Platelets are crucial in maintaining hemostasis and therefore preventing blood loss upon vessel injury. However, platelets also drive pathological processes, most importantly arterial thrombosis, which are worldwide still a leading cause of death, due to stroke, myocardial infarction, and thromboembolism. Patients at recurrent risk of these diseases receive antiplatelet agents, in many cases they are effective, but can also cause bleeding side effects. More targeted approaches of antiplatelet medication are needed, for which the platelet-specific collagen receptor glycoprotein VI (GPVI) is a promising candidate.

Chapter 1 introduces basic principles of the multi-step processes of platelet activation in arterial thrombus formation as it occurs under flow conditions. The chapter follows with a more in-depth description of the for this thesis most relevant platelet agonist collagen and of the two main collagen receptors expressed on platelets, GPVI and integrin α2β1. The next part introduces the current options to intervene with platelet-collagen interactions. These are in particular: i) the recombinant GPVI fusion protein, Revacept, which masks GPVI-binding motifs on exposed collagen fibers; ii) the Fab fragment, 9O12, directed against human GPVI to directly prevent receptor agonist interaction; iii) camelid heavy chain antibodies, called nanobodies (Nb), which have been selected to target GPVI on the platelet receptor level. Since this thesis extensively describes the use of whole blood microfluidics for the assaying of platelet activation and thrombus formation under flow, the last part of the chapter is dedicated to reported capabilities of this technique.

Chapter 2 provides a commentary on a paper by Staessens & De Meyer (Platelets, 2021; 32:331), in which 177 thrombi from stroke patients were collected and their structure was evaluated by histological staining. The detailed thrombus composition appeared to be linked to the efficiency of surgical removal after the stroke. For the analyzed thrombi, the authors described red blood cell zones and platelet-rich zones, which were linked to a looser or a denser structure, respectively. The chapter brings these findings in context with other literature and discusses some shortcomings of the study. For instance, the absence of quantification of the immunohistochemistry data and the unavailability of patient-related parameters or clinical intervention outcomes, which would have been important for comparison with the composition of the thrombi. Nevertheless, at present this is the only study marrying an impressive ex vivo thrombus sample size with a broad panel of histological stains. It does validate textbook knowledge and gives better incentives to improve the treatment of patients with ischemic stroke.

Chapter 3 describes an effort to investigate and compare the inhibition patterns of four clinically relevant interventions, aimed to block the interactions of platelets with collagens. As a central test system, microfluidic chambers were used, in which whole blood was flowed over
a series of collagens or collagen-like substrates. Microscopic images were taken to assess the level of inhibition on thrombus formation parameters. As GPVI inhibitors were used the recombinant GPVI construct Revacept, the anti-human GPVI Fab fragment 9O12 (resembling Glenzocimab), and the Syk protein tyrosine kinase inhibitor PRT-060318; furthermore, as an integrin α2β1 antagonist the antibody 6F1 was utilized. In the chapter, it is revealed that all interventions led to an overall downregulation of the thrombus formation on most collagen-like surfaces, although the precise patterns of inhibition varied greatly between the individual treatments and the collagen substrates. Differences seen between Revacept and 9O12 Fab related to the GPVI-activating potential of the specific collagen, with Revacept being more active on GPO-enriched substrates. This finding may be relevant for the ongoing clinical trials with Revacept and the 9O12-based antibody Glenzocimab. Among all antagonists, universally and most potent was the Syk inhibitor PRT-060318, followed by moderate effects of the 6F1 antibody. This especially was the case for human vascular-type of collagen preparations, in which cases 6F1 outperformed Revacept. Our results imply that caution is needed when selecting a certain collagen substrate for the testing of antiplatelet drugs for effects in flow-dependent thrombus formation.

Chapter 4 examines the approach to prevent GPVI-collagen interactions with a novel anti-human GPVI nanobody Nb2. The data presented in this chapter show that Nb2 impairs collagen-dependent thrombus formation as well as the GPVI-dependent signaling. This nanobody effect was observed with a fibrillar collagen and with collagen-containing patient derived atherosclerotic plaque homogenate. The chapter also points out that the two substrates were different concerning the degree of α2β1 engagement, which was high for the fibrillar collagen and was low with the plaque homogenate. For plaque homogenate, only the combined blockage of αIIbβ3 and α2β1 was able to inhibit platelet adhesion and thrombus formation to the same extent as Nb2 alone. Furthermore, Nb2 prevented GPVI signaling, observed as a loss of Syk, Lat and PLCγ2 phosphorylation, especially upon plaque stimulation. Additional work was directed to find the mode of action of Nb2, which likely is through the disruption of GPVI clustering and a consequently restricted receptor signaling. Overall, this work emphasizes the critical difference in GPVI-mediated platelet activation by atherosclerotic plaque or by isolated collagen. Collectively, the data warrant further investigation of Nb2 as a potential anti-thrombotic agent.

As a continuation of the Nb work, Chapter 5 describes how nanobodies can be used as novel tools to investigate the clustering of GPVI on activated platelets. For this purpose, the non-inhibitory anti-GPVI Nb28 was fluorescently labeled, and then used in whole blood flow studies over collagens. For four different collagen types, the distribution of GPVI on the platelet surface was examined with Nb28 AF488 using an automated image analysis. Labeled
Nb28 appeared to indicate the formation of macro-clusters of GPVI on platelets that adhered to various substrates. This cluster formation corresponded with the extent of thrombus formation and the platelet procoagulant activity (phosphatidylserine exposure). High staining and high platelet responses were only observed on the most active fibrillar collagen and on the collagen-related peptide with a GPVI binding motif. No GPVI cluster formation was observed of platelets that adhered to less active substrates, such as collagen III and collagen-related peptide without GPVI binding motif. Interestingly, the clustering could be disrupted by direct receptor inhibition, but not by pharmacological inhibition of downstream signaling molecules, although this partly reduced the thrombus formation. In general, the work shows that GPVI (macro-) clustering is a process that occurs not only in static but also in flow conditions and is then linked to increased platelet activation.

Chapter 6 investigates the role of platelet Syk on thrombus formation under flow as well as on platelet signaling via cytosolic Ca\(^{2+}\) mobilization, in response to various vascular-type of collagens and collagen-related peptides. It is demonstrated that the selective inhibition of Syk with PRT-060318 suppressed parameters of thrombus formation for all collagen-like substrates. The compound furthermore reduced platelet Ca\(^{2+}\) responses with those collagens or collagen-related peptides that were able to activate the platelets in suspension. The obtained thrombus formation data was also used to build a prediction model, based on regression analysis, for the GPVI dependency of collagen preparations. The model indicated a mixed role of GPVI in thrombus formation on vascular-derived collagen surfaces, which was abolished upon inhibition of Syk. This gave further proof that the Syk pathway in platelets is essential for the thrombus formation with all platelet-activating collagens, even with those lacking the GPVI-specific recognition sequence GPO.

In Chapter 7, investigates to what extent protein tyrosine phosphatases are able to downregulate GPVI-dependent thrombus formation, also under conditions where integrin \(\alpha_2\beta_1\) is blocked. For this purpose, modulatory effects were examined on thrombus formation of the ITIM-linked receptor PECAM1, which couples to the protein tyrosine phosphatase non-receptor type PTPN11. Experiments using collagens with high or low GPVI dependency showed that the impairment of thrombus formation upon blockage of \(\alpha_2\beta_1\) could be restored when also PECAM1 was inhibited using a selective antibody. The supposedly negative regulation by PECAM1 was further examined using blood samples from patients with a gain-of-function mutation in PTPN11 - presenting with the Noonan syndrome, which includes a small to moderate bleeding phenotype. Flow studies with blood from seven Noonan patients showed a variable, but overall partial reduction of the collagen-induced platelet activation that became enforced upon \(\alpha_2\beta_1\) blockage. However, the gain of PTPN11 activity did not enhance the rescuing effect caused by PECAM1 inhibition. Taken together, the results of this chapter
indicate that the PECAM1 and PTPN11 restraining mechanisms on collagen-induced thrombus formation are independent of the extent of GPVI activation, but dependent on other factors such as the PTPN11 activity and the engagement of integrin α2β1.

The general discussion Chapter 8 places the findings presented in this thesis in context with the current literature. Discussed are the different platelet-activating effects of collagen preparations, also in comparison to atherosclerotic plaque material. The relative role of GPVI appears to be higher in plaque-induced thrombus formation than in vascular collagen-induced thrombus formation, where integrin α2β1 is more active. Furthermore, I discuss the relevance of GPVI clustering for platelet activation, and debate to which extent microfluidic whole blood assays can proximate (patho)-physiological flow conditions in thrombosis and hemostasis. In the final part of Chapter 8, I compare the earlier developed and the newly arising GPVI inhibitors in terms of action and mechanism. Conclusively, this thesis has deepened the insight into the variable role of GPVI as a collagen receptor and into the many ways to interfere with its platelet-activating function.