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Role of the serotonin transporter and the 5-HT2A and 5-HT4 receptors for platelet function in blood

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Dedicated to my parents

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1. State of the art

1.1 Introduction: Serotonin, the happiness hormone

Serotonin, also known as 5-hydroxytryptamine (5-HT), is a molecule well known for its positive influence on the mood and its protective properties against the onset of various mental and physical disorders (1). Therefore it is popularly known as the "happiness" or the "happy" hormone.

5-HT is a monoamine synthesized in the serotonergic neurons of the central nervous system and the enterochromaffin cells of the gastrointestinal tract (2-4). It binds and activates G-protein coupled 5-HT receptors. These are diverse, and ubiquitously expressed in the human organism, and possess a wide range of functions (2, 5, 6). This is not surprising since 5-HT is probably one of the oldest neurotransmitters/hormones in the evolution of life (6). In the last 20 years, seven families of 5-HT receptors have been identified and various subtypes have been described. The increasing number of 5-HT receptors and the lack of suitable selective agents, have made it difficult to unravel the roles of the 5-HT receptor subpopulations(5).

Since 5-HT receptors are widely expressed in the central nervous system (CNS), 5-HT takes part in plentiful brain functions (7, 8). Until now 5-HT has established its role in significant psychiatric disorders, such as depression, anxiety, obsessive-compulsive disorder, eating disorders, nausea and centrally-induced emesis, dependence, cognitive disorders and migraine (2, 5, 7, 9).

In the periphery 5-HT is involved in numerous physiological functions. These include hypoglycemic effects, stimulation of lipid accumulation, gastrointestinal motility, intestinal secretion, inflammation, liver regeneration, uterine contraction, pulmonary artery remodeling, vascular tone and permeability, platelet aggregation, and chronotropic and inotropic myocardial effects (2, 7, 10-14).

The understanding of the 5-HT related physiological functions are of clinical importance, especially with regard to the treatment of depressive disorders, irritable bowel disease and chemotherapy–induced nausea with medications that inhibit 5-HT re-uptake and 5-HT receptors (7). Further clinical importance relates to the association of peripheral 5-HT with autoimmune diseases, liver diseases, and asthma, conditions which 5-HT-modifying medication might target in the future (15-20).

1.2 Biosynthesis and degradation of 5-HT

5-HT is a common monoamine in the human body. 5-HT is produced by enterochromaffin cells of the gastrointestinal tract and part of it is secreted into the blood. Then, 5-HT is taken up via the serotonin transporter (SERT) and stored in platelets and pulmonary neuroepithelial bodies. 5-HT is also produced by the serotonergic neurons, which almost exclusively originate in the raphe nuclei located in the midline of the brainstem (3, 4, 7).

5-HT is synthesized by initial hydroxylation of the essential amino acid L-tryptophan. This first step is also the rate-limiting step in 5-HT biosynthesis. L-tryptophan is transported through the blood-brain barrier by the neutral amino acids transmitter, on which it competes with other amino acids such as phenylalanine,

leucine and methionine. Tryptophan hydroxylase catalyzes the conversion of tryptophan into 5-hydroxytryptophan. Two isoforms of tryptophan hydroxylase can be distinguished, one expressed in the brain in serotonergic neurons and the other one in the periphery (2, 5, 21). 5-hydroxytryptophan is then further converted to 5-hydroxytryptamin (5-HT) by the aromatic L-amino acid decarboxylase (Figure 1.1) (2, 5).

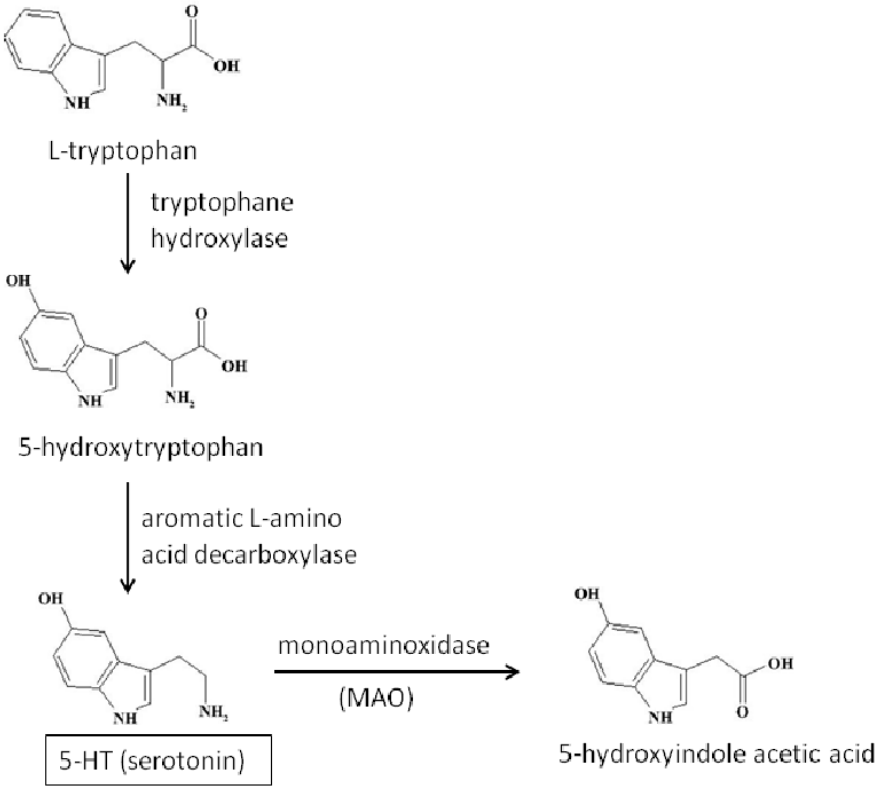

Figure 1.1: Biosynthesis and degradation of serotonin.

5-HT is metabolized by the monoaminoxidase enzyme (MAO) into 5-hydroxyindole acetic acid (5-HIAA) by oxidative deamination. Two types of MAO, MAO-A and MAO-B exist in neurons, but only MAO-B in platelets. Circulatory 5-HT is

metabolized mainly in the liver. Also the endothelium of pulmonary capillaries is able to degrade 5-HT. Brain 5-HIAA is actively transported into the periphery and secreted along with the 5-HIAA of non-CNS origin in urine (2).

Notably 5-HT is not able to pass the blood-brain barrier; thus the 5-HT systems of the CNS and the periphery are considered to be mutually independent (11).

1.3 5-HT transporter (SERT) and 5-HT receptors

The 5-HT transporter (**ser**otonin **t**ransporter; SERT) is a monoamine transporter consisting of 12 transmembrane regions (74 kDa). SERT demonstrates high affinity for the uptake of 5-HT (3, 22-24). SERT has been located on neuronal cells and takes part in regulating the strength and duration of the serotonergic signal transmission. SERT is also expressed in peripheral cells on the plasma membrane of platelets, lymphoblasts, monocytes, enterochromaffin cells, endothelial cells and placental syncytiotrophoblasts (22). Peripheral and neuronal SERTs are considered largely identical in structural and functional properties (23, 25).

5-HT interacts with a diversity of receptors which are mainly G-protein-coupled receptors (GPCRs). The 5-HT receptors are currently classified into 7 different families, some of them containing further subtypes (6). An overview of the 5-HT receptor classification, the coupling to G-proteins or ion channels, and the main signaling mechanisms is shown in Table 1.

Table 1: 5-HT receptor families

5-HTreceptor family	Subtypes	Coupling	Signaling mechanism
$5\text{-}HT_1$	$5\text{-}HT_{1A}$, $5\text{-}HT_{1B}$, $5\text{-}HT_{1D}$, $5\text{-}HT_{1E}$, $5\text{-}HT_{1F}$	$G_{i/0}$-protein coupled	Decreasing cellular cAMP levels via inhibition of adenylate cyclase activity
$5\text{-}HT_2$	$5\text{-}HT_{2A}$, $5\text{-}HT_{2B}$, $5\text{-}HT_{2c}$	$G_{q/11}$-protein coupled	Increases cellular levels of IP_3 and DAG via activation of phospholipase C
$5\text{-}HT_3$	$5\text{-}HT_{3A}$, $5\text{-}HT_{3B}$	Ligand-gated ion channel	Membrane depolarization via Na^+- and Ca^{++}-influx, K^+ efflux
$5\text{-}HT_4$	$5\text{-}HT_{4A}$-$5\text{-}HT_{4N}$ (at least 9 variants)	G_s-protein coupled	Increasing cellular cAMP levels via stimulation of adenylate cyclase activity
$5\text{-}HT_5$	$5\text{-}HT_{5A}$, $5\text{-}HT_{5B}$	$G_{i/0}$-protein coupled	Decreasing cellular cAMP levels via inhibition of adenylate cyclase activity
$5\text{-}HT_6$	$5\text{-}HT_6$	G_s-protein coupled	Increasing cellular cAMP levels via stimulation of adenylate cyclase activity
$5\text{-}HT_7$	$5\text{-}HT_{7A}$, $5\text{-}HT_{7B}$, $5\text{-}HT_{7C}$, $5\text{-}HT_{7D}$	G_s-protein coupled	Increasing cellular cAMP levels via stimulation of adenylate cyclase activity

Before the current knowledge on the role of 5-HT in platelets is presented, a short insight into the platelet morphology and function will be provided.

1.4 Platelets and platelet function

1.4.1 Platelet origin and morphology

Platelets originate from a type of large nucleated hematopoietic stem cells, the megakaryoblast, within the bone marrow. The mature megakaryocytes create long

cytoplasmic processes, designated as proplatelets, through junctions in the lining of blood sinuses. Platelets are released from the tips of these proplatelets into the circulation. Each megakaryocyte can release up to 1000-3000 platelets. Many aspects of platelet formation mechanisms by megakaryocytes are still poorly understood though (26, 27).

Platelets are small, 2-3 μm in diameter, anucleate disc-shaped cells. Normally about two thirds of platelets are present in the circulating blood at concentrations of 150.000-400.000 platelets/μl; one third of platelets is reversibly sequestered in the spleen. The circulating platelet population is replaced every 7-10 days. The site of platelet degradation is the reticulo-endothelial system of the liver and spleen (27, 28).

Platelets have a complex morphology consisting of distinct membrane systems, and cytoskeletal structures, and containing different granules and organelles.

The three distinct membrane systems are the following:

- The plasma membrane contains specific GPCRs and is densely packed with glycoprotein receptors which regulate platelet activation, adhesion and aggregation. (27-29).
- The open canalicular system (OCS) is connected to the cell surface and although its role has not been entirely defined, it possibly serves as (a) a mechanism for entry of external elements into the platelets, (b) a mechanism for release of the dense and α-granule constituents into the extracellular space, (c) a depository of surface glycoproteins and (d) an internal membrane reservoir for the formation of filopodia and spreading of platelets upon adhesion and activation (27).

- The dense tubular system is a closed channel network of residual endoplasmic reticulum, in which ionized calcium (Ca^{++}) is stored and released upon platelet activation (27, 30).

The resting platelet cytoskeleton maintains the integrity and shape of the platelet, even under high shear forces in the circulation, and consists of:

- The spectrin-based membrane skeleton, which supports the plasma membrane and the OCS (31).
- The actin cytoskeleton. Forty percent of actin is organized as F-actin into filaments, whilst the rest (G-actin) is stored in the cytoplasm and forms filaments upon platelet activation. Actin filaments bind with the glycoprotein Ib. They add to the stability of the platelet shape and regulate the topological distribution of surface glycoproteins in activated platelets (27, 28, 32).
- The marginal band of microtubules consisting of α- and β- tubulin, supports the discoid shape of resting platelets, and depolymerizes rapidly upon platelet activation (27).

The platelets contain also the following granules and organelles:

- The α-granules contain a diversity of biologically active proteins, which are involved in platelet adhesion and aggregation (vWf, thrombospondin), chemotaxis of other blood cells (β-TG, PF4), angiogenesis at sites of vascular injury (VEGF, endostatin), smooth muscle cell proliferation and migration (PDGF), inflammation (P-selectin, IL-1, CD40-ligand) and coagulation (factor V, fibrinogen, PAI-1) . Their diameter is typically 200-500 nm and upon platelet activation they merge with the plasma membrane or the OCS (27, 28).

- The dense granules are a little smaller in diameter (200 nm), and contain ATP, ADP, Ca^{++}, polymers of inorganic phosphate and, the main molecule of our study, 5-HT. They have also been reported to contain epinephrine. The secretion of dense granule constituents serves mainly as a feedback for platelet activation (27, 33-35).
- The lysosomes release degrading enzymes which may play a role in the restructuring of vessel walls after injury (27, 28).
- The peroxisomes contain the enzyme catalase (27).
- The mitochondria supply the platelet with energy during its 7-10 days lifespan (27).

1.4.2 Platelet adhesion

Platelets circulate in blood in a resting state and do not adhere to undamaged vessel walls (36). They are circulating monitors of the vessel integrity and at sites of vascular injury, they adhere to the exposed subendothelial extracellular matrix (28, 36, 37). The adhesion of the still resting platelets is known as primary adhesion, in contrast to the adhesion of already activated platelets, which is known as secondary platelet adhesion (28). Platelet adhesion occurs under venous and arterial flow conditions, with their lower and higher shear stress rates, respectively (36). At low shear rates (<500 s^{-1}), such as in veins and larger arteries, platelets adhere primarily to collagen, fibronectin and laminin. At higher shear rates (>1000 s^{-1}) as in smaller or stenotic arteries, the platelet surface glycoprotein Ibα (GPIbα), part of the glycoprotein Ib-V-IX complex, has a crucial role. Its high affinity interaction with von Willebrand factor (vWF), which binds to and is immobilized on collagen of the

subendothelial matrix, slows down the fast flowing platelets, enabling primary platelet adhesion (28, 36, 37). The reduced platelet velocity allows interactions with further platelet receptors, thereby stabilizing platelet adhesion. Such interactions occur between the platelet integrin $\alpha_2\beta_1$ and glycoprotein VI (GPVI) with collagen and between the integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIbIIIa ; GPIIbIIIa) with fibrinogen (38) (Table 2). Platelets can also adhere to vWf via $\alpha_{IIb}\beta_3$, to laminin via $\alpha_6\beta_1$, to fibronectin via $\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$, to thrombospondin via GPIba, to collagen via GPIV, and to vitronectin via $\alpha_v\beta_3$, interactions that perhaps regulate platelet adhesion to different compositions of extracellular matrix. Yet, the contribution of each of these interactions remains to be investigated (36, 37) (Table 2). Subsequently, platelets undergo activation leading to shape change, secretion, and spreading on the surface of the extracellular matrix, a process regulated mainly by the integrin $\alpha_{IIb}\beta_3$. This allows more extensive interaction of the platelet receptors with the extracellular matrix and comprises a mechanism to better withstand the shear forces of the flowing blood (28, 37).

Table 2: Main integrins and glycoproteins, their ligands and copies per platelet.

Name	Ligand	Copies per platelet	References
$\alpha_{IIb}\beta_3$	fibrinogen (vWf, fibronectin)	~ 60.000-100.000 (part of this population is stored in internal pools and is externalized upon platelet activation)	Gawaz(28), Michelson(39)
$\alpha_2\beta_1$	collagen (vWf)	~ 1000-4000	Gawaz(28), Broos et al.(36)
$\alpha_5\beta_1$	fibronectin	~ 1000	Gawaz(28)
$\alpha_6\beta_1$	laminin	~ 1000	Gawaz(28)
$\alpha_v\beta_3$	vitronectin	~ 100	Gawaz(28)
GP Ib/V/IX complex	vWf	~ 25.000	Gawaz(28)
GP IV	collagen	~ 15.000-25.000	Gawaz(28)
GP Iba	thrombospondin	~ 25.000	Gawaz(28)
GP VI	collagen	~ 1000-9000	Burkhardt et al. (40), Nieswandt et al.(41)

1.4.3 Platelet activation and aggregation

After platelet deceleration by the GPIb-vWF- interaction, the binding of GPVI and integrin $\alpha_2\beta_1$ to collagen triggers specific signaling pathways leading to platelet activation (42-44). The mild bleeding diathesis of patients with GPVI or integrin $\alpha_2\beta_1$ deficiency might indicate that these two activation pathways are mutually independent, rather than the dependence of integrin $\alpha_2\beta_1$ activation on GPVI, although this is a matter of controversy (36). GPVI is more efficient in signaling than the integrin $\alpha_2\beta_1$. Signaling includes the activation of Src-family and Syk-tyrosine kinases and leads to an explosion of activation events. These include the phospholipase Cγ2 (PLCγ2)-mediated increase of IP_3 (inositol 1,4,5-trisphosphate) and DAG (diacylglycerol) leading to cytosolic Ca^{++} increase and protein kinase C activation respectively (36).

Phospholipase A_2 activated by increased cytosolic Ca^{++} and protein kinase C catalyzes the liberation of arachidonic acid (AA) from membrane phospholipids. AA in turn is converted to thromboxane A_2 (TxA_2), a potent platelet activator. TxA_2 is released from platelets to generate feedback amplification of platelet activation and also to provoke smooth muscle cell contraction. The binding of TxA_2 to its G_q-coupled platelet surface receptor activates PLCβ2, whose pathway is similar to PLCγ2. Alternatively, the platelet thromboxane receptor couples to the G_{13}-protein, whose activation stimulates the Rho/ Rho-kinase pathway mediating further platelet activation (28, 36).

Another crucial step is the preparation of platelets to form bridges with other platelets (i.e. platelet aggregation). The integrin responsible for this function, $\alpha_{IIb}\beta_3$, has a low affinity for its ligands (fibrinogen and vWF) on resting platelets. The cytosolic increases of Ca^{++} and DAG mediate a conformational change of the integrin $\alpha_{IIb}\beta_3$ enabling it to bind its ligands (mainly fibrinogen). A molecule of fibrinogen can then bind with two $\alpha_{IIb}\beta_3$ receptors on two activated platelets and forms a bridge between them (36).

1.4.4 Secretion of platelet granule contents and amplification of platelet activation

The rise of cytosolic Ca^{++} and activation of protein kinase C (PKC) in stimulated platelets lead to fusion of the granules with the cytoplasmic membrane. Lipid components, soluble N-ethylmaleimide sensitive attachment protein receptors (SNARE) proteins, that form a fusion machinery, and chaperone proteins, which

modulate the SNARE-proteins, have been shown to take part in the secretion process (45).

As mentioned above, α-granules contain a diversity of biologically active proteins, which serve to foster platelet adhesion, platelet aggregation, chemotaxis and angiogenesis at sites of vascular injury. Fibrinogen and vWF released from α-granules reinforce platelet activation, platelet recruitment and platelet-platelet interactions (36). The expression of the α-granule proteins integrin $\alpha_{IIb}\beta_3$ and P-selectin on the platelet surface also supports platelet aggregation and adhesion (28). Furthermore, the coagulation factors V, XI and XIII and prothrombin are also released and enhance thrombus stabilization (36). Thrombin, in particular, which is generated from prothrombin in the vicinity of activated platelets, interacts with the G_q-coupled PAR-1 and PAR-4 receptors stimulating PLCβ, and recruits circulating non-activated platelets to the platelet thrombus (46).

Notably recent studies indicate that the population of α-granules is heterogeneous. For example the vascular endothelial growth factor (VEGF; angiogenesis stimulator) and endostatin (an angiogenesis inhibitor) have been found to be segregated into separate and distinct α-granules and to be differentially secreted: VEGF in PAR-1 and endostatin in PAR-4activated platelets, respectively (47, 48).

The dense granules play a major role in amplification of the platelet response. The released ADP binds to the G_q-coupled receptor $P2Y_1$ activating the PLCβ-dependent signaling pathway, and to the Gi-coupled $P2Y_{12}$ receptor suppressing intracellular levels of cAMP. The second messenger cAMP is a well-known inhibitor of platelet activation (36, 46, 49). Secreted ATP binds to the $P2X_1$ channel provoking

influx of extracellular Ca^{++} and resulting in potentiation of platelet shape change and granule secretion (36, 50). Epinephrine has also been found to be released from dense granules and to stimulate the α2-adrenergic receptor on the platelet surface. This receptor is like the $P2Y_{12}$ receptor G_i-coupled, and potentiates platelet activation (34, 51). 5-HT secreted from dense granules can be taken back up by the 5-HT transporter (SERT) of platelets or it interacts with the 5-HT2A receptor on the platelet surface (6, 23, 52). The role of 5-HT in platelet activation will be discussed below.

Lysosomes secrete degrading enzymes that may take part in clot remodeling and further platelet activation (53). The lysosomal enzymes loosen the subendothelial structure and play a pathophysiological role in restructuring the vessel wall during atherosclerosis (28).

An overview of the main platelet G-protein coupled receptors and their ligands is shown in Table 3.

Table 3: Main platelet G-protein coupled receptors (GPRCs), their ligands and copies per platelet.

Name	Coupling	Ligand	Copies per platelet	Reference(s)
Thromboxane receptor	G_q, $G_{12/13}$	Thromboxane	~ 1000-2000	Hechler et al. (54)
PAR-1	G_q, G_i, $G_{12/13}$	Thrombin	~ 500-2000	Hechler et al. (54)
PAR-4	G_q, $G_{12/13}$	Thrombin	unknown	Michelson (39), Molino et al. (55)
$P2Y_1$ receptor	G_q	ADP	~ 150	Purvis et al. (56)
$P2Y_{12}$ receptor	G_i	ADP	~ 400	Ohlmann etal. (57)
PAF receptor	G_i, G_q	PAF	~ 300	Michelson (39)
α_2-adrenergic receptor	G_i	Epinephrine	~ 300	Pozgajova et al. (58)
β_2-adrenergic receptor	G_s	Epinephrine	unknown	
5-HT2A receptor	G_q	5-HT	unknown	Khait et al.(59), Spiegset et al. (60), Coccaro et al. (61)
5-HT4 receptor	G_s	5-HT	unknown	
IP receptor	G_s	PGI_2	unknown	Colman (62)
EP3 receptors	G_i	PGE_2	unknown	Michelson (39)
EP2 and EP4 receptors	G_s	PGE_2	unknown	Michelson (39)

1.4.5 Platelet aggregation

Platelet aggregation is defined as the process of co-adhesion between two platelets; the platelet aggregate is the final step of primary hemostasis and stops blood loss from damaged vessels (28).

At lower shear rates (<1000 s^{-1}) the activation of the integrin $\alpha_{IIb}\beta_3$ (fibrinogen receptor) by platelet agonists and the successive bridging of two $\alpha_{IIb}\beta_3$ receptors with one fibrinogen molecule predominates the aggregation process (36). At higher shear rates (1000 – 10000 s^{-1}) non-activated discoid platelets form transient adhesive contacts with other adherent platelets through interactions of the glycoprotein Ib with vWF, and of integrin $\alpha_{IIb}\beta_3$ with fibrinogen. This initial step of aggregation is further supported by the narrow space between aggregating platelets

which facilitates the accumulation of soluble platelet agonists such as ADP, TxA_2, thrombin (36). Platelet inside-out signaling leads to integrin $\alpha_{IIb}\beta_3$ activation, and to its binding of fibrinogen, but also of vWF and fibronectin, which results in the formation of stable platelet aggregates. Besides integrin $\alpha_{IIb}\beta_3$, other surface molecules have been recently implicated in thrombus stability (36). Finally, the platelet thrombus is consolidated by a network of fibrin during the processes of secondary hemostasis (36, 37). At very high shear (>10.000 s^{-1}) platelets aggregate almost exclusively due to vWF-GPIb interactions (36).

1.5 Uptake, storage and release of 5-HT by platelets

The concentration of 5-HT in platelet-poor or platelet-depleted plasma is very low: 179 nM and 0.6 nM, respectively (63, 64). Circulating 5-HT is taken up by SERT into the platelet cytoplasm (65, 66). Cytosolic 5-HT is then transported into the dense granules by the vesicular monoamine transporter (VMAT) (67, 68), where it is stored along with ATP, ADP, high levels of Ca^{++} and inorganic phosphate polymers (33). 5-HT in the dense granules is not freely diffusible and forms large molecular aggregates with ATP, which are stabilized by the divalent cations found in the same granules (69). The dense granule concentration of 5-HT is very high, and varies in different studies between 65 mM and 0.5 M (33, 69). The mass of 5-HT has been approximately estimated as 589 ng (3.34 nmol) 5-HT per 10^9 platelets (15). Upon exposure to subendothelial matrix and upon stimulation by agonists, including thrombin, thrombin receptor activating peptide (TRAP), thromboxane analogues and ADP, platelets undergo degranulation, which leads to 5-HT secretion into the

extracellular space (33, 70). 5-HT can then interact with platelet 5-HT receptors or it is taken up again by the platelet 5-HT transporters**.**

Few studies have studied the 5-HT content in other blood components. Until now, 5-HT has also been found in erythrocytes, lymphocytes, monocytes and mast cells. The existence of 5-HT in granulocytes is still controversial (71-75).

1.6 The platelet 5-HT transporter (SERT) and selective serotonin reuptake inhibitors (SSRI)

1.6.1 The platelet 5-HT transporter (SERT)

The 5-HT transporter (SERT) is a Na^+/Cl^--dependent solute carrier transporter (SLC6 family) consisting of 12 transmembranous regions (23, 76). The uptake of extracellular 5-HT takes part in two steps. In the first one, an extracellular 5-HT molecule binds with SERT and is transported into the cell along with an ion of Na^+. In the second step K^+ is countertransported across the membrane to the outside of the cell (23).

SERT is a membrane-skeleton-associated protein, and its regulation in platelets is complex. Studies of washed platelets have shown that extracellular 5-HT concentrations as low as 1 nM increase SERT expression on the plasma membrane and 5-HT uptake; whereas higher concentrations of extracellular 5-HT decreased both SERT expression and 5-HT uptake even below the baseline (63, 65, 76, 77). Moreover, platelet activation by the PKC pathway also causes a rapid (1 min) loss of transport activity, followed by a more delayed (10-30 min) internalization of SERT; these effects may be mediated by the interaction of SERT with regulatory proteins,

such as the adaptor protein Hic-5 (77). On the other hand, a direct interaction between SERT and the $\alpha_{IIb}\beta_3$ integrin has been shown to increase the level of SERT on the platelet surface and its transporter activity (78).

SERT expression may also be genetically regulated. A frequent polymorphism in the 5'-flanking region of the SERT gene has been identified, which leads to the creation of short (S) and long alleles (L). The S allele has been associated with decreased SERT expression and 5-HT uptake (79-83). It is unclear, how this polymorphism is related to platelet reactivity, and cardiac risk. One study reported that patients carrying the S allele had an increased risk for cardiac events and a higher frequency of depressive symptoms (79). However, another study found that the existence of two S alleles in an individual appears to have a protective effect, delaying the onset of the first myocardial infarct, and this effect is more relevant among smokers (81). Abdelmalik et al. showed that, under SSRI treatment, carriers of two SS alleles had a lower platelet 5-HT concentration and an attenuated platelet aggregation (in PRP) in comparison to carriers with at least one L allele (80). However, two studies failed to associate this specific SERT polymorphism with platelet 5-HT concentrations among healthy individuals and with changes in platelet aggregation, bruising and spontaneous bleeding events during selective 5-HT reuptake inhibitor (SSRI) therapy (82, 83).

1.6.2 Effects of selective serotonin reuptake inhibitors (SSRIs) on platelets

Treatment with SSRIs leads to reduced platelet 5-HT content by inhibiting the SERT-mediated 5-HT uptake; they could therefore affect platelet function (84, 85)

and impede vasoconstriction mediated by platelet-secreted 5-HT. Both effects could cause an increase of bleeding time.

Epidemiological studies

A cohort study by Dalton *et al* reported increased risk of upper gastrointestinal bleeding in patients receiving SSRIs, especially when combined with non-steroidal anti-inflammatory drugs (NSAIDs) and low-dose aspirin (86). A case-control study by Opatrny *et al* demonstrated a small increased risk of gastrointestinal hemorrhage with SSRI use (87). Another cohort study in the Danish population also showed an increased post-surgical bleeding risk in breast cancer patients under SRRI treatment (88). Two retrospective case-control studies in the UK and Denmark also supported the statement that SSRIs, especially in combination with NSAIDs or low dose aspirin, increase the risk of gastrointestinal bleeding (89, 90). Similarly, a case-control study showed a protective effect of SSRI use against myocardial infarction (MI) (91), while a cohort study reported a reduced MI risk among depressive patients receiving SSRIs for at least 3 months (92). In a meta-analysis, depressive patients with coronary heart disease receiving SSRIs also demonstrated lower coronary heart disease readmission and mortality rates compared to placebo or no treatment (93).

Yet other epidemiological studies did not confirm an increased bleeding risk during SSRI intake or a cardio-protective effect of SSRI treatment. In particular, a case control study by Carvajal *et al* failed to demonstrate a significant increase in gastrointestinal bleeding by SSRI treatment (94). Moreover, two other studies, a case-control study with short-term exposure to SSRIs and a cohort study in elderly patients, reported an increased MI risk after medication with SSRIs (95, 96).

In vitro studies

Carneiro *et al* demonstrated that acute blockade of the platelet SERT with the SSRI citalopram *in vitro* attenuated the ADP-induced aggregation of washed platelets with normal intracellular stores of 5-HT (78). In another study, platelet incubation with citalopram *in vitro* also attenuated 5-HT dependent platelet aggregation in PRP, as measured by light transmittance aggregometry, and in flow experiments (97). Moreover, incubation with citalopram attenuated the peak of thrombin generated by 5-HT stimulation of platelets, and showed a tendency to inhibit 5-HT stimulated blood coagulation (97). On the other hand, a study using human washed platelets showed that fluoxetine augmented platelet aggregation induced by ADP, thrombin and PAR-1 and PAR-4 activators (98).

1.7 Platelet protein serotonylation

In 2002 Dale et al. showed that upon activation with a combination of strong agonists, such as collagen and thrombin, a fraction of platelets expressed on their surface α-granule factor V (FV) and other α-granule proteins, including fibrinogen/fibrin, vWf, fibronectin, α2-antiplasmin and thrombospondin (defined as COAT-platelets or more recently as coated-platelets) (99, 100). It was proposed that COAT-platelets form by 5-HT–mediated stabilization of the receptor-mediated binding of adhesive and procoagulant proteins on the platelet surface. It was reported that procoagulant proteins such as factor V and fibrinogen are covalently bound to 5-HT via a transglutaminase reaction (“serotonylation”, see below), and that both fibrinogen and thrombospondin are capable of binding serotonylated proteins. The interactions between these proteins and their platelet surface

receptors have been suggested to result in a stabilized network of proteins ("COAT") on the platelet surface (99-101).

Additionally, Walther et al. found evidence of protein serotonylation inside the platelets. They reported a rapid covalent transglutaminase-mediated binding of cytoplasmic 5-HT to the GTPases RhoA and Rab4, rendering the latter active and inducing α-granule secretion through cytoskeletal rearrangements. As compared with wild-type platelets, α-granule secretion was decreased in platelets from tryptophan hydroxylase 1 isoenzyme knock-out mice, which lack the ability to synthesize peripheral 5-HT but have normal levels of 5-HT in the CNS. The protein transamidation of 5-HT was termed by Walther et al as serotonylation (102).

These proposed roles of platelet 5-HT by attaching covalently to platelet surface and cytoplasmic proteins are interesting, but they need to be confirmed by further investigations. If true, SSRI intake should reduce the number of COAT-platelets and inhibit α-granule secretion. Indeed, in an *ex vivo* study chronic blockade of SERT by SSRIs resulted in a lower number of COAT-platelets; this effect was however, barely significant ($p=0.046$) (103). Moreover, the reduction of COAT-platelets was not restricted to patients taking SSRIs, but was also found in patients treated with aspirin (103). Together these results should be considered with caution since the number of COAT-platelets varies in different patients very much (99, 103). Considering the possible functional role of intracellular protein serotonylation as suggested by Walther et al (102), a selective inhibition of α-granule secretion by SSRI intake has not been reported so far.

1.8 Platelet 5-HT receptors

1.8.1 The platelet 5-HT2A receptor

As with the SERT, 5-HT2A receptor mRNA has been found to be expressed in platelets with identical splicing to the brain 5-HT2A receptor (52). 5-HT2A receptors are located on the platelet surface and are coupled preferentially via the G_q proteins, which signal to the IP_3/PKC pathway leading to calcium mobilization (6, 52, 104). Therefore, 5-HT stored in platelet-dense granules and released from them upon platelet stimulation might serve as a feedback mediator by binding to 5-HT2A receptors on the platelet surface and support platelet aggregation (49).

5-HT, when added alone *in vitro*, is a weak platelet agonist and has been reported to activate or not to activate platelets in blood (105, 106). However, 5-HT potentiates platelet aggregation when added together with other platelet stimuli such as ADP, epinephrine, collagen, thrombin and thromboxane A2 (97, 106, 107). Therefore, it is possible that 5-HT released from platelet dense granules could together with other platelet-released mediators (ADP, Thromboxane A2) synergistically mediate and amplify platelet aggregation.

1.8.2 Effects of 5-HT2A receptor antagonists on platelets

5-HT2A receptor antagonists block the binding of 5-HT to the 5-HT2A receptor and antagonize the subsequent 5-HT-dependent calcium mobilization and platelet activation. Various 5-HT2A receptor antagonists have been known for a long time to inhibit 5-HT-dependent platelet aggregation (105, 108-111). 5-HT2A receptor antagonists have unequivocally been shown to inhibit 5-HT-potentiated platelet

aggregation of PRP in humans and many other species *in vitro* and *ex vivo*: Two studies demonstrated an inhibition of 5-HT-potentiated platelet aggregation of human platelets by various 5-HT2A receptor antagonists by using light transmittance aggregometry (LTA) in PRP and by measuring platelet count in blood (109, 110). R-96544 is a potent and selective 5-HT2A receptor antagonist, in comparison to sarpogrelate, a well-known 5-HT2A receptor antagonist, which is not as potent as R-96544 and also has a high affinity for receptors other than 5-HT2A, namely α1-adrenergic and histamine H1 receptors. R-96544 inhibited 5-HT-potentiated aggregation *in vitro* in a series of species, namely human, monkey, cat, rabbit, rat and mice platelets (112). The same study showed *ex vivo* inhibition of 5-HT–potentiated platelet aggregation by intravenously administered R-96544 and R-102444, another structurally similar 5-HT2A receptor antagonist, in rabbits and in rats (112). In a recent study, cyproheptadine and pizotifen, two antidepressant non-selective 5-HT2A antagonists, reversed serotonin-enhanced ADP-induced platelet aggregation *in vitro* in humans and *ex vivo* in mice (113). Two other 5-HT2A receptor antagonists, SR 46349 and APD791, also inhibited the 5-HT-potentiated platelet aggregation in human, rabbit and dog platelets *in vitro*, as measured by LTA. Oral administration of these two agents also inhibited rabbit and dog platelets *ex vivo* (114, 115). Additionally, 5-HT2A antagonists attenuated 5-HT-potentiated thrombosis in an *in vivo* model in mice (116). Hence, platelet aggregation potentiated by exogenous 5-HT administration is sensitive to 5-HT2A receptor blockade in human and animal platelets, both *in vitro and ex vivo*. Platelet aggregation potentiated by exogenous 5-HT administration was usually the main

parameter measured when the antithrombotic effect of a 5-HT2A receptor antagonist was examined.

Do 5-HT2A receptor antagonists inhibit platelet aggregation when no exogenous 5-HT is added, after platelet activation with physiological stimuli? As far as animals are concerned, in one study a selective 5-HT2A antagonist (DV-7028) inhibited collagen-induced 5-HT secretion in washed platelets and collagen-induced platelet aggregation in PRP in rats *in vitro* (117). Furthermore, the 5-HT2A antagonist ketanserin alone or in combination with aspirin, inhibited the collagen-dependent platelet aggregation in cats *ex vivo* (107). Interestingly, quite a few studies have been conducted *in vivo*. Four studies reported an inhibitory effect of three different 5-HT2A receptor antagonists in the Folts model of coronary occlusion in the dog (114, 118-120). Herbert *et al* also reported that the 5-HT2A receptor antagonist SR 46349 prolonged bleeding time and suppressed thrombosis in an arterio-venous shunt model in rabbits, where thrombosis was induced by vessel ligation plus administration of i.v. tissue thromboplastin (114). Similarly, in a study involving rats, Pawlak *et al* reported that the 5-HT2A receptor antagonist DV-7028 prolonged bleeding time and inhibited thrombus formation in the arterial occlusion model, though it did not prevent venous thrombosis (117). In mice a 5-HT2A receptor antagonism also prolonged the occlusion time of mesenteric arterial thrombosis induced by electric stimulation (116). Two antidepressant 5-HT2A antagonists, cyproheptadine and pizotifen, prolonged tail bleeding time and prolonged occlusion time in a carotid artery thrombosis model in mice *in vivo* (113). Thus, the 5-HT2A receptor plays an important role *in vivo* in various animal thrombosis models.

Studies investigating the inhibition of stimulus-induced human platelet activation by 5-HT2A receptor antagonists have provided conflicting results. Ketanserin did not affect ADP-, epinephrine-, thromboplastin-, and collagen-induced platelet aggregation in PRP in one study, but inhibited ADP-, epinephrine- and thrombin-induced platelet aggregation in PRP in another (107, 121). In patients with Raynaud's phenomenon long term intake of ketanserin left *ex vivo* collagen-, ADP- and even 5-HT-induced aggregation in PRP unaffected (122). On the other hand, another set of patients with Raynaud's phenomenon had prolonged bleeding time under ketanserin treatment, although no change in agonist-induced platelet aggregation before and during treatment was observed (123). Last but not least, treatment with ketanserin surprisingly reduced the rate of intraoperative blood loss during total hip arthroplasty in elderly patients (124). It may be that in human physiology it is only the vascular action and not the pro-aggregative action of 5-HT that is inhibited by 5-HT2A antagonists. In summary, under physiologically relevant conditions, the antagonism of 5-HT2A receptors on animal platelets has provided us with results that in many cases were not confirmed in studies with human platelets.

It should be noted that the 5-HT2A receptor antagonist ketanserin, although clinically introduced in the 1980s for the treatment of hypertension, was soon abandoned because of its pro-arrhythmic properties. It was never used as an antiplatelet drug (108, 125). Nevertheless, research concerning new 5-HT2A receptor antagonists continues until now and suggests possible clinically relevant anti-platelet properties based mostly on animal studies (115, 118).

1.8.3 The platelet 5-HT4 platelet receptor

Recently evidence for the expression of 5-HT-4 receptors has been described in human platelets (126). 5-HT4 receptors are G_s-coupled receptors and activate adenylyl cyclase, thus increasing the intracellular content of cAMP (127). This second messenger cAMP inhibits platelet activation (36, 46) and therefore the stimulation of platelet 5-HT4 receptors should lead to inhibition of platelet aggregation. However, tegaserod, a potent 5-HT4 receptor agonist enhanced ADP-, collagen-, epinephrine- and 5-HT-induced aggregation in PRP in vitro as measured by light transmittance aggregometry (LTA) (126). A more recent study using the same methodology failed to reproduce the stimulating effect of tegaserod on ADP-induced aggregation (128).

1.9 Topic and question of the dissertation

Serotonin is stored in platelets and may alter their function; therefore, we aimed to investigate the effect of SSRIs on platelet aggregation, and to understand the role of platelet 5-HT2A and 5-HT4 receptors for platelet function.

SSRIs are at present the most commonly prescribed antidepressive drugs worldwide. Many, but not all studies have shown an increase in bleeding complications and a decrease in the incidence of myocardial infarction following SSRI therapy (94-96). *In vitro,* few studies using PRP demonstrated an inhibitory effect of SSRIs on platelet aggregation, whilst another one using washed platelets showed the opposite effect (78, 97, 98). We decided to study the *in vitro* effects of SSRIs on platelet aggregation under more relevant physiological conditions, i.e. in human blood.

Although orally effective platelet inhibitors such as aspirin and $P2Y_{12}$ receptor antagonists (clopidogrel and prasugrel), are currently available for the treatment of atherothrombosis and its sequelae (myocardial infarction, ischemic stroke), new antiplatelet therapies are needed that lack the bleeding complications of the currently used anti-platelet drugs. The findings of early and recent animal studies *ex vivo* and *in vivo* suggest that the blockade of the platelet 5-HT2A receptors might be a good target for future anti-platelet therapy. However, studies with human blood and physiological platelet stimuli provided conflicting results. We, therefore, decided to investigate for the first time, in a comprehensive study the effects of 5-HT2A antagonists on platelet function in human blood *in vitro* under static and arterial flow conditions, after stimulation with physiologic agonists and a pathophysiologically relevant platelet agonist, i.e. human atherosclerotic plaque homogenate.

Very recently the expression of 5-HT4 receptors on platelets has been reported. Their role for platelet function is unclear. We therefore investigated the effect of 5-HT4 receptor agonists and antagonists in human blood. The aims and experimental work program of the dissertation are divided into three sections:

1) Effect of SSRI on platelet aggregation in human blood

2) Role of the 5-HT2A receptor for platelet function in human blood

3) Role of the 5-HT4 receptor for platelet aggregation in human blood.

The overall goal of the dissertation is to provide a comprehensive, better understanding of the 5-HT related platelet physiology, in order to identify possible adverse effects of 5-HT-related medications targeting other systems and to explore

whether platelet 5-HT receptors could be possible new targets for anti-platelet therapy.

2. Materials and methods

2.1 Materials

2.1.1 Devices

Type of device	Name and origin
Light Transmission Aggregometer	Lumi-Aggregometer (Chronolog, Havertown, PA)
Impedance-Aggregometer	Multiplate®, (Dynabyte Medical, München, Germany)
Microscope	Inverted fluorescence microscope (Nikon TE2000E-PFS, Tokyo, Japan)
Microscope camera	CCD camera (CooLSNAP HQ2, Tuscon, AZ, USA)
Shear-stress platelet function analyzer	Cone and plate analyzer Impact-R, (Matis-Medical, Brussels, Belgium)
Parallel plate flow chamber	Sticky-Slide $I^{0.1}$ Luer (ibidi GmbH, Martinsried, Germany)
Flow analysis pump system	ibidi pump system (ibidi GmbH, Martinsried, Germany)

2.1.2 Disposable materials

Type of material	Origin
Butterfly needle (19-Gauge)	Intermedica GmbH (Mainz, Deutschland)
Lumi-aggregomter cuvettes, 450 µl	Chronolog, Havertown, PA
Lumi-aggregometer disposable siliconized stir bars	Chronolog, Havertown, PA
Multiplate®-test cells	Dynabyte Medical (München, Germany)

2.1.3 Chemicals

Chemical	Origin	Stock concentration, dilution buffer
(±)-epinephrine	Sigma-Aldrich (Taufkirchen, Germany)	1 M epinephrine in 1 M HCl, further dilution in NaCl 0.9%
Adenosine 5′-triphosphate disodium salt hydrate (ATP)	Sigma-Aldrich (Taufkirchen, Germany)	0.9 % NaCl
Adenosine 5'-diphosphate sodium salt (ADP)	Sigma-Aldrich (Taufkirchen, Germany)	0.9 % NaCl
ASA control (Acetyl salicylic acid)	Dynabyte (Munich, Germany)	Distilled H_2O; 5.4 mM
CJ-033,466 (5-HT4 receptor agonist)	Tocris bioscience (Ellisville, MO, USA)	DMSO to 100 mM, further dilution in 0.9% NaCl
Collagen Horm®	Nycomed Pharma (Munich, Germany)	SKF solution (supplied within Collagen Horm® package)
Fluoxetine hydrochloride	Sigma-Aldrich (Taufkirchen, Germany)	0.9 % NaCl
GR113808 (5-HT4 receptor antagonist)	Tocris bioscience (Ellisville, MO, USA)	25 mM GR 113808 in 1 M HCl, further dilution in 0.9%NaCl
Ketanserin (+)-tartrate salt	Sigma-Aldrich (Taufkirchen, Germany)	10 mM ketanserin in 0.1 M HCl, further dilution in 0.9% NaCl
Luciferin-luciferase reagent	Chronolog (Chrono-Lume®, Havertown, PA, USA)	Distilled H_2O
Quinacrine dihydrochloride (mepacrine)	Sigma-Aldrich (Taufkirchen, Germany)	0.9 % NaCl
R-96544 hydrochloride (5-HT2A receptor antagonist)	Tocris bioscience (Ellisville, MO, USA)	0.9 % NaCl
Recombinant lepirudin (hirudin, Refludan®)	Schering AG(Germany)	0.9 % NaCl

RS 23597-190 hydrochloride (5-HT4 receptor antagonist)	Tocris bioscience (Ellisville, MO, USA)	0.9 % NaCl
Sarpogrelate hydrochloride (5-HT2A receptor antagonist)	Tocris bioscience (Ellisville, MO, USA)	0.9 % NaCl
Serotonin hydrochloride (5-HT)	Sigma-Aldrich (Taufkirchen, Germany)	0.9 % NaCl
TRAP-6 (PAR-1 agonist)	Bachem (Weil am Rhein, Germany)	0.9 % NaCl

2.1.4 Buffers

Buffer	Origin
Sodiumchloride (NaCl) 0.9 %	
Dimethylsulfoxid (DMSO)	Calbiochem
Dulbecco's Phosphate buffered Saline (PBS)	Sigma-Aldrich (Taufkirchen, Germany)
Hydrochloric acid (HCl) fuming 37%	Merck (Darmstadt, Germany)

2.2 Methods

2.2.1 Blood collection

Blood was obtained from healthy volunteers, who denied taking any medication affecting platelet function for at least two weeks preceding the experiments. Informed consent was obtained in accordance with the Helsinki protocol. Recombinant hirudin was dissolved in 0.9% NaCl solution to a concentration of 2000

U/ml (130 µg/ml). Blood was obtained by venipuncture using a 19-gauge needle and a 10 – 50 ml plastic syringe containing one tenth volume of recombinant hirudin (final concentration in blood ~200 U/ml; 13 µg/ml). The syringe was then gently moved in order to allow a homogeneous distribution of blood and anticoagulant. In order to avoid platelet activation by subendothelial tissue thromboplastin, the first 3 ml of blood were drawn in a separate syringe and were subsequently discarded (129).

Aggregation measurements, dense granule secretion measurements and flow experiments were performed between 30 minutes and 4 hours after venipuncture. Only in one set of experiments we examined the aggregation induced by 5-HT alone early after venipuncture. In this set of experiments aggregation measurements were conducted between 5 min and 30 min after venipuncture, as indicated in the Results section.

2.2.2 Preparation and preservation of human atheromatous plaque homogenate

Atherosclerotic plaques were obtained from patients who underwent endarterectomy for high grade carotid artery stenosis with *en bloc* preservation of the tissue structure (130). Patient consent was obtained and approved by the Ethics Committee of the Faculty of Medicine of the University of Munich. After intra-operative removal, plaque specimens were immediately shock frozen in liquid N_2, and stored at -80°C. Under sterile conditions, atheromatous plaques were carefully dissected from other regions of the atherosclerotic tissue specimens (130). The plaque samples were then weighed, homogenized and the concentration was

adjusted to 100 mg wet weight/ml. The plaque homogenates were kept at –80°C until further experimentation (131, 132). Plaque homogenates from 5 to 7 individual patients were pooled and used for the experiments.

2.2.3 Whole blood platelet aggregation

Whole blood platelet aggregation was determined by multiple electrode aggregometry (MEA) using the Multiplate® device from Dynabyte Medical (Munich, Germany) (Figure 2.1)

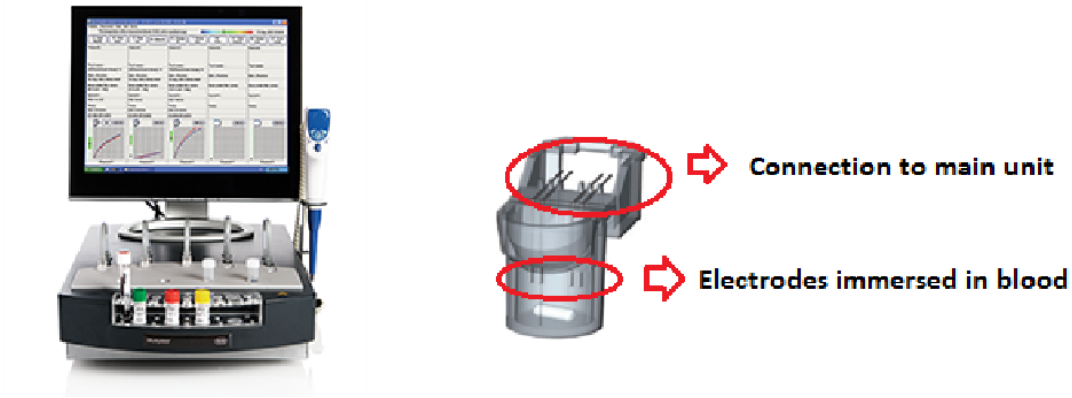

Figure 2.1: The Multiplate® device. The central unit of the Multiplate®-system has 5 positions for measuring plaletet aggregation. Blood can be warmed at 37°C by a heated metalic plaque containing the 5 cuvette positions. A screen enables the control and the visualization of the experiments (left). The Multiplate®-cuvette has a stir bar and two pairs of electrodes (right). For measurement of aggregation, one end of the electrodes is connected to the main unit of the Multiplate®-system, and the other end of the electrodes is immersed in blood. The measurement of electric impedance between the anode and cathode of the two electrode pairs provides two independent values of aggregation in the same cuvette. Modified image from www.multiplate.net (133).

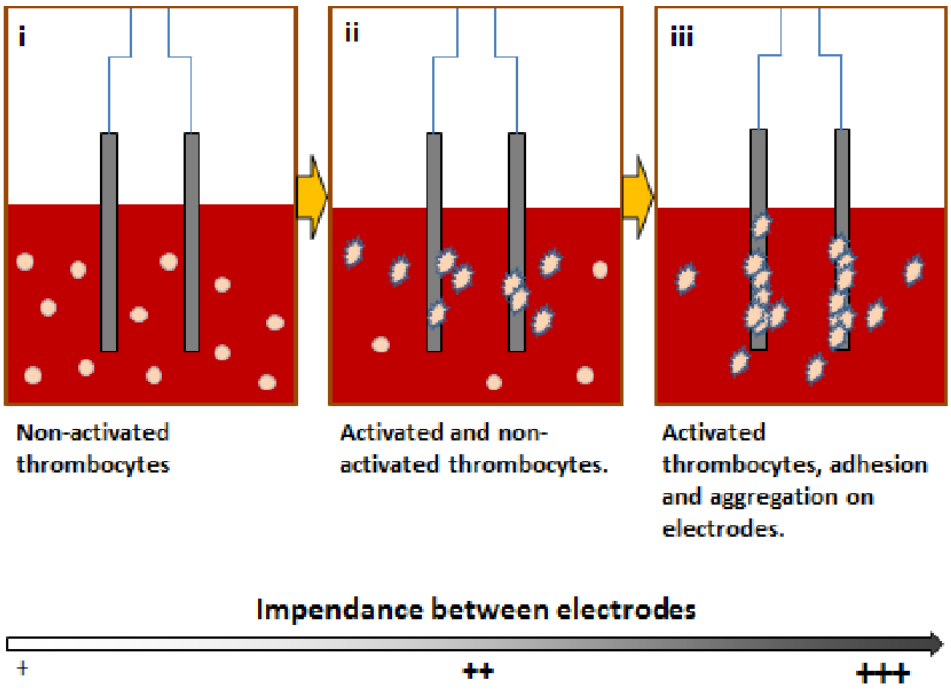

Figure 2.2: Principle of impedance aggregometry. Inactive platelets (i) that get activated by the presence of platelet stimuli (ii) adhere and aggregate onto the electrodes (iii) thereby creating a platelet layer which elevates the electric impedance between the two electrodes.

This device detects the increase of electrical impedance due to the adhesion and aggregation of platelets on the surface of two independent electrode pairs in the test cuvette (Fig 2.2).

Samples (0.6 ml) contained a 1:1 mixture of 0.9% NaCl and anticoagulated blood which were stirred in the presence or absence of SSRIs, 5-HT2A receptor antagonists, 5-HT4 receptor antagonists or solvent at 37°C for 3 min in the cuvettes. In some experiments different pre-incubation periods of 13 minutes or 5 seconds were used as described in the results section. At the end of pre-incubation, platelet stimuli (serotonin, epinephrine, TRAP, arachidonic acid, collagen, plaque homogenate) or solvent were added, and the measurements were started. The increase in electrical impedance was recorded continuously for 5 min. The mean value of the two independent determinations is expressed in arbitrary units over time (AU* min).

2.2.4 Measurement of ATP secretion

ATP secretion was measured using the Lumi-Aggregometer from Chronolog. This device measures the luminescence emitted when ATP interacts with the luciferin-luciferase reagent. The firefly luciferase catalyzes a two-step chemical reaction (134):

$$\text{Luciferin} + \text{ATP} \xrightarrow{\text{Luciferase}} \text{Luciferil-adenylate} \qquad (1^{st}\ \text{step})$$

$$\text{Luciferil-adenylate} \xrightarrow{\text{Luciferase} + O_2} \text{Oxyluciferin} + CO_2 + \text{AMP} + \text{light} \qquad (2^{nd}\ \text{step})$$

In this way, ATP secreted from platelet dense granules upon platelet activation in response to various stimuli is measured. The measurement is normally applied in platelet-rich plasma, but can also be performed in blood (135, 136).

Hirudin-anticoagulated blood (200 µl) was diluted 1:1 with normal saline solution in a Chronolog cuvette containing a stir bar and gently mixed with a 200 µl pipette. Stirring speed was set to 800 rpm. The diluted hirudinized blood was incubated for 30 sec at 37°C with 50 µl luciferin-luciferase reagent. Then ketanserin (1 µM) or solvent was added, the samples were incubated for 3 min at 37°C and then exposed to platelet stimuli or solvent. The increase of luminescence over time was recorded by a 2 channel recorder. For each experiment, the maximal increases of luminescence were translated into ATP concentration by performing a standard curve with increasing concentrations of ATP added to the blood/NaCl samples.

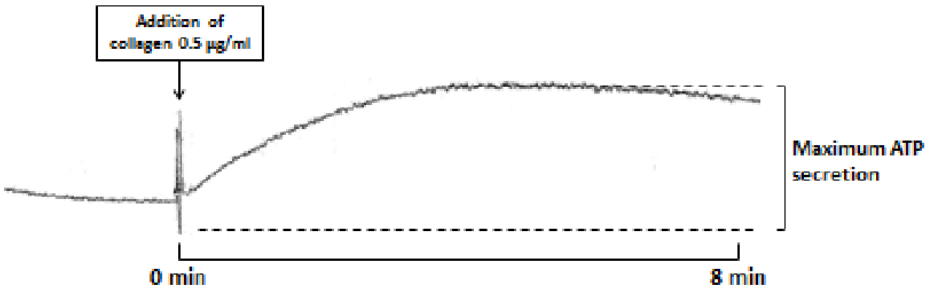

Figure 2.3: Tracing of ATP secretion as measured by luminescence. Platelets were activated by addition of a low dose of collagen (0.5 µg/ml) to diluted blood. The rise of activated platelets correlates with platelet ATP secretion from dense granules, which is measured by luminescence in the presence of luciferin-luciferase reagent. After a plateau of maximal ATP secretion the ATP concentration decreases.

2.2.5 Plaque-induced platelet adhesion and thrombus formation under flow

In order to assess the effect of the 5-HT2A receptor antagonist ketanserin on platelet aggregation under more relevant physiological conditions, we performed platelet adhesion and aggregation studies under arterial flow conditions. For this purpose the pump system and parallel plate flow chambers from ibidi GmbH (Martinsried, Germany) were used. The pump applies a computer controlled amount of air pressure through a plastic tube into an upright syringe containing whole blood. The blood flows through a parallel plate flow chamber with a defined flow rate which determines the shear rate and shear stress, and is collected in a second syringe.

As parallel plate flow chamber, the Ibidi Sticky-Slide $I^{0.1}$ Luer) of 0.1 mm height, 5 mm width and 50 mm length was used. It consists of a U-shaped glass slide with self-adhesive underside onto which a thin glass coverslip is attached by applying firm pressure. The glass coverslip was coated before with plaque homogenate (Fig. 2.5).

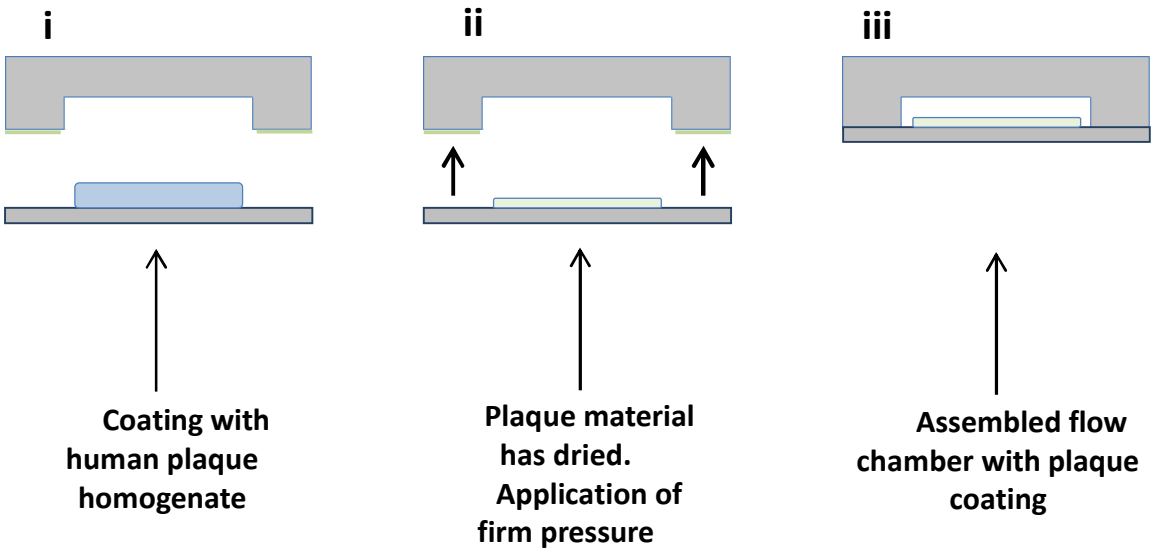

Figure 2.5: Preparation and profile of the parallel plate flow chamber containing human plaque.

The chamber consists of two parts: A thin glass coverslip coated with human plaque homogenate (lower part), and a bottomless ibidi slide with self-adhesive underside (upper part). The thin glass coverslip was coated with 20µl of human atheromatous plaque (5mg/ml) (i) and was left to dry (ii). The two parts of the slide were joined by applying firm pressure (ii) to assemble the flow chamber(iii).

Each glass coverslip (24x60 mm, thickness 0.16-0.19mm; Menzel Gläser, Braunschweig, Germany) was first washed with isopropranolol and dried. The slides were then coated with 20 µl of diluted human atheromatous plaque homogenate (concentration 5 mg/ml; diluted with PBS) and was left to dry at room temperature overnight.

The plaque-coated chambers were mounted on the stage of an inverse microscope (Nikon TE2000E-PFS, Tokyo, Japan) equipped with an incubation chamber (37°C). Before the experiment, the chambers were perfused with PBS for 2 min at a wall shear rate of 500 s^{-1}. Mepacrine (10 µM) was added to blood (37°C, 3 min) in order to visualize platelets by fluorescence. Then, the hirudin-anticoagulated blood was transferred to the inlet syringe, and chambers were perfused for 10 min at a wall shear rate of 500 s^{-1} (flow 247 µl/min). Blood was preincubated with or

without the 5-HT2A receptor antagonist ketanserin (1 µM) at 37°C for 3 min prior to start of flow.

Fluorescence microscopy (excitation: 485 +/- 25 nm; emission: 528 +/- 38nm; x10 objective) was used for visualization of mepacrine-labeled platelet adhesion and aggregation onto plaque homogenate in flowing blood. Platelet deposition was recorded in real-time with a CCD camera. For each flow experiment, surface fields of the size of 538.000 μm^2 were recorded. Video fluorescent images were analyzed off-stage with the software NIS-element 3.1 version (Nikon). In each field, the area covered by atherosclerotic plaque prior to the flow, and the area covered by platelet thrombi plus atherosclerotic plaque after each minute were analyzed. The area covered by atherosclerotic plaque was subtracted from the total fluorescent area in order to calculate the platelet coverage.

2.2.6 Shear-induced platelet adhesion and aggregation

Shear-induced platelet adhesion and aggregate formation was measured in the cone and plate analyzer Impact-R (Matis-Medical, Brussels, Belgium) (137). Blood samples (0.2 ml), in the presence or absence of 10 µM ketanserin, were placed on polystyrene surface plates and subjected to flow using a rotating teflon cone. The rotation induced a constant fluid shear stress over the entire plate surface. Wells were washed with PBS and stained with May–Grünwald stain, and analyzed with an inverted light microscope (137). The shear-induced platelet adhesion and aggregation was evaluated by platelet surface coverage and average size of platelet aggregates. In two sets of experiments two different shear rates of 600 s^{-1} or 1200 s^{-1} were applied.

These experiments were conducted in cooperation with Dr Ela Shai and Prof. David Varon (Coagulation Unit, Hadassah-Hebrew University Medical Center, Jerusalem, Israel).

2.2.7 Statistical analysis

Results are given as mean ± SD or ± SEM from n experiments conducted with blood from different donors. Statistical significance was assessed by paired two-sided t-test, unpaired two-sided t-test or unpaired rank sum test (Mann-Whitney), where applicable. Statistical analysis was performed using Sigma-Plot 11.0. For all analyses a level of significance 5% ($p<0.05$) was adopted.

3. Results

3.1 Role of the serotonin reuptake transporter (SERT) for platelet aggregation in blood

3.1.1 5-HT potentiates the epinephrine-induced platelet aggregation

We examined the stimulatory effect of 5-HT, epinephrine and its combination on platelet aggregation as measured by MEA in whole blood. A set of experiments after 3 min of platelet pre-incubation at 37^{o}C was conducted. The results are presented in Figure 3.1.

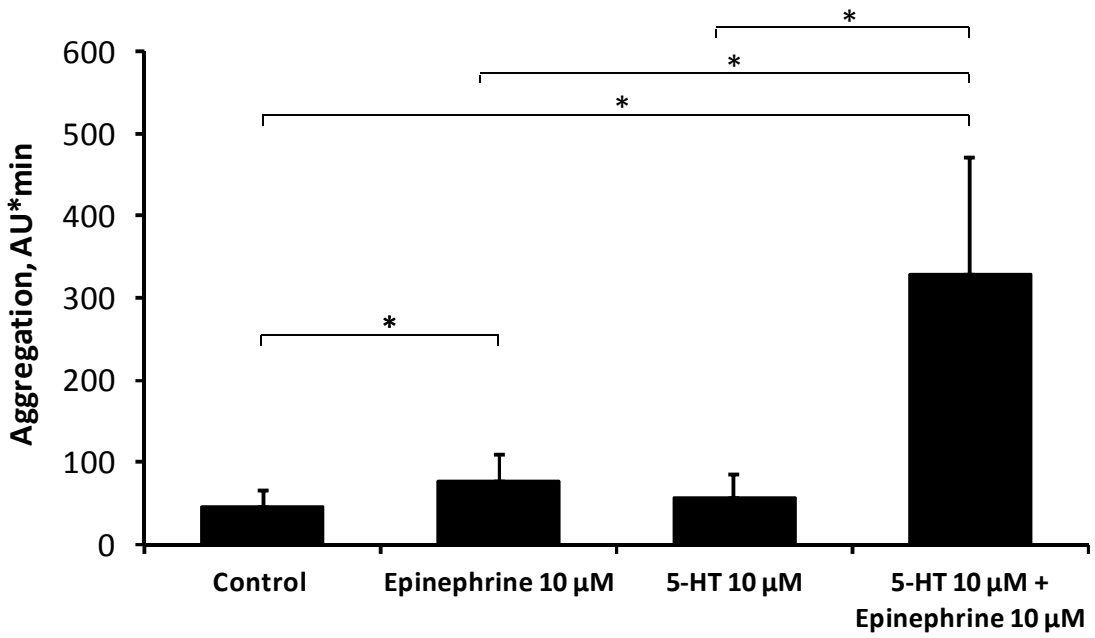

Figure 3.1: Potentiation of epinephrine-induced platelet aggregation by 5-HT. Hirudin-anticoagulated blood was incubated for 3 min under stirring conditions at 37°C before addition of platelet stimuli. The platelet aggregation response was recorded by MEA for 5 min. Results are expressed as mean values ± SD (n=9 with different blood donors). * refers to $P < 0.01$.

Blood that is not agonist-stimulated (column designated as control) shows only a small amount of aggregation. This response is commonly described as spontaneous platelet aggregation.

Platelet aggregation measured after addition of 10 µM 5-HT did not differ significantly from control (56 ± 29 vs 47 ± 18 AU*min respectively, values are mean ± SD; P = 0.24). Addition of 10 µM epinephrine alone induced a small increase of platelet aggregation in comparison to control (76 ± 34 vs. 47 ± 18 AU*min respectively, values are mean ± SD; P < 0.01) (Figure 3.1). 5-HT (10µM) added simultaneously with epinephrine (10 µM) was able to potentiate the epinephrine-induced aggregation from a mean ± SD of 76 ± 34 AU*min to 327 ± 143 AU*min (P < 0.01) (Figure 3.1).

Commonly, as described in the Methods section, blood is left after venipuncture for 30 min at room temperature before measuring platelet aggregation. Hypothetically, released 5-HT from platelets activated by the venipuncture might cause homologous desensitization of the 5-HT receptor(s) over time and, therefore, a direct platelet response is not detectable after 30 min. Yet, some response to 5-HT might be detectable early after venipuncture, where receptor desensitization might be less pronounced. Therefore, we further examined if 5-HT alone induced aggregation early after venipuncture. Experiments were conducted 5, 15 and 30 min after venipuncture. However, 5-HT did not stimulate platelet aggregation at any time after venipuncture. Spontaneous aggregation (control) and platelet aggregation after 5-HT admission did not differ during the first 30 min after venipuncture (Figure 3.2).

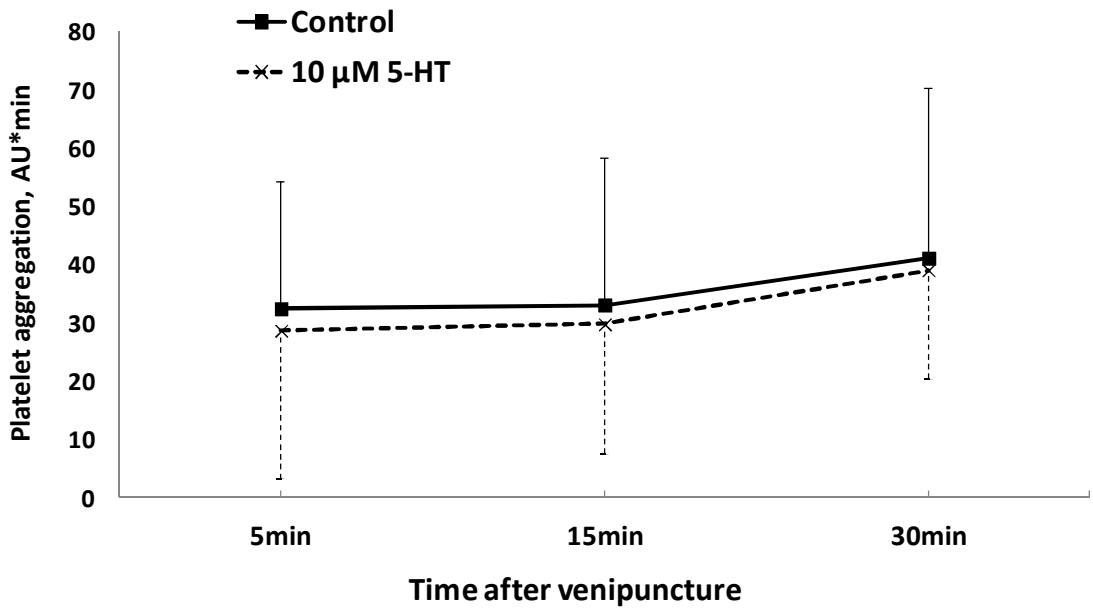

Figure 3.2: Effect of 5-HT on whole blood platelet aggregation 5, 15 and 30 minutes after venipuncture. Hirudin-anticoagulated blood was incubated for 3 min with stirring at 37°C before addition of 10 µM 5-HT. Experiments were started 5, 15 and 30 minutes after venipuncture. Results are expressed as mean values ± SD (n=10 with different blood donors).

The good potentiation of epinephrine-induced platelet aggregation by 5-HT, combined with the fact that epinephrine is a weak mediator of platelet aggregation with a distinct signal transduction pathway from 5-HT, suggests that the concomitant use of 5-HT + epinephrine is a good model to study inhibitors of the serotonergic pathway of platelet aggregation.

3.1.2 The SERT-inhibitor fluoxetine inhibits the 5-HT-induced potentiation of platelet aggregation

In order to assess the effect of 5-HT reuptake transporter inhibitors (SSRIs) on 5-HT-dependent platelet aggregation *in vitro*, we used a 10 µM concentration of the SSRI fluoxetine, the highest achievable plasma concentration after clinical

administration (138). Fluoxetine is one of the oldest and best studied SSRIs with high affinity for the 5-HT reuptake transporter (89, 91).

Pre-treatment of whole blood with 10 µM fluoxetine for 3 minutes inhibited partially 5-HT-dependent platelet aggregation from 327 ± 143 to 195 ± 90 AU*min (mean ± SD; $P < 0.01$) (Figure 3.3).

In another set of experiments, we addressed the question whether extending the time of blood pre-incubation with fluoxetine might enhance its inhibition of 5-HT-dependent platelet aggregation. Whole blood was incubated for 10 minutes at 37°C without stirring with fluoxetine or solvent, prior to dilution with 0.9% NaCl and stirring for 3 min. Additional pre-treatment of whole blood with 10 µM fluoxetine for 10 minutes also reduced 5-HT-dependent platelet aggregation: from 163 ± 76 AU*min to 101 ± 45 AU*min (mean ± SD; $P < 0.05$) (Figure 3.3). Since the synergistic effect of 5-HT and epinephrine on platelet aggregation was clearly less in this set of experiments, it cannot be concluded that inhibition of 5-HT-dependent platelet aggregation by fluoxetine was more pronounced. In a third set of experiments, we tested if fluoxetine had an immediate effect on platelet aggregation. In such a case, the inhibitory effect on platelet aggregation should not be attributed to the modification of SERT-function by fluoxetine (and the subsequent change in intra- and extracellular 5-HT concentrations), but rather to the antagonism of 5HT2A receptors. To test this hypothesis 10 µM fluoxetine was added just 5 seconds prior to addition of 5-HT plus epinephrine. This minimal incubation time with fluoxetine inhibited aggregation from 357 ± 209 to 216 ± 179 AU*min (mean ± SD; $P < 0.01$) (Figure 3.4).

The mean inhibition of aggregation after incubating whole blood with 10 µM fluoxetine for either 5 seconds, 3 min or 13 min, was 49 ± 31 %, 38 ± 17 % and 34 ±

21 %, respectively (mean ± SD, no statistical significance between different incubation periods for unpaired two-sided t test and Mann-Whitney rank sum test). The corresponding spontaneous platelet aggregation (controls) for the three sets of experiments were 21 ± 22, 47 ± 18 and 41 ± 15 AU*min (mean ± SD) and the difference was statistically significant between the first two sets ($P<0.05$, unpaired two-sided t test). After subtracting the amount of spontaneous platelet aggregation from all other experiment results, inhibition was 53 ± 34 %, 45 ± 19 % and 53 ± 35 % for 5 seconds, 3 min or 13 min respectively (mean ± SD, no statistical significance between different incubation periods for unpaired two-sided t test and Mann-Whitney rank sum test). Thus, the inhibition of 5-HT potentiated platelet aggregation was not different for the three incubation periods with 10µM fluoxetine.

A concentration response curve of fluoxetine on aggregation induced by 10 µM 5-HT plus epinephrine 10 µM was performed (Figure 3.5). Concentrations of 1 to 100 µM fluoxetine were used. The minimal concentration of fluoxetine that significantly inhibited 5-HT potentiated platelet aggregation was 5 µM and the inhibition by 100 µM fluoxetine was almost complete. The IC_{50} of fluoxetine was calculated with a four parameter sigmoidal model at 20 µM. In conclusion, the results indicate that fluoxetine, an SSRI, immediately inhibits 5-HT-potentiated platelet aggregation in whole blood *in vitro* most likely by an antagonism of platelet 5HT2A receptors.

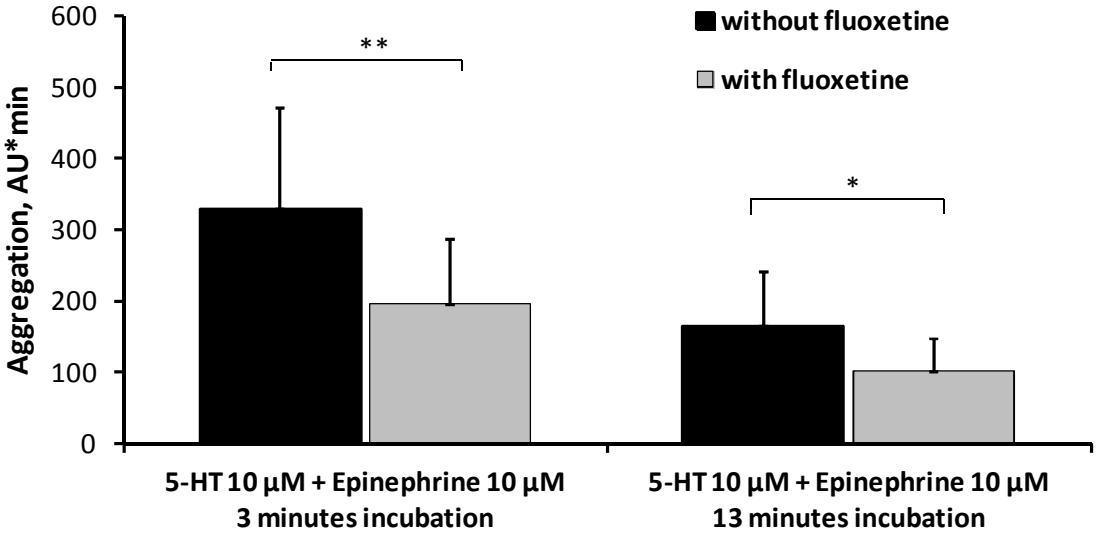

Figure 3.3: Effect of 3 and 13 minutes pre-incubation with 10 µM fluoxetine on 5-HT-potentiated platelet aggregation in whole blood. Hirudin-anticoagulated blood was diluted 1:1 with saline, and incubated in the absence or presence of 10 µM fluoxetine for 3 min (3 minutes incubation). Alternatively, in the absence or presence of 10 µM fluoxetine, hirudin-anticoagulated blood was incubated for 10 min at 37°C without stirring, then diluted 1:1 with saline, and incubated for 3 min with stirring at 37°C (13 minutes incubation). 5-HT (10 µM) plus epinephrine (10 µM) were added thereafter. Results are expressed as mean values ± SD (n=9 for and n=8 with different blood donors for 3 and 13 min incubation respectively). * refers to $P < 0.05$ and ** refers to $P < 0.01$.

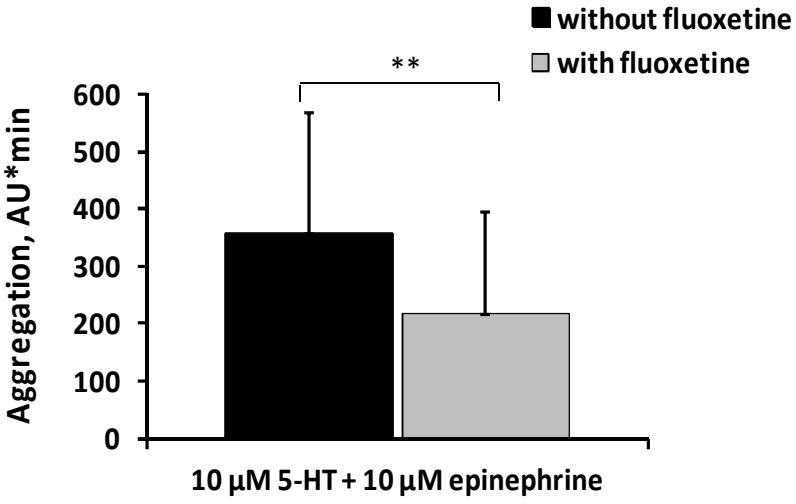

Figure 3.4: Effect of 5 seconds incubation with 10 µM fluoxetine on 5-HT-potentiated platelet aggregation in whole blood. Hirudin-anticoagulated blood was incubated for 3 min at 37°C; 10 µM fluoxetine or solvent was added 5 seconds before addition of the platelet stimuli. The results are expressed as mean values ± SD (n=6 with different blood donors). ** refers to $P < 0.01$.

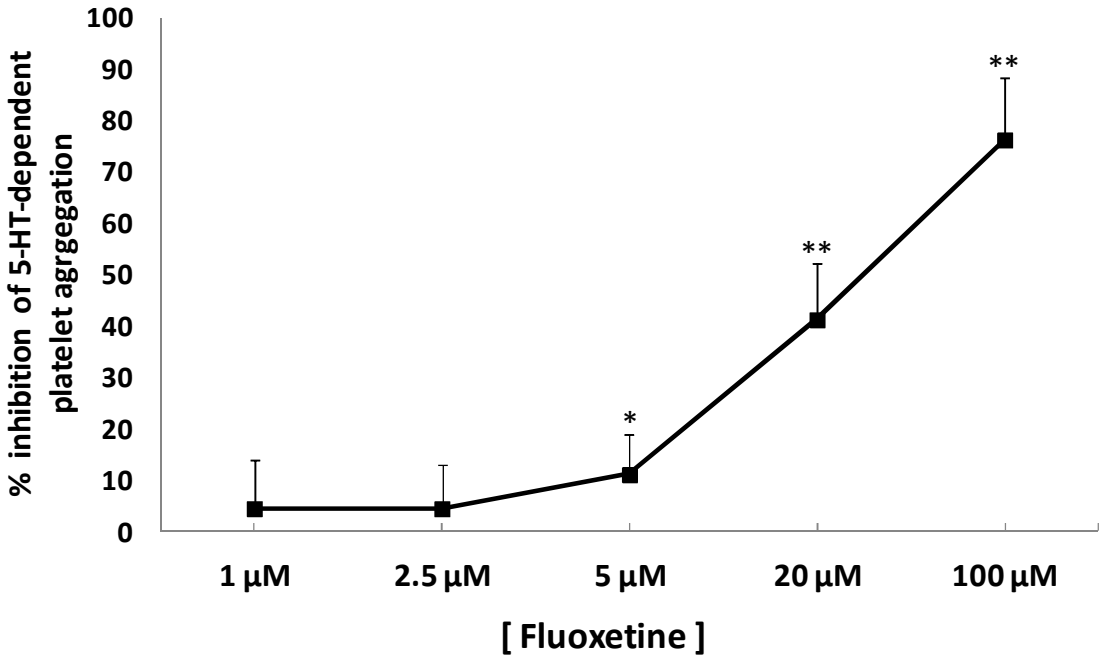

Figure 3.5: Effect of different concentrations of fluoxetine on 5-HT-potentiated platelet aggregation in whole blood. Hirudin-anticoagulated blood was incubated in the absence or presence of different concentrations of fluoxetine for 3 min at 37°C before addition of platelet stimuli. Results are expressed as percent of inhibition of platelet aggregation, after spontaneous aggregation was subtracted. * refers to $P < 0.05$ and ** refers to $P < 0.01$ for paired two sided t-test.

3.1.3 No effect of fluoxetine on stimulus-dependent platelet aggregation

Next we investigated the effects of the SSRI fluoxetine on a variety of physiologically and pathophysiologically relevant platelet agonists, namely ADP, TRAP, collagen and human atherosclerotic plaque homogenate. All these stimuli are known to increase the secretion of 5HT from platelet dense granules; fluoxetine could interfere with the action of released 5HT.

Addition of ADP induced a moderate platelet aggregation at a concentration of 1 µM and high platelet aggregation at a concentration of 5 µM. In both cases, incubation of whole blood with fluoxetine for 3 minutes did not inhibit the platelet aggregation induced by these two different concentrations of ADP (Figure 3.6).

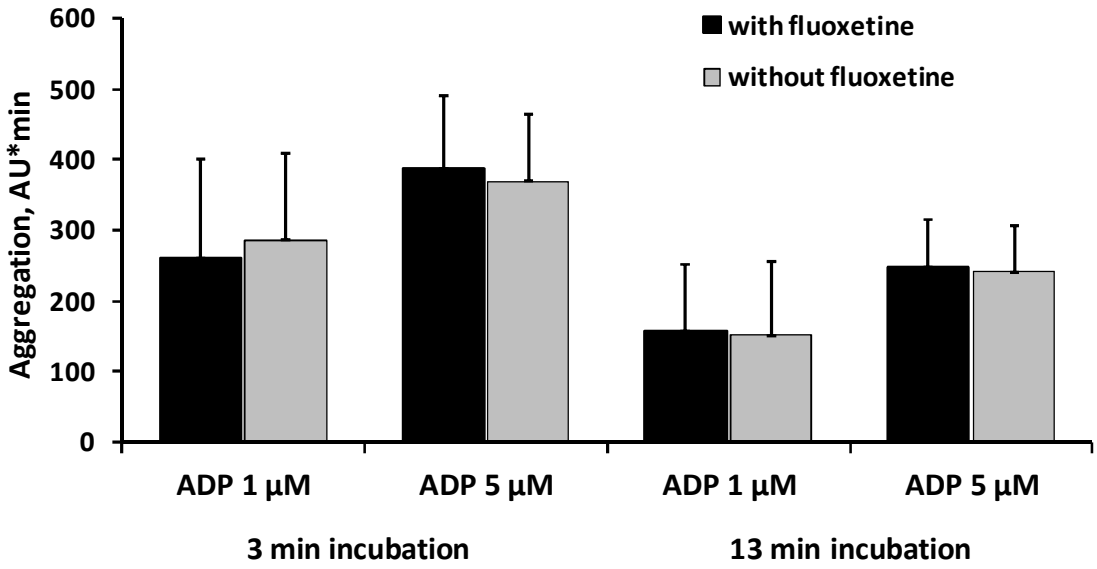

Figure 3.6: Effect of fluoxetine on ADP-induced platelet aggregation in whole blood. Hirudin-anticoagulated blood was diluted 1:1 with saline, and incubated in the absence or presence of 10 µM fluoxetine for 3 min (3 minutes incubation). Alternatively, in the absence or presence of 10 µM fluoxetine, hirudin-anticoagulated blood was incubated for 10 min at 37°C without stirring, then diluted 1:1 with saline, and incubated for 3 min with stirring at 37°C (13 minutes incubation). ADP was added thereafter. Results are expressed as mean values ± SD (n=5 with different blood donors). No effect of fluoxetine was observed.

When whole blood was incubated additionally for 10 minutes without stirring at 37°C, platelet aggregation induced by 1 µM and 5 µM ADP was less as compared to the experiments with a shorter incubation period. There was again no effect of fluoxetine in this set of experiments (Figure 3.6).

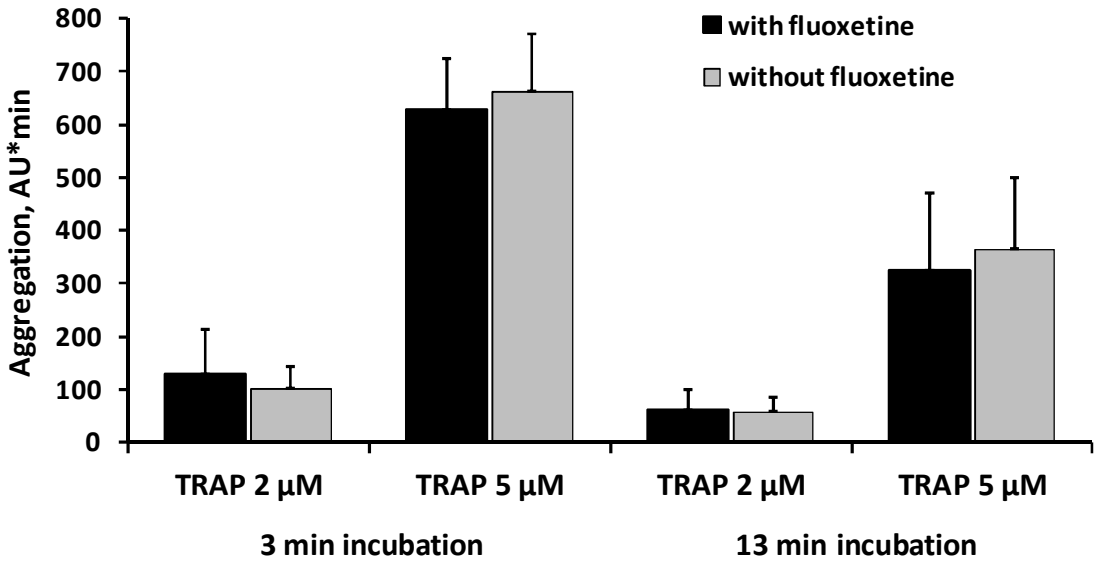

Figure 3.7: Effect of fluoxetine on TRAP-induced platelet aggregation in whole blood. Hirudin-anticoagulated blood was diluted 1:1 with saline, and incubated in the absence or presence of 10 µM fluoxetine for 3 min (3 minutes incubation). Alternatively, in the absence or presence of 10 µM fluoxetine, hirudin-anticoagulated blood was incubated for 10 min at 37°C without stirring, then diluted 1:1 with saline, and incubated for 3 min with stirring at 37°C (13 minutes incubation). TRAP was added thereafter. Results are expressed as mean values ± SD (n=5 with different blood donors). No effect of fluoxetine was observed.

Next, the effect of fluoxetine on platelet aggregation induced by the thrombin receptor activating peptide (TRAP) was tested (Figure 3.7). TRAP activates PAR-1, the main thrombin receptor of human platelets. It was found that 2 µM TRAP induced a modest platelet aggregation, whilst 5 µM TRAP induced a potent platelet aggregation. No effect of fluoxetine was observed on the aggregation induced by either concentration of TRAP used (2 µM and 5 µM) with both incubation periods (3 and 13 minutes respectively) (Figure 3.7).

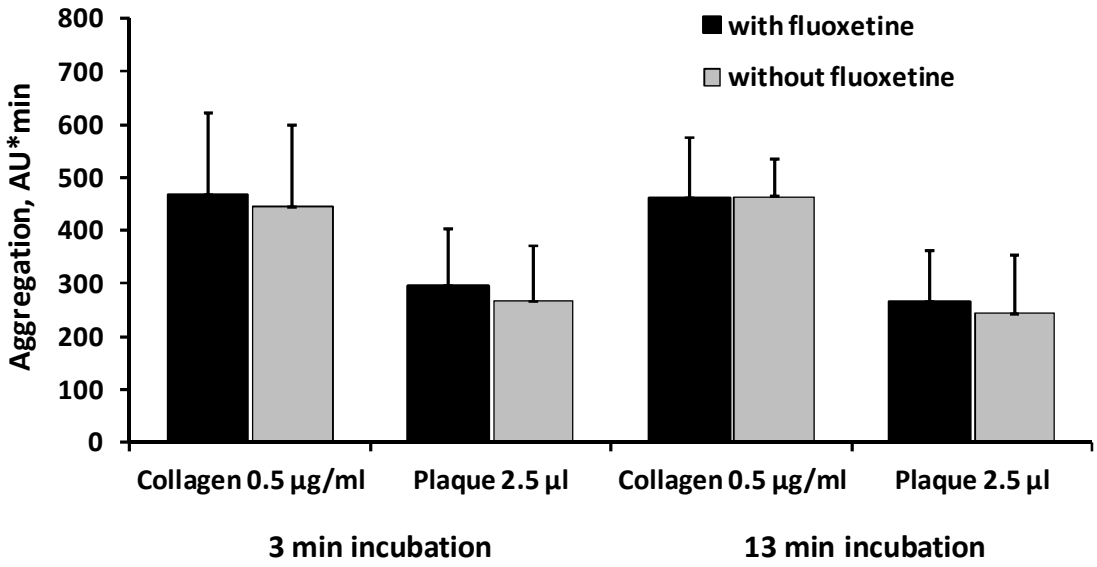

Figure 3.8: Effect of fluoxetine on collagen and plaque-induced platelet aggregation in whole blood.
Hirudin-anticoagulated blood was diluted 1:1 with saline, and incubated in the absence or presence of 10 µM fluoxetine for 3 min (3 minutes incubation). Alternatively, in the absence or presence of 10 µM fluoxetine, hirudin-anticoagulated blood was incubated for 10 min at 37°C without stirring, then diluted 1:1 with saline, and incubated for 3 min with stirring at 37°C (13 minutes incubation). Thereafter 0.5 µg/ml collagen or 2.5 µl human plaque homogenate (final concentration of 0.42 mg/ml) were added. Results are expressed as mean values ± SD (n=5 with different blood donors). No effect of fluoxetine was observed.

We also investigated the effect of the SSRI fluoxetine on platelet aggregation induced by collagen, a main substance of the subendothelial matrix and a main initiator of platelet aggregation. The relatively high platelet aggregation induced by 0.5 µg/ml collagen could not be inhibited by incubation with 10 µM fluoxetine for 3 or 13 minutes (Figure 3.8).

Last but not least, we investigated the effect of fluoxetine on the platelet aggregation induced by the pathophysiologically relevant platelet agonist, human atheromatous plaque homogenate. Platelet aggregation induced by 2.5 µl of plaque homogenate (final concentration of 0.42 mg/ml) was again not inhibited by incubation with 10 µM fluoxetine for 3 or 13 minutes (Figure 3.8).

In conclusion, fluoxetine was able to inhibit only the 5-HT-potentiated platelet aggregation in blood, but it did not interfere with the aggregation response induced by a variety of other platelet stimuli, which play a significant physiological and pathophysiological role on platelet aggregation.

3.1.4 Effects of the combination of acetylsalicylic acid and fluoxetine on platelet aggregation

Fluoxetine alone did not inhibit platelet aggregation, except the one dependent on exogenous 5-HT administration. Subsequently, we investigated if fluoxetine could potentiate platelet inhibition caused by the clinically relevant antiplatelet acetylsalicylic acid (ASA, aspirin). Such an interaction could be of clinical significance, especially if it occurred when atherosclerotic plaque was used as a platelet stimulus.

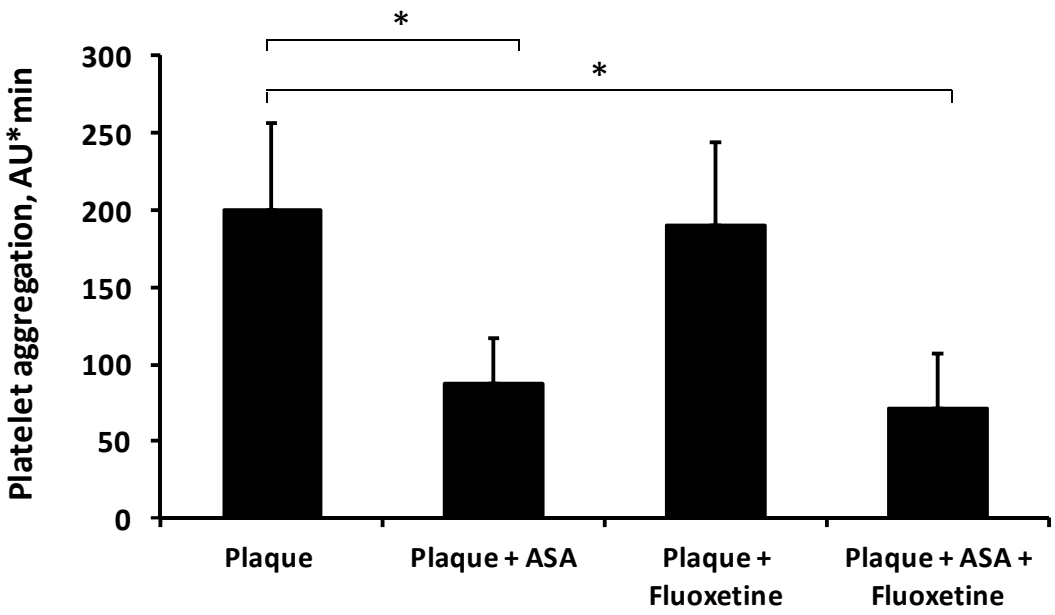

Figure 3.9: Fluoxetine does not potentiate the inhibition of plaque-induced aggregation by acetylsalicylic acid (ASA). Hirudin-anticoagulated blood was incubated in the absence or presence of 10 µM fluoxetine, 5.4 mM acetylsalicylic acid (ASA) or their combination for 3 min at 37°C before addition of 2.5 µl of human atherosclerotic plaque homogenate (final concentration of 0.42 mg/ml). Results are expressed as mean values ± SD (n=6 with different blood donors). Fluoxetine did not inhibit plaque-dependent aggregation alone or in the presence of ASA. * refers to $P < 0.05$.

Incubation with ASA inhibited the plaque-induced aggregation significantly. Platelet aggregation response was 200 ± 55 to 86 ± 29 AU*min in the absence or presence of ASA respectively (mean ± SD; $P < 0.05$) (Figure 3.9). As observed in previous experiments, 10 µM fluoxetine did not inhibit the plaque-induced platelet aggregation. Moreover the plaque-induced platelet aggregation observed with inhibition by ASA plus fluoxetine did not differ from that observed by ASA alone (70 ± 35 and 86 ± 29 AU*min respectively, $P = 0.26$) (Figure 3.9).

We also investigated the inhibitory effect of ASA alone and fluoxetine plus ASA on the 5-HT-potentiated aggregation in order to assess any possible synergism.

As expected, 10 µM fluoxetine reduced platelet aggregation induced by 5-HT plus epinephrine from 379 ± 156 to 307 ± 166 AU*min (mean ± SD; $P < 0.05$) (Figure 3.10). ASA had no effect on 5-HT-potentiated aggregation (366 ± 166 to 379 ± 156 AU*min with and without 5.4 mM ASA, $P = 0.32$) (Figure 3.10). Moreover, the inhibitory effect of fluoxetine alone did not differ from that of the combination of ASA plus fluoxetine (307 ± 166 to 320 ± 158 AU*min respectively, $P = 0.48$) (Figure 3.10).

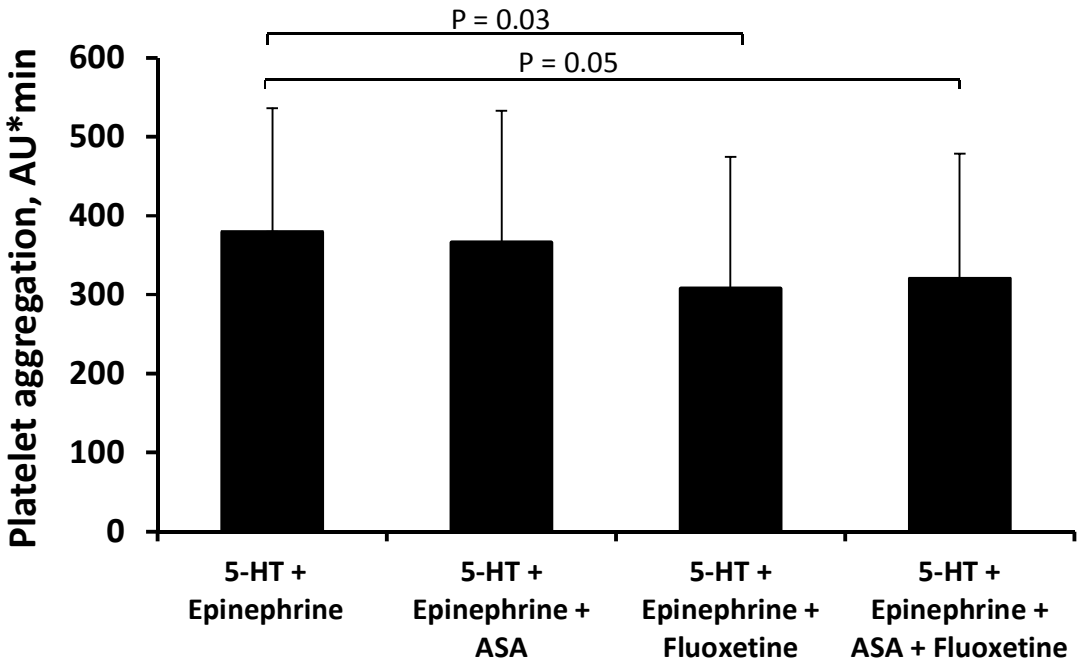

Figure 3.10: Aspirin does not potentiate the inhibition of 5-HT-dependent aggregation by fluoxetine. Hirudin-anticoagulated blood was incubated in the absence or presence of 10 µM fluoxetine, 5.4 mM acetylsalicylic acid (ASA) or their combination for 3 min at 37°C before addition of 10 µM 5-HT plus 10 µM epinephrine. Results are expressed as mean values ± SD (n=6 with different blood donors). ASA did not inhibit 5-HT-dependent aggregation alone or in the presence of fluoxetine. P refers to paired two-sided t-test.

In conclusion, plaque-induced platelet aggregation was inhibited by ASA, and 5-HT dependent platelet aggregation was inhibited by fluoxetine, but their combination did not result in a further inhibition on either type of stimulus-induced platelet aggregation.

3.1.5 Role of different blood pre-incubation time periods on spontaneous and stimulus-induced platelet aggregation

As described in the previous sections, we conducted two sets of experiments to examine the effect of fluoxetine on each different platelet agonist. In the first set of

experiments we used the standard procedure of multiple electrode aggregometry, that is we incubated blood diluted 1:1 with saline at 37°C for 3 minutes under stirring conditions prior to addition of the platelet agonist. In the second set of experiments whole blood was pre-incubated for 10 minutes without stirring at 37°C, and then diluted 1:1 with saline, and incubated for 3 minutes whilst stirring prior to addition of the platelet agonist. Thus, in the second set of experiments blood was exposed at 37°C for an additional time period of 10 minutes. We observed that for certain platelet stimuli the additional incubation time period of blood at 37°C resulted in a decrease of platelet aggregation.

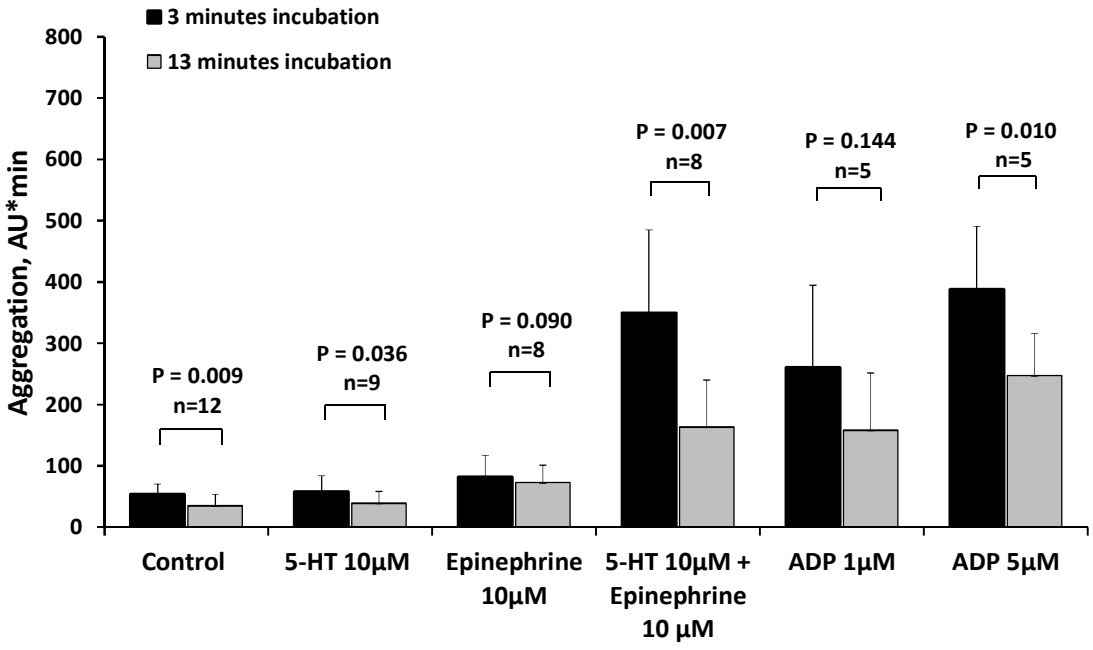

Figure 3.11: Effect of different incubation periods at 37°C on platelet aggregation after addition of buffer (control), 5-HT, epinephrine, 5-HT plus epinephrine and ADP. Hirudin-anticoagulated blood was diluted 1:1 with saline, and incubated for 3 min with stirring at 37°C (3 minutes incubation), or hirudin-anticoagulated blood was incubated for 10 min at 37°C without stirring, then diluted 1:1 with saline, and incubated for 3 min with stirring at 37°C (13 minutes incubation). Platelet stimuli were added thereafter. Results are expressed as mean values ± SD (n=5-12 with different blood donors). P values refer to paired two-sided t-test.

The additional pre-incubation period of blood at 37^{o}C led to reduction of spontaneous aggregation (control) from 54 ± 16 to 34 ± 18 AU*min ($P < 0.01$, n=12 with different donors) (Figure 3.2). 5-HT alone, as described above, did not induce platelet aggregation compared to control. The 10 min pre-incubation at 37^{o}C also reduced the aggregation observed in the presence of 10 µM 5-HT from 58 ± 25 to 39 ± 19 AU*min ($P < 0.05$ n=9 with different donors) (Figure 3.11). The epinephrine-induced platelet aggregation tended to be inhibited by the 10 min pre-incubation at 37^{o}C, but statistical significance was not reached (82 ± 34 to 72 ± 28 AU*min, $P = 0.09$, n=8 with different donors) (Figure 3.11). The combined effect of 10 µM HT plus 10 µM epinephrine was inhibited by 10 min pre-incubation at 37^{o}C from 350 ± 134 to 163 ± 76 AU*min ($P < 0.01$, n=8 with different donors) (Figure 3.11).

The relatively high platelet aggregation induced by 5 µM ADP was also significantly decreased from 388 ± 101 to 247 ± 68 AU*min by 10 min pre-incubation at 37^{o}C ($P = 0.01$, n=5 with different donors) (Figure 3.11). The same pre-incubation inhibited the moderate platelet aggregation induced by 1 µM ADP (261 ± 133 versus 158 ± 93 AU*min for 3 and 13 minutes incubation respectively), but no statistical significance was reached ($P = 0.14$, n=5 with different donors) (Figure 3.11).

Similarly, the high amount of platelet aggregation induced by 5 µM TRAP was significantly inhibited from 627 ± 96 to 325 ± 143 AU*min by 10 min pre-incubation at 37^{o}C, while the inhibition of moderate platelet aggregation induced by 2 µM TRAP, from 128 ± 86 to 62 ± 36 AU*min, did not reach statistical significance ($P = 0.01$ and $P = 0.08$ with 5 and 2 µM TRAP respectively, n=5 with different donors) (Figure 3.12).

In contrast, the 10 min pre-incubation at 37°C had no effect on the platelet aggregation induced by collagen and human plaque (P = 0.96 and P = 0.52 respectively, n=5 with different donors) (Figure 3.12).

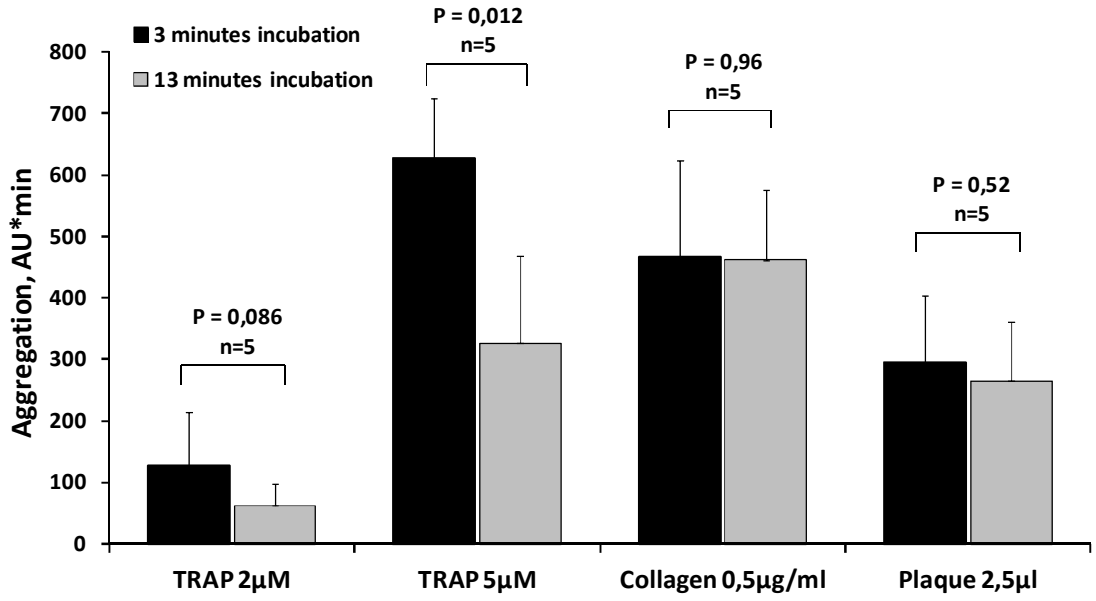

Figure 3.12: Effect of different incubation periods at 37°C on TRAP-, collagen- and plaque-induced platelet aggregation. Hirudin-anticoagulated blood was diluted 1:1 with saline, and incubated for 3 min with stirring at 37°C (3 minutes incubation), or hirudin-anticoagulated blood was incubated for 10 min at 37°C without stirring, then diluted 1:1 with saline, and incubated for 3 min with stirring at 37°C (13 minutes incubation). Platelet stimuli were added thereafter. Results are expressed as mean values ± SD (n=5-12 with different blood donors). P values refer to paired two-sided t-test.

In conclusion, pre-incubation of whole blood at 37°C for 10 minutes prior to the standard 3-min incubation in MEA, inhibited significantly the spontaneous aggregation, as well as the platelet aggregation observed after addition of 5-HT, 5-HT plus epinephrine, high concentrations of TRAP and ADP, and tended to inhibit the platelet aggregation induced by epinephrine and small concentrations of TRAP and ADP. All the receptors of the above stimuli, even of spontaneous aggregation which

is ADP-dependent, are coupled to G-proteins. On the other hand, whole blood platelet aggregation induced by collagen and atherosclerotic plaque, whose receptors are not G-protein coupled, were not affected by incubation of whole blood at 37^{O}C for 10 minutes.

3.2 Role of the platelet 5-HT2A receptor for platelet adhesion, aggregation and secretion in blood

3.2.1 Role of 5-HT2A receptor antagonists on 5-HT induced potentiation of platelet aggregation

The main 5-HT receptor on the platelet surface is the G_q-coupled 5-HT2A receptor (6). Since exogenously administered 5-HT can potentiate human whole blood platelet aggregation induced by a variety of agonists, we investigated the effect of 5-HT2A receptor antagonists on 5-HT-dependent aggregation. Once again, we used the model of 5-HT plus epinephrine-induced platelet aggregation, where the potentiating effect of 5-HT on the weak aggregation induced by epinephrine is quite pronounced.

Three different 5-HT2A antagonists were used: ketanserin, R-96544 and sarpogrelate (112, 139, 140). The 5-HT-potentiated platelet aggregation was dose dependently inhibited to levels of 64 ± 42 and 76 ± 29 AU*min with 10 μM ketanserin and 10 μM R-96544, respectively, levels that are similar to the amount of aggregation induced by 10 μM epinephrine alone. Statistical significance was reached at concentrations of $\geq$ 10 nM for ketanserin and of $\geq$ 1nM for R-96544. The IC_{50} values of ketanserin and R-96544 hydrochloride were 5.6 and 1.8 nM,

respectively. Statistical significance of inhibition by sarpogrelate required a much higher concentration (100μM) (Figure 3.13).

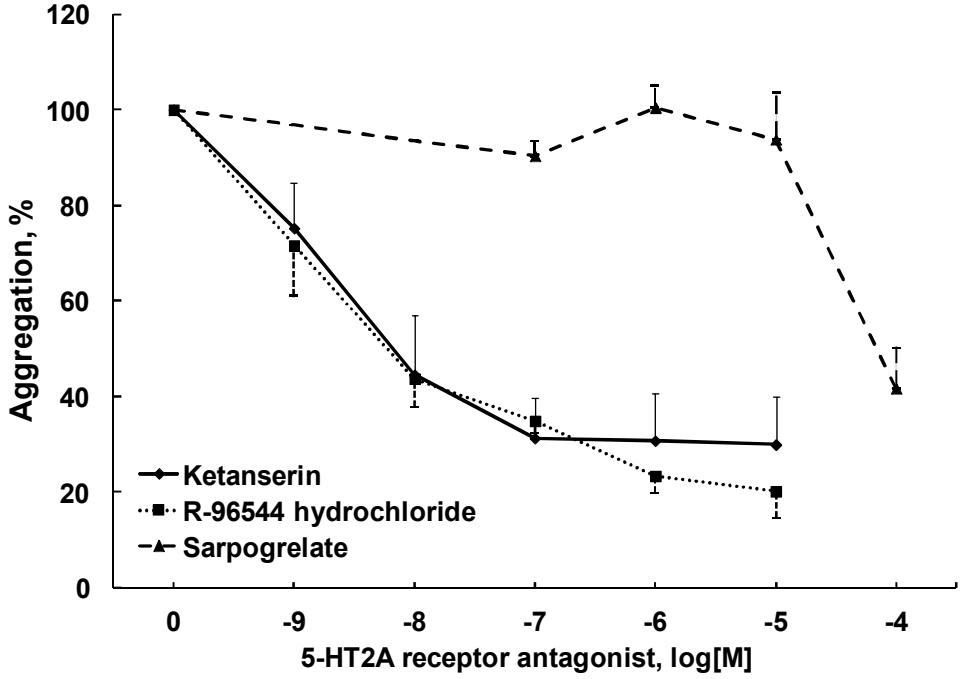

Figure 3.13: Effect of three different 5-HT2A receptor antagonists on 5-HT-dependent platelet aggregation in whole blood. Hirudin-anticoagulated blood was incubated with different concentrations of ketanserin, R-96544 and sarpogrelate for 3 min with stirring at 37°C before addition of 10 μM 5-HT plus 10 μM epinephrine. The platelet aggregation response was recorded by MEA for 5 min. Aggregation induced by 5-HT plus epinephrine was set to 100% in individual experiments. Results are expressed as mean values ± SE (n=5 with different blood donors).

3.2.2 Effect of ketanserin on stimulus-induced whole blood platelet aggregation

After examining the effect of 5-HT2A antagonists on 5-HT-dependent platelet aggregation, we examined the effect of ketanserin on aggregation induced by physiological and pathophysiological platelet stimuli, such as collagen, TRAP and plaque.

In 5 experiments with different blood donors a supramaximal concentration of 1 µM ketanserin inhibited potently the 5-HT-dependent platelet aggregation, but did not have any effect on platelet aggregation induced by collagen (0.1 µg/ml and 0.5 µg/ml), plaque (0.42 mg/ml) and TRAP (5 µM) (Figure 3.14).

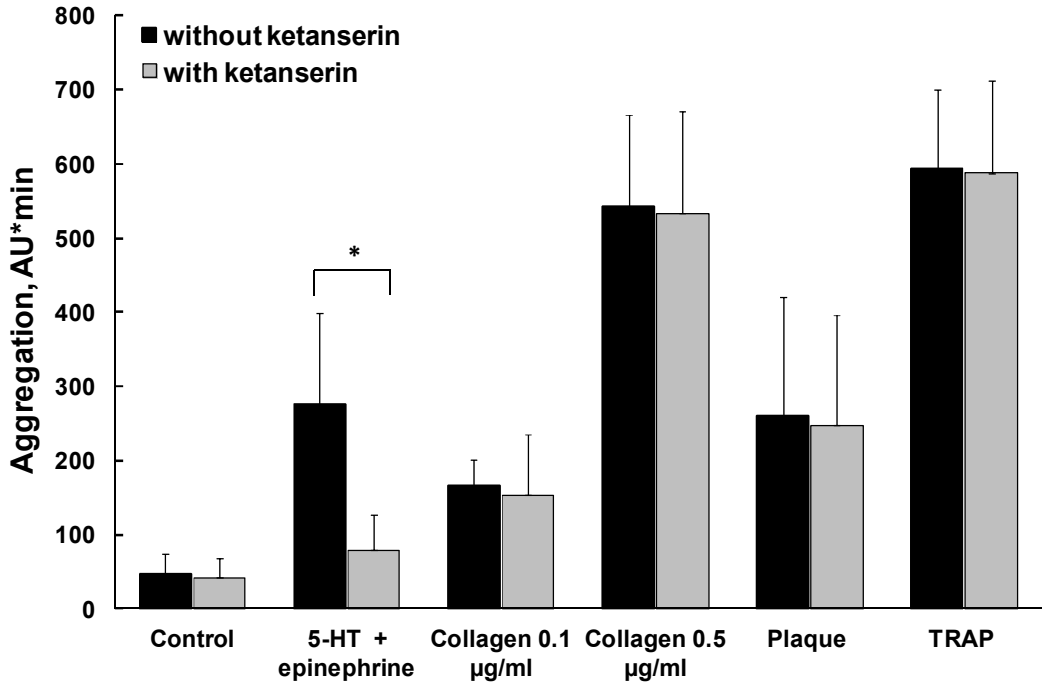

Figure 3.14: Effect of ketanserin on stimulus-induced platelet aggregation in whole blood. Hirudin-anticoagulated blood was incubated in the absence or presence of 1 µM ketanserin for 3 min at 37°C before addition of 10 µM 5-HT plus 10 µM epinephrine, collagen (0.1 µg/ml and 0.5 µg/ml), plaque (0.42 mg/ml) or TRAP (5 µM). The platelet aggregation response was recorded by MEA for 5 min. Results are expressed as mean values ± SD (n=5 with different blood donors). * $P < 0.05$

3.2.3 Effect of ketanserin on stimulus-induced ATP secretion in whole blood

Aside from the effects on platelet aggregation, we examined the effects of ketanserin on ATP secretion in the same blood donors. After platelet activation, ATP secretion originates from dense granules as 5-HT secretion does (33).

No ATP secretion was observed without addition of platelet stimuli (Figure 3.15). The small amount of ATP secretion observed in blood stimulated with 10 µM 5-HT plus 10 µM epinephrine (22 ± 21 nM, mean ± SD) was completely inhibited by 1 µM ketanserin (Figure 3.5).

The low ATP secretion induced by 0.1 µM collagen was not inhibited by 1 µM ketanserin. At higher concentrations of collagen (0.5 µg/ml), an ATP secretion of 237 ± 135 nM ATP without ketanserin and 152 ± 80 nM ATP with ketanserin was observed, but the difference was not statistically significant (P=0.11, n=5) (Figure 3.15).

The plaque-induced ATP secretion reached 57 ± 29 nM ATP and the TRAP-induced ATP-secretion reached 227 ± 215 nM ATP. Ketanserin did not inhibit ATP-secretion induced by either human plaque or ATP (Figure 3.15).

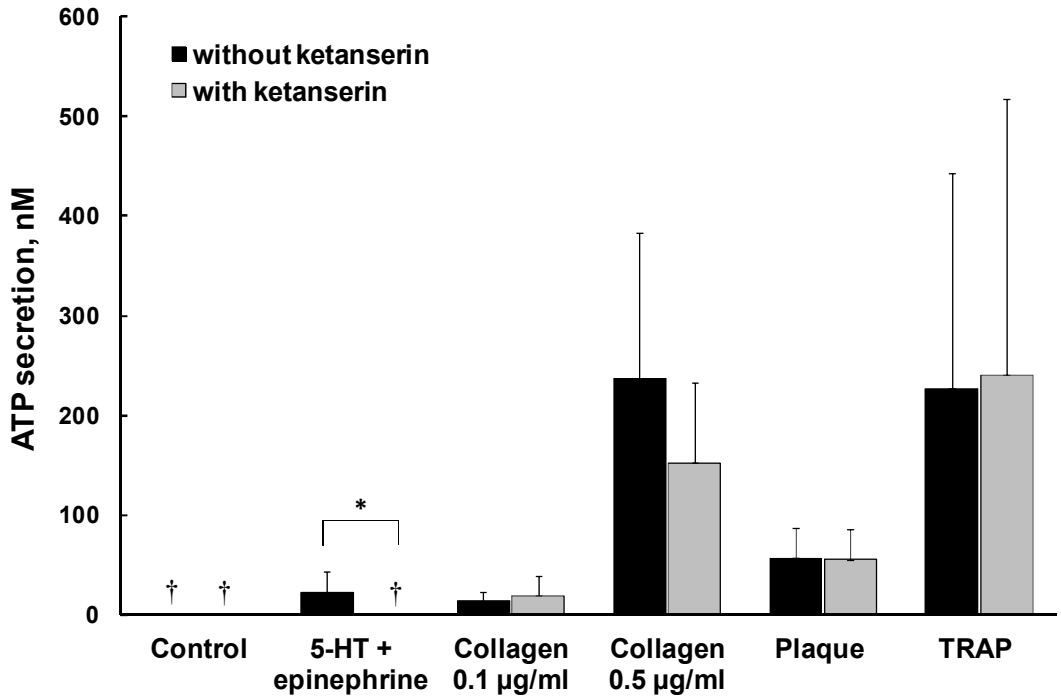

Figure 3.15: Effect of ketanserin on stimulus-induced ATP secretion in whole blood. Diluted hirudin-anticoagulated blood (1:1) was incubated for 30 seconds with luciferin-luciferase reagent, then 1 µM ketanserin or solvent was added and blood was further incubated for 3 min at 37°C before addition of 10 µM 5-HT plus 10 µM epinephrine, collagen (0.1 µg/ml and 0.5 µg/ml), plaque (0.42 mg/ml) or TRAP (5µM). The maximum ATP response was recorded by luminescence. Results are expressed as mean values ± SD (n=5 with different blood donors). * $P < 0.05$ for Mann–Whitney rank sum test. † Values are 0 ± 0 nM ATP (mean ± SD).

In conclusion, ketanserin inhibited only the 5-HT-dependent ATP secretion, but not the ATP secretion induced by three important pathophysiological relevant platelet agonists, namely TRAP, collagen and plaque.

3.2.4 Effect of ketanserin on plaque-induced platelet aggregate formation under arterial flow

Ketanserin did not affect platelet aggregation and dense granule secretion after stimulation with various platelet stimuli in static assays. To explore our question in

under more relevant pathophysiological conditions we examined the effect of ketanserin under arterial flow conditions using human plaque-homogenate coated surfaces, a model that resembles *in vivo* atherothrombus formation. The shear rate applied was 500 s^{-1}, which corresponds to blood flow in medium sized arteries. The platelet surface coverage was measured by fluorescence (Figure 3.16).

A maximal concentration of ketanserin (1 µM) had neither at early, nor at late stages after start of blood flow, a significant effect on platelet adhesion and aggregation to human atherosclerotic plaques (Figure 3.17). Thus 5-HT2A receptor antagonism does not affect the plaque-induced platelet aggregation under flow.

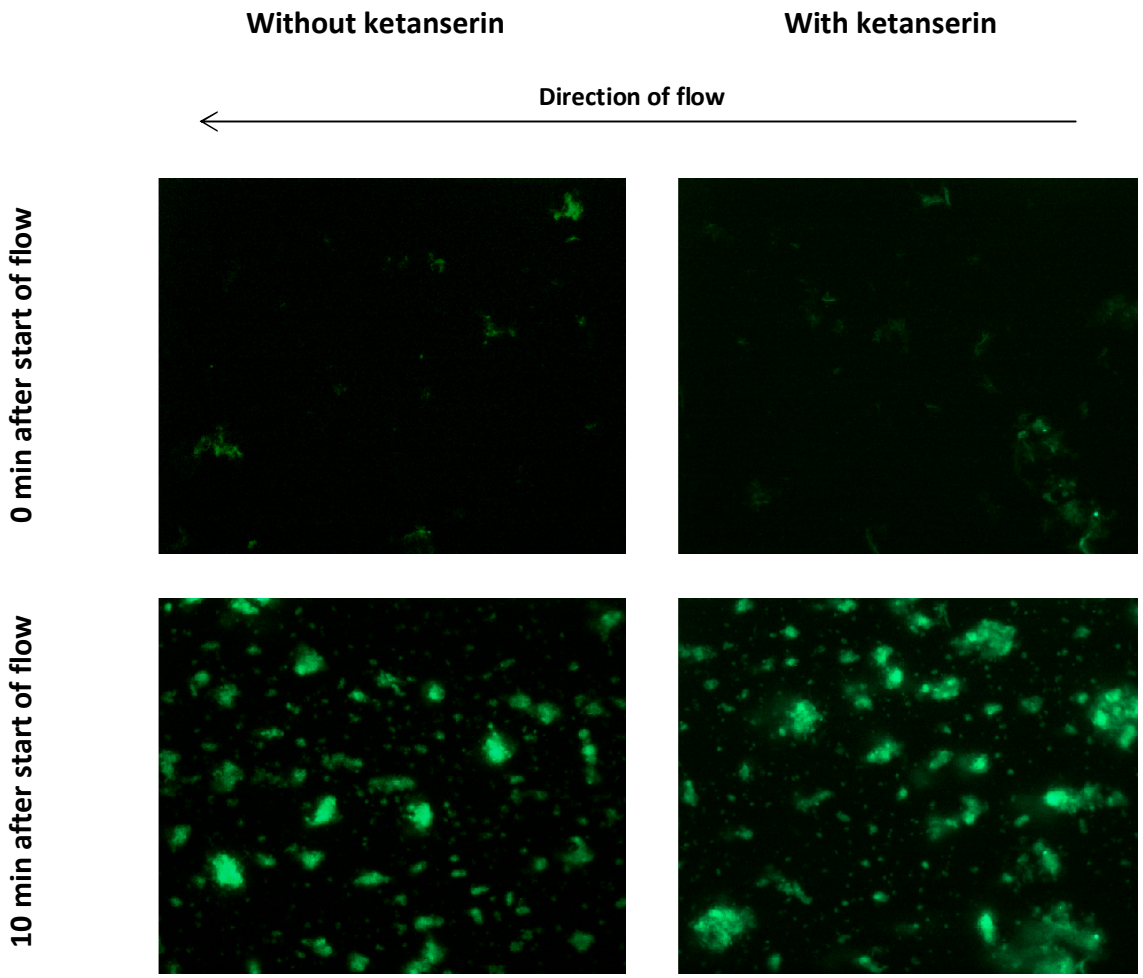

Figure 3.16: Human plaque-induced platelet adhesion and aggregation under arterial flow with and without ketanserin. Fluorescence micrographs. Hirudin-anticoagulated blood was incubated for 3 min at 37^{o}C with solvent (control) or ketanserin (1 µM) along with 10 µM mepacrine to stain platelets. Blood was then perfused over plaque-coated surfaces for 10 min at 37^{o}C at a shear rate of 500 s^{-1}. Fluorescent images of one representative experiment at the start (0 min) and at the end (10 min) in the presence or absence of 1 µM ketanserin are shown. The light-green areas at 0 min are due to autofluorescence of the plaque material, while the brighter green areas at 10 min is fluorescence emitted from the mepacrine-stained platelets and platelet aggregates.

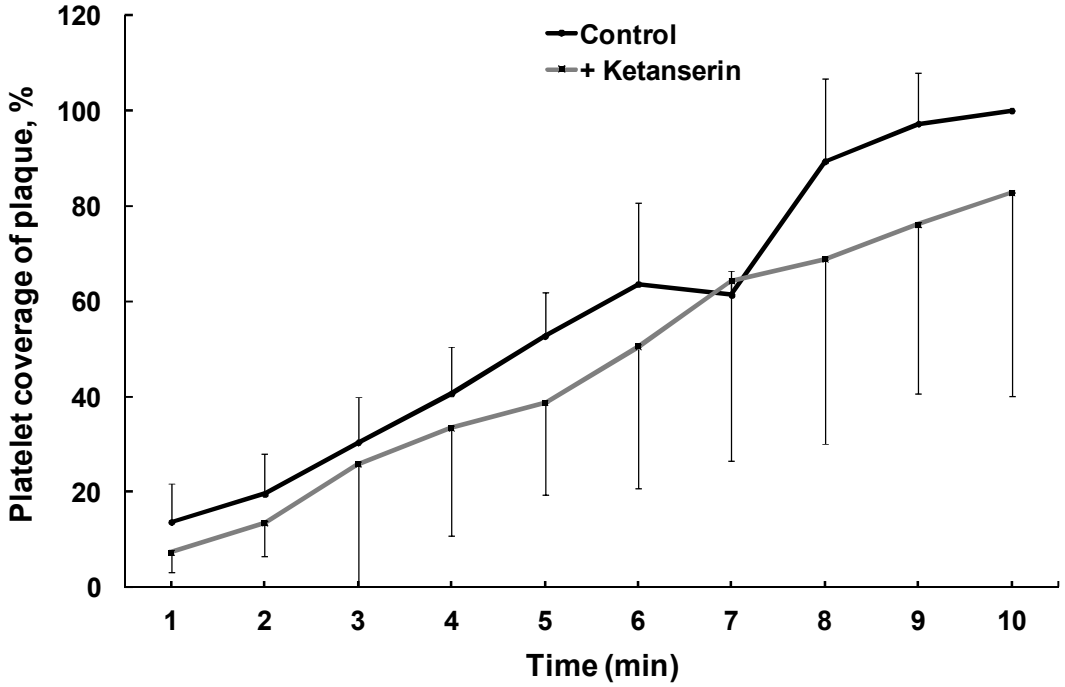

Figure 3.17: Effect of ketanserin on human plaque-induced platelet adhesion and aggregation under arterial flow. Line diagram. Blood was treated as in Fig. 3.16. Platelet coverage was quantified each minute. Values (% of platelet coverage of control at 10 min) are mean ± SD (n= 3 with different blood donors).

3.2.5 Effect of 5-HT2A receptor antagonism on shear-induced platelet adhesion and aggregate formation

We also examined the effect of ketanserin on shear-induced platelet adhesion and aggregation using a different experimental model, the cone and plate analyzer (137). Two different shear rates, 600 s^{-1} and 1200 s^{-1} were applied. The experiments at 1200 s^{-1} expanded the applicability of our findings at shear stress rates found in slightly stenotic arteries.

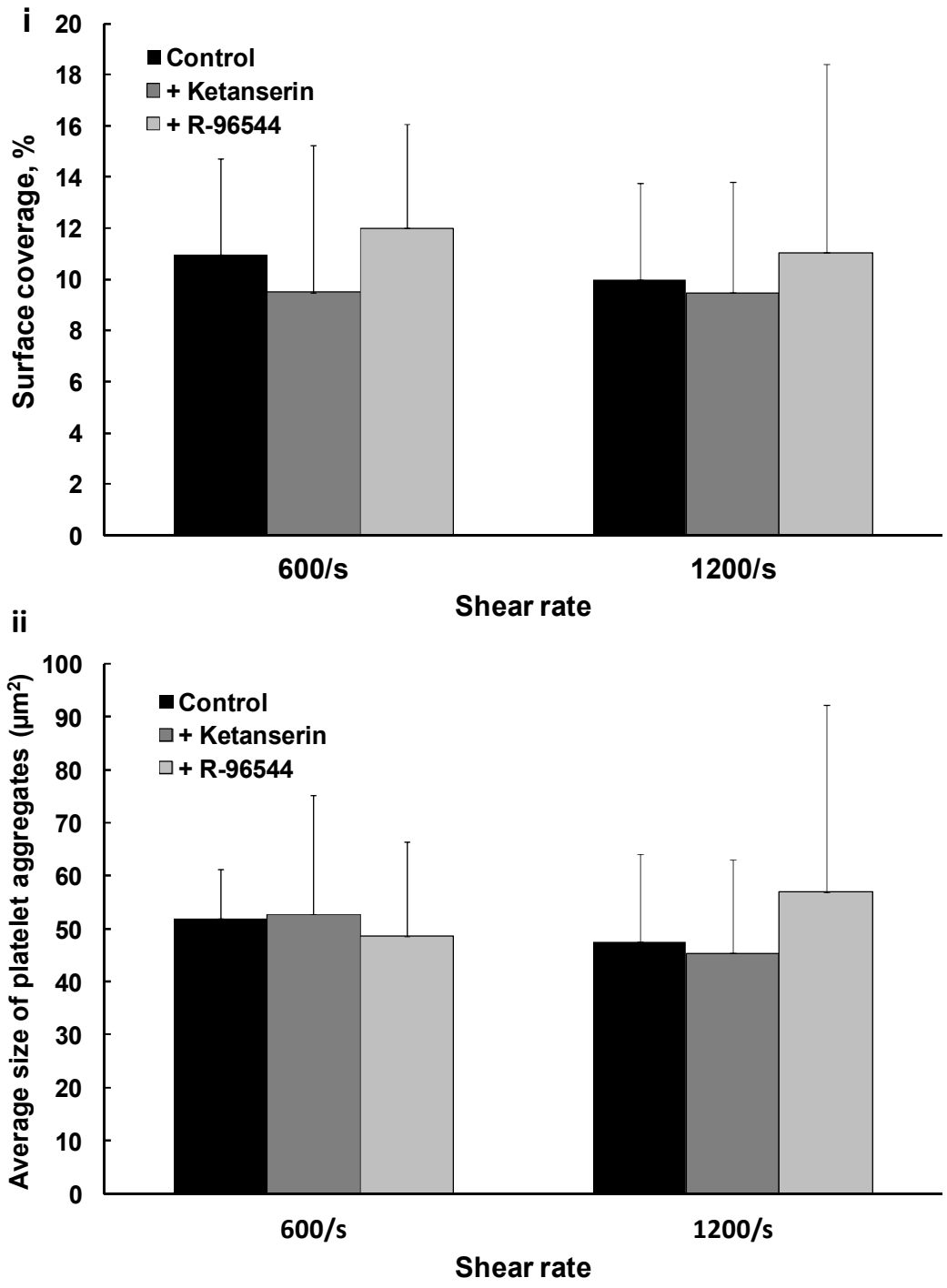

Figure 3.18: Shear stress-induced platelet adhesion and aggregate formation is not affected by ketanserin. Blood (0.25 mL) was incubated with solvent (control), ketanserin (1 µM) or R-96544 (1 µM) for 3 min before application of two different shear rates for 2 min in a cone and plate analyzer. (i) Platelet surface coverage. (ii) Average size of platelet aggregates. Values are mean ± SD (n= 5 with different blood donors).

The two potent 5-HT2A receptor antagonists ketanserin and R-96544 did not inhibit shear stress induced platelet adhesion and aggregate formation, either at a shear rate of 600 s^{-1} or at a shear rate of 1200 s^{-1} (Figure 3.18).

3.3 Role of the platelet 5-HT4 receptor for platelet aggregation in blood

3.3.1 Role of 5-HT4 receptor agonism on platelet aggregation

Recently the expression of 5-HT-4 receptors was described in human platelets (126). In two contradictive studies, a potent, partial 5-HT4 receptor agonist was found either to enhance or not to affect stimulus-induced platelet aggregation in PRP *in vitro* (126, 128). In view of these results, we investigated the effect of 5-HT4 receptor activation on platelet function in blood by using multiple electrode aggregometry.

A stimulation of a physiologically relevant G_s-coupled 5-HT4 receptor would theoretically raise the intracellular levels of cAMP leading to inhibition of platelet aggregation. We explored the effect of CJ-033,466, a potent and selective 5-HT4 receptor agonist (141) on inhibition of platelet aggregation induced by ADP. However, pre-incubation of blood (3 min) with 5 different concentrations of CJ-033,466 had no effect on platelet aggregation induced by two concentrations of ADP (1 µM and 5 µM) (Figure 3.19).

Next, we examined the effect of 5-HT4 activation on spontaneous platelet aggregation, which is also dependent on ADP. This originates from erythrocytes

which are mechanically altered by the stirring process, and is released to the extracellular space (142). As in the experiments of ADP-induced aggregation, CJ-033,466 had no effect on spontaneous aggregation (Figure 3.20, lower curve).

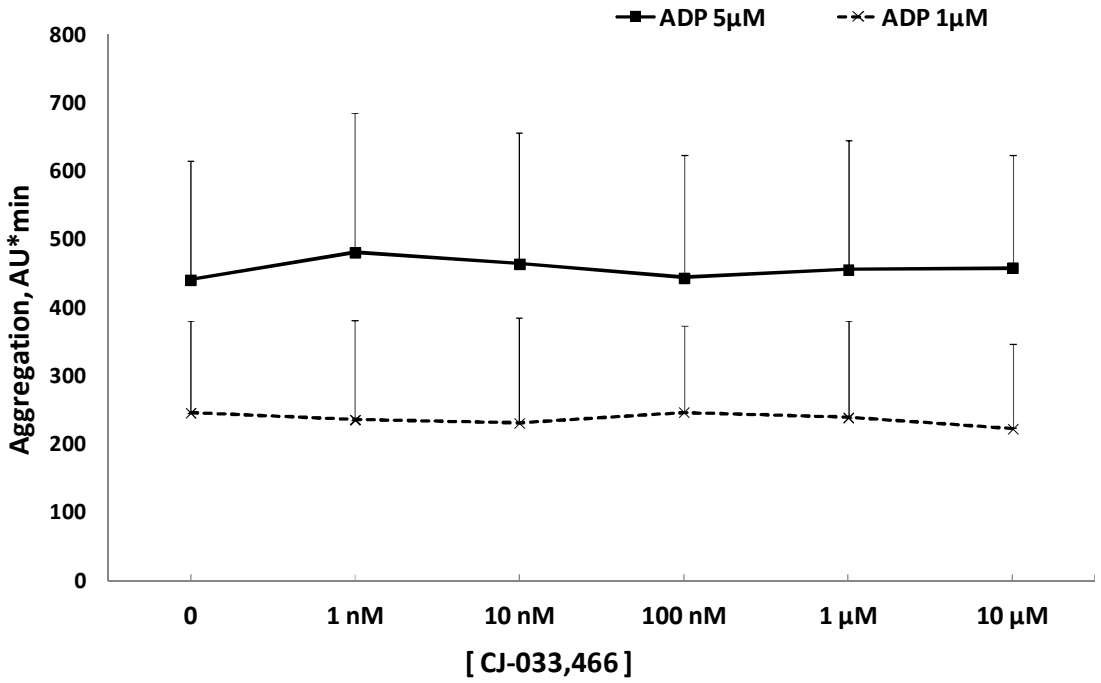

Figure 3.19: Effect of 5-HT4 activation on ADP-induced platelet aggregation in whole blood. Hirudin-anticoagulated blood was incubated with different concentrations of CJ-033,466 for 3 min with stirring at 37°C before addition of two different concentrations of ADP. Results are expressed as mean +SD (n=5 with different blood donors).

Last, we examined the effect of 5-HT4 activation on the small increase of aggregation induced by epinephrine, which activates platelets through an α_2-adrenergic receptor coupled G_i-mediated pathway (51). If active 5-HT4 receptors coupled to G_s existed on the platelet surface, CJ-033,466 would directly counteract epinephrine's weak platelet stimulatory action. Indeed, CJ-033,466 dose-dependently reduced the epinephrine-induced aggregation. It was significant at 1 and 10 µM concentrations of CJ-033,466, respectively ($P < 0.05$, n=6 with different

blood donors) (Figure 3.20, upper curve). This indicates that active 5-HT4 receptors may exist on the platelet membrane surface.

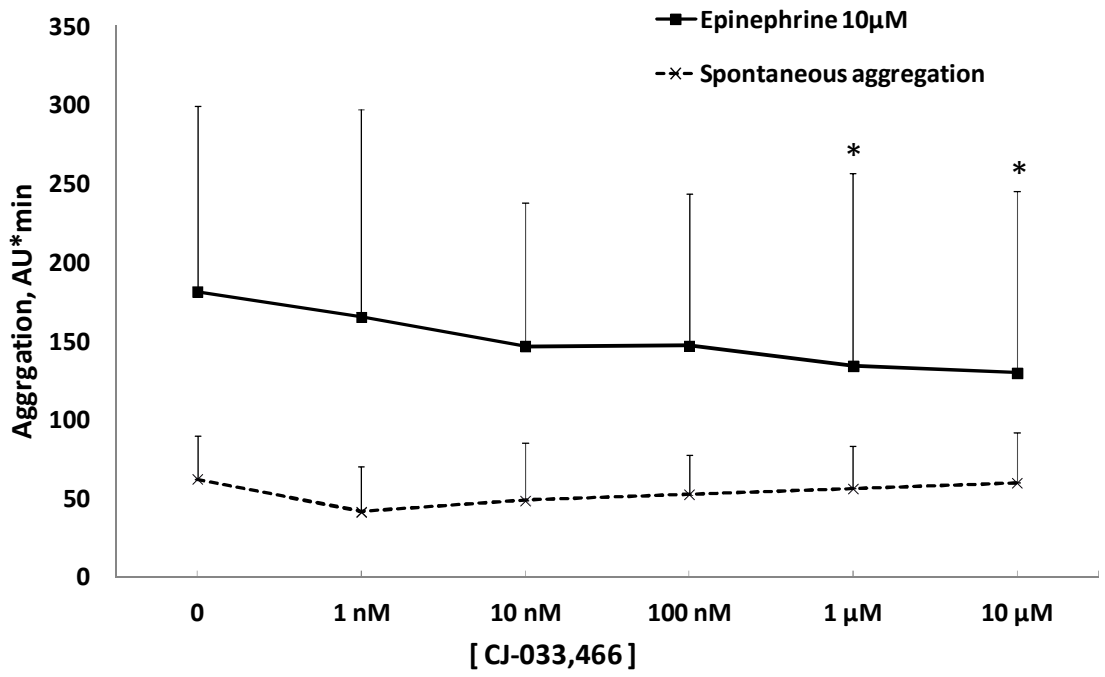

Figure 3.20: Effect of 5-HT4 activation on spontaneous and epinephrine-induced platelet aggregation in whole blood. Hirudin-anticoagulated blood was incubated with different concentrations of CJ-033,466 for 3 min with stirring at 37°C before addition of 10 µM epinephrine or solvent. Results are expressed as mean values + SD (n=6 and n=7 with different blood donors for epinephrine-induced and spontaneous aggregation respectively). * $P < 0.05$ for paired two sided t test versus control (0 nM CJ-033,466).

3.3.2 Role of 5-HT4 receptor antagonism on platelet aggregation

Furthermore, the role of 5-HT4 receptor antagonism was examined using two potent and selective structurally distinct 5-HT4 receptor inhibitors, GR113808 and RS 23597-190 (143, 144).

If 5-HT activated both a stimulatory 5-HT2A receptor and an inhibitory 5-HT4 receptor on platelets, the inhibition of active 5-HT4 receptors on the platelet surface

would blunt the 5-HT4-mediated increase of intracellular cAMP and potentiate 5-HT-dependent platelet aggregation induced by 5-HT alone (via the G_q-dependent 5-HT2A pathway) or in combination with epinephrine (activation of the G_i pathway).

However, incubation of blood with 5 different concentrations of the two 5-HT4 receptor antagonists GR113808 and RS 23597-190 did not have any effect on whole blood platelet aggregation induced by 10 µM 5-HT alone or in combination with 10 µM epinephrine (Figure 3.21). Hence 5-HT does not activate the platelet 5-HT4 receptor in blood.

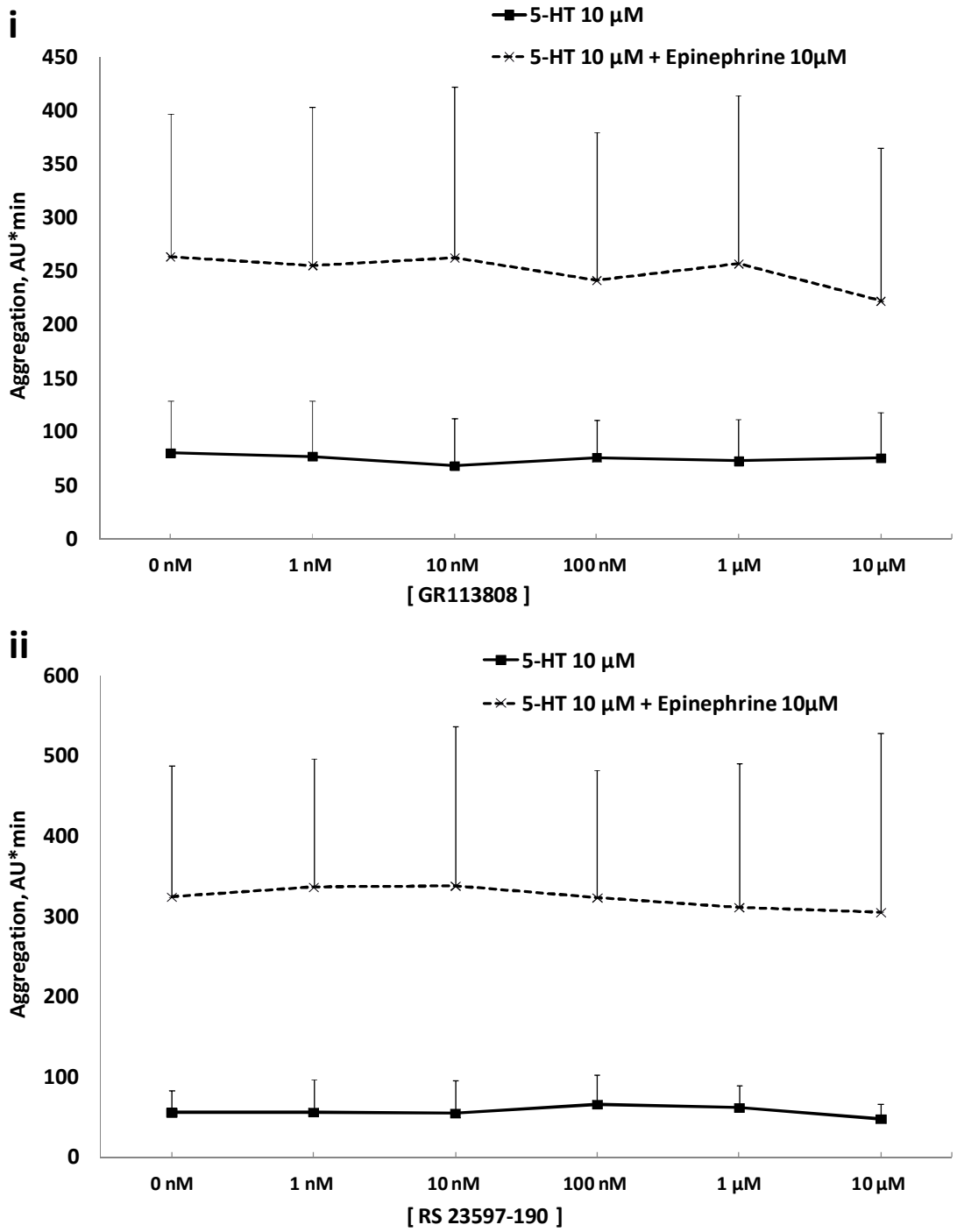

Figure 3.21: Effect of 5-HT4 antagonism on 5-HT-dependent platelet aggregation in whole blood. Hirudin-anticoagulated blood was incubated with different concentrations of GR113808 (i) and RS 23597-190 (ii) for 3 min with stirring at 37°C before addition of 10 µM 5-HT alone or 10 µM 5-HT plus 10 µM epinephrine. Results are expressed as mean values ± SD (n=5 with different blood donors).

4. Discussion

4.1 Role of exogenous 5-HT on whole blood platelet aggregation

We found that 5-HT on its own did not induce platelet aggregation in blood. We hypothesized that 5-HT released from some platelets activated during venipuncture might cause homologous desensitization of the 5-HT receptor(s) and therefore, a platelet response to 5HT might not be detectable, if measured 30 min after venipuncture. Platelets might respond to 5-HT very early after venipuncture, where receptor desensitization might not yet have occurred. Therefore, we measured 5-HT induced platelet aggregation as early as 5 min after venipuncture and compared the values with the aggregation values obtained 15 and 30 minutes after venipuncture. However, no 5-HT-induced aggregation was observed in these experiments either.

Our results do not agree with Bevan et al. who showed a small reversible proaggregatory effect of 5-HT by measuring the decrease of platelet count in whole blood (105). Our observations, however, confirm the results of Li et al. who measured 5-HT-induced platelet aggregation in blood by using impedance aggregometry, the decrease in platelet count and *in vitro* filtragometry (the time taken for platelet aggregates to occlude a microfilter), and who observed no platelet-stimulating effect of 5-HT alone on platelets in whole blood (106).

In contrast, 5-HT is known to potentiate platelet aggregation in blood induced by thrombin, ADP, epinephrine, lysophosphatidic acid (LPA) and collagen (106, 145-147). Usually pairs of platelet stimuli with different signaling mechanisms show good potentiation. For example 5-HT, whose platelet 5-HT2A receptor is coupled to G_q proteins and ultimately activates PKC and mobilizes Ca^{++} (145, 146), synergizes well

in PRP with epinephrine, which decreases intracellular cAMP through G_i-coupled α_2-adrenergic receptors (147). This potentiation of epinephrine-induced aggregation by 5-HT was confirmed in our experiments in whole blood. Data from Shah et al in PRP show that the synergism of 5-HT plus epinephrine can be inhibited at different steps of the 5-HT- or epinephrine-dependent signaling cascades (147). On the other hand, 5-HT cannot potentiate platelet aggregation induced by ATP, whose main mechanism is the same as 5-HT, the mobilization of Ca^{++} (148). The synergism of 5-HT with LPA is dependent on dense granule secretion of ADP, which binds to a G_i-coupled $P2Y_{12}$ receptor (probably the synergizing receptor) and to a $P2Y_1$ receptor which couples to G_q as 5-HT does. (146). Yet, not all different aggregation signaling pathways potentiate each other: the synergism of 5-HT with collagen (145) does not seem to depend on the collagen-induced GPVI/tyrosine kinase signaling cascade (149), but rather seems to be mediated by the ADP- or thromboxane A2-pathways, which are activated in collagen-induced platelet activation (146, 149).

4.2 Role of the platelet 5-HT transporter for platelet aggregation

In order to shed light on the possible role of SERT for platelet activation, we conducted *in vitro* experiments with the SSRI fluoxetine in whole blood. The SSRIs are nowadays the most commonly prescribed antidepressant drugs (89). They have been shown to deplete 5-HT from the platelets' dense granules (84, 85). Nevertheless, the implications of the SSRI pharmacotherapy for the platelet aggregation response are unclear.

The results demonstrated that fluoxetine inhibited the 5-HT (10 µM)-dependent potentiation of epinephrine-induced aggregation. The effects were shown in 3

different sets of experiments with 3 different pre-incubation periods of fluoxetine (5 sec, 3min and 13 min). The degree of inhibition did not differ significantly between these 3 sets. Dose-inhibition curves showed IC_{50} values of 10-20 µM fluoxetine which are in the range of the highest blood concentrations of fluoxetine (about 10 µM) reported after clinical administration (138). If the synergistic effect of 5-HT were important for the *in vivo* platelet aggregation, clinically administered fluoxetine should provide cardioprotective effects.

Fluoxetine had no effect on the platelet aggregation induced by platelet stimuli which activate GPCRs (ADP or TRAP). Furthermore, no inhibition of platelet aggregation by fluoxetine was observed with collagen and human plaque, which activate glycoprotein VI (GPVI) (36, 131). Our results clearly indicate that fluoxetine in blood does not impair the platelet aggregation process itself, as it has recently been suggested based on the observed interaction of SERT with the integrin $\alpha_{IIb}\beta_3$ at the platelet surface (78). Furthermore, fluoxetine does not impair GPVI and G-protein dependent pathways of platelet aggregation.

Our results indicate that fluoxetine only inhibits the potentiation of platelet aggregation mediated by addition of exogenous 5-HT to blood. A possible explanation is that fluoxetine inhibits the 5-HT induced platelet activation of the 5-HT2A receptor. Since the SSRI fluoxetine has an appreciable affinity for 5-HT2A receptors (150, 151), and 5-HT2A receptors are solely responsible for the synergistic effect of exogenously administered 5-HT on platelet aggregation, the anti-aggregatory effect of fluoxetine on 5-HT-potentiated platelet aggregation can be attributed to the blockade of 5-HT2A receptors. Our findings regarding the fact that fluoxetine pre-incubation of 5 sec, 3 min or 13 min has a similar inhibitory effect on

platelets stimulated by 5-HT plus epinephrine also supports that fluoxetine inhibits platelet aggregation by antagonizing the 5-HT2A receptors.

Moreover our findings show that fluoxetine did not attenuate non-5-HT-dependent platelet aggregation. This result indicates that protein serotonylation mediated by released 5-HT which is re-taken up by SERT, is not involved in platelet activation induced by physiological platelet stimuli.

Another previously proposed mechanism of 5-HT dependent platelet activation is the covalent binding of 5-HT to fibrinogen and thrombospondin on COAT-platelets, a subpopulation of platelets which express on their surface a variety of α-granule proteins in a stable manner (see Introduction 1.7) (99-101). If COAT-platelets were formed in our experiments and played a pro-aggregatory role as proposed (99-101), an acute blockade of 5-HT uptake activity by fluoxetine leading to a local high extracellular concentration of 5-HT should have resulted in a higher percentage of COAT-platelets and higher platelet aggregation values. Such an effect was, however, not observed in our experiments. Thus, we conclude that either no COAT-platelets were produced or that their existence did not affect platelet aggregation. We should, nevertheless, mention that the stimuli used in our experiments were rather weak in comparison to the combination of strong stimuli (e.g. collagen plus thrombin) previously used to produce COAT-platelets (99-101).

Our findings do not confirm a previous *in vitro* study showing augmentation of PAR-1- or PAR-4-induced platelet aggregation by fluoxetine (98). They are also in contrast to two other *in vitro* studies, which used washed platelets and showed inhibition of ADP-induced aggregation and collagen-mediated platelet activation and aggregation by the SSRI citalopram (78, 152). Our results are in agreement with an *in*

vitro study in PRP showing inhibition of 5-HT-enhancement of ADP-induced aggregation by citalopram (97). As far as clinical studies are concerned, our results do not support two studies reporting increased cardiovascular risk after initiation of SSRI treatment in elderly (95, 96). In contrast, our findings might lend some indirect support of epidemiological studies showing increased bleeding tendency and protection against cardiovascular disease under SSRI treatment (86-88, 91-93). This effect could be more pronounced with concomitant use of NSAIDs or aspirin, which inhibit a different pathway, the cyclooxygenase-dependent aggregation pathway. To examine this possibility, we studied next the combined effect of aspirin and fluoxetine on platelet aggregation *in vitro* in whole blood.

4.3 Effect of the combination of fluoxetine plus acetylsalicylic acid on inhibition of platelet aggregation

Two epidemiological studies have reported an increased risk of upper gastrointestinal bleeding in patients receiving SSRIs in combination with NSAIDs or aspirin (86, 89). Another study, however, failed to demonstrate platelet inhibition *ex vivo* in patients treated with a SSRI plus aspirin (153).

We therefore examined the possible synergistic action of SSRIs and aspirin on the pathophysiological relevant plaque-induced platelet aggregation and on the 5-HT-dependent platelet aggregation (5-HT + epinephrine) in whole blood *in vitro*. Similar to our previous findings, fluoxetine inhibited potently the 5-HT-dependent, but not the plaque-induced platelet aggregation. On the contrary, aspirin inhibited potently the plaque-induced, but not the 5-HT-dependent platelet aggregation. Neither the inhibition of 5-HT-dependent platelet aggregation, nor the inhibition of plaque-

induced aggregation by aspirin was increased by the combination of aspirin and fluoxetine. Therefore, we conclude that it is not the combined effects of SSRIs and aspirin on platelet aggregation that can be held responsible for bleeding complications.

Interestingly, a recent study has shown that SSRIs are associated with uncomplicated peptic ulcers (154). A previous study in mice had also shown that injected 5-HT aggravates peptic ulcers in mice, probably by damaging the regenerative microvessels involved in the healing process of these ulcers (155). A transient increase of plasma 5-HT by some monoamine transporter inhibitors has also been shown (156). Hence, the combination of attenuated platelet aggregation by aspirin and NSAIDs with an aggravation of peptic ulcers by increased free plasma 5-HT under SSRI treatment could be a possible explanation of an increased upper gastrointestinal bleeding risk, but further studies are needed.

4.4 Role of blood pre-incubation on stimulus-induced platelet aggregation

During our experiments with fluoxetine we also observed that both platelet aggregation induced by ADP, TRAP, epinephrine, 5-HT plus epinephrine, which act on G-protein coupled receptors, and the spontaneous platelet aggregation, which is ADP-dependent (157), were lower, when blood was pre-incubated for 10 min at 37°C compared to 0 min pre-incubation time. In contrast, platelet aggregation induced by collagen and atherosclerotic plaque, which do not activate GPCRs were not affected by the additional 10 min incubation at 37^{o}C.

Although there is no simple explanation for these results, I would like to comment that these findings are in accordance with previous findings that show lower affinity of G-protein coupled receptor for their ligands at temperatures ranging from 37^{o} to 50^{o}C compared to room temperatures (158-161), and with a previous study that showed enhanced platelet aggregation and activation at 28^{o}C, a temperature attained during hypothermia in cardiac surgery (162).

4.5 Role of the platelet 5-HT2A receptor for platelet activation under static and flow conditions

The next step in our research concerned the role of 5-HT2A receptors on platelet aggregation. Until now, human and animal studies have shown unequivocally that antagonism of the 5-HT2A receptors attenuates platelet aggregation mediated by exogenous 5-HT *in vitro* and *ex vivo* (106, 108-112, 114, 115).

We ran a set of experiments examining the effect of different concentrations of three 5-HT2A receptor antagonists (ketanserin, R-96544 and sarpogrelate) on 5-HT-dependent platelet aggregation in whole blood. Platelet aggregation induced by epinephrine plus 5-HT was potently inhibited by ketanserin and R-96544. Sarpogrelate, on the other hand, required much higher concentrations to inhibit 5-HT-potentiated platelet aggregation. Moreover, high concentrations of 10 μM of ketanserin and 10 μM R-96544 inhibited platelet aggregation to levels of 64 ± 42 and 76 ± 29 AU*min, which are similar to levels induced by epinephrine alone. In additional experiments on ATP secretion, the little amount of ATP secretion induced by epinephrine plus 5-HT was completely inhibited by 1 μM ketanserin.

According to these results, a potent blockade of 5-HT2A antagonists leads to complete inhibition of 5-HT-dependent platelet aggregation. Finally, we can infer that the 5-HT2A receptors are solely responsible for the potentiation of platelet aggregation by exogenously administered 5-HT.

Moreover, the complete inhibition of 5-HT-potentiated platelet aggregation by the 5-HT2A receptor antagonist, discards the possibility that serotonylation of cytoplasmic proteins plays any role in platelet activation, since according to Walther et al added 5-HT would enhance platelet activation through SERT mediated cytosolic 5-HT increase and subsequent protein serotonylation (102).

The next step was to investigate the role of endogenous 5-HT in stimulus-induced platelet aggregation. The various concentrations of endogenous 5-HT in human blood compartments are as follows: The 5-HT concentration of platelet depleted plasma is very low, and ranges between 10 and 44 nM (163-166). Platelet 5-HT (measured in PRP) is between 3.22 and 6 nmol/10^9 platelets (164, 165, 167, 168). In the hypothetical scenario of complete exocytosis of platelet 5-HT, an individual with a high platelet count (400.000/µl) and the maximum platelet 5-HT concentration (6 nmol/10^9 platelets), could reach a maximal blood 5-HT concentration of 2.4 µM after platelet stimulation. According to another study the average total 5-HT content of blood (including platelets) is 0.74 µM (169). Since 5-HT is not completely released even after stimulation with a strong platelet stimulus such as collagen (170), the extracellular 5-HT blood concentration is expected to be lower than 0.74 µM. This level of 5-HT concentration is much lower than the 10 µM 5-HT used for potentiation of platelet aggregation in our experiments. However, in a previous study using

human PRP a 5-HT concentration as low as 0.5 μM could potentiate ADP-induced aggregation (171).

Our next aim was to study the possible role of released 5-HT as a feedback mediator of human platelet activation. In order to reach a solid, reliable conclusion we assessed the effect of 5-HT2A receptor antagonism in four different types of experiments using whole blood; namely MEA, platelet ATP secretion, plaque-induced platelet adhesion and aggregation under arterial flow conditions, and shear-induced platelet adhesion and aggregate formation.

We found that a supramaximal concentration of 1 μM ketanserin had no effect on platelet aggregation induced by collagen, TRAP and plaque measured by MEA. Similarly, ketanserin did not inhibit platelet ATP secretion induced by the same platelet agonists. Ketanserin had also no effect on human plaque-induced platelet adhesion and aggregation under arterial flow. Moreover, neither ketanserin nor R-96544 affected platelet adhesion and aggregate formation induced by two different shear rates, 600 s^{-1} and 1200 s^{-1} as measured by the cone and plate analyzer (137). These results demonstrate clearly that endogenous serotonin released upon platelet stimulation does not support platelet aggregation and secretion. Our experiments on platelet adhesion and aggregate formation stimulated by different shear rates and by atherosclerotic plaque under arterial flow conditions extend the applicability of our findings to pathophysiological situations of rupture of atherosclerotic plaques in normal sized and stenotic arteries. Our findings are in contrast to several studies performed in dogs that reported, in the absence of exogenous serotonin, an inhibition of stimulus-induced platelet aggregation by 5-HT2A receptor antagonists *in vitro, ex vivo* and *in vivo* (118-120). Also, in other species, inhibition of stimulus-

induced platelet aggregation by 5-HT2A receptor antagonists has been observed in the absence of added serotonin (112, 114, 117, 172). In animal *in vivo* thrombosis models the effect 5-HT2A antagonists on platelets and VSMCs (vasoconstriction) cannot be separated. In these models the inhibition of vasoconstriction induced by 5-HT, and not attenuated platelet aggregation, might be the reason, why 5-HT2A receptor antagonism is effective in thrombosis models.

Thus, it seems that in species other than human, serotonin released upon platelet stimulation might serve as positive feedback mediator of platelet activation. Why it does not have this function in human platelets is intriguing. It could be that the amount of serotonin released from human platelets is not sufficient to activate the human platelet 5-HT2A receptor. The theoretical maximum extracellular 5-HT concentration in human blood, as calculated above is 2.4 µM, whilst in an *in vivo* experiment in dogs, the maximum 5-HT plasma concentration in the vicinity of a coronary occlusion measured was approximately 2.3 µM (173). According to these results, there is no substantial difference between the 5-HT concentrations in men and dogs that could explain the difference in the potentiation of aggregation by secreted 5-HT.

A second possibility is that 5-HT released from stimulated platelets does not reach its 5-HT2A receptor, perhaps since it is taken up before by SERT. The blockade of SERT should then enhance stimulus-induced aggregation. However, the results of our experiments do not support this suggestion: fluoxetine did not enhance aggregation induced by epinephrine, ADP, TRAP, plaque and collagen.

A third possibility is a different structure and pharmacology of 5-HT2A receptors of humans as compared to other species (174, 175). Human, monkey and pig 5-HT2A

receptors have a higher affinity for tryptamines without N1-substitution than rat 5-HT2A receptors (174-176); thus human 5-HT2A receptors should have had a greater affinity for 5-HT, a N1-unsubstituted tryptamine, and enhanced platelet response to 5-HT as compared to rats. However, 5-HT2A blockade inhibited collagen-induced platelet aggregation in rats and not men (117, 177). Thus, it is not the different affinity to the 5-HT2A receptor that enables rat platelets to respond to endogenously released 5-HT. The reason behind the difference of human platelets and the platelets from other species (mice, rats, cats, dogs) concerning their response to endogenously released 5-HT, might be sought in the signaling activity of the 5-HT2A receptor. If the porcine or monkey platelet response to 5-HT was similar to that of human platelets, they would provide a better animal model for future research.

The findings of this study lead to the conclusion that 5-HT2A receptor antagonists do not provide a promising strategy for antiplatelet therapy. Our findings also suggest that inhibition of platelet aggregation will not be a side effect of 5-HT-related medications that target other organ systems.

4.6 Role of the platelet 5-HT4 receptor for platelet aggregation

The expression of 5-HT-4 receptors (serotonin type 4 receptors) has been recently described in human platelets (126). The 5-HT4 receptors are G_s-coupled receptors and, therefore, their activation would theoretically lead to inhibition of platelet activation. The opposite was demonstrated in a study, where tegaserod, a 5-HT4 receptor agonist, enhanced platelet aggregation induced by other stimuli in PRP (126). Yet, in another study tegaserod did not alter ADP-induced platelet aggregation in PRP (128).

Our results were the first that used whole blood to test the effect of 5-HT4 receptor agonists and antagonists on platelet aggregation. They show that the stimulation of platelet 5-HT4 receptors by a wide range of 5-HT4 agonist (CJ 033,466) concentrations had no effect on whole blood platelet aggregation. Moreover, 5-HT4 agonism had no effect on spontaneous platelet aggregation. Nevertheless, 1 μM and 10 μM CJ 033,466 inhibited significantly the weak platelet aggregation induced by epinephrine. We then moved on to examine the effects of 5-HT4 receptor antagonism. Incubation with five different concentrations of two structurally different 5-HT4 antagonists, GR113808 and RS 23597-190, did not affect whole blood platelet aggregation observed in the presence of 10 μM 5-HT or induced by 10 μM 5-HT and 10 μM epinephrine.

Since 5-HT4 antagonists did not enhance platelet aggregation in the presence of exogenous 5-HT, we cannot attribute the lack of effect by exogenous 5-HT alone on human platelet aggregation to a possible co-existence of two antagonizing receptors, 5-HT2A and 5-HT4, on the platelet surface. We, therefore, conclude that 5-HT4 receptors have no role on 5-HT-dependent platelet aggregation.

We found that stimulation of 5-HT4 platelet receptors did not inhibit spontaneous platelet aggregation and aggregation induced by the ADP, however 5-HT4 stimulation inhibited the small platelet aggregation induced by epinephrine. We should keep in mind that epinephrine receptors and 5-HT4 receptors antagonize each other on the cAMP intracellular pathway, by lowering and raising the intracellular cAMP concentrations respectively. The effect of 5-HT4 receptor agonism on epinephrine-induced aggregation is an indication that 5-HT4 receptors exist on the platelet surface. However, the effect of 5-HT4 receptors may be so weak that

their potential can be demonstrated only in special conditions, i.e. in the presence of weak platelet aggregation that depends on the cAMP-dependent pathway, as that induced by epinephrine. Therefore, we can conclude that platelet 5-HT4 receptors are of minor importance for platelet aggregation and do not constitute a notable target for antiplatelet drugs.

4.7 Roles of platelet 5-HT in various diseases

The roles of platelet 5-HT in various diseases are constantly being unraveled.

Platelet-derived 5-HT plays a role in autoimmune disorders. Decreased contents of platelet 5-HT, possibly released during inflammatory episodes, have been found in patients with rheumatoid arthritis, systemic lupus erythematosus, progressive systemic sclerosis and Sjögren's syndrome (15, 16, 178). In a mouse model of autoimmune arthritis platelet 5-HT has been implicated in joint inflammation by enhancing the permeability of the synovial microvasculature, an action antagonized by SSRIs (178). Similarly, a microvascular leak mechanism has been implicated in the aggravation of acute lung injury by platelet 5-HT (179). In addition, platelet-secreted 5-HT promotes the recruitment of neutrophils to sites of acute inflammation (14), where it activates inflammatory signaling pathways (180).

Platelet 5-HT has been reported to be important for liver regeneration after liver resection in rats and humans (12, 17, 181). However, platelet 5-HT is also involved in pathological conditions of the liver, such as liver fibrosis, steatohepatitis, liver carcinomas and aggravation of viral hepatitis (17-19).

Administration of the drug tianeptine, an antidepressive agent, has been shown to enhance the uptake of plasma 5-HT by platelets and to diminish asthma attacks

(20). Platelet- derived 5-HT has further been shown to enhance migration of rat aortic smooth muscle cells, a function which might affect atherosclerosis (182).

A recent study showed that elevated plasma 5-HT altered the expression of 723 murine genes, which must reflect a wide range of 5-HT mediated functions (183). As mentioned in the introduction, a wide variety of 5-HT mediated functions exist, yet only the experiments mentioned above refer to the effects of 5-HT originating from platelets. Summing up, platelets serve as an important carrier of peripheral 5-HT and may distribute it upon demand to injured or inflamed organs and tissues.

5. Conclusion and perspectives

Exogenous 5-HT potentiates platelet aggregation and this potentiation can be unequivocally inhibited by 5-HT2A receptor antagonists in many mammalian species, including man (109, 110, 112-116). In the absence of exogenous 5-HT, 5-HT2A antagonism also inhibits platelet activation in mice, rats, cats, rabbits and dogs (107, 113, 114, 116-120). Therefore, 5-HT2A antagonists have been considered as possible anti-platelet drugs, although 5-HT2A antagonists have until now failed to inhibit platelet aggregation *in vitro* and *ex vivo* in man (107, 121-123).

Our results show that 5-HT2A receptors mediate in blood the potentiation of human platelet aggregation by exogenous 5-HT (177), but they are not involved in the response of endogenously 5-HT released upon platelet activation by physiological stimuli, shear stress or by flow over plaque-coated surfaces (177). Hence, endogenous 5-HT does not play a role in human platelet aggregation.

SSRIs on the other hand, have been held responsible for bleeding complications and protection from myocardial ischemia (86-93), effects which have not been confirmed by a few other studies (94-96). In our experiments the acute administration of the SSRI fluoxetine, alone or in combination with aspirin, did not inhibit platelet aggregation induced by physiological stimuli (157) and therefore, we conclude that fluoxetine does not interfere with physiological platelet aggregation mechanisms. Nevertheless, SSRIs could enhance bleeding complication by decreasing platelet 5-HT content leading to a reduced 5-HT mediated vasoconstriction or interference with the 5-HT mediated healing of peptic ulcers (2, 90, 154, 155). Our experiments showing that fluoxetine inhibits stimulus-induced platelet aggregation

in the presence but not absence of exogenous 5-HT (177), leave no room to support serotonylation of cytoplasmic or platelet surface proteins as a pro-aggregative mechanism (99-102). Additionally, we provide functional evidence for the presence of recently proposed platelet inhibitory 5-HT4 receptors (126, 128) on platelets, but they may play only a minor role in regulating human platelet aggregation.

The reason why platelet 5-HT plays a role in mice-, rat-, cat-, rabbit- and dog-, but not human platelet aggregation is intriguing and cannot be explained based on our current knowledge. The higher affinity for 5-HT of the human, monkey and porcine 5-HT2A receptor compared to that of the rat (174-176), does not explain the pro-aggregative effect of 5-HT in mice; but it suggests structural differences in the 5-HT2A between species.

Based on the current knowledge and on our study, we can conclude that platelet function is not affected by endogenous 5-HT, but platelets are important by serving as a carrier for 5-HT which is distributed to diseased organs and tissues. The absence of platelet pro-aggregative effects by endogenous 5-HT may prohibit the use of 5-HT2A receptor antagonists as antiplatelet drugs, but it may also suggest a reduced adverse events profile for drugs targeting the platelet 5-HT uptake. Such drugs could be helpful for treatment of disorders that depend on platelet-derived 5-HT, like autoimmune disorders, liver diseases and asthma (12, 14-20, 178-181) and further platelet 5-HT-dependent disorders whose discovery is anticipated in the future.

6. Summary

6.1 Summary

The field of serotonin (5-HT) research continues to expand. A variety of physiological and pathophysiological functions regulated by 5-HT has been identified. Selective serotonin reuptake inhibitors (SSRIs) and 5-HT-3-receptor antagonists are used in medical therapy, and more 5-HT-related medicaments are expected in the future.

Circulating 5-HT is stored mainly in the dense granules of platelets. 5-HT stimulates platelets, which has been observed, apart from humans, in various other species. Therefore, the stimulatory effect of exogenous 5-HT in high concentrations (> 0.5 µM) on human platelets in blood is unambiguous. However, results of the platelet-stimulating effect of the endogenous 5-HT, which is stored in dense granules and released upon platelet activation, are contradictory.

The aim of this thesis was to investigate the acute effect of SSRIs on human platelet aggregation, the role of the 5-HT2A receptor for platelet function in human blood, and the role of the newly discovered 5-HT4 receptor for platelet function.

In some—but not all—clinical studies, SSRIs on the one hand have been suspected to cause bleeding complications, on the other hand they may protect against ischemic cardiovascular diseases. In our studies, the acute addition of the SSRI fluoxetine to blood, alone or in combination with aspirin, indeed inhibited the potentiation of platelet aggregation by exogenous 5-HT (probably by unspecific inhibition of 5-HT2A receptors), the platelet aggregation induced by physiological

stimuli was, however, not affected. Therefore, we conclude that the serotonin transporter is not involved in the physiological platelet aggregation process. Yet, SSRIs could cause bleeding complications by reducing the platelet 5-HT content, consequently leading to a reduced 5-HT-mediated vasoconstriction, or contribute to stomach ulcers by disruption of the 5-HT-mediated wound healing.

Our results indicate that 5-HT2A receptors mediate the potentiation of aggregation of human platelets in the blood by exogenous 5-HT. However, 5-HT2A receptor antagonists do not inhibit platelet activation by physiological stimuli, shear stress, or flow over atherosclerotic plaque material. Therefore, endogenous 5-HT, which is released upon platelet activation, plays no role in human platelet aggregation.

Finally, our studies support the expression of platelet-inhibiting 5-HT4 receptors. However, these played only a minor role in the regulation of human platelet aggregation induced by epinephrine.

The question why platelet 5-HT plays a role in platelet aggregation of mice, rats, cats, rabbits, and dogs, but not of men, is intriguing, but has no explanation according to our present knowledge. Species-specific structural differences of the 5-HT2A receptors are suggested.

Based on the current knowledge and our experiments, it can be concluded that human platelet function is not influenced by endogenous 5-HT, but platelets are important for transporting 5-HT in the blood to diseased organs and tissues. The absence of a stimulating action of endogenous 5-HT on platelets precludes the use of 5-HT2A receptor antagonists as anti-thrombotic medications.

6.2 Zusammenfassung

Der Bereich der Serotonin- (5HT-) Forschung erweitert sich ständig. Eine Vielzahl von physiologischen und pathophysiologischen Funktionen, die durch 5-HT reguliert werden, wurde identifiziert. Selektive Serotonin-Wiederaufnahmehemmer (SSRIs) und 5-HT3-Rezeptor-Antagonisten werden in der medizinischen Therapie angewendet, und zukünftig könnten weitere 5-HT-bezogene Medikamente dazukommen.

Zirkulierendes 5-HT ist vor allem in den dichten Granula von Thrombozyten gespeichert. Es stimuliert Thrombozyten von Menschen als auch von verschiedenen Tierspezies. Dabei ist die stimulierende Wirkung von exogenem 5-HT in hoher Konzentration (> 0,5 µM) auf humane Thrombozyten eindeutig. Jedoch sind die Ergebnisse bezüglich der Thrombozyten-stimulierenden Wirkung des endogenen, in dichten Granula gespeicherten 5-HT, welches nach Thrombozytenaktivierung freigesetzt wird, widersprüchlich.

Das Ziel der vorliegenden Arbeit war es, die akute Wirkung von SSRIs auf die Aggregation humaner Thrombozyten, und die Rolle des 5-HT2A-Rezeptor für die Thrombozytenfunktion im menschlichen Blut *in vitro* zu untersuchen, sowie die Rolle des 5-HT4-Rezeptors, eines neu-entdeckten Thrombozytenrezeptors, für die Plättchenfunktion zu erforschen.

SSRIs wurden aufgrund mancher, aber nicht aller klinischer Studien einerseits für Blutungskomplikationen verantwortlich gemacht, andererseits könnten sie bei ischämischen kardiovaskulären Erkrankungen protektiv wirken. In unseren Untersuchungen wurde durch die kurzfristige Zugabe des SSRI Fluoxetin zu Blut die Potenzierung der Thrombozytenaggregation durch exogenes 5-HT zwar gehemmt

(wahrscheinlich durch unspezifische Inhibierung des 5-HT2A-Rezeptors), die durch physiologische Stimuli induzierte Thrombozytenaggregation jedoch nicht beeinflußt. Daher folgern wir, dass thrombozytäre Serotonin-Transporter bei der physiologischen Thrombozytenaggregation nicht involviert sind. Dennoch könnten SSRIs zu Blutungskomplikationen führen (durch Verringerung des thrombozytären 5-HT-Gehalts, und in Folge einer reduzierten 5-HT-vermittelten Vasokonstriktion), oder zu Magengeschwüren durch Störung der 5-HT-vermittelten Wundheilung beitragen.

Unsere Ergebnisse zeigen, dass 5-HT2A-Rezeptoren die Potenzierung der Aggregation humaner Thrombozyten im Blut durch exogenes 5-HT vermitteln. Antagonisten des 5-HT2A-Rezeptors hemmen aber nicht die Thrombozyten-Aktivierung induziert durch physiologische Stimuli, Scherstress oder Fluss über atherosklerotisches Plaquematerial. Endogenes 5-HT welches nach Thrombozyten-Aktivierung freigesetzt wird, spielt daher keine Rolle in der menschlichen Thrombozytenaggregation.

Zuletzt unterstützen unsere Untersuchungen die Expression von Thrombozyten-Aggregationshemmenden 5-HT4 Rezeptoren. Diese spielten jedoch nur eine geringe Rolle bei der Regulation der Adrenalin-induzierten Thrombozytenaggregation.

Die Frage, warum thrombozytäres 5-HT zwar eine Rolle bei der Thrombozytenaggregation von Mäusen, Ratten, Katzen, Kaninchen und Hunden, aber nicht bei der humanen Thrombozytenaggregation spielt, ist fesselnd, aber lässt sich gemäß unseren derzeitigen Kenntnissen nicht klären. Vermutlich liegen Spezies-spezifische strukturelle Unterschiede der 5-HT2A-Rezeptoren vor.

Basierend auf dem aktuellen Wissen und auf unseren Experimenten kann man schlussfolgern, dass die menschliche Thrombozytenfunktion durch endogenes 5-HT

nicht beeinflüsst wird, aber Thrombozyten sind wichtig, um das 5-HT im Blut zu erkrankten Organen und Geweben zu transportieren. Die Abwesenheit einer stimulierenden Wirkung von endogenem 5-HT auf Thrombozyten schliesst die Verwendung von 5-HT2A Rezeptor-Antagonisten als antithrombotische Medikamente jedoch aus.

7. Own publications

Bampalis VG, Khandoga AL, Siess W. Fluoxetine inhibition of 5-HT-potentiated platelet aggregation in whole blood. Thromb Haemost. 2010 Dec; 104(6):1272-4

Bampalis VG, Dwivedi S, Shai E, Brandl R, Varon D, Siess W. Effect of 5-HT2A receptor antagonists on human platelet activation in blood exposed to physiological stimuli and atherosclerotic plaque. J Thromb Haemost. 2011 Oct; 9(10):2112-5

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10. Abbreviations and units

10.1 Abbreviations

5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine, serotonin
AA	arachidonic acid
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ASA	acetylsalicylic acid
ATP	adenosine triphosphate
AU	arbitrary units
cAMP	cyclic adenosine monophosphate
CCD camera	charge coupled device camera
CD40-ligand	cluster of differentiation 40-ligand
CNS	central nervous system
COAT-platelet	collagen-and-thrombin platelets or coated-platelets
DAG	Diacylglycerol
Epi	Epinephrine
F-actin	filamentous-actin
G-actin	globular-actin
GPIbα	glycoprotein Ibα
GPCR	guanosine nucleotide-binding protein coupled receptors
G-protein	guanosine nucleotide-binding protein
GPVI	glycoprotein VI
IL-1	interleukin 1
IP3	inositol trisphosphate
LPA	lysophosphatic acid
LTA	light transmittance aggregometry
MAO	Monoaminooxidase
MEA	multiple electrode aggregometry
MI	myocardial infarct
OCS	open canalicular system

PAI-1	plasminogen activator inhibitor-1
PAR-1	proteinase-activated receptor 1
PAR-4	proteinase-activated receptor 4
PDGF	platelet-derived growth factor
PF-4	platelet factor 4
PKC	protein kinase C
PLC	phospholipase C
PPP	platelet poor plasma
PRP	platelet rich plasma
SERT	serotonin transporter
SNARE	soluble NSF attachment protein receptor
SSRI	selective serotonin re-uptake inhibitors
TPH	tryptophan hydroxylase
TRAP	thrombin receptor activating peptide
TxA_2	thromboxane A2
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle constriction
vWf	von Willebrand factor
β-TG	beta-thromboglobulin

10.2 Units

g	gram
L	liter
M	molar (=mol/liter)
µg	microgram
µl	microliter
µM	micromolar
mg	miligram
ml	mililiter
mm	milimeter
mM	milimolar
min	minute
ng	nanogram

nM	nanomolar (=nmol/l)
nm	nanometer
rpm	revolutions per minute
s, sec	second

11. Eidesstattliche Versicherung

Eidesstattliche Versicherung

Vasileios Bampalis

Name, Vorname

Ich erkläre hiermit an Eides statt,

dass ich die vorliegende Dissertation mit dem Thema

Role of the serotonin transporter and the 5-HT2A and 5-HT4C receptors for platelet function in blood.

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

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