Review

Platelet, Antiplatelet Therapy and MasId: A Narrative Review

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Posted Date: 1 March 2024

doi: 10.20944/preprints202403.0006.v1

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Platelet, Antiplatelet Therapy and Masld: A Narrative Review

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Abstract: Metabolic-associated fatty liver disease (MASLD) is not only associated with traditional cardiovascular risk factors such as T2DM and obesity, but it is also an independent risk factor for the development of cardiovascular disease. MASLD has been shown to be independently related to endothelial dysfunction and atherosclerosis. MASLD is characterized by a chronic proinflammatory response that, in turn, may induce a prothrombotic state. Several mechanisms such as endothelial and platelet dysfunction, changes in the coagulation cascade, decreased fibrinolytic activity can contribute to induce the prothrombotic state. Platelets are players and addresses of metabolic dysregulation; obesity and insulin resistance are related to platelet hyperactivation. Furthermore, platelets can exert a direct effect on liver cells, particularly through the release of mediators from granules. Growing data in literature support the use of antiplatelet agent as a treatment for MASLD. The use of antiplatelets drugs seems to have potential clinical implication for the prevention of HCC patients with MASLD, since platelets contribute to fibrosis progression and cancer development. This review aims to summarize the main data on the role of platelets in the pathogenesis of MAFLD and its main complications such as cardiovascular events and the development of liver fibrosis. Furthermore, we will examine the role of antiplatelet therapy not only in the prevention and treatment of cardiovascular events but also as a possible anti-fibrotic and anti-tumor agent.

Keywords: cancer; NAFLD; MASLD; aspirin; thromboxane

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of liver disease worldwide [1]. Recently, researchers and scientific societies moved towards changing the terminology. The novel nomenclature for a metabolic-associated fatty liver disease (MAFLD) has been proposed in 2020 by a group of experts to overcome the issues related to the old terminology [2]. The diagnosis of MAFLD is based on the presence of hepatic steatosis and at least one between these three conditions: type 2 diabetes mellitus (T2DM), obesity or metabolic dysregulation [2]. In 2023, a multi-society Delphi consensus statement proposed a new nomenclature about steatotic liver disease (SLD) [3]. SLD can

 doi:10.20944/preprints202403.0006.v1

be diagnosed histologically or by imaging, and it displays many potential aetiologies [3]. Metabolic dysfunction associated steatotic liver disease (MASLD) is defined as the presence of hepatic steatosis in conjunction with one cardiometabolic risk factor and no other discernible cause [3]. Persons with MASLD and steatohepatitis will be designated as metabolic dysfunction associated steatohepatitis (MASH) [3].

Recent evidence demonstrated that MASLD is not only associated with traditional cardiovascular risk factors such as T2DM [4] and obesity [5], but it is also an independent risk factor for the development of cardiovascular disease [6,7].

NAFLD has been shown to be independently related to endothelial dysfunction and atherosclerosis [8]. NAFLD is characterized by a chronic proinflammatory response that, in turn, may induce a prothrombotic state [9]. Several mechanisms such as endothelial and platelet dysfunction, changes in the coagulation cascade, decreased fibrinolytic activity can contribute to induce the prothrombotic state [10]. The "thrombophilic" state correlated with liver diseases may induce macrovascular and microvascular events; in particular, the formation of microthrombi in the hepatic venules can affect the course of liver disease [11]. The process of hepatic injury induced by the microthrombi in cirrhotic patients has been called "parenchymal extinction" [12,13]. The obliteration of hepatic and portal venules by microthrombi induce the disruption of the normal blood flow, thus leading to congestion, local ischemia and parenchymal injury [12,13]. The subsequent hepatocyte apoptosis induces the extinction of the parenchyma, which is replaced by fibrous septa [12].

Moreover, hepatic stellate cells can be activated directly by increased amounts of thrombin and coagulation proteases, even in the absence of intrahepatic thrombosis [11,13]. Furthermore, a growing body of evidence demonstrated that antithrombotic treatment not only prevents thrombotic events in patients with liver diseases, but also potentially slows down liver disease progression [14].

This review aims to summarize the role of platelets in the pathophysiology of patients with NAFLD and its complications, and to examine the evidence on the use of antiplatelet therapy in this category of patients.

2. Coagulation Cascade in Non-Alcoholic Fatty Liver Disease

There are currently conflicting data on the dysregulation of the coagulation cascade in patients with NAFLD, especially in the early stages. Kotronen et al performed a study on 54 subjects with NAFLD demonstrating that higher levels of coagulation factors VIII, XI, and XII were related to liver fat content [15]. Another study on 273 participants with histologically diagnosed NAFLD showed increased levels of fibrinogen, factor VIII, and von Willebrand factor (vWF) factor and decreased levels of antithrombin than healthy subjects [16]. Otherwise, levels of coagulation factors were not related to histological changes, and PAI-1 levels were the only parameter correlating with NAFLD severity [16]. Tripodi et al. demonstrated a significant prothrombotic state in 113 subjects with different stages of NAFLD, that was more evident in patients with more severe liver disease (cirrhosis) than in those with simple steatosis [17]. Many other works in NAFLD/NASH patients demonstrated higher levels of factors VII, VIII, IX, XII, vWF, and tissue factor (TF), probably related to higher C-reactive protein (CRP), plasminogen activator inhibitor 1 (PAI-1), and fibrinogen levels (9, 11, 13, 15). Otherwise, two works performed on biopsy-proven NAFLD patients argued that the prothrombotic dysregulation was more likely to be related to obesity or insulin resistance than to liver fat content [18,19]. Moreover, Assy et al. showed that some factors in the coagulation cascade are initially increased in NAFLD, but tend to decrease with liver disease progression, thus resulting in hemorrhagic diathesis [20]. Eventually, the concept of "rebalanced hemostasis" is actually well established in advanced forms of the disease that have progressed to cirrhosis [21].

3. Role of Platelet in Masld (NAFLD)

3.1. Platelet, Metabolism Dysregulation & Liver Disease

NAFLD/NASH often develops in the context of obesity, metabolic syndrome and T2DM [22] (Figure 1). NAFLD/NASH induces a chronic inflammatory state on the liver, which is characterized

 doi:10.20944/preprints202403.0006.v1

by a complex pathophysiology. Lipid species can induce an inflammatory response in the liver by activating infiltrating and resident immune cells. There is a relevant correlation between liver fat and markers of systemic inflammation and oxidative stress [23].

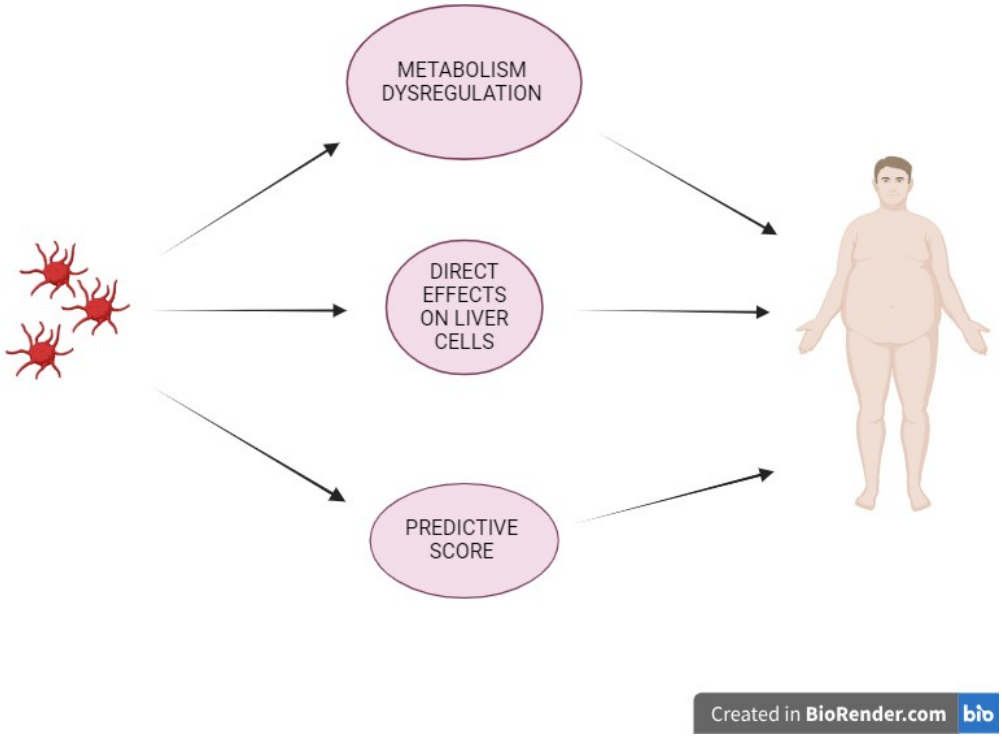

Figure 1. Summary figure on the role of platelets in patients with MASLD (NAFLD). Platelets are players and addresses of metabolic dysregulation; obesity and insulin resistance are related to platelet hyperactivation. Furthermore, platelets can exert a direct effect on liver cells, particularly through the release of mediators from granules. Furthermore, platelets are included in the majority of clinical scores linked to liver disease and risk of evolution towards cirrhosis.

NAFLD patients are characterized by low levels of plasma endogenous secretory receptor for advanced glycation endproducts (esRAGE), interleukin (IL)-10 and adiponectin, and higher CD40 ligand, endogenous thrombin potential and oxidized low-density lipoproteins (LDL) [24]. The RAGE axis is involved in a wide spectrum of diseases, including T2DM, atherothrombosis, chronic renal failure, neurodegeneration, cancer and aging [25,26]. Circulating soluble forms of RAGE, arising from receptor ectodomain shedding and splice variant esRAGE secretion, can counteract RAGE-mediated pathogenesis, by acting as a decoy [25,26]. Several works showed that low levels of esRAGE can be useful as a biomarker of ligand-RAGE pathway hyperactivity and inadequate endogenous protective response [25,26].

Liver fat is directly related to CRP, isoprostanes, IL-6, intercellular adhesion molecule-1 (ICAM-1) and P-selectin levels [23]. ICAM-1 and P-selectin levels are significantly greater in subjects with liver steatosis and elevated ALT in comparison with those without steatosis [23,27]. *In vitro* studies demonstrated that exposure of hepatocytes to fatty-acids induces the expression of tumor necrosis factor (TNF)-α [28], IL-6, ICAM-1, [29] and isoprostanes, [30] through the nuclear factor-κB (NF-kB) [31]. Chronic hepatic activation of the NF-κB pathway can induce IL-6-mediated insulin resistance; TNF-α inhibition decreases liver fatty acid oxidation and insulin resistance by Kupffer cell activation [32,33].

In obese subjects, platelets are characterized by increased aggregability and activation [34] (Figure 2). The adipokine leptin provides a potential link between platelets, obesity and NAFLD. Leptin levels are related to NAFLD degree, and atherothrombotic events can be triggered in a platelet

 doi:10.20944/preprints202403.0006.v1

leptin receptor-dependent manner in those patients [35]. Indeed, leptin induces adenosine diphosphate (ADP)-related platelet aggregation at clinically relevant levels [36]. Thromboxane (TX)A_2 release as well as hepatic TXA_2 receptor expression are increased in NAFLD patients than healthy subjects [37]. In obese and insulin resistant patients, plasmatic levels of P-selectin are increased, and decrease after weight loss [38].

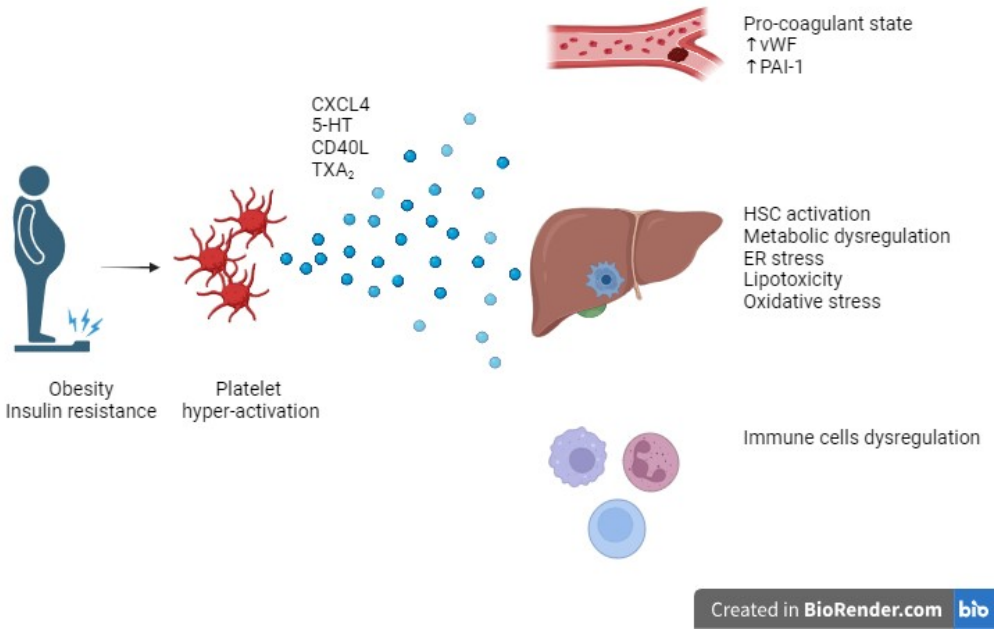

Figure 2. Obesity and insulin resistance are correlated with platelet hyper-activation. Upon activation, platelets release some molecules and mediators harbored in their granules such as 5-HT, CD40L and TXA_2. Those molecules are involved in determining a pro-thrombotic and pro-coagulant state. Moreover, many pathways are involved in direct liver damage by HSC activation, ER stress, lipotoxicity and oxidative stress. Eventually, there is a cross-talking between platelets and immune cells, thus inducing a dysregulated immune response involved in liver damage and in determining coagulative disorder. Abbreviations: chemokine (C-X-C motif) ligand 4, CXCL4; 5-hydroxytryptamine, 5-HT; CD40 ligand, CD40L; thromboxane A_2, TXA_2; von Willebrand factor, vWF; plasminogen activator inhibitor 1, PAI-1; hepatic stem cell, HSC; endoplasmic reticulum, ER.

In obese patients, high levels of circulating platelet-derived microvesicles (PMVs) are directly related to BMI and waist circumference. In obese patients, PMVs are heterogeneous in size and distribution, with different amounts of molecules related to thrombosis and tumorigenesis [39]. Notably, weight reduction can decrease the circulating levels of PMVs [40]. PMVs may bear functional receptors from platelet membranes, thus exerting different effects. The exposure of phosphatidylserine is related to pro-thrombotic and inflammatory milieu [41]. PMVs can regulate the expression of cyclooxygenase-2 (COX-2) and prostacyclin (PGI_2) in endothelial cells [42]. Moreover, PMVs are involved in monocytes and endothelial cells interaction by regulating the expression of ICAM-1 [43], and the recruitment of neutrophils by P-Selectin and IL-1 expression [44]. Furthermore, they can induce the production of pro-inflammatory molecules [e.g. CD40-ligand (CD40L), IL-1, IL-6 and TNF-α] [45], thus increasing the activation of the classic complement pathway [46].

Platelet hyper-activation is also observed in patients with hypercholesterolemia, together with higher expression of fibrinogen binding, P-selectin, superoxide anion and enhanced TXA_2 production. Plasma from patients with high cholesterol levels is characterized by increased levels of platelet activation markers, such as CD40L, soluble P-selectin, platelet factor 4 (PF-4) and thromboglobulin [47]. Notably, triglycerides-rich particles can directly induce platelet activation [48].

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In patients with insulin resistance, the adipokines resistin, leptin, PAI-1 and retinol binding protein 4 (RBP4) can dysregulate insulin receptor substrate-1 (IRS-1) expression in megakaryocytes, thus disrupting insulin signaling in platelets [49,50]. High glucose levels in diabetes are related to platelet hyperactivation, enhanced fibrinogen binding and TXA_2 production (51-53). Platelets from obese, insulin-resistant subjects demonstrated an impaired response to nitric oxide (NO) and dysregulated cyclin guanosine monophosphate (cGMP)-dependent protein kinase (PKG) signaling system.

Moreover, the inhibition of platelet activation by PGI_2 and the activation of the cyclin adenosine monophosphate (cAMP)-dependent protein-kinase (PKA) pathways are dysregulated [54]. In that context, platelet activation signals are overexpressed including increase in free intracellular calcium and the release of platelet activation molecules such as PMVs [52,55].

High glucose levels are correlated with the release of pro-oxidant molecules, which can enhance cytosolic phospholipase A2 signaling, thus catalyzing arachidonic acid release and TXA_2 generation. Aldose reductase pathway activation can enhance TXA_2 biosynthesis amplified by exposure to collagen [52].

In diabetes, TXA_2-mediated platelet hyper-activation is driven by protein kinase C (PKC)/p38 mitogen activated protein kinase (MAPK) pathway, and it also related to increased CD40L release [52,56]. CD40L belongs to the TNF superfamily, and it is increased in NAFLD platelet surface [57]. In animal studies on insulin resistance, the genetic or antibody mediated disruption of CD40L signaling have been shown to decrease the effects of diet on steatosis, adipose tissue accumulation and insulin resistance, acting on hepatic very low density lipoprotein (VLDL) secretion and genes regulating lipid balance [58].

Moreover, diabetes is related to the loss of function and damage of mitochondria in platelets, cytochrome c release, caspase-3 activation, thus inducing platelet apoptosis [59].

3.2. Platelet & Liver

A growing body of evidence demonstrated that platelets play an active and direct role in the pathogenesis of liver disease and inflammation. Activated platelets contribute to cytotoxic T lymphocyte (CTL)-mediated liver damage in a model of viral hepatitis [60]. Kupffer cells can recruit platelets to the liver in early and late stages of NAFLD/NASH [61]. In the early stages of NASH this involved hyaluronan and platelet CD44 [61]. Glycoprotein Ib platelet subunit alpha (GPIbα) is a platelet surface membrane glycoprotein, and it is involved in the interaction of platelets with Kupffer cells at late but not at early phases of NASH development [61]. Furthermore, there is no evidence for a role of platelet GPIIb/IIIa in NASH [61]. Once recruited at the liver level, platelets can release granules containing several molecules [61]. Alpha and delta (dense) granules may release in the microenvironment the pro-aggregatory factors ADP, serotonin and thrombin along with inflammatory cytokines, chemokines and growth factors [62,63]. Platelets can store and even synthesize IL-1, PAI-1 and tissue factor (TF). Platelets release factors can change gene expression in endothelial cells, leukocytes, stromal cells and fibroblasts thus directly participating to inflammation [64]. Patients and mice with NAFLD have increased blood levels of molecules present in granules from platelets. Thrombospondin (TSP-1) is present in platelets, but it is synthesized by hepatic stellate cells, Kupffer cells, endothelial cells, and adipocytes also, and it can exert a beneficial effect on NAFLD due to inhibition of genes promoting lipid production [65].

Malehmir et al demonstrated that several cytokines and platelet-secreted factors are decreased upon anti-GPIbα antibody treatment thus arguing that molecules contained in α granules yield an increase of immune cell attracting chemokines/cytokines [61]. The same authors argued that three main ligands of GPIbα (vWF, macrophage integrin-1 and P-selectin) are not relevant for NASH, but they reinforced the concept of the pro-inflammatory function of α-granules in intrahepatic immune cell attraction [61]. Selectins have been demonstrated to be involved in leukocyte recruitment to liver microvasculature upon inflammatory response [66]. Eventually, GPIbα can play a role in disease development independent of a ligand [67]. Notably, anti-GPIbα antibody treatment can exert a therapeutical anti-NASH effect (61, 68, 69).

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Indeed, both ticagrelor and anti-GPIbα antibody treatment can partially revert fibrosis on liver [61]. Therefore, P2Y12 antagonist treatment, depletion of functional GPIbα or lack of α-granules not only abolish activation, accumulation and adhesion of platelets to the liver endothelium but can also decrease intrahepatic immune-cell recruitment, thus reducing liver damage and disease development [61].

In models of liver disease, platelets have been shown to regulate gene expression in hepatocyte and deliver genetic signals to target cells. *In vitro* study with hepatoblastoma cell line (HepG2) demonstrated the direct transfer of mRNA from platelets to hepatocytes by internalizing platelets. Platelets internalization has been also demonstrated in animal model after a partial hepatectomy, and it was related to hepatocyte proliferation. On the other hand, enzymatic removal of platelet-derived ribonucleic acid (RNA) decreases hepatocyte proliferation [70]. Moreover, micro-RNA (miRNA) can be transferred from platelets to hepatocytes through the release of microparticles. PMP bearing miR-25-3p induces hepatocyte proliferation by changing gene expression [71].

Platelets may cross-talk with hepatic stellate cells by some molecules with both pro- and anti-fibrotic effects [72]. Adenine nucleotides and hepatocyte growth factor contained in platelets granules display antifibrotic effects [73], and those beneficial effects are proved by the reduction of liver fibrosis after treatment with platelet-rich plasma (PRP) [74].

Activated platelets exert a profibrotic effects by inducing hepatic micro-thrombosis [75] and through TGF [76], platelet-derived growth factor subunit B (PDGF-B) [77], vWF [78], platelet-derived sphingosine-1-phosphate signaling [79] which increase collagen secretion by hepatic stellate cells [80], thus becoming myofibroblasts [81].

The contribution of platelets to liver inflammation was confirmed by immunohistochemical staining on liver biopsies, which demonstrated the accumulation of platelet and neutrophil extracellular traps (NET) in liver, with a correlation with NAFLD activity score. Circulating platelets from NAFLD subjects were demonstrated to have significant increase of inflammatory transcripts, while leukocytes did not [82].

4. Platelet and Predictive Score of Fibrosis

NAFLD patients are characterized by higher values of platelet distribution width (PDW) compared to healthy subjects [83,84]. Larger platelets display higher amounts of granules and adhesion receptors, thus leading to increased platelet activation [84]. PDW is directly correlated with platelet size thus reflecting the heterogeneity in platelet morphology, and it changes upon platelet activation [83]. Cao et al. demonstrated that platelet count and PDW are negatively related to the stage of fibrosis [85].

Moreover, lower platelet count and higher mean platelet volume (MPV) have been shown to be independent NAFLD predictors [86]. Several previous studies reported that steatosis was related to an increase in MPV values [86,87]. A recent Korean study showed a relevant correlation between the presence of NAFLD and higher MPV values in 628 obese volunteers [87]. A recent meta-analysis combining data from eight cross-sectional and cohort studies confirmed that NAFLD patients compared with controls had significantly increased MPV [88]. A study performed on 100 patients with biopsy-proven NAFLD demonstrated a significant stepwise increase in MPV levels from subjects with normal histology through patients with simple steatosis to those with NASH; MPV was significantly related to the histological features of NASH, such as steatosis, inflammation, ballooning, and fibrosis [89]. Those observations can be explained by the fact that an increased systemic inflammatory response with the release of cytokines induced by NAFLD can change the platelet size, with larger platelets consequently secreting more granules and prothrombotic factors [88,90].

The platelet count is based on the balance between the production and destruction of platelets. In chronic liver diseases, the increase in spleen volume induces the sequestration and the destruction of platelets [91]. In NAFLD patients, platelet count was negative related to the degree and the severity of liver fibrosis (92-95). Indeed, fibrosis arises around the central veins, and portal hypertension seems to be primarily the result of central vein occlusion in NAFLD progression to NASH [96].

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Moreover, platelets play a role in the process of liver fibrosis by decreasing expression of the main fibrogenic cytokine TGF-β and by enhancing the expression of matrix metalloproteinases [95].

Shah et al. evaluated the diagnostic accuracy of different scoring system in 541 NAFLD patients [97]. The area under the receiver operating characteristic (AUROC) of the fibrosis index based on 4 Factors (FIB4) index, NAFLD fibrosis score, the aspartate aminotransferase to platelet ratio index (APRI) and AST-to-platelet ratio to evaluate the diagnostic performance for hepatic fibrosis stages ≥ stage 3 were 0.802, 0.768, 0.730 and 0.720, respectively [97]. The AUROC of the platelet count to detect NAFLD patients with hepatic fibrosis ≥ stage 3 was 0.774 [92]. Indeed, the platelet count shows almost the same diagnostic accuracy than other biomarkers of liver fibrosis [92]. Therefore, it would be interesting to establish a threshold or range of platelet count that could suggest evolution towards hepatic fibrosis in patients with NAFLD.

In the work of Yoneda et al., the most accurate platelet count threshold for the detection of severe fibrosis (stage 3–4) was 19.2 x 10^4 /μl, and that for the diagnosis of cirrhosis (stage 4) was 15.3 x 10^4/μl in patients with NAFLD [92]. Therefore, NAFLD patients with platelet counts of less than 19.2 x 10^4/μl should be closely monitored, because it is likely that they may progress to NASH [92].

In a recent work, MPV has been shown to be decreased in women with MASLD associated with obesity and closely related to early liver inflammation in NASH [98]. Specifically, platelet count and plateletcrit have been related to hepatic ballooning [98]. Duran-Bertran et al. presented a new predictive model by using MPV, ALT levels and the presence of DM and MS to predict MASLD in obese women, displaying an area under the ROC curve of 0.84 [98].

In conclusion, patients with hepatic steatosis and NAFLD are characterized by higher values of PDW and MPV, while low platelet counts would be indicative of progression towards hepatic fibrosis.

5. Antiplatelet Therapy in NAFLD

A growing body of evidence demonstrate how antiplatelet therapy can mitigate fibrosis development in NAFLD, thus identifying it as a potential antifibrotic agent (81, 99-102). In murine models, aspirin has been shown to exert an anti-inflammatory effect by limiting the activation of hepatic stellate cells through the inhibition of the pro-inflammatory enzyme COX-2 and by interfering with the PDGF signaling [81,102]. Fujita et al. performed a study on fisher 344 male rats fed with a choline-deficient, l-amino acid-defined (CDAA) diet or a high-fat high-calorie (HF/HC) diet, and treated with or without the antiplatelet agents, aspirin, ticlopidine or cilostazol for 16 weeks [103]. All three antiplatelet drugs significantly decreased liver steatosis, inflammation and fibrosis in the CDAA diet group [103]. Cilostazol was the most effective, and it reduced liver steatosis also in HF/HC diet group [103]. Cilostazol exerts its beneficial effect against NAFLD by suppressing MAPK activation induced by oxidative stress and PDGF by intercepting signal transduction from a Akt to c-Raf [103]. Those findings are consistent with the results of a recent study conducted on human patients showing the association between the use of acetylsalicylic acid with reduced liver steatosis and fibrosis [104].

The association between acetylsalicylic acid and histological features of NAFLD has been further investigated in a prospective study conducted on a cohort of patients with histological diagnosis of NAFLD, followed by an extensive follow-up [50]. It has been observed that daily intake of acetylsalicylic acid is associated with a 46% reduction in the risk of developing fibrosis compared to non-users. Notably, this effect is specific to the molecule itself and not to the class of drugs, as other NSAIDs, lacking antifibrotic action, do not reduce the incidence of advanced fibrosis [50].

A cross-sectional analysis using data from the National Health and Nutrition Examination Survey III (NHANES III) specifically showed that the effect is linked to acetylsalicylic acid, unlike ibuprofen [104]. This study highlighted how aspirin use is significantly associated with lower fibrosis indices (e.g. FIB4, APRI), especially in individuals at risk of chronic liver diseases, with a consistent negative association across different types of chronic liver diseases (chronic viral hepatitis, suspected alcoholic liver disease and NASH) [104].

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The possibility of a correlation between regular aspirin intake and a reduction in the prevalence of NAFLD was demonstrated also by Shen and colleagues in a study of the general population in the United States involving over 11000 adults [105]. Regular aspirin use, compared to not using it, was significantly related to a 38% lower probability of developing NAFLD [105]. The probability of developing NAFLD among men was less than half in those who reported occasional use of aspirin and two-thirds in those who used it regularly [105]. Aspirin use and NAFLD were not found to be correlated in women and younger participants, possibly because women have higher urinary excretion of aspirin and its metabolites than men, and younger subjects have higher aspirin clearance than older subjects [105]. Therefore, it can be argued that younger or female patients may require higher doses of aspirin to achieve a pharmacological effect against NAFLD. However, the lack of information on exact doses of aspirin made it difficult to evaluate dose-dependent associations between aspirin and NAFLD. Furthermore, the diagnosis of NAFLD was based on ultrasound but was not confirmed by liver biopsy; therefore, some participants may have been misclassified as having or not having NAFLD. According to the findings, taking aspirin regularly may lead to a lower risk of NAFLD, especially in men or older adults [105]. However, as a cross-sectional study, it was not possible to assess whether the use of aspirin could prevent the incidence or reduce the progression of NAFLD [105].

It also been proven that aspirin use was associated with significantly lower liver fibrosis indexes among U.S. adults with suspected chronic liver disease [104]. According to a systematic review and meta-analysis, there is a protective relationship between antiplatelet therapy and the prevalence of advanced liver fibrosis in patients with NAFLD [106].

In the work of Simon et al, longer duration of aspirin use was associated with progressively reduced risk for incident advanced fibrosis, and this association was similar for any age and sex [50].

In a prospective study of patients with bioptically proven NAFLD, daily use of aspirin was associated with less severe histological characteristics of NAFLD and NASH and a lower risk of progression towards advanced fibrosis [50]. Compared to non-regular use, daily use of aspirin has been associated with significantly lower odds of NASH [odds ratio adjusted (aOR), 0.68; 95% confidence interval (CI), 0.37-0.89] and fibrosis (aOR, 0.54; 95% CI, 0.31-0.82) [50]. Daily consumers of aspirin had a significantly lower risk of developing advanced fibrosis than nonhabitual consumers [hazard ratio adjusted (aHR) [107], 0.63; 95% CI. 0.43-0.85] [50]. This relationship appeared to depend on duration, with the greatest benefit found with at least 4 years of aspirin use (aHR, 0.50; 95% CI, 0.35-0.73) [50].

Several molecular mechanisms have been suggested to explain the possible efficacy of antiplatelet therapy against NAFLD and its progression to NASH. Aspirin may stimulate NO and prostacyclin production through the expression of endothelial NO synthase and vascular endothelial growth factor, resulting in antioxidant activity [108]. Moreover, aspirin can inhibit the production of TNF-α, which causes inflammation and liver fibrosis [109]. Furthermore, aspirin reduces the expression of PDGF, thus decreasing hepatic inflammation, steatosis and fibrosis [110]. Antiplatelet therapy can stimulate the insulin signaling pathway and improving IR by PDGF-induced activation of the mitogen-activated protein kinase pathway [111,112]. Eventually, aspirin-induced lipoxins in the liver regulate cytokine-chemokine axes in liver cells [113]. Malehmir et al demonstrated that several cytokines and platelet-secreted factors are decreased upon anti-GPIbα antibody treatment [61]. Moreover, P2Y12 antagonist treatment, as well as depletion of functional GPIbα or lack of α-granules can decrease activation, accumulation and adhesion of platelets to the liver endothelium, thus ameliorating liver damage and attenuating fibrosis development [61].

Furthermore, in presence of hyperlipidemia and metabolic dysregulation, many of the biological substrates and conditions, such as IR and pro-inflammatory cytokines, are involved in the pathogenesis of both NAFLD and atherosclerosis [114]. Aspirin has been shown to improve both atherosclerosis and NAFLD simultaneously [115]; this mainly occurs through the following two pathways: activating catabolic lipid metabolism (decreased lipid accumulation in HepG2 cells, aorta and liver) and reducing inflammation (decrease in NF-κB and TNF-α) [115].

 doi:10.20944/preprints202403.0006.v1

6. Antiplatelet Therapy and Cancer

Hepatocellular carcinoma (HCC) incidence is increasing most rapidly than any cancer, with an age-adjusted annual increase of 3,8% and 2,8% in men and women in the U.S., respectively [116,117]. Several epidemiological studies confirmed the increasing incidence and risk of NAFLD-associated HCC [118,119]. The increasing rates of NAFLD-associated HCC is also demonstrated by changes in liver transplantation (LT) indications [120]. The European Liver Transplant Registry database from 2002-2016 showed that 8,4% of transplant patients were for NASH in 2016 (compared to 1,3% in 2002), 39% of whom had HCC [121].

Pharmaceutical inhibition of platelet function by using antiplatelet treatment have been previously shown to correlate with better outcome in cancer patients (122-124). Therefore, the potential cancer chemo-preventive role of antiplatelet drugs in NAFLD/NASH patients, especially aspirin and other NSAIDs, represents an intriguing research area [125].

A recent meta-analysis by AISF (Associazione Italiana per lo Studio del Fegato) HCC Special Interest Group including 20 studies demonstrated that patients treated with antiplatelet therapy show a 40% reduced risk of HCC incidence and a halved risk for all-cause mortality in patients with HCC treated with either curative or palliative strategies [126]. In this meta-analysis patients with a clear indication for antiplatelet therapy were evaluated, but there was no specific evaluation for subgroups of patients nor for the NAFLD/NASH field specifically.

Another study analyzing prospective data of the National Institutes of Health– AARP Diet and Health Study cohort demonstrated that aspirin users had statistically significant reduced risk of HCC incidence and mortality due to chronic liver disease (CLD) compared to those who did not use aspirin [127]; otherwise, users of non-aspirin nonsteroidal anti-inflammatory drugs (NSAIDs) had a reduced risk of mortality due to CLD but did not have lower risk of HCC incidence in comparison with those who did not use non-aspirin NSAIDs [127]. This finding may reflect differences in COX-inhibitory effects of aspirin and non-aspirin NSAIDs, because aspirin irreversibly inhibits and modifies both isoforms of COX, the constitutive COX1, which is expressed in most normal tissues, and the inducible COX2, which is highly expressed in response to a broad spectrum of proinflammatory stimuli, including those that mediate hepatic carcinogenesis [127]; moreover, while aspirin irreversibly inhibits COX isoenzymes, NSAIDs do so reversibly, with transient effects that may reduce anti-fibrotic activity [127].

Lee et al. evaluated 35,898 cirrhotic patients dividing them into two groups (those treated with aspirin for at least 84 days vs controls without treatment) [128]. Daily aspirin use was independently associated with a reduced risk of HCC (three-year HR 0.57; 95% CI 0.37-0.87; p = 0.0091; five-year HR 0.63, 95% CI 0.45-0.88; p = 0.0072) inversely correlated with the treatment duration [3-12 months: HR 0.88 (95% CI 0.58-1.34); 12-36 months: HR 0.56 (0.31-0.99); and ≥ 36 months: HR 0.37 (0.18-0.76)] [128]. Overall mortality rates were significantly lower among aspirin users compared with untreated controls [three-year HR 0.43 (0.33-0.57); five-year HR 0.51 (0.42-0.63)] [128]. The incidence of GI bleeding was not increased in daily aspirin users compared to untreated cirrhotic patients without a previous history of GI bleeding or with a previous history of GI bleeding [128].

Furthermore, Choi et al. evaluated LT recipients with pre-transplant HCC and divided them into two groups according to the use of antiplatelet agents for > 90 days or not [129]. The 5-year cumulative incidences of HCC recurrence and HCC-specific death were similar between the antiplatelet and non-antiplatelet groups [129]. All-cause and non-HCC deaths were also similar between the two groups [129].

A recent study compared the outcomes between 33,484 patients with NAFLD who continuously received a daily dose of aspirin for 90 days or more, and 55,543 subjects who had not received antiplatelet therapy, by using Taiwan's National Health Insurance Research Database [130].

The 10-year cumulative incidence of HCC in the treated group was significantly lower than that in the untreated group (0.25% [95% CI, 0.19-0.32%] vs. 0.67% [95% CI, 0.54-0.81%]; P < 0.001) [130]. Aspirin therapy was significantly associated with a reduced HCC risk (aHR 0.48 [95% CI, 0.37-0.63]; P < 0.001) [130]. In older (age > 55 years) patients with serum ALT elevation (high risk patients), the 10-year cumulative incidence of HCC in the treated group was significantly lower than that in the

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untreated group (3.59% [95% CI, 2.99-4.19%] vs. 6.54% [95% CI, 5.65-7.42%]; P < 0.001) [130]. Moreover, HCC risk was significantly lower by using aspirin for ≥ 3 years (aHR 0.64 [95% CI, 0.44-0.91]; P = 0.013), when compared with short-term use (< 1 year) [130].

Tan et al. performed a metanalysis on 147 283 participants by including 19 studies evaluating patients with hepatitis B virus (HBV), hepatitis C virus (HCV), alcohol-related liver disease (ALD) or NASH that were administered at least one NSAID or antiplatelet therapy for a defined period of time and were followed for at least 6 months [131]. Aspirin use reduced the risk of HCC incidence (HR: 0.51, 95% CI: 0.36-0.72); and improved liver-related mortality (OR: 0.32, 95% CI: 0.15-0.70), with a small increased risk of gastrointestinal bleeding events (OR: 1.32, 95% CI: 1.08-1.94) [131]. With respect to HCC recurrence following treatment, analysis of all aspirin and NSAID treatment was associated with a decreased risk of HCC recurrence (HR: 0.80, 95% CI: 0.75-0.86) [131]. By stratified analysis, only the non-aspirin NSAID group showed significant risk reduction (HR: 0.73, 95% CI: 0.63-0.84) [131].

Therefore, there is evidence that the use of antiplatelet drugs (both aspirin and others) is correlated with a reduced incidence of HCC. Regarding the type of drug (aspirin vs other NSAIDs) there are non-univocal data, in some cases conflicting [127,131], which deserve studies based on specific molecular mechanisms.

Molecular Mechanisms

Antiplatelet drugs, in particular aspirin, have been investigated as cancer preventatives, particularly in the context of colorectal cancer [125,132]. A recent survey demonstrated that among subjects who had previously taken aspirin, 1.9% had taken it for cancer prevention [133]. Participants with a personal or family history of cancer were more likely to perceive aspirin as necessary for cancer prevention [133]. Otherwise, concerns about taking aspirin at higher doses and its side effects, such as gastrointestinal bleeding, were common [133].

The beneficial effects of antiplatelet therapy on cancer can be explained by the involvement of TX-dependent platelet activation and cyclooxygenase-2-driven inflammatory response in the early stages of carcinogenesis [134,135] (Figure 3). Joharatnam-Hogan et al measured urinary 11-dehydro-thromboxane B_2 (U-TXM), a biomarker of *in vivo* platelet activation, in patients with breast, colorectal, gastro-oesophageal and prostate cancers after radical therapy [136]. Aspirin 100 mg daily decreased U-TXM similarly across all tumour types; however, aspirin 300 mg daily provided no additional suppression of U-TXM compared with 100 mg [136].

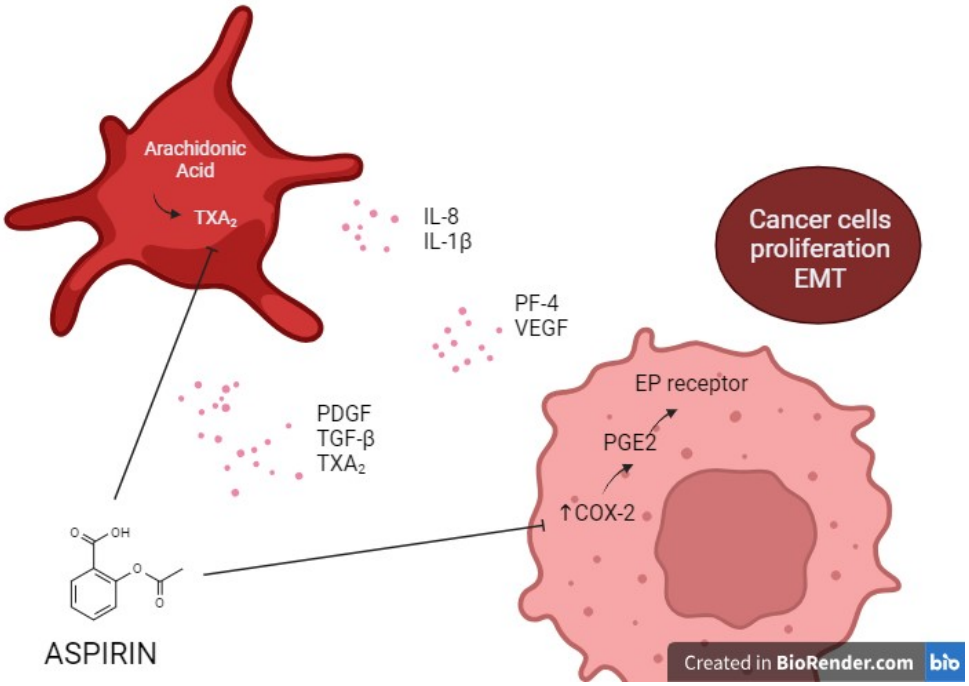

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Figure 3. Summary figure on the anti-cancer mechanisms of antiplatelet therapy (aspirin). Aspirin can block COX-2 both at the platelet level and in tumor cells. At the platelet level, COX blockade reduces platelet activation and the release of mediators that may be involved in tumorigenesis (e.g. TGF-β, VEGF, TXA2). At the tumor cell level, COX-2 blockade appears to be able to reduce cell proliferation and epithelial-mesenchymal transition. Abbreviations: thromboxane A_2, TXA_2; interleukin, IL; platelet-factor-4, PF-4; vascular endothelial growth factor, VEGF; platelet-derived growth factor, PDGF; transforming growth factor β, TGF-β; cyclooxygenase,COX; prostaglandin E, PGE; prostaglandin E receptor, EP; epitelio-mesenchimal transition, EMT.

The beneficial effects of antiplatelet drugs against the development of HCC in patients with NAFLD can be divided into two main mechanisms. On the one hand, the slowdown in the progression towards liver fibrosis and NASH (as previously described), and on the other a possible direct effect of platelets and their mediators on carcinogenesis mechanisms.

A recent work by Ma et al. demonstrated that the inhibition of the P2Y12 receptor on platelets can promote tumor growth via CD40L in mice with NAFLD [137]; however, there are conflicting data on this mechanism in the literature. Indeed, in the context of NAFLD and HCC, platelets can exert both pro-hepatocarcinogenesis function by driving NASH pathology, and anti-tumor activity against established tumors by driving CD8+ T cell responses (122-124, 138, 139).

Previous studies showed that platelets can enhance adaptive immunity [138,139], and activated platelets can release several molecules from α-granules, such as CD40L (Table 1). Several studies in literature showed CD40L to exert a role in anti-tumor immunity, and platelets are the main source of circulating CD40L [140]. Platelet-derived CD40L is greater released in both NAFLD mouse models and patients with NASH [137]. Moreover, TGF-β released from platelets can impair T cell anti-tumor function [141].

Table 1. Comparison between different evidences on the role of the CD40-CD40L pathway on carcinogenesis.

CD40 pathway ANTI-TUMOR ACTIVITY	CD40 pathway PRO-TUMOR ACTIVITY
CD40L is related to a strong CD8+ T cells responses through CD40 licensing of dendritic cell (61, 122, 124, 136, 137, 138, 146).	Platelet-derived CD40L is greater released in both NAFLD mouse models and patients with NASH [136]
Co-stimulation with CD40L-expressing dendritic cells (DC) significantly improves vaccination by inducing an early and strong Th1-shift in the tumor environment as well as higher tumor apoptosis [141].	In NAFLD patients CD40L protein production is induced in megakaryocytes rather than NAFLD or tumors causing a transfer of CD40L pre-mRNA or mRNA into platelets [145].
Platelet-derived CD40L can increase CD8+ T cell activation and their recruitment to liver in NAFLD (61, 122, 124, 136, 137, 138, 146).	IL-12 dependent increase of CD40L production in bone marrow megakaryocytes in NAFLD models [136,143]
	Megakaryocytes harbored in lung can produce higher CD40L amount [136,144]
	Inhibition of the P2Y12 receptor on platelets can promote tumor growth via CD40L in mice with NAFLD [136]

A recent study in mice by Vogt et al showed that intratumoral co-stimulation with CD40L-expressing dendritic cells (DC) significantly improves vaccination with murine (m) alpha-fetoprotein (AFP)-transduced DC in pre-established HCC *in vivo* [142]. Combined therapy induced an early and strong Th1-shift in the tumor environment as well as higher tumor apoptosis, leading to synergistic tumor regression of HCC [142].

Different platelet inhibition strategies can affect platelet CD40L release differently, causing opposite effects on HCC tumor growth in NAFLD [137]. Actually, Santilli et al previously showed

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TX-dependent CD40L release in patients with T2DM, showing dose-dependent inhibition of CD40L circulating levels upon aspirin administration and after slow recovery after aspirin withdrawal [56].

Indeed, COX inhibition fails to block platelet CD40L release depending on internal Ca2+ and protein kinase C but not involving tyrosine kinases, extracellular signal-regulated kinase (ERK) or p38, and COXs are downstream of ERK and p38 during platelet activation [143]. Therefore, P2Y12 inhibition related beneficial effect on HCC development in NAFLD may not be found using COX or phosphodiesterase 3 (PDE3) inhibitors [137]. Blocking platelet aggregation while sparing CD40L release using non-P2Y12 inhibitors such as aspirin may be more suitable for HCC patients with NAFLD [61].

Recent data demonstrated an IL-12 dependent increase of CD40L production in bone marrow megakaryocytes in NAFLD models [137,144]. Particularly, macrophages can induce an increase of IL-12 in the liver and CD40L production from bone marrow megakaryocytes [137]. A recent work demonstrated that the expansion of hepatic dendritic cells in NASH is induced by increased production of dendritic cell progenitors inside the bone marrow and enhanced liver recruitment [145]. Moreover, megakaryocytes harbored in lung can produce higher CD40L amount [137,145]. Intriguing, the lung can potentially contribute to liver tumor regulation in NAFLD by modulating platelets and CD40L release.

Moreover, data from a recent study on obese patients suggested that in NAFLD patients CD40L protein production is induced in megakaryocytes rather than NAFLD or tumors causing a transfer of CD40L pre-mRNA or mRNA into platelets [146].

Eventually, CD40L is related to a strong CD8+ T cells responses through CD40 licensing of dendritic cells [147]. Platelet-derived CD40L can increase CD8+ T cell activation [137] and their recruitment to liver in NAFLD [61]. Notably, to depletion of CD8+ T cells has been showed to decreased antiplatelet beneficial effect on HCC development in NAFLD, thus suggesting that the anti-HCC platelet function is at least in part mediated by CD8+ T cells [137,148].

7. Implications for Antiplatelet Therapy in Primary Prevention

The use of antiplatelet therapy in primary prevention has been scaled down in recent years on the basis of 3 large trials which highlighted a greater risk of bleeding complications compared to a benefit in terms of reduction of cardiovascular events (149-151). Subsequent works and expert opinions reworked this type of evidence, thereby suggesting that in patients at higher cardiovascular risk (e.g. T2DM patients) the benefit of antiplatelet therapy exceeds the bleeding risk [152]. Nowadays, several international guidelines report different types of indications for the use of antiplatelet therapy in primary prevention, thus creating heterogeneity in prescription methods [153].

The recent “DCRM Multispecialty Practice Recommendations for the management of diabetes, cardiorenal, and metabolic diseases” argued that the use of aspirin is not generally recommended for patients without a history of ASCVD or other risk factors [154]. The Task Force believes prescribing aspirin for patients with two or more cardiovascular risk factors (i.e., advanced age, elevated non-HDL-C, elevated LDL-C, low HDL-C, diabetes, hypertension, CKD, cigarette smoking, family history of ASCVD, elevated CAC score > 100) may benefit those who are not at increased risk of bleeding [154].

Nowadays, the prescription of antiplatelet therapy in patients with MASLD is based mainly (solely) on the evaluation of cardio-metabolic comorbidities, with no reference to the degree of hepatic alteration; this is due to the lack of strong evidence and adequate studies.

A recent work randomized two thousand four hundred participants with NASH to a strategy with polypill containing aspirin, atorvastatin, hydrochlorothiazide, and valsartan vs a control group with the standard or care, with a 5-years follow-up [144]. As expected, the adjusted relative risk of major cardiovascular events in participants with NASH (0.35, 95% CI 0.17-0.74) was under half that for participants without NASH (0.73, 95% CI 0.49-1.00), even if the difference did not reach statistical significance [155]. In this study, NAFLD patients demonstrated a significant decrease in liver enzymes after 60 months of follow-up (intragroup -12.0 IU/L, 95% CI -14.2 to -9.6) [155].

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Furthermore, growing evidence in the literature refers to an inter-individual variability in response and efficacy to the use of antiplatelet therapy, particularly in some categories of subjects with specific comorbidities [156,157]. More specifically, antiplatelet therapy can be less effective in patients with a faster recovery of platelet COX-1 activity during the 24-hour dosing interval [157].

A recent study by Simeone et al. showed that patients with accelerated COX-1 recovery are characterized by reduced platelet glycoprotein (GP)$_{Ib\alpha}$ shedding, and this can contribute to higher thrombopoietin (TPO) production and higher rates of newly formed PLT, escaping aspirin inhibition over 24 hours [158]. Intriguing, NAFLD and visceral obesity have been identified among clinical markers, together with younger age and higher TPO/GC ratio, predictive for the likelihood of faster COX-1 recovery and suboptimal aspirin response [158].

8. Conclusions

Growing data in literature support future research on the use of an antiplatelet agent as a new treatment for MASLD. Nowadays, there are limited data from prospective studies on the effects of aspirin in patients with MASLD. Most studies available on the association between antiplatelet drugs and NAFLD prevalence are observational. Prospective studies and randomized and controlled clinical trials are necessary to determine if antiplatelet therapy can prevent or delay the onset or progression of NAFLD, whether this effect is restricted to certain population subgroups, and what type and dose range of antiplatelet agent is most effective. The use of antiplatelets drugs seems to have potential clinical implication for the prevention of HCC patients with MASLD, since platelets contribute to fibrosis progression and cancer development. Therefore, MASLD patients may require anti-platelet agents to prevent cardiovascular diseases, fibrosis progression and cancer development.

Conflicts of Interest: authors declare no conflict of interest.

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