Chapter 1
Introduction and review of literature

1.1 Hemostasis

Hemostasis is a physiological response to tissue injury leading to arrest of blood loss from ruptured vessels, while maintaining normal blood flow elsewhere in the circulation [1]. The anti-coagulant surface of the endothelium in blood vessels maintains blood in its fluid state, but if the vascular tissue is damaged components of the sub-endothelial matrix activate the two main processes of hemostasis to initiate blood clotting, composed primarily of platelets and fibrin [2]. This is a tightly regulated process – activated instantly on injury, but localized to the site of injury. The two main components of hemostasis are – (i) Primary hemostasis, a process of platelet aggregation and platelet plug formation and (ii) Secondary hemostasis, formation of insoluble fibrin generated by the proteolytic coagulation cascade.

1.1.1 Primary hemostasis

Platelets or thrombocytes are small anuclear cell fragments, derived from megakaryocytes, and are present at 150-400 million ml^{-1} of blood circulating in the vascular system for about ten days [3,4]. When blood vessels are damaged platelets are exposed to sub-endothelial matrix, followed by adhesion to collagen and activation [3,5]. Glycoprotein (GP) receptors, integrins or non-integrins, are expressed on the surface of platelets which interact with exposed collagen and adhesion proteins on the subendothelial matrix. GP Ib-IX-V binds to immobilized von Willebrand factor (vWF), a large multimeric protein, through GPIbα-vWF A1 domain interaction [6]. The platelet membrane has several collagen receptors, including the integrin $\alpha_2\beta_1$ [7], GPVI [8], GPIV (CD-36) [9,10], and p65, the 65 kDa protein specific for type I collagen [11]. GPVI and GPIb-IX-V are vital for adhesion and subsequent activation of platelets [12].

Many agonists, such as ADP, thrombin, platelet-activating factor, and lysophosphatidic acid play a critical role in platelet activation and thrombus formation [13], while other platelet agonists, such as thromboxane A_2 (TXA_2), Adenosine diphosphate (ADP), and serotonin, amplify platelet activation and recruit circulating platelets. These agonists activate platelets through G-protein–coupled receptors (GPCRs). GPCRs transmit signal though heterodimeric G Proteins which consist of α, β, and γ subunits and bind to GPCRs in an α/β/γ complex. Platelets express Gq, G12/G13, Gi/Gz, and Gs, subfamilies of G proteins, that couple to agonist receptors and stimulate platelet activation, with the exception of Gs. Gs is coupled to receptors for prostacyclin and adenosine (physiological platelet inhibitors) that stimulate adenylyl cyclase–dependent cAMP synthesis and mediate inhibitory signals [13,14]. Thrombin-induced platelet activation takes place by the cleavage of a dual system of G-protein–coupled protease-activated receptors (PARs): PAR1 and PAR4 (coupled to Gq and G12/G13) in humans [15] and PAR3 and PAR4 in mice [16]. TXA_2 activates platelets via the Gq and G13 coupled TXA_2/prostaglandin H2 receptor (TP) [17,18]. Platelets are activated by ADP via $P2Y_1$ (Gq coupled) and $P2Y_{12}$ (Gi coupled) [13,19].

Platelet activation induces a conformational transition in several receptors such as αIIbβ3, α2β1, αvβ3, α5β1 and α6β1 on the surface of platelets, resulting in exposing of ligand binding sites [20,21]. These receptors bind to multiple ligands including fibrinogen, VWF, collagen, fibronectin, vitronectin, or laminin, that promote platelet-platelet aggregation [22-24]. The platelet plug is further stabilized by deposition of insoluble fibrin which is generated by the coagulation cascade.

1.1.2 Secondary hemostasis

Secondary hemostasis involves a cascade of coagulation serine proteases culminating in an insoluble fibrin which finally forms a crosslinked fibrin mesh by thrombin cleavage of soluble fibrinogen. This complex process of activation and cleavage of serine proteases leading to fibrin generation occurs at the same instant to platelet aggregation [25]. The coagulation cascade is inactive in intact and healthy blood vessels; anti-coagulant mechanisms which include thrombomodulin and

heparan sulfate proteoglycans on vascular endothelium maintain continuous blood flow. When the endothelial tissue is injured, factor VII (FVII) comes in contact with tissue factor (TF), which non-proteolytically activates FVII to FVIIa [26], and forms a VIIa-TF complex. This complex activates factor X (FX) and factor IX (FIX) to FXa and FIXa, respectively ("a" suffix stands for *activated*). This activation pathway is known as the extrinsic pathway of coagulation. FIXa, in the presence of its cofactor factor VIIIa, also activates factor X to FXa, which in turn activates prothrombin to generate thrombin [27].

Factor XII (FXII, Hageman factor), in contact with negatively charged membrane of activated platelets, is autocatalytically converted to a small amount of FXIIa [28]. This factor initiates the intrinsic (blood-borne) pathway of the coagulation pathway, in a reaction which involves high molecular weight kininogen (HMWK) and plasma kallikrein (PK) – together these constitute the plasma contact system [29]. FXIIa cleaves prekallikrein to kallikrein, which reciprocally accelerates the activation of FXII [30]. FXIIa triggers fibrin generation by activating factor XI (FXI) and also releases the inflammatory mediator bradykinin (BK) from HMWK through cleavage by PK [31]. BK binds to the kinin B2 receptor (B2R) activating proinflammatory signaling pathways that result in blood vessel dilation, chemotaxis of neutrophils, and increased vascular permeability [32]. FXIa converts factor IX (FIX) to FIXa by proteolytic cleavage [33]. Traces of thrombin cleaves FVIII to generate FVIIIa [34]. FVIIIa forms a tight non-covalent complex with FIXa on the phospholipid membrane and further binds to FX, which is subsequently activated to form FXa [35,36]. The ternary complex of FIXa, FVIIIa, and FX, bound to the phospholipid membrane, is usually referred to as Tenase complex, and is the main component of the positive feedback loop in the coagulation cascade [37].

Parallelly, FXa binds to FVa to form the prothrombinase complex with enzymatic activity – a positive feedback reaction. This complex converts the proenzyme prothrombin to its enzyme form, thrombin [38]. Thrombin cleaves fibrinogen to generate the fibrin monomer, which then rapidly polymerizes to form the insoluble fibrin clot [39]. Thrombin also activates FXIII and converts it to FXIIIa, thus mediating the covalent cross-linking of the fibrin polymers to form a mesh of

stable fibrin, which is less soluble than fibrin polymers [36]. Thrombin also catalyzes the formation of the cofactors FVa and FVIIIa, resulting in efficient amplification of coagulationc [40].

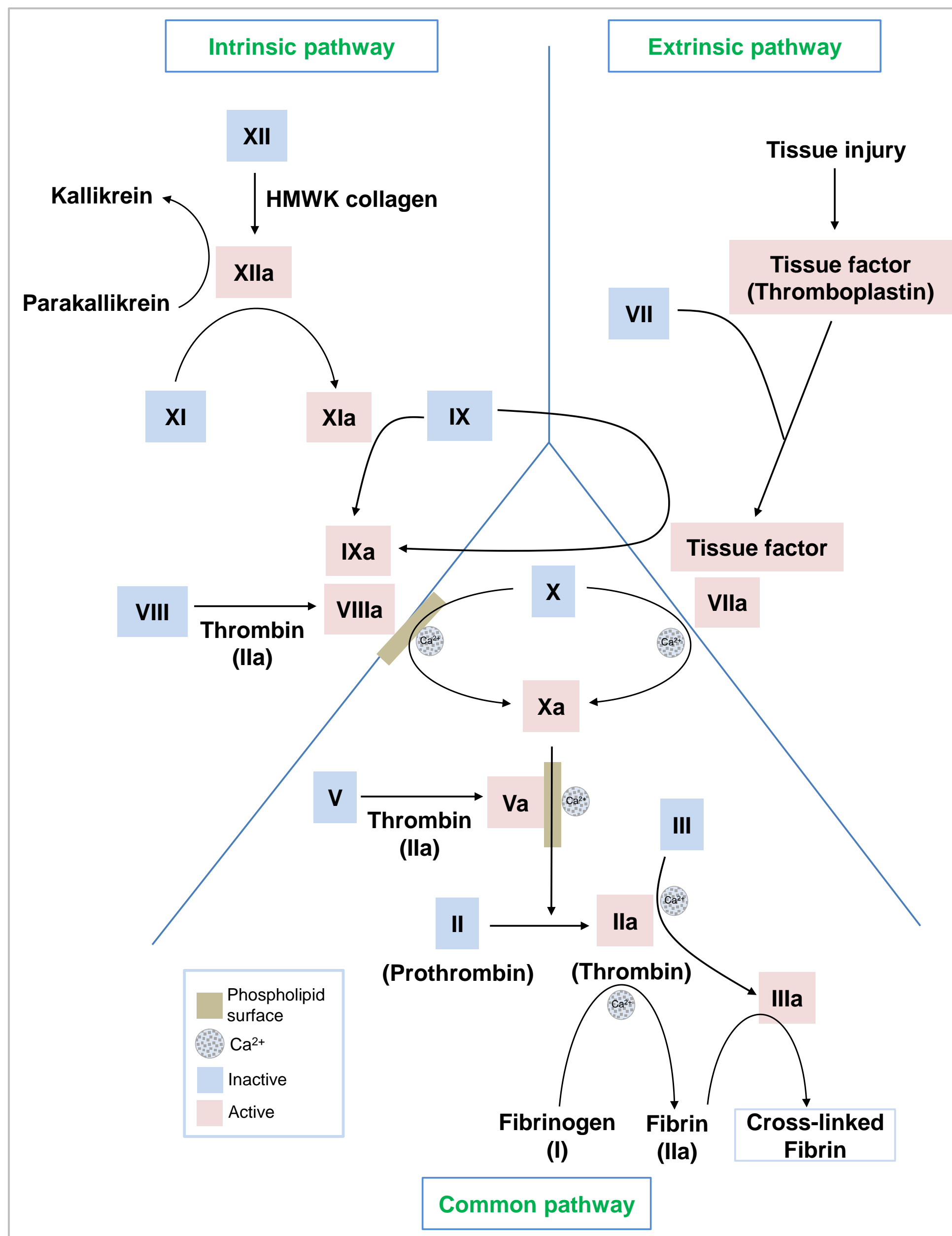

Figure 1.1 The coagulation cascade [26].

1.1.3 Fibrinolysis

The fibrinolytic system prevents blood clots in healthy blood vessels by dissolving blood clots during the process of wound healing. Plasmin is the major

fibrinolytic protease that cleaves and breaks down fibrin. Plasmin is generated from plasminogen (zymogen) by the proteolytic activity of tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). Plasmin, through a positive feedback mechanism, cleaves tPA and uPA from single chain to more active two-chain polypeptides [41]. Fibrin regulates its own degradation by binding tPA and plasminogen; thus localizing plasmin generation and activity at clot site. Plasmin cleaves fibrin polymers, yielding soluble degradation products, and exposing carboxy-terminal lysine (Lys) residues. Kringle 2 of tPA and Kringle 1 and 4 of plasminogen contain Lys-binding sites, that mediates further binding to fibrin and thus enhances plasmin generation and fibrin removal [41]. These serine proteases are down-regulated by serpins present in blood; α_2-plasmin inhibitor (α_2-PI) inhibits plasmin, and plasminogen activator inhibitors 1 and 2 inhibit tPA and uPA [42].

1.2 Thrombosis: prevention and treatment

Hemostasis is a dynamic and tightly regulated system and disturbance or instability of the system could lead to hemorrhagic disorders or thrombosis. Thrombotic disorder may be classified into two major groups – (i) hereditary (hereditary thrombophilia, antithrombin III, Protein C and Protein S deficiency, Factor V mutation, and Prothrombin (Gene 20210A) mutation) and (ii) acquired (antiphospholipid antibody syndrome, increased level of factors VIII, IX, XI or fibrinogen, fibrinolysis defects, and homozygous homocystinuria) [43]. Thrombosis particularly results in high morbidity and mortality due to vascular occlusion with the consequence of myocardial infarction (MI), stroke, pulmonary embolism (PE), or deep-vein thrombosis (DVT) [44]. Cardiovascular diseases (CVDs) conduce greatly to the mortality, morbidity and economic encumbrance of illness throughout the world and approximately 17.5 million people (31% of total deaths worldwide) die annually due to CVDs [45,46]. Anticoagulants such as heparin are the drugs of choice for the prevention and treatment of venous thromboembolism (VTE) [47], the acute medical care of patients with acute coronary syndromes (ACS) and for stroke prevention in patients with atrial fibrillation (AF) or mechanical heart valves [48]. Heparin has a number of major limitations including narrow therapeutic window and highly variable dose-response [49]. Vitamin K antagonists (VKAs) (e.g. warfarin) are effective for

the prevention of recurrent ischemia after acute myocardial infarction, but are not widely used for their limitations such as narrow therapeutic window, delayed action, multiple food and drug interactions and metabolic variation [50,51]. Antiplatelet drugs are used for arterial thrombotic events (e.g. in the treatment of ACS and prophylactic management of coronary, cerebral and peripheral artery disease), but is not as efficient as anticoagulants in the prevention of VTE [52]. Limitations of these drugs have driven the continual and intense search for newer, more efficacious and safe anticoagulants, especially those targeting specific factors of the coagulation cascade [49]. Several direct thrombin inhibitors such as hirudin [53], bivalirudin [54], argatroban [55], and dabigatran etexilate [56] are currently in the market. Recombinant hirudin used for the treatment of patients with heparin-induced thrombocytopenia (HIT) and thrombosis prophylaxis after major orthopedic surgery, has few major drawbacks such as risk of bleeding, dependence of pharmacokinetics upon renal function, lack of antidote, immunogenicity and rebound hypercoagulability [57]. Unlike hirudin, bivalirudin has negligible immunogenic potential and is eliminated by non-organ mechanism (proteolysis) and renal route (20%), an advantage over hirudin (renally excreted) and argatroban (hepatobiliary excretion mechanism) [58]. Argatroban is limited mainly in the treatment of HIT [59]. Dabigatran etexilate is the latest oral drug developed for the prevention of venous thromboembolic events during hip or knee replacement surgery [60]. Dabigatran has several advantages as compared to warfarin and other oral vitamin K antagonists, which include rapid onset of action, predictable anticoagulant effect, specific target, and low potential food and drug interactions. However, there are several unresolved issues with use of dabigatran etexilate, such as its safety and efficacy of inhibiting thrombin generation in the long run, safety and utility of the lower dose in elderly patients with moderate renal impairment or patients with low body weight (<50 kg), monitoring its anticoagulant effect, the safety of its administration dabigatran in combination with antiplatelet drugs and p-glycoprotein–affecting drugs and the reversibility of the anticoagulant effect of dabigatran in the event of acute bleeding or urgent surgery [61,62]. It is obvious that newer and safe anticoagulants with different pharmacological and pharmacokinetic properties are needed to be developed and thus,

the search for novel therapeutic leads for development of better anticoagulant drug is still pertinent.

1.3 Evolution of hematophagy

Hemostasis is an efficient and well-regulated process of subverting any vascular injury and preventing loss of blood. The vertebrate blood coagulation system has originated since approximately 400 MYA and evolved to acquire the present form about 200 MYA [63]. To feed on the vertebrate blood requires that the animal overcome the host hemostatic response, including platelet activation and aggregation, local vasoconstriction, and coagulation of blood [64]. The evolution of hematophagy or blood-feeding behavior in animals occurred independently at least six times in the approximately 15,000 species and 400 genera of hematophagous arthropods during the Jurassic and Cretaceous eras, 145–65 MYA [65,66]. Hematophagous arthropods thus adapted to an efficient and already existing hemostatic system. It is also noteworthy that similar inhibitory mechanisms against host hemostatic system have evolved several times in due course of time [67]. Hematophagy is believed to evolve via two major routes – prolonged association with vertebrate hosts and morphological pre-adaptation to incisive penetration [68]. It is very likely for insects to be attracted to the nests or burrows of vertebrate host for the favorable humid and warm environment, protected habitat and/or abundance of food supply. Behavioral adaptation of these insect may have allowed occasional feeding from the host skin, in addition to their phoretic behavior; this could have led to morphological adaptation too [68]. The present-day mallophagans which feed on feathers and skin of birds do sometimes feeds on blood. The insect while feeding often breaks the dermis and gets access to blood, thus feeding on blood [69]. The high nutritional value of blood may have favored the physiological, behavioral and morphological adaptations in many insects, first to facultative and eventually, in some insects, to obligate hematophagy [68]. Second route of evolution suggests that blood-feeding behavior developed in some insects from ancestral insects that were morphologically pre-equipped with mouthparts for piercing surfaces [70-72]. Entomophagous insects gave rise to some of the hematophagous insect groups, which is to a great extent evident from the lifestyles of several present-day insects [70]. Hematophagy is also thought to have arisen from

plant-feeding ancestors which possessed mouthparts specialized for piercing and sucking, that would pre-adapt them for hematophagy [68,73].

1.4 Ticks and their economic importance

Ticks are obligate, non-permanent hematophagous ectoparasites of terrestrial vertebrates. Ticks comprise the suborder Ixodida of the order Parasitformes [74]. They are unique in the sense that they have large body (unfed adult ticks: 2-20 mm; engorged females: 25-30 mm), specialized mouthparts (hypostome), and a peculiar highly specialized assemblage of sensory structures on tarsus I (Haller's organ) [75,76]. The body of tick is divided into two parts, consisting of the capitulum (gnathosoma) and the main body (idiosoma) to which the legs are attached [76]. These comprise of about 883 species and are divided into three families, the Ixodidae (hard ticks; 696 species), Argasidae (soft ticks; 186 species), and Nuttalliellidae (1 species) [77-80]. The family Nuttalliellidae is monotypic and shares characters of both Argasidae and Ixodidae, in addition to other derived features [81].

Argasidae ticks are nest, burrow, or roost parasites, and usually mate in the habitat. Argasids feed on the same host individual or species throughout their life, though their host range spans widely diverse terrestrial vertebrates, including turtles, squamates (lizards and snakes), amphibians, birds, and mammals [82]. They are found in all major biogeographical regions, tropical and temperate. Nymps and adults in this family possess a ventral position of the capitulum and a highly sculptured integument and do not have dorsal scutum. Their life cycle consist of an egg, a hexapod larva, octopod nymphal instars, and the adult. The number of nymphal instars is variable and is unique among known Acari. Argasid larvae may require few days to feed, but most nymphs and adults feed to repletion in a few hours or less (rapid feeding) [82]. Females feed intermittently, depositing a small batch of eggs after each feeding session. Ixodidae ticks differ from argasids in that they have an anterior capitulum, a striate integument and a scutum [82]. Ixodids developmental stages include egg, larvae, nymph and adult; these have a single nymphal stage. Life cycle of hard-ticks may be classified as one-host, two-host, or three-host, according to the number of times the three feeding stages change their hosts [75,83]. Ixodids feed to repletion

slowly for over several days, after which they drop off the host. Despite their slow feeding behavior, these ticks may spend 96-97% of their life off-host [84]. Fully-engorged adult females drop off their host and produce a single, large batch of eggs, after which they die.

Ticks and tick-borne diseases (TTBDs) are of immense economic importance. These blood-feeding parasites acquire blood meal from their host, continuously deteriorating the animal health leading to decrease in productivity such as milk production [85]. Moreover, they are serious vectors of many disease-causing viruses and bacteria. For example, they transmit several arboviruses (e.g., tick-borne encephalitis virus, several Reoviridae, Bunyaviridae, and Iridoviridae), protista (*Babesia* and *Theileria*), and bacteria (*Rickettsia, Ehrlichia, Borrelia*) [83]. TTBDs affect 80% of the cattle throughout the globe, particularly tropical and subtropical countries, including India [86]. India share nearly 57% of world's buffaloes, 16.5% cattle, 16.3% goats, and 5.7% sheep and 70% of the population depend on agriculture for their livelihood [85]. TTBDs cause very large damage to livestock in India (cost of TTBDs estimated to be US$ 498.7 million) [87], affecting the livelihood of a large fraction of the population [88]. In India, tick belonging to *Amblyomma* (including *Aponomma*), *Haemaphysalis*, *Dermacentor*, *Rhipicephalus* (including *Boophilus*), *Hyalomma*, *Ixodes*, *Nosomma*, *Argas*, *Ornithodoros*, and *Otobius* genera are found across the various states [89], among which *Amblyomma*, *Haemaphysalis*, *Dermacentor*, *Rhipicephalus*, *Hyalomma*, and *Ixodes* ticks are found in the state of Assam [88].

Ticks are also serious vectors of human infectious diseases and are considered second only to mosquitoes as vectors, and these cause several serious diseases in humans [85]. Ticks are known to transmit several pathogens which are causal agents of diseases like Texas cattle fever [90], tick relapsing fever [91], Rocky Mountain spotted fever [92], Mediterranean spotted fever [93], tularemia [94], Lyme borreliosis [95], and rickettsioses [96]. Ticks also act as reservoirs of tick-transmitted bacteria, including spotted fever group rickettsiae, recurrent fever borreliae, and *Francisella tularensis* [97]. At least 222 species of the known tick species have been reported to feed blood from human, but only few commonly feed on people [98-

100]. Anderson and Magnarelli (2008) identified 33 species that commonly feed on people, of which 28 species harbor and transmit human disease-causing pathogens [76]. With the increase in incidence of tick-borne diseases and expansion of the geographic areas of occurrence of ticks, it becomes increasingly important for the health workers and researchers to be able to distinguish the diverse, and often overlapping, clinical manifestations of these diseases and finds effective means to control tick and tick-borne diseases.

1.5 Tick salivary gland: goldmine of anti-hemostatic proteins

Ticks possess a pair of salivary glands positioned anterolaterally on both sides of the tick's hemocoel which is a multifunctional complex organ [101,102]. Each gland has a main salivary duct, which pass antecranially into the salivarium, a passive saliva storage area, and fuse with the pharynx of the tick to form the oral cavity through which the blood meal (inwards) and the saliva (outwards) flow [102,103]. The salivary glands consists of an anterior region of acini consisting of multiple cell types (Type I, II, and III in females and all three plus Type IV in males) which are arranged more caudally in lobules connected by intra- and inter-lobular ducts to the main salivary ducts; the anterior acini are generally agranular and primarily involved with osmo-regulation, while the caudal acini are involved in secretion of bioactive components of the saliva [101,104-106].

To acquire blood meal, ticks insert their hypostome through the host's skin and anchor in the site of bite with cementing material and/or backwards pointing barbs. This damages the host tissue, capillary and small blood vessels, and consequently activates the hemostatic system and, subsequently, the immune system of the host [107]. For successful feeding, ticks counteract the host hemostatic system by injecting via saliva a vast range of bioactive molecules into the feeding lesion. These biologically active molecules include immunomodulators, vasodilating molecules, anticoagulants, inhibitors of platelet adhesion and aggregation and fibrin(ogen)olytic agents [107].

1.5.1 Inhibitors of blood coagulation

Blood coagulation cascade is activated by sequential proteolysis of circulating zymogens of serine proteinases [108]. Tick saliva contains various inhibitory components that target the various proteases or complexes of the coagulation cascade. These include inhibitors of thrombin, Factor Xa, Tissue factors or Tenase complex.

1.5.1.1 Thrombin inhibitors

Alpha-Thrombin is the key enzyme in the coagulation cascade, common to both pathways, and most of the blood feeding arthropods target this enzyme. About 17 tick thrombin inhibitors have been identified, of which the sequence information of seven is known [107]. Most of the thrombin inhibitors characterized from ticks are Kunitz-type proteinase inhibitors. These are found in both soft (ornithodorin from *Ornithodoros moubata*, savignin form *O. savignyi*, monobin from *Argas monolakensis*) and hard ticks (boophilin from *R. (B.) microplus*, amblin from *Amblyomma hebraeum*, haemalin from *Haemaphysalis longicornis* [109-115]. These are double domain Kunitz-type thrombin inhibitors and function by inhibiting the active-site of thrombin non-canonically with the N-terminal domain and binding the thrombin exosite-I with their C-terminal domain [110,115]. Tick thrombin inhibitors are kinetically slow, tight-binding, competitive inhibitors, although soft tick inhibitors are more potent and specific for thrombin [102]. A very potent thrombin inhibitor, variegin was isolated from a tropical bont tick, *Amblyomma variegatum*. Variegin is a tight-binding competitive inhibitor of thrombin and binds to thrombin active site through residues 8–14 and residues 15–32 binds to thrombin exosite-I [116]. Another novel thrombin inhibitor, madanin, was isolated from *H. longicornis*. Madanin is a cysteine-less, cleavable competitive inhibitor of thrombin and the founding member of the unique class of MEROPS I53 superfamily of inhibitors [117,118]. The family I53 is a new family of inhibitors of the serine-type endopeptidase thrombin (created on 4 August 2008 – MEROPS 8.2). The *MEROPS* database is a manually curated information resource of proteolytic enzymes, their inhibitors and substrates. The hierarchical classification for peptidase inhibitors was established in 2004 and these are clustered into families, which are in turn clustered into clans [119,120]. Each clan,

family or holotype inhibitor is assigned to an identifier 'I'. The clans are assigned two letters identifier (e.g. IA, IB) with the letter 'I' and a second letter 'A' to 'Z'. Each family is assigned to an identifier with the letter 'I' and a serial number (e.g. I1, I12). Hyalomin-1, a competitive thrombin inhibitor, from *H. marginatum rufipes* was characterized recently and it blocked the thrombin-mediated activation of factor XI and factor V and thrombin-mediated platelet aggregation [121].

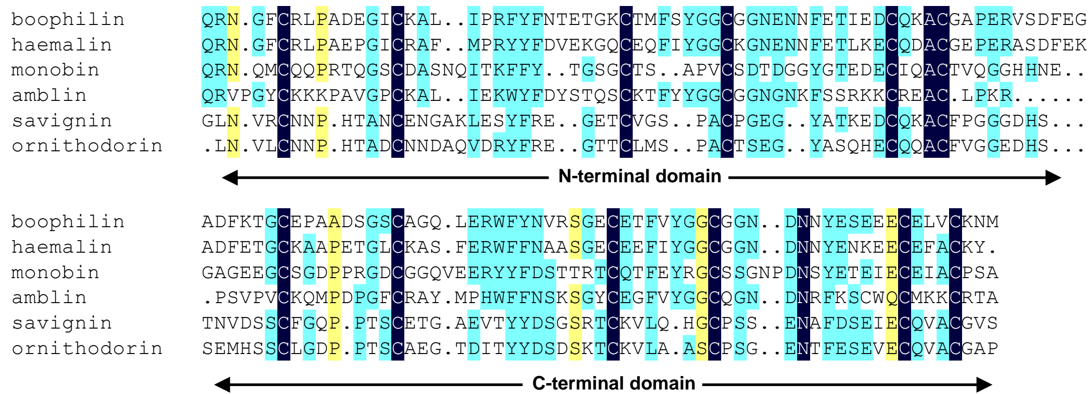

Figure 1.2 Amino acid sequence alignment of several Kunitz-type inhibitors from ticks – boophilin variant G2 (Uniprot entry Q8WPI3), haemalin (Uniprot entry B6ZIW0), monobin (Uniprot entry Q09JW6), amblin (Uniprot entry Q5VJG2), savignin (Uniprot entry Q8WPG5) and ornithodorin (Uniprot entry P56409). Shaded regions represent identical amino acid residues.

1.5.1.2 Factor Xa inhibitors

Both the intrinsic and extrinsic pathways converge at FX, making it a good target for blood feeding animals. The well-characterized Factor Xa inhibitor from tick is the Tick Anticoagulant Peptide (TAP) isolated from *O. moubata*, which is a single domain Kunitz-type natural inhibitor of FXa [122]. This single domain Kunitz-type proteinase inhibitor with a highly distorted reactive-site loop inhibits FXa active-site non-canonically; the N-terminal TAP bind to the active site and its two other segments bind to exosite of FXa [123]. *O. savagyni* also produces a competitive and slow tight-binding FXa inhibitor (fXaI; AAN76827) with 46% identity to TAP and a molecular mass of 7183 Da [124]. Recombinant salp14 from *I. Scapularis* has FXa inhibiting activity and RNAi of Salp14 resulted in reduction of anti-FXa activity of *its* saliva [125]. FXa inhibitors have also been reported from the saliva of *R. appendiculatus* [126], *A. americanum* [127] and *H. truncatum* [128].

1.5.1.3 Tissue factor pathway inhibitors

Tissue factor (TF) plays a central role in initiation of coagulation and therefore are good targets for tick's antithrombotic strategy. TF pathway inhibitors (TFPIs) inhibit the coagulation pathway through the inhibition of factor VIIa (FVIIa)-TF complex, which is FXa-dependent [107]. A two-Kunitz domain TFPI, Ixolaris, identified in *I. scapularis,* binds to the FX heparin-binding site and inhibits prothrombinase assembly *in vitro* [129,130]. In vivo administration of Ixolaris in rat model showed effective antithrombotic activity, without hemorrhage or bleeding [131]. Another inhibitor of FVIIa-TF complex identified in *I. scapularis*, Penthalaris (Penthalaris 1, 2, and 3), contains five Kunitz-domains [132]. Penthalaris 1 inhibits FVIIa-TF complex by binding to FX and FXa. Penthalaris has also been identified in *I. pacificus* [133].

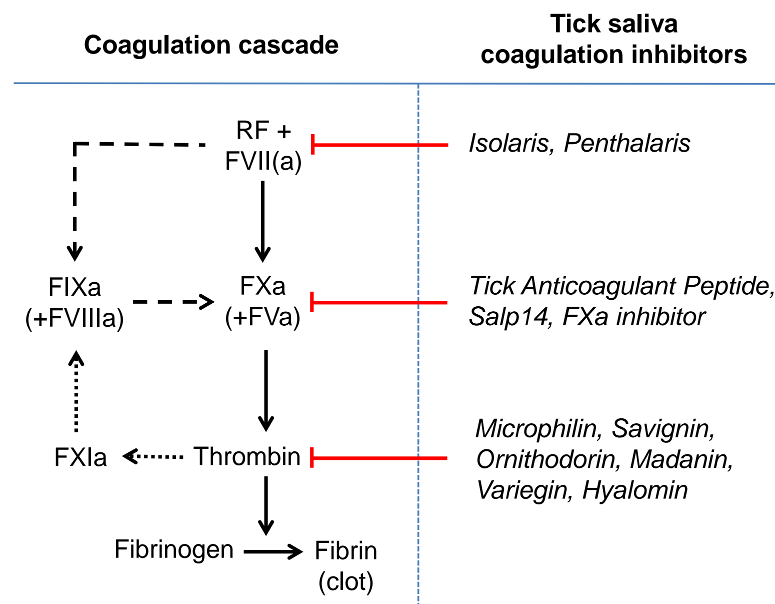

Figure 1.3 Tick salivary gland proteins and their targets on coagulation factors [134].

1.5.1.4 Contact system proteins inhibitors

Haemaphysalin, a two tandem Kunitz-type proteinase inhibitor, identified from *Haemaphysalis longicornis* inhibits the reciprocal activation of FXIIa and kallikrein [135]. The C-terminal domain of haemaphysalin prevents the association of high molecular-weight kininogen (HMWK) and FXII with activating cell surfaces by

binding to them in a Zn^{2+}-dependant manner [136]. *R.(B.) microplus* trypsin inhibitor A (BmTI-A), a larval BPTI–Kunitz serine protease inhibitor, inhibits human plasma kallikrein and affects the intrinsic pathway [137]. BmTI-D from the same species is a more potent BPTI–Kunitz family kallikrein inhibitor [138]. In addition, *Rhipicephalus sanguineus* trypsin inhibitor, RsTIQ2, which belongs to the Kunitz-type serine proteinase inhibitor family, inhibits kallikrein and elastase [139].

1.5.2 Inhibitors of platelet aggregation and adhesion

Vascular injury facilitates the immediate adherence of platelets to the site of injury, which is activated by agonists like collagen, thrombin, ADP, Thromboxane A_2, and von Willebrand factor (vWF). Platelet activation and aggregation can be inhibited though the disruption of agonists [102]. Apyrase, an ATP-diphosphohydrolase (E.C. 3.6.1.5) that hydrolyses ATP and ADP into AMP and phosphate, is found in almost all hematophagous arthropods where it inhibits ADP-induced platelet aggregation by hydrolysing ADP released by damaged cells at the site of feeding [103]. Apyrase activity has been found in *Ixodes dommini/scapularis*, *Ornithodoros moubata* and *Ornithodoros savignyi* [140-142].

Other inhibitors of platelet activation and aggregation include moubatin from *O. moubata* [143], and longicornin from *Haemaphylasis longicornis* [144] which inhibit collagen-induced aggregation. Some of the platelet aggregation inhibitors from tick that inhibit receptors on the surface of platelets are TAI, (Tick Adhesion Inhibitor) isolated from *O. moubata* [145] (inhibits integrin ∞2β1-collagen adhesion), disagregin from *O. moubata* [146], savignygrin from *O. savignyi* [147], variabilin from *Dermacentor variabilis* [148] and ixodegrin from *I. pacificus* [133] (inhibit integrin $\infty_{IIb}\beta_3$-fibrinogen mediated aggregation).

1.5.3 Fibrin(ogen)olytic agents

A novel proteinaceous metallo-carboxypeptidase inhibitor was isolated from ixodid tick *Rhipicephalus bursa*, and named as tick carboxypeptidase inhibitor (TCI) [149]. The recombinant TCI (rTCI) binds to plasma carboxypeptidases B in a double-headed manner and inhibits it, stimulating fibrinolysis *in vitro* [150]. Another

carboxypeptidase inhibitor called HlTCI was isolated from *H. longicornis* with 63.9% similarity to the carboxypeptidase inhibitor from *R. Bursa* [151]. An immunomodulator, iris, from *Ixodes ricinus* is another serpin (serine protease inhibitor) that inhibits elastase-like proteases and effects fibrinolysis *in vitro* [152]. The saliva of *I. scapularis* has also been reported to contain a metal-dependent proteolytic activity towards gelatin, fibrin(ogen), and fibronectin [153].

1.5.4 Immunomodulatory components

Apart from anti-hemostatic compounds, tick secretes immunomodulatory molecules in their saliva to suppress the host immunity for successful feeding and completion of life cycle. Prostaglandin E_2 (PGE_2), which promotes vasodilation and inhibits platelet aggregation, mast cell degranulation, dendritic cell maturation and T-lymphocyte activation has been identified in *I. scapularis*, *A. americanum*, *B. microplus*, *H. longicornis* and *I. holocyclus* [141,154-157]. *I. scapularis* saliva contains a kininase, a metallo dipeptidyl carboxypeptidase, that hydrolyses kinins (e.g. bradykinin), and thus prevents irritation and pain [141,158]. An angiotensin-converting enzyme like protein (ACE-like protein; AAB04998) has been reported in the salivary glands of *Rhipicephalus (Boophilus) microplus* [159]. This protein has sequence homology with the mammalian angiotensin converting enzymes and may function as host cardiovascular system modulator or anti-inflammatory agent through non-specific kininase activity [103,160].

Ticks also produce anti-inflammatory mediators, which scavenge the biogenic amines such as histamine and 5-Hydroxytryptamine (5-HT), to prevent host rejection during long period-feeding. Histamine-binding proteins, lipocalins, have been isolated from *R. appendiculatus*, *Argas monolakensis*, *I. scapularis* and *A. americanum* [161-164]. Other anti-inflammatory components include anti-serotonin protein from *D. reticulatus* [165], complement inhibitors such as Isac from *I. scapularis* [166], OmCI from *O. moubata* [167] and Salp20 from *I. scapularis* [168] and bradykinin-binding protein from *I. scapularis* [141,158].

1.6 Tick sialome

Ticks have evolved complex saliva for successful and continuous feeding. With the emergence of high-throughput molecular techniques like transcriptomics and proteomics, a large variety of tick salivary transcripts and proteins have been identified. Surprisingly, it has been found that tick saliva contain a much broader complexity of protein than thought before; it contains hundreds of proteins, a substantial fraction of which has no homology to any protein in the database [169,170]. Most of the tick salivary proteins are members of the multi-gene families and it is difficult to anticipate the function of the majority of the tick salivary proteins [170]. So far, less than 5% of the tick salivary proteins have been expressed and their function described for any tick species with known transcriptome. Hence, it is important to understand the proteins of tick salivary gland.

Salivary transcriptomes or sialotranscriptomes ("sialo"; Greek: saliva, spittle, foam from the mouth; the salivary glands) have been described and characterized from several hard ticks such as *A. americanum* [169], *Amblyomma cajennense* [171], *Amblyomma variegatum* [172], *Dermacentor andersoni* [173], *Rhipicephalus appendiculattus* [174], *Ixodes scapularis* [161,175], *Ixodes pacifics* [133], *Ixodes ricinus* [176], *Hyalomma marginatum rufipes* [177] etc, as well as from the soft ticks *Ornithodoros parkeri* [178], *Argas monolakensis* [112,179], *Ornithodoros coriaceu* [180] etc. Francischetti *et. al.* (2010) assembled the EST collections and extracted their open reading frames (ORFs) which are curated as gene families. Some of the families of tick salivary gland proteins have been described in Table 1.1; however, most of the functions of the protein are yet to be deciphered.

Table 1.1 Putative secreted salivary proteins from ticks curated as gene families [170].

Gene families	No. of proteins identified	Functions
Glycine-rich, or proline-rich, collagen-like Superfamily	446	Housekeeping proteins, proteins associated with tick-cement function, or immunity.
Mucins	58	Tick feeding

Antigen 5 (AG5) protein family	17	Functionally not characterized
Ixodegrin superfamily	37	Functionally not characterized
Ixostatins	28	Functionally not characterized
Families containing protease inhibitor domains		
1. Kunitz-type	297; e.g. Ixolaris, savignin, savignygrin, disagregin	Anti-thrombin, fXa inhibitor, TF pathway inhibitors, platelet inhibitors (not all have been functionally characterized)
2. Serpin domain family	49; e.g. Iris	Except IRIS Serpin which is immunosuppressive, no other salivary Serpin has been functionally characterized
3. Cystatins	16; e.g. Sialostatin L and Sialostatin L2	Immunosuppressive and anti-inflammatory functions
4. Thyropin family	7	Function unknown
5. TIL domain-containing peptides	41; e.g. ixodidin	Anti-trypsin, anti-elastase, antimicrobial
6. Hirudin-like/Madanin/ Variegin sperfamily	18; e.g. madanin 1	Metastriate specific
7. Basic tail and 18.3-kDa superfamily	102 and 72	Anti-clotting, may be platelet aggregation inhibitors, function unknown for 18.3-kDa superfamily
8. Carboxypeptidase inhibitor family	9	May effect fibrinolysis
Lipocalins	307; e.g. monomine and monotonin	Histamine- and serotonin-binding, anti-complement, immunoglobulin binding, toxicosis
8.9 kDa polypeptide family	60	Unique, function unknown
23-kDa family	10	Unique, function unknown
13-kDa family	5	Unique, function unknown
12-kDa family	3	Unique, function unknown
PGFG repeat family	4	Function unknown
IS4 family	3	May belong to lipocalin

		superfamily
Cytotoxin-like family	8	Function unknown
16 kDa family	14	Function unknown
Enzymes	110 metalloproteases, 34 trypsin-like serine proteases, 13 serine carboxypeptidases, 2 prolyl carboxypeptidases, 20 carboxy esterases, 20 chitinases, 7 lipases, 7 phospholipase A_2, 8 sphyngomyelinases, 1 leukotriene hydrolase, 14 5′nucleotidases/apyrases, 1 ectonucleotide pyrophosphatase/ phosphodiesterase, 2 multiple inositol phosphatases, 9 kininases, 3 alkaline phosphatases, 4 ribonucleases, 1 epoxy hydrolase, 1 pyrophosphatase, and 8 endonucleases	Some may be lysosomal and housekeeping. Anti-platelet aggregation, anti-coagulants, antifungal activity, effect fibrinolysis pathway etc.
Immune-related products 1. Pattern recognition proteins	 36; e.g. Ixoderins	 Pattern-recognition , molecules, anti-microbial, innate immunity, lipid metabolism
2. Thioester/alpha2 macroglobulin family	13	Fragmented coding sequences
3. Antimicrobial peptides and proteins	49; lysozyme, defensins	Antimicrobial
Metastriate-specific families	196; e.g. Da-p36, evasin (19 orphan protein families)	Immunosuppressor, chemokine-binding, housekeeping, fibronectin, endostatin or insulin growth-factor binding
Prostriate-specific families	6 + 1 orphan family	Isac protein family (18 proteins) have anti-complement activity, rest 101 not characterized
Argasidae-specific families	57	Function unknown
Secreted conserved proteins	126	May be housekeeping or involved in feeding
Possible housekeeping proteins	4531	Housekeeping

The sialomes (transcripts and proteins expressed in the salivary glands) of ticks are very complex, with about 100-200 secreted proteins in soft ticks of which approximately 60% could be confirmed by proteomics method and about 500 in hard ticks, the presence and abundance of which at a given point of time is not clear [170,178,179]. Besides, proteins in the salivary glands are differentially expressed at different feeding stages, which makes it difficult to make a comparative studies of the hard and soft tick sialomes to obtain a fair idea of the evolution of salivary gland protein complexity [170]. It has been found that gene duplication and most probably genome duplications, play a major role in tick salivary gland protein evolution and evolution of novel functions during their adaptation to hematophagy [175,179]. Exploration of more sialomes, covering wider tick genera will help in addressing the current problems of understanding tick salivary gland protein evolution and also discovery of novel therapeutic leads.

Most importantly, understanding the role of tick salivary gland proteins in attachment to host and transmission of disease pathogens may help in discovery and development of new anti-tick vaccines for preventing tick infestation and tick-borne diseases [134]. We know tick saliva contains a pool of anti-hemostatic, anti-inflammatory, and immunomodulatory substances. For instance, at the bite site, apart from controlling blood flow, the saliva blocks the activation of histamine, ATP, bradykinin, serotonin, leukotriene B4 which trigger inflammatory response such as pain and itching in the host [181]. Also the site of injury contains high Interleukin 4 and other immunomodulatory substances which inhibit dendritic cell maturation and lymphocyte activation. This environment favors the transmission of pathogens. Anti-tick vaccine developed from tick salivary proteins targets the molecules that aid in pathogen transmission, as compared to traditional vaccines which target the proteins on the surface of pathogens [181]. Antibody raised against Salp 15 which inhibits CD4(+) T cell activation, protected mice against *Borrelia burgdorferi* [182,183]. Mice (infected with tick-borne encephalitis virus) vaccinated with 64TRP, a recombinantly produced truncated salivary cement protein, 64P from *R. appendiculatus* were protected from lethal encephalitis [184-186]. Some of the tick-protective antigens are listed in Table 1.2. With the advent of high-throughput technologies, the identification of more orthologous families of proteins has become

possible for developing potential vaccine candidate with high probability of cross-reactivity [107]. Omics and system biology has facilitated the functional assignment and identification of ESTs that are potential vaccine candidates [134,187,188].

Table 1.2 Some tick-protective proteins.

Tick protein	Tick species	Function	Reference
BmTI	*Boophilus microplus*	Trypsin inhibitor	[189]
Bm86/Bm95	*B. microplus*	Unknown	[190-192]
64P	*R. appendiculutus*	Cement protein	[184,186]
P29	*H. longicornis*	Putative extracellular matrix protein	[193]
Serpin	*H. longicornis* *R. appendiculutus*	Serine protease inhibitor	[194,195]
4F8	*I. scapularis*	Nucleotidase	[196,197]
Subolesin	*I. scapularis*	Tick feeding and reproduction	[198,199]
Voraxin	*A. hebraeum*	Male engorgement factor	[200]

1.7 *Haemaphysalis bispinosa*

The genus *Haemaphysalis* is the second largest in the world and consists of 165 species [201,202]. *Haemaphysalis bispinosa*, a hard tick, was first described by Neumann in 1897 from Ramanathapuram, Tamil Nadu, India and is characterized by the absence of well-developed cornua [203,204]. It does not possess salient lateral borders on the palpae and the palpal dorsomedial spur is posteriorly tilted or is on its basal border. The male is about 1.5 mm x 1.1 mm to 2.1 mm x 1.46 mm in size, while unfed female measures 2.25 mm x 1.7 mm and replete female measures 7.0 mm x 4.0 mm [203]. *H. bispinosa* ticks are found to infest cattle, goats, sheep, buffalo, birds, wild mammals and horses, but are more prevalent in goats [88]. These occur almost throughout Indian subcontinent (Recorded form Andhra Pradesh, Arunachal Pradesh, Assam, Bihar, Goa, Gujarat, Himachal Pradesh, Jammu – Kashmir, Jharkhand, Karnataka, Madhya Pradesh, Maharashtra, Manipur, Mizoram, Orissa, Punjab, Sikkim, Tamil Nadu, and West Bengal), and Nepal, Bhutan, Bangladesh, Pakistan, Myanmar, Sri Lanka and Malaysia [88,205-207]. *H. bispinosa* has been found to harbor Kyasanur forest disease (KFD) virus, though it is not proven if it transmits the pathogen [203,208]. The ticks were reported to be infected by *Bartonella bovis*, the causal agent of a zoonotic disease, bartonellosis [209]. This species is a vector of

Babesis motasi and *Babesia ovis* in sheep and goats, *Babesia canis* and *Babesia gibsoni* in dogs and *Babesia equi* in horses and donkeys [210]. It is also potential vector of *Borrelia burgdorferi*, *Theileria sergenti* and *Babesia bigemina* [211,212] and has been found to feed on human blood too [213]. Thus *H. bispinosa* is an economically and medically important tick species.

The first molecular characterization of *Haemaphysalis bispinosa* ticks was reported from Sonitpur, Assam, India [214]. It was found to be phylogenetically closest to *H. longicornis* and was found to infest cattle and goats. The morphological characterization, biology, distribution, immunology and control of *H. bispinosa* and disease prevalence in it has been studied by various groups [209,210,215-221]. Ticks are rich source of anti-hemostatic proteins and several anti-hemostatic proteins have been isolated and characterized which has been reviewed by many researchers [103,107,170,222]. Thrombin, the central player in hemostatic system, is the primary target of ticks and many thrombin inhibitors have been isolated and characterized from soft ticks and hard ticks, which has been discussed in earlier sections. Thrombin inhibitors have been characterized from some *Haemaphysalis* ticks. Madanin, a thrombin inhibitor of an unique family I53 of inhibitors, was identified and characterized from the salivary gland of *H. longicornis* [117,118]. Another thrombin inhibitors, chimadanin and hemalin, was isolated from the salivary and midgut cDNA library, repectively of *H. longicornis* [223,224]. However, there is no report of anti-hemostatic proteins or compounds from the saliva, salivary gland or hemolymph of *H. bispinosa*. Hence, in the present study following objectives have been undertaken for identification of anti-thrombin and to understand its mechanism of action.

Objectives are:

1. ***Collection and identification of cattle ticks***
2. ***Extraction and characterization of salivary gland extract***
3. ***Identification and molecular characterization of anti-thrombin transcript from the salivary gland***
4. ***Molecular cloning and over-expression of anti-thrombin protein***
5. ***Characterization of the recombinant anti-thrombin protein***
6. ***Structure-function relationship of the recombinant protein***