The authors declare no conflict of interests.

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