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Immuno-Thrombotic Effects of Platelet Serotonin

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Abstract

Platelets transport and store serotonin at a high concentration in dense granules and release it upon activation. Abnormal serotonin concentrations in the blood plasma or increased platelet serotonin release promote the development of thrombosis, sepsis, allergic asthma, myocardial infarction, and stroke. Consequently, experimental data suggest possible benefits of serotonin receptor blockade or inhibition of platelet serotonin uptake in the indicated human diseases. Here, we highlight the current state of basic biological research regarding the role of platelet serotonin in normal and pathophysiological conditions focusing on thrombotic and inflammatory diseases. We also describe the possible clinical applicability of targeting thrombo-immune-modulatory effects of platelet serotonin to treat common health problems.

Keywords: platelets, serotonin, inflammation, thrombosis, selective serotonin reuptake inhibitors

1. Introduction

Serotonin (5-HT) is a well-known neurotransmitter, which regulates neural activity and a variety of neuropsychological processes [1]. As it has been shown to be involved in the regulation of systemic and cellular functions, alterations in serotonin concentration in the body are associated with many different diseases, such as irritable bowel syndrome, restless legs syndrome, sudden infant death syndrome, autism, headache, insomnia, anxiety, depression, anorexia, schizophrenia, Parkinson's and Alzheimer's disease, pulmonary hypertension, and

Figure 1. 5-HT biosynthesis and receptor distribution in brain (A) and periphery (B). Serotonin (5-HT), serotonin transporter (SERT), monoamine oxidase (MAO), 5-hydroxyindole acetic acid (5-HIAA), 5-hydroxytryptophan (5-HTP), tryptophan (TRP), and vesicular monoamine transporter (VMAT). For the details, see the text.

myocardial infarction. 5-HT was first described in 1930 by Vitttorio Ersparmer who isolated it from enterochromafin cells of the gut [1]. Only a small amount of 5-HT is synthesized in brain (5%), whereas 95% is produced by the enterochromafin cells of the gastrointestinal (GI) tract. 5-HT is synthesized from the essential amino acid L-tryptophan (TRP) to 5-hydroxytryptophan (5-HTP) by the enzyme L-tryptophan hydroxylase (TPH)-1 in the brain and

TPH-2 in the periphery [2, 3]. The activity of these TPH enzymes is the rate-limiting step in the production of 5-HT in both organs. After its synthesis in the gastrointestinal tract, 5-HT is released into the bloodstream. 5-HT can bind and activate several isoforms of 5-HT receptors expressed throughout the body (**Figure 1**). 5-HT receptors were identified on different blood cells and in the vessel wall including lymphocytes, endothelial, and smooth muscle cells, respectively, which can respond to 5-HT under certain physiological conditions. These receptors constitute a family of seven different receptor sub-classes: 5-HT₁ (A-F, P, S), 5-HT₂ (A-D), 5-HT₃, 5-HT₄, 5-HT₅, 5-HT₆, and 5-HT₇ [3, 4]. All these receptors belong to the GPCR superfamily with the exception of 5-HT₃ [3], which is a member of nicotinic acetylcholine receptor superfamily and is a ligand-gated ion channel.

5-HT can also be taken up from plasma into several cells—such as platelets—via the 5-HT transporter (5-HTT, SERT). After uptake, 5-HT can be then stored in vesicles and granules through the action of vesicular monoamine transporter (VMAT)-1/2 which is expressed in neurons, neuroendocrine cells, and platelets. The largest quantity of serotonin is believed to be stored in platelets, from where it can be released upon platelet activation, for example, during thrombus formation or inflammatory reactions. Interestingly, chemical precursors of 5-HT can pass across the blood-brain barrier, but 5-HT cannot, thereby effectively isolating the brain 5-HT pool from the periphery and vice versa. In the brain, 5-HT regulates several complex networks, such as mood, perception, reward, anger, memory, appetite, attention, and sexuality. There are two major routes of 5-HT metabolism, which convert 5-HT to melatonin and 5-HIAA. 5-HT is metabolized by neurons and endothelial cells by monoamine oxidases (MAOs) and the products of this breakdown are then excreted by the kidney [3, 5–7].

Peripheral 5-HT regulates heart development and rate, valvulopathy, pain, nociception, embryonic development, vasoconstriction/vasodilatation, blood flow, hemostasis, and many other important processes. Platelets are not able to synthesize serotonin, but take it up from plasma via 5-HTT, store it in dense granules (via VMAT-1), and release it into the blood during their activation. Platelet serotonin has not only well-established autocrine functions during platelet activation and thrombus growth but also paracrine functions in the vasculature including modulation of endothelial, smooth muscle, and immune cell function.

2. Autocrine-regulatory mechanisms of platelet serotonin

Platelets store 5-HT in their dense granules at millimolar range and secrete it after activation [8]. Dense granule and 5-HT release support the recruitment of circulating platelets to preformed thrombi, thereby leading to thrombus growth. This process is mediated through the interaction between 5-HT and its receptor 5HT_{2A} expressed on circulating platelets. Activated 5-HT_{2A} receptor transduces the signal to G_q-phospholipase C (PLC) β -signaling cascade. Enhanced PLC β activity results in intracellular Ca²⁺ mobilization from the store through inositol 3-phosphate (IP3) receptor and mediates 1,2-diacylglycerol (DAG)-dependent protein kinase C (PKC) activation, thereby amplifying platelet reactivity (**Figure 2**).

In addition to the mobilization of cytosolic Ca²⁺ [9, 10], receptor-ligand interactions are also known to regulate 5-HT uptake kinetics. In human platelets, the rise of cytoplasmic Ca²⁺ in

Figure 2. Autocrine effects of platelet 5-HT. Activated platelets release 5-HT, thereby amplifying platelet activation and the recruitment of circulating platelets. Binding of platelet 5-HT to the 5-HT_{2A} receptor induces activation of $\text{PLC}\beta$ -signaling cascade and upstream effectors which support platelet reactivity. Receptor-ligand interactions also regulate 5-HTT uptake kinetics by interconnecting several signaling pathways. For the details, see the text.

the absence of exocytosis reduces 5-HT transport into the cytoplasm, thereby decreasing the release of 5-HT [9]. Interestingly, rabbit platelets activated in the presence of the extracellular Ca^{2+} chelator ethylene tetraacetic acid also displayed a decrease in 5-HT transport activity [11, 12]. Consistently, human platelets treated with the membrane permeant Ca^{2+} chelator BAPTA-AM also had reduced 5-HT transport in the presence of extracellular Ca^{2+} [9]. Activation of the Orai1 Ca^{2+} channel induces a robust Ca^{2+} influx called store-operated Ca^{2+} entry (SOCE), which is triggered through the release of Ca^{2+} from intracellular stores.

This process is controlled by functional coupling of activated stromal interaction molecule 1 (STIM1) to Orai1 [13]. Interestingly, strongly reduced SOCE was found in *5HT^{-/-}* platelets [14]. This suggests that secreted platelet 5-HT contributes to the regulation of SOCE through binding to 5-HT_{2A} which activates Gq-PLC β -mediated Ca²⁺ store release, thereby further activating STIM1/Orai1 complex. Interestingly, SOCE-induced signal can strongly inhibit 5-HT uptake in human platelets via 5-HTT [9, 11]. This could be an important step to keep 5-HT outside of platelets, thereby increasing extracellular 5-HT concentration and permanently activating 5-HT_{2A} on the platelet surface. Therefore, 5-HT cannot enter the platelet cytosol during SOCE. Interestingly, 5-HTT contains several consensus sites for PKC. It has been shown that PKC activity is required for the internalization of the transporter suggesting a link between 5-HT uptake and intracellular Ca²⁺ level [15–18]. Altogether, Ca²⁺ signaling, Ca²⁺ store release, and Ca²⁺ influx through SOCE play an important regulatory role for 5-HT cycling in human and mouse platelets.

After Ca²⁺ store release and PKC activation, integrins exposed and activated on the platelet surface support aggregation and thrombus formation. In β 3 integrin-deficient platelets, 5-HT uptake was strongly reduced, indicating a functional crosstalk between 5-HTT and β 3 integrin [19]. Integrin activation defect in response to glycoprotein VI (GPVI) or C-type lectin-like receptor 2 (CLEC-2) stimulation was found in *5HT^{-/-}* mouse platelets, which was fully rescued in the presence of extracellular 5-HT [14]. The physical interaction between 5-HTT and β 3 seems to be dispensable for β 3 integrin activation. The observed integrin activation defect is due to the lack of the secreted platelet 5-HT which further amplifies “inside-out” activation of integrins through Ca²⁺-dependent and independent pathways mediated by Ca²⁺- and DAG-regulated guanine exchange factor-1 (CalDAG-GEFI) and PKC, respectively.

Although 5-HT is mainly stored in dense granules, intracellular-free 5-HT in the cytoplasm has been proposed to activate diverse biological processes called serotonylation. It has been shown that small-guanosine triphosphate-binding protein (GTP)-ases covalently bind 5-HT, thereby changing the structure and activity of GTPase, leading to α -granule exocytosis from platelets. This process requires tissue transglutaminase and factor XIIIa, both activated by mobilized Ca²⁺. Transglutaminase may mediate the transamidation of small GTPases, like cytoplasmic Ras homolog gene family member A (RhoA) and a small GTP-binding protein Rab4. Serotonylation in turn blocks the inactivation of both molecules. A complex composed of Ca²⁺ and calmodulin (CaM) may also activate guanine exchange factors (GEFs), which induce the exchange of guanosine di- (GDP) to triphosphate (GTP) on RhoA and Rab4 and thus stimulates activation of the respective protein. These two active molecules play an important role in cytoskeleton rearrangement, exocytosis of α -, and dense granule contents. Some bioactive molecules stored in platelet granules, such as fibrinogen and factor V, are also known to be serotonylated [8]. Upon platelet activation, these proteins are exposed at the platelet surface and are used to mark a subpopulation of highly activated, pro-coagulant platelets, the so-called collagen and thrombin-activated (COAT) platelets. Coated platelets express high levels of phosphatidylserine and strongly support prothrombinase activity [8, 20].

Besides the dopamine transporter (DAT), the noradrenaline transporter (NET), and the organic transporter (OCT), 5-HTT is an important 5-HT transporter to regulate 5-HT uptake from the blood plasma and reuptake of the released platelet 5-HT in certain physiological

conditions. 5-HTT is encoded by the SLC6A4 gene containing 14 exons. The protein structure of 5-HTT contains 12 transmembrane domains. In humans, the splice variants of 5-HTT and their mutations are associated with several pathologies, such as anxiety, suicide, depression, substance abuse, autism, and neurogenic disorders [21–24]. 5-HTT is abundantly expressed not only on neurons, endothelial cells, mast cells, immune cells, in intestine, and vasculature, but also in platelets [25, 26]. It is well established that in platelets 5-HTT plays an important role in the uptake of 5-HT from the circulation. Monoamine transporters are thought to be able to compensate for one another where they are co-expressed. For example, 5-HT may be taken up in venous vessels independently of 5-HTT expression [25, 27]. Interestingly, and in sharp contrast to venous vessels, genetic ablation of *5Htt* in mice completely abolished 5-HT uptake in platelets, since no detectable secreted 5-HT was observed upon platelet activation, indicating an essential role of 5-HTT for 5-HT uptake into platelets, which cannot be compensated by other transporters [14]. Altogether, these results highlight the cell-type-specific regulation of 5-HT uptake in mammalian cells.

5-HTT can be targeted by several antidepressants, such as selective serotonin reuptake inhibitors (SSRIs) (cf., Section 5), which are widely used in the treatment of psychiatric diseases to increase 5-HTT concentrations in the synaptic space. The blockade of 5-HTT with the SSRI citalopram reduces the aggregation response to collagen in human platelets [28] due to reduced phosphorylation of a tyrosine-protein kinase Syk in the GPVI signalosome. Syk can also bind and phosphorylate 5-HTT suggesting an Syk-mediated functional crosstalk between 5-HTT and GPVI complex. Interestingly, *5Htt*^{-/-} mouse platelets could not show any abnormalities in the tyrosine phosphorylation cascade of the GPVI signalosome, as Syk phosphorylation was normal after GPVI stimuli. Consequently, Syk and 5-HTT interaction seems to be dispensable for the initial activation of GPVI complex, but enhanced Syk activity may regulate the 5-HT uptake in platelets [29].

3. Paracrine-regulatory mechanisms of platelet serotonin

During degranulation, activated platelets secrete a significant amount of 5-HT from dense granules which is clinically relevant to induce acute thrombotic events [30, 31] by promoting vasoconstriction and cellular activation of neighboring platelets and lymphocytes through their 5-HT receptors.

5-HT receptors expressed on endothelial, smooth muscle, and immune cells respond to platelet-derived 5-HT (**Figure 3**). 5-HT has growth-promoting effects on endothelial cells, which may facilitate tissue healing after vascular damages [32]. However, 5-HT may also exert dual effects either stimulating constriction or dilatation of microvasculature. In the liver, 5-HT appears to mainly promote constriction of hepatic sinusoid vessels, since mice lacking peripheral 5-HT display elevated sinusoidal perfusion under physiological and pathological conditions [33]. By contrast, platelet-derived 5-HT coordinates the formation of gaps between endothelial cells in the joint microvasculature, which in arthritic conditions may contribute to inflammation [34]. How these processes are regulated is still not clear but presumably may involve differential signaling pathways through specific 5-HT receptors expressed on vascular endothelial and smooth muscle cells.

Figure 3. Paracrine effects of platelet 5-HT. Secretion of platelet 5-HT modulates the function of endothelial and smooth muscle cells either promoting vessel constriction or dilatation. Platelet 5-HT influences several functions of immune cells, indicating their importance in the regulation of immune cell response and activities under pathophysiological conditions. For the details, see the text.

Platelet-derived 5-HT can regulate the function of T- and B-cells, natural killer cells, monocytes, and neutrophils under certain conditions [35–38]. In the spleen, 5-HT increases monocyte differentiation into dendritic cells and early naive T-cell activation via the 5-HT_{2A} receptor [38, 39]. Furthermore, it also has been shown that lymphocytic cytokine levels in mice are reduced after treatment with SSRI [40]. In a mouse model of viral hepatitis, the release of 5-HT by platelets was responsible for tissues damage caused by CD8 (+) T-cells, microcirculatory events, and reduced clearance of infiltrated viruses [33]. Moreover, specific antagonism of 5-HT receptors in mice attenuated asthmatic attacks and sepsis [37, 41].

5-HT released from dense granules upon activation by the inflamed endothelium also contributes to the recruitment of immune cells to the vascular wall [37]. Indeed, platelet-derived 5-HT promotes leukocyte migration, possibly via activation of endothelial cells, thereby enhancing P-selectin exposure and IL-8 release [37], which trigger neutrophil rolling, adhesion, and extravasation. Moreover, locally increased levels of platelet-released 5-HT had paracrine effects on endothelial cells, thereby inducing microvasculature leakage through the activation of transglutaminase and the phosphorylation of vimentin [42]. By contrast, in solid tumors platelet-released 5-HT has been described as a major regulator of the tumor vascular homeostasis that continuously prevent bleeding. Interestingly, tumor-infiltrating leukocytes have been identified as the cause of tumor bleeding [43, 44]. Altogether, these studies suggest that under specific conditions, platelet-released 5-HT promotes clot formation and modulates immune cell functions.

In humans, 5-HT levels appear elevated in infection and autoimmune diseases, suggesting that SSRI could be applicable for vascular and immune system modulation. Since platelets are the major 5-HT store in the blood, pharmacological blockage of 5-HT uptake in platelets increases the level of 5-HT in the blood plasma transiently. Unexpectedly, *5HT^{-/-}* mice display reduced 5-HT levels in plasma [14]. In *5HT^{-/-}* mice, elevated urinary 5-HIAA levels were detected suggesting a faster 5-HT metabolism in the peripheral blood. Consequently, platelet 5-HT uptake and storage play an important regulatory role for controlling systemic 5-HT metabolic cycles. Future studies are needed to specify the exact mechanisms of platelet-derived 5-HT on vascular and immune system modulation in normal physiology and diseases.

4. Pathophysiological consequences of abnormal platelet serotonin release

5-HT plasma concentration was analyzed in several pathological contexts. It became widely recognized that 5-HT is an independent risk factor for platelet aggregation and for thrombus formation in animal models (cf., Section 6) and human patients [19, 45–49]. Plasma 5-HT can support platelet aggregation and thrombus growth through 5-HT_{2A}-dependent or independent signaling pathways. Pharmacological blockade of 5-HT_{2A} receptor increases the 5-HT uptake rates in animal models of hypertension, as well as ex vivo platelet aggregation. Vikenes et al. detected a 10-fold increase of plasma 5-HT in patients undergoing angiography after admission for myocardial infarction [50]. In these patients, high plasma 5-HT was associated with cardiac events. In another study, more than 10-fold rise in 5-HT has been noticed in coronary vessels of patients following angioplasty. Importantly, in these patients the level of 5-HT in the systemic plasma was normal [51]. Together, these studies suggest that in vivo the interplay between circulating 5-HT and platelet function could be a predictive factor.

5-HT levels are drastically increased during myocardial ischemia, and blockade of the 5-HT₂ receptor improves the outcome after myocardial infarction in different mouse models [52, 53]. 5-HT also enhances the survival of cardiomyocytes via the 5-HT_{2B} receptor. In hepatic ischemia models, platelets promote tissue repair [54], and proliferation of hepatocytes was shown to be partly mediated by platelet 5-HT after liver ischemia [55]. 5-HT also contributes to intratumoral homeostasis by dysbalancing permeability factors [44]. 5-HT-induced growth of human hepatocellular carcinoma cells and specific blockade of the 5-HT₂ receptor decreased recruitment of circulating tumor cells [56, 57]. It has been suggested that the inhibition of platelet granule contents might be effective to induce intratumoral bleeding, thereby decreasing tumor viability and growth. Additionally, plasma 5-HT levels are increased in patients with colorectal, liver, and intestinal cancers [58, 59].

Allergic airway inflammation provokes a local release of 5-HT in mouse models and human patients [41]. Interestingly, after challenge with an allergen, 5-HT increased 10-fold in broncho-alveolar lavage of predisposed patients, inducing asthmatic attacks. In line with these studies, 5-HT is known as a key regulator of pulmonary vascular resistance and vessel wall integrity [60, 61].

5. Clinical applications: effects of selective serotonin reuptake inhibitors on platelet functions

Selective serotonin reuptake inhibitors are commonly used drugs for the treatment of patients with severe depressive and anxiety disorders [62]. SSRIs were developed to selectively inhibit the uptake of 5-HT through the 5-HTT transporter in the brain, while having minimal side effects on DAT and NET proteins which can also transport 5-HT [63]. The action of SSRIs relies on the modulation of the allosteric region of the transporter, thereby leading to a conformational change and blocking of the uptake of 5-HT [63]. The uptake of 5-HT into neurons is very important for the clearance of the synaptic cleft, preventing firing rates and overstimulation of receptors [64]. This uptake and the later release are blocked upon treatment with SSRIs, such as fluvoxamine, fluoxetine, nortryptiline, citopram, and escitalopram [65]. The different SSRIs vary in kinetics being competitive and non-competitive inhibitors. Two distinct binding sites on 5-HTT have been identified, a low-affinity allosteric site, mediating the dissociation of SSRIs from their high-affinity site, which induces the blockade of 5-HT uptake [64].

There is evidence that targeting 5-HT receptors or using serotonin-like molecules is effective in the treatment of non-neuronal diseases. The use of tricyclic antidepressants, but not SSRIs, is associated with an increased risk of myocardial infarction. SSRIs have shown no cardiac toxicity, even in patients with heart disease. Several epidemiologic studies reported lower cardiovascular morbidity and mortality in patients treated with SSRI [66–68].

Depression is a significant risk factor for ischemic heart and cerebrovascular disease as well as mortality following myocardial infarction. The potential effects of SSRIs upon the cardiovascular system may therefore play an important role. These drugs had potential benefit in hypertensive patients after myocardial infarction and hypertensive responses to depression were reduced in patients who had been prescribed SSRIs [30]. In blood samples of depressive patients taking fluoxetine, the platelet aggregation response to submaximal collagen stimulation was decreased [69]. In this study, a significant decrease in 5-HT concentration was observed in platelet-rich plasma associated with the use of fluoxetine but not with the tricyclic antidepressant amitryptiline. It is intriguing whether lowered platelet 5-HT content translates into less 5-HT release during platelet activation in patients with thrombotic diseases. Enhanced platelet reactivity was observed in patients suffering from depression and chronic heart disease due to the upregulated β -thromboglobulin (β -TG) and platelet factor 4 (PF4) levels [70]. Lowered PF4 and β -TG levels have been observed upon treatment with SSRI paroxetine [71], suggesting that reduced platelet aggregation *in vivo* may impact coronary artery-related mortality. SSRI treatment also decreases platelet reactivity in patients with heart failure. Other SSRIs, sertraline, and N-desmethylsertraline were also shown to dampen platelet responses [72].

SSRIs have been shown to increase the risk of bleeding in patients with liver cirrhosis and liver failure. Importantly, SSRIs may also directly increase gastric acidity with ulcerogenic effect resulting in GI bleeding. The risk of SSRI-associated GI bleeding is increased with the concurrent use of nonsteroidal anti-inflammatory drugs, anticoagulants, and antiplatelet agents,

and is decreased by concurrent proton pump inhibitors [73, 74]. In conclusion, SSRIs appear to be protective against cardiovascular diseases and may enhance the risk for GI bleeding. However, to date this evidence is not yet conclusive.

6. Experimental studies on the role of platelet serotonin in arterial thrombosis and stroke

Over the past decades, the functions of peripheral 5-HT have received increasing attention. It has been shown that peripheral 5-HT plays a major role in a variety of important processes, including hemostasis and immune defense. This has been addressed by using *Tph1*^{-/-} mice, which lack peripheral 5-HT in the circulation, due to the lack of the enzyme that converts hydroxylases tryptophan to 5-HT in the gut [75]. In humans, abolished or decreased level of TPH1 is associated with impulsive behavior, aggression, irritable bowel syndrome, anxiety, and other pathologies [76–79]. Genetic ablation of TPH1 function in mice not only leads to several disorders, such as mild anemia, cardiomyopathy, and diabetes, but also to other defects in hemostasis, erythropoiesis, pulmonary hypertension, and lung regeneration. The lack of 5-HT in this mouse model is associated with decreased neutrophil recruitment to inflammatory sites, diabetics, and mild anemia [37, 80].

Recent studies using wild-type mice infused with 5-HT or *Tph1*^{-/-} mice have demonstrated that peripheral 5-HT is required for platelet aggregation [14]. Additionally, *in vivo* 5-HT infusion generates hyperreactive platelets with reduced bleeding time and shortened occlusion time of the carotid arteries in wild-type mice. *5Htt*^{-/-} mice have prolonged bleeding time, reflecting the increased bleeding risk described to occur using long-term SSRI treatment in human patients. In comparison to this relatively mild hemostatic defect, *5Htt*^{-/-} mice were not able to form occlusive thrombi in response to mechanical injury of the abdominal aorta as compared to wild-type animals [14].

Platelets contribute to the progression of infarct growth after transient brain ischemia by thrombo-inflammation with platelet-immune cell interactions. SSRI treatment of stroke patients has been described to enhance brain function recovery, indicating a therapeutic benefit of the direct blockade of 5-HTT function. Neuroblast proliferation and cell migration have been shown to be enhanced and associated with increased microvessel density during SSRI treatment, explaining the possible role of 5-HTT in tissue repair after ischemic insults [81–83]. *5Htt*^{-/-} mice have been studied in the tMCAO (transient intraluminal filament model of middle cerebral artery occlusion) model of ischemic stroke. Unexpectedly, these mice developed similar brain infarcts to wild-type controls and the 5-HTT neurological outcome was indistinguishable [14]. In line with this study, SSRI treatment could not reduce infarct size or cerebral edema in mice [82], suggesting that this treatment cannot protect neurons or other cells in the ischemic brain. Altogether, these results indicate that SSRI treatment may have a long-term effect in the ischemic brain tissue which positively influences post-stroke recovery. Further investigation is necessary to understand the specific role of peripheral and brain 5-HT in thrombo-inflammation during stroke and infarct progression.

7. Conclusions

5-HT is an ancient molecule that is better known for its functions in the brain than in the periphery. However, literature describing the contribution of peripheral 5-HT, including platelet 5-HT, is rapidly growing. It became evident that platelet 5-HT has a complex role involving many bidirectional interactions with tissue microenvironment to regulate platelet and immune cell functions. SSRI treatment in animal models appears to improve thrombotic and inflammatory diseases. Further fundamental and preclinical studies are needed for a better understanding of platelet 5-HT functions in humans. In conclusion, targeting thrombo-immune-modulatory functions of platelet serotonin may provide new important therapeutic approaches.

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