The expanded universe of thrombin: a comprehensive review of its hemostasis-independent roles and structure-function relationships

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ABSTRACT

Thrombin, the central protease of the coagulation cascade, has long been recognized for its essential role in hemostasis, converting fibrinogen to fibrin and activating platelets. However, a growing body of evidence over the past two decades has firmly established thrombin as a pluripotent mediator operating at the intersection of coagulation, inflammation, immunity, and tissue repair. This manuscript provides a comprehensive review of the hemostasis- and coagulation-independent functions of thrombin, integrating updated evidence with the sophisticated structure-function relationships that govern these diverse activities. We explore the molecular basis of thrombin's versatility, focusing on its allosteric regulation by sodium ions, its two anion-binding exosites, and its primary signaling mechanism via protease-activated receptors. The discussion extends to thrombin's critical roles in neurobiology, where it exhibits a dualistic nature in stroke and neurodegeneration, its profound influence on inflammation and immunity, and its paradoxical involvement in cancer progression and angiogenesis. Furthermore, we highlight emerging concepts, including the discovery of its tissue-specific roles. Understanding these multifaceted functions is crucial for developing targeted therapeutic strategies that can modulate thrombin's pathological actions without compromising its essential hemostatic functions.

Key words: thrombin, PARs, thrombomodulin, thrombin-induced cell activation.

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Introduction: the Jekyll and Hyde of the coagulation system

Hemostasis, the physiological response to vascular injury, is meticulously orchestrated to prevent blood loss while maintaining fluidity within the vasculature. At the heart of this cascade lies α-thrombin (henceforth referred to as thrombin), a trypsin-like serine protease generated from its zymogen precursor, prothrombin.[1] Traditionally, thrombin has been revered as the *king* of coagulation due to its procoagulant functions: it cleaves fibrinogen to form the fibrin clot,[2] activates factor XIII to stabilize that clot,[3] amplifies its own generation by activating cofactors V and VIII,[4] and potently activates platelets.[5-7] The structural basis for these interactions has been a subject of intense study, revealing a sophisticated molecular machine.[8]

This classical view, however, represents only a fraction of thrombin's biological repertoire. It is now unequivocally clear that thrombin is a *Janus-headed proteinase*, capable of exerting a multitude of effects that extend far beyond clot formation.[8,9] Depending on its concentration, its binding partners, and the cellular context, thrombin can also act as an anticoagulant (via the thrombomodulin-protein C system),[10-13] an antifibrinolytic (via TAFI activation),[14] a potent inflammatory or anti-inflammatory mediator,[15,16] a neuronal protectant or toxin,[17] and a driver of angiogenesis and tumor metastasis.[18] The growing recognition of its role in conditions like neurodegeneration,[19] underscores the broad clinical relevance of these non-canonical functions.

This review aims to synthesize current knowledge on these hemostasis- and coagulation-independent functions. We will dissect how thrombin's unique structure enables its functional diversity, explore its multifaceted roles in inflammation, immunity, and cancer, and delve into its critical, dualistic actions within the cen-

tral nervous system. Finally, we will touch upon emerging evidence that redefines our understanding of how thrombin operates in the complex milieu of the vasculature and tissues.

The molecular toolkit: structure-function relationships governing thrombin's versatility

To appreciate the breadth of thrombin's functions, one must first understand the structural features that allow a single enzyme to recognize and interact with such a diverse array of substrates, receptors, and cofactors. Thrombin's versatility is not a consequence of a promiscuous active site but rather of highly evolved regulatory domains and allosteric control mechanisms. The intricate interplay between its exosites, as highlighted by Fredenburgh & Weitz,[9,20] forms a complex communication network that fine-tunes substrate specificity.

The active site and the sodium switch

The active site of thrombin is typical of trypsin-like serine proteases, containing the catalytic triad (His57, Asp102, Ser195). However, its specificity is profoundly modulated by an allosteric mechanism involving the binding of a single sodium ion (Na^+) (Figure 1). Di Cera and colleagues have elegantly demonstrated that thrombin exists in at least two distinct conformations: a Na^+-bound fast form and a Na^+-free slow form, with a third inactive form (E*) also identified.[21] This allosteric communication is a classic example of how a small ion can dictate macromolecular function.[22] The fast form (E:Na^+) is the procoagulant, prothrombotic form of the enzyme. It has a high specificity for cleaving fibrinogen and activating Protease-Activated Receptors-1 (PAR-1),[22] driving clot formation and platelet activation.[6] In contrast, the slow form (E), which predominates in the absence of Na^+, exhibits a significantly reduced catalytic efficiency towards procoagulant substrates. However, given thrombin's roles in inflammation and neuronal plasticity,[23,24] sodium-mediated regulation of its activity in non-blood environments remains possible.

This *sodium switch* is therefore a critical determinant of thrombin's functional duality, allowing it to toggle between procoagulant and anticoagulant roles based on the ionic environment and allosteric modulators.

The anion-binding exosites: docking stations for specificity

Thrombin's surface is adorned with two positively charged regions, remote from the active site, known as anion-binding exosites.[25] These exosites act as recognition and docking sites for the numerous ligands that give thrombin its multifunctional character.[26] Previous studies emphasized how these exosites do not function in isolation but communicate with each other and the active site to modulate overall enzyme activity.[27-30]

Anion Binding Exosite-I (ABE-I, or the *fibrinogen-recognition site*): this site mediates the interaction with substrates like fibrinogen, the cofactor thrombomodulin, the platelet receptor PAR-1, the leech anticoagulant hirudin, and FV/FVIII.[31-33] The binding of thrombomodulin ABE-I is a classic example of functional plasticity; it not only blocks fibrinogen binding but also in-

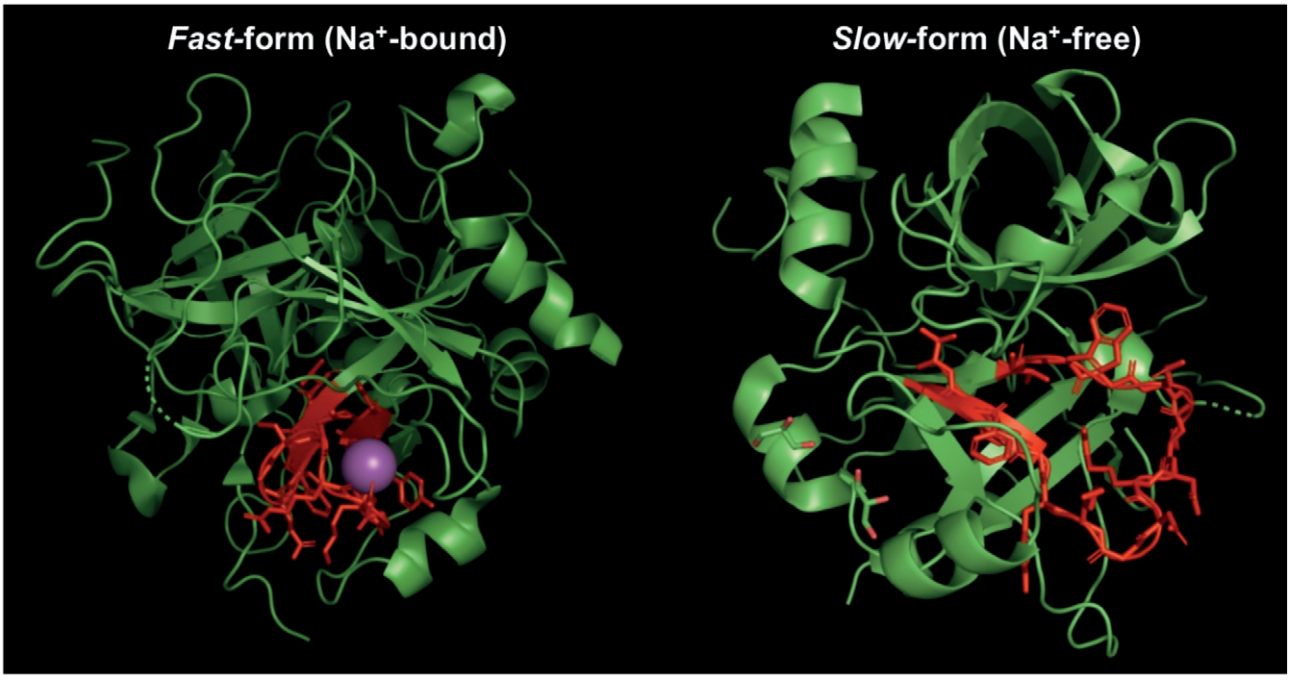

Figure 1. Structural comparison between active (fast-form) and inactive (slow-form) forms of thrombin. The active E form (pdb 3LU9) and the inactive form (pdb 3BEI, red) are depicted in green. They mainly differ in the Na+ binding loop (V213-T229) depicted as red sticks. The sodium ion is depicted as a magenta sphere. Note how the 220-loop is partly destructurated in the slow-form, being unable to bind the sodium ion.

duces a conformational change that enhances thrombin's ability to activate protein C, flipping the switch from procoagulant to anticoagulant.[30,34,35]

Anion Binding Exosite II (ABE-II or the *Heparin-binding site*): this site binds glycosaminoglycans like heparin, as well as the platelet receptor GPIbα.[36-39] Heparin binding to exosite II facilitates the inhibition of thrombin by antithrombin III, a process whose allosteric communication network has been further elucidated in recent studies.[40] Furthermore, the interaction with GPIbα on platelets localizes thrombin to the platelet surface, favoring its own generation and platelet activation via PAR-1.[6]

Protease-activated receptors: the signaling gateway

While thrombin can act on soluble proteins, its effects on cells are predominantly mediated through the proteolytic activation of PARs, a family of G-protein-coupled receptors.[5] Thrombin cleaves the N-terminal extracellular domain of PAR-1, PAR-3, and PAR-4 (in humans), unmasking a new N-terminus that acts as a tethered ligand, binding intramolecularly to the receptor to initiate transmembrane signaling.[5] The specific roles of these receptors in platelet function and beyond continue to be an area of active investigation, with recent reviews detailing their involvement in the release of platelet-derived microparticles.[41]

Types of protease-activated receptors

Protease-activated receptors-1

PAR-1 is the high-affinity thrombin receptor, activated by picomolar to nanomolar concentrations of thrombin.[5] It is expressed on platelets, endothelial cells, neurons, fibroblasts, and immune cells, mediating a vast array of responses from platelet aggregation to neuroinflammation.[42] Although PAR1 is expressed throughout the cell membrane, its localization within distinct microdomains dictates signaling outcomes by different proteases.[43-46] For instance, PAR1 localization within caveolin-1-rich lipid rafts, where it colocalizes with Endothelial Protein C Receptor (EPCR) in endothelial cells,[43,46] and with integrin Mac-1 in macrophages,[47] is critical for Activated Protein C (APC)-mediated cytoprotective signaling. For instance, PAR1's presence in caveolin-1-rich lipid rafts, specifically its colocalization with EPCR in endothelial cells,[43,46] or with integrin Mac-1 in macrophages,[47] is critical for cytoprotective signaling by APC. On endothelial cells, constitutive PAR1 colocalization with β-arrestins provides a scaffold for disheveled-2, linking PAR1 to β-catenin/Wnt signaling.[48] Knockdown of either scaffold protein prevents PAR1-driven Rac1 activation by APC, without affecting thrombin-mediated RhoA signaling.[48] Thus, PAR1 localization within caveolin-rich microdomains is a key determinant of its signaling bias toward cytoprotective outputs in both cell types. All these experimental evidences led to explore the therapeutic potential of targeting PAR-1 is being explored in various disease models, including neurodegeneration.[49]

Protease-activated receptors-4

PAR-4 activation requires higher thrombin concentrations and plays a role in sustained platelet activation and inflammatory responses.[50] Human PAR-4 is activated when thrombin cleaves its amino-terminal exodomain at the Arg_{47}/Gly_{48} peptide bond to unmask the tethered ligand GYPGQV,[50,51] being responsible for control of sustained cytoplasmic calcium mobilization.[52] PAR-3, another cell membrane thrombin receptor, is thought to act primarily as a cofactor for PAR-4 activation in mice, though its role in humans is less defined.[53]

The *tethered ligand* mechanism, occurring upon PAR-1 and PAR-4 hydrolysis, means that thrombin's signaling is irreversible; once cleaved, the receptor is activated until it is desensitized and internalized. This underscores the potency and delicacy of thrombin-mediated cellular regulation.

Thrombin as a master regulator of inflammation and immunity

The intersection of coagulation and inflammation is now a well-established field, and thrombin sits at its epicenter (Figure 2). By activating PARs on vascular and immune cells, thrombin orchestrates a complex inflammatory response that is essential for pathogen containment and tissue repair but can become detrimental in conditions like sepsis and chronic inflammatory diseases.[54,55] The signaling pathways involved are complex, involving multiple protein kinases within the endothelium.[56]

Activation of the endothelium and innate immunity

Upon injury or inflammatory challenge, thrombin activates endothelial cells via PAR-1. This triggers a cascade of events: cells retract, increasing vascular permeability and contributing to edema formation; they upregulate adhesion molecules like P-selectin, which promotes the rolling and adhesion of leukocytes; and they release von Willebrand factor (VWF), further supporting platelet adhesion.[57,58] This thrombin-driven change in endothelial phenotype converts the vessel lining from a quiescent, anti-adhesive surface to a pro-inflammatory one, facilitating the recruitment of immune cells to the site of injury or infection. The regulation of VWF, a key player in this process, is itself a complex field, with recent studies showing how its unfolded form can bind protein S and modulate anticoagulant pathways.[59]

Direct effects on immune cells

The influence of thrombin extends directly to cells of the immune system, many of which express PARs. Thrombin acts as a potent chemotactic agent for monocytes and neutrophils, drawing them into inflamed tissues.[60] It stimulates macrophages and monocytes to produce and release a host of pro-inflammatory cytokines, including interleukin-1β, interleukin-6, and tumor necrosis factor-α.[60] These cytokines, in turn, further amplify the coagulation response, creating a positive feedback loop that can spiral into pathological disseminated intravascular coagulation in severe sepsis. Recent evidence also highlights a direct link between thrombin and the complement system, a key arm of innate immunity. Thrombin can directly cleave complement component C5 to generate the potent anaphylatoxin C5a, bypassing the classical complement activation pathways and directly linking thrombosis to complement-mediated inflammation.[61]

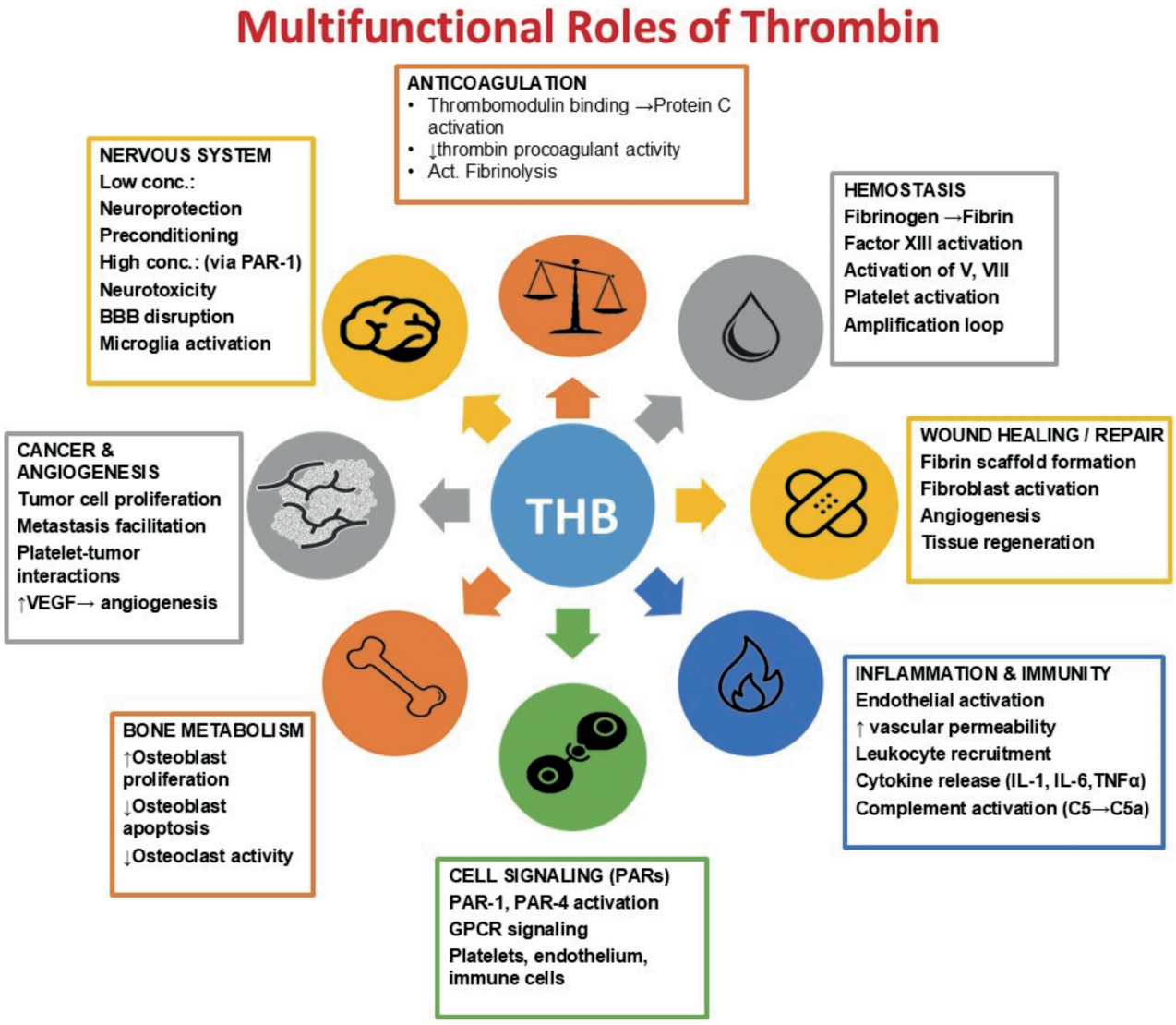

Figure 2. Thrombin (THB) as a multifunctional protease integrating coagulation and cellular signaling. It promotes hemostasis by generating fibrin, activating coagulation factors, and stimulating platelets, while also exerting anticoagulant effects via thrombomodulin-dependent protein C activation. Through PAR receptors, thrombin regulates inflammation by activating endothelium, recruiting leukocytes, and inducing cytokine release. Its actions are context-dependent in the nervous system, ranging from neuroprotection to neurotoxicity. Thrombin also supports tumor progression and angiogenesis, enhances wound healing, and modulates bone remodeling. Overall, its diverse effects depend on concentration, cofactors, and cellular environment, highlighting its central role in physiology and disease.

Platelets as immune effectors

Beyond their role in hemostasis, platelets are critical immune cells. Thrombin is the most potent activator of platelets, acting through PAR-1 and PAR-4. This activation not only causes aggregation but also triggers the release of a plethora of immunomodulatory molecules from platelet granules, including CD40 ligand (CD40L), P-selectin, and various chemokines.[62] CD40L, in particular, can engage with CD40 on endothelial cells, prompting them to secrete chemokines and express adhesion molecules, further promoting leukocyte recruitment.[62] This crosstalk firmly places thrombin-activated platelets as central players in the thrombo-inflammatory continuum. Furthermore,the platelet-derived microparticles generated upon activation are now recognized as important vectors for inflammatory signaling.[63]

Thrombin in the nervous system: a double-edged sword

The brain was once considered an "immune-privileged" site, isolated from the coagulation system. This view has been radically overturned. It is now recognized that thrombin, entering the brain parenchyma after blood-brain barrier (BBB) disruption or hemorrhage, exerts powerful and dose-dependent effects on all neural

cell types, playing a significant role in the pathology of stroke, traumatic brain injury, and neurodegenerative diseases.[64]

Neurotoxicity *vs.* neuroprotection: the concentration paradigm

High concentrations

The effects of thrombin in the brain are highly concentration-dependent, a phenomenon often referred to as the *thrombin paradox*. Following intracerebral hemorrhage or significant BBB breakdown, large amounts of blood-derived prothrombin enter the brain and are converted to thrombin. This high concentration of thrombin is unequivocally detrimental.[64] It induces apoptosis in neurons and astrocytes,[65] disrupts the BBB (leading to vasogenic edema), and activates microglia, the brain's resident immune cells, triggering a potent and damaging neuroinflammatory response.[66] These effects are primarily mediated by PAR-1 and PAR-4 on neurons and glial cells. Studies are now exploring the potential of PAR-1 antagonists to mitigate such damage in models of Huntington's disease.[67]

Low concentrations

At picomolar to low nanomolar concentrations, thrombin exerts a neuroprotective effect.[45] This phenomenon, known as *preconditioning*, can protect neurons from subsequent hypoxic or ischemic insults. Low-dose thrombin has been shown to promote cell survival, reduce apoptosis, and even aid in tissue repair and remodeling after injury. The mechanisms may involve the upregulation of protective factors, as seen with thrombopoietin's role in mitigating neuronal injury.

This duality presents a major therapeutic challenge. While inhibiting thrombin is a logical strategy to limit brain damage after a hemorrhagic stroke, complete inhibition could potentially abrogate its beneficial, protective functions.

Contribution to neurodegenerative and inflammatory diseases

Emerging evidence implicates thrombin in the pathogenesis of chronic neurological conditions. In Alzheimer's disease, thrombin levels are elevated in the brain, and it has been shown to promote the deposition of amyloid-β peptides and contribute to tau hyperphosphorylation, hallmarks of the disease.[68] In multiple sclerosis, thrombin-mediated disruption of the BBB and promotion of neuroinflammation are thought to exacerbate disease progression. Furthermore, thrombin can contribute to seizure activity and memory impairment by altering neuronal electrophysiology.[69]

Thrombin in cancer, angiogenesis, and tissue repair

The role of thrombin extends into oncology and regenerative medicine, where it influences tumor growth, the formation of new blood vessels, and the healing of damaged tissues. The tumor microenvironment is a particularly rich area for studying these effects, where coagulation proteases like thrombin can modulate immune responses.

Tumor progression and metastasis

A strong link exists between the coagulation system and cancer, a relationship famously highlighted by Trousseau's syndrome (migratory thrombophlebitis as a sign of occult malignancy). Thrombin is a key driver of this pro-tumorigenic environment.[70] Many tumor cells express PAR-1. Thrombin-mediated activation of PAR-1 on these cells can stimulate their proliferation, enhance their motility, and promote an invasive phenotype, facilitating local invasion and metastasis. For a tumor to grow beyond a certain size, it must recruit its own blood supply, a process called angiogenesis. Thrombin is a potent inducer of angiogenesis.[71] It can directly act on endothelial cells to promote sprouting and tube formation. Indirectly, it stimulates tumor cells and stromal cells to release potent pro-angiogenic factors, most notably vascular endothelial growth factor.[71] Moreover, thrombin promotes metastasis by several mechanisms. It facilitates the adhesion of circulating tumor cells to the endothelium or to platelets (forming tumor cell-platelet emboli), which shields them from immune surveillance and aids in their extravasation into distant tissues.[18] New research also suggests that coagulation proteases can modulate nucleic-acid sensing pathways like the cGAS-STING system in the tumor microenvironment, potentially affecting anti-tumor immunity.[72]

Wound healing and tissue regeneration

Beyond its pathological roles in cancer, thrombin's ability to promote cell proliferation and angiogenesis is fundamental to physiological wound healing and tissue regeneration. In the initial phases of wound repair, the thrombin-generated fibrin clot provides a provisional matrix for cell migration. Subsequently, by activating PAR-1 on fibroblasts and endothelial cells, thrombin stimulates their proliferation and the production of new extracellular matrix, orchestrating the granulation tissue formation and revascularization essential for healing.[73] This principle is being harnessed in tissue engineering, with biomaterials being developed that incorporate thrombin receptor-activating peptides to promote hemostasis and accelerate the healing of infected wounds.[74]

Effect on bone cells

Bone health depends on a finely regulated balance between the resorptive activity of osteoclasts, cells derived from monocytic precursors, and the bone-forming activity of osteoblasts. A recent *in vitro* study demonstrated that thrombin inhibits both osteoclast differentiation and activity, while promoting osteoblast proliferation and reducing apoptosis in bone tissue.[75] These findings suggest that, beyond its role in hemostasis, thrombin may help prevent bone erosion associated with chronic synovitis and arthritis in several hemorrhagic conditions, including hemophilia. These effects are mediated by the presence of PAR-1 receptors on the membranes of both osteoclasts and osteoblasts. If confirmed by future clinical trials, this knowledge could contribute to the development of effective strategies to prevent arthropathy in patients with inherited bleeding disorders.

Discussion and future directions

The expanding body of evidence reviewed here reinforces the concept of thrombin as a multifunctional protease that operates at

the interface of coagulation, inflammation, immunity, and tissue remodeling. This functional versatility is grounded in a well-defined and extensively validated structural framework. The sodium-dependent allosteric regulation, the coordinated interplay between anion-binding exosites I and II, and the modulation of substrate specificity through cofactors such as thrombomodulin collectively represent the most robust and experimentally substantiated aspects of thrombin biology.[9,20-22,29] These mechanisms provide a coherent molecular basis for thrombin's dualistic behavior, enabling it to switch between procoagulant and anticoagulant functions in a context-dependent manner.

Beyond these consolidated principles, thrombin exerts a wide range of cellular effects primarily mediated through PARs, influencing endothelial activation, leukocyte recruitment, cytokine production, and platelet-driven immune responses.[54-62] These pathways place thrombin at the center of the thromboinflammatory axis, where coagulation and innate immunity are tightly interconnected. In parallel, thrombin contributes to processes such as angiogenesis, tumor progression, and tissue repair, further underscoring its role as a master regulator of adaptive biological responses to injury and stress.[18,70,71,73]

At the same time, recent studies have extended the functional repertoire of thrombin into emerging and less well-characterized areas. These include its potential involvement in nucleic acid sensing pathways such as cGAS-STING signaling within the tumor microenvironment,[72] its capacity to directly modulate the complement system through cleavage of C5,[61] and its participation in neurodegenerative and neuroinflammatory conditions, including Alzheimer's disease and multiple sclerosis.[68,69] While these findings are biologically intriguing and supported by growing experimental evidence, it is important to recognize that they are still largely derived from *in vitro* systems or preclinical models. As such, they do not yet reach the same level of validation as the structural and canonical functional properties of thrombin.

A similar consideration applies to the emerging role of thrombin in bone remodeling. Available data indicate that thrombin can influence both osteoclast and osteoblast function through PAR-mediated signaling, potentially shifting the balance toward bone formation.[75] Moreover, its broader role in inflammation suggests an indirect contribution to the regulation of the bone microenvironment. However, these observations remain predominantly experimental, and a clear link to clinical outcomes has not yet been established. Therefore, while the involvement of thrombin in skeletal biology represents an intriguing extension of its functional spectrum, it should currently be regarded as a hypothesis-generating area requiring further validation.

Taken together, these considerations highlight the importance of distinguishing between well-established mechanisms and emerging hypotheses within the rapidly evolving field of thrombin biology. Equating highly validated structural and biochemical evidence with preliminary or preclinical findings may lead to an overinterpretation of the current state of knowledge. A more nuanced perspective, acknowledging different levels of evidentiary strength, is essential to maintain scientific rigor and to guide future research directions.

From a translational standpoint, the increasing complexity of thrombin signaling presents both challenges and opportunities. Therapeutic strategies targeting thrombin have traditionally focused on its inhibition to prevent thrombosis. However, the recognition of its pleiotropic and context-dependent functions suggests that more selective approaches may be required. These could include the development of exosite-specific inhibitors, allosteric modulators that influence the sodium-binding equilibrium, or biased ligands targeting PAR signaling pathways to selectively modulate downstream effects without compromising essential hemostatic functions. Such strategies may allow for a more precise intervention within the thromboinflammatory network and enhance the clinical impact of targeting thrombin.

Ultimately, the future of this field will depend on the ability to bridge the gap between mechanistic insights and clinical application. This will require not only the validation of emerging concepts in relevant *in vivo* models and human studies but also a clearer integration of thrombin's diverse functions into unified pathophysiological frameworks. By maintaining a critical distinction between established knowledge and evolving evidence, thrombin can be more effectively positioned as a target for innovative therapeutic strategies in a wide range of thromboinflammatory, degenerative, and possibly skeletal disorders

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