Post-transcriptional control of hemostatic genes: mechanisms and emerging therapeutic concepts in thrombo-inflammatory disorders

Sven Danckwardt[1,2,3,4,5 *], David-Alexandre Trégouët[6], Elisabetta Castoldi[7*]

[1] Centre for Thrombosis and Hemostasis (CTH), University Medical Centre Mainz; Mainz, Germany.

[2] German Centre for Cardiovascular Research (DZHK); Berlin, Germany.

[3] Posttranscriptional Gene Regulation, University Medical Centre Mainz; Mainz, Germany.

[4] Institute for Clinical Chemistry and Laboratory Medicine, University Medical Centre Mainz; Mainz, Germany.

[5] Center for Healthy Aging (CHA); Mainz, Germany.

[6] University of Bordeaux, INSERM, Bordeaux Population Health Research Center, UMR 1219, Department of Molecular Epidemiology of Vascular and Brain Disorders (ELEANOR), Bordeaux, France

[7] Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht, The Netherlands

*Correspondence

E-mail: Sven.Danckwardt@unimedizin-mainz.de

E-mail: e.castoldi@maastrichtuniversity.nl

The hemostatic system is pivotal to maintaining vascular integrity. Multiple components involved in blood coagulation have central functions in inflammation and immunity. A derailed hemostasis is common in prevalent pathologies such as sepsis, cardiovascular disorders and, lately, COVID-19. Physiological mechanisms limit the deleterious consequences of a hyperactivated hemostatic system through adaptive changes in gene expression. While this is mainly regulated at the level of transcription, co- and posttranscriptional mechanisms are increasingly perceived as central hubs governing multiple facets of the hemostatic system. This layer of regulation modulates the biogenesis of hemostatic components, for example in situations of increased turnover and demand. However, they can also be 'hijacked' in disease processes, thereby perpetuating and even causally entertaining associated pathologies. This review summarizes examples and emerging concepts that illustrate the importance of posttranscriptional mechanisms in hemostatic control and crosstalk with the immune system. It also discusses how such regulatory principles can be used to usher in new therapeutic concepts to combat global medical threats such as sepsis or cardiovascular disorders.

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Introduction

In light of the current SARS-CoV2 pandemic, the mechanisms underlying the crosstalk between the hemostatic system and the immune system have received unprecedented attention. This interplay plays a central role in many pathological processes, ranging from sepsis to cardiovascular disease.

Perturbations of the hemostatic system are common in sepsis, the leading cause of death in critically ill patients worldwide[1]. As a systemic inflammatory response to severe infections, sepsis involves excessive activation of the coagulation system[2]. This can result in severe complications such as disseminated intravascular coagulation (DIC), which eventually leads to tissue necrosis, multiple organ failure and death, illustrating that inappropriate amplification of protective host-defense mechanisms can become a devastating alliance of harm[3].

Cardiovascular disorders including myocardial infarction, ischemic stroke and venous thromboembolism are the leading global cause of mortality with over 17 million deaths annually[4]. The incidence of cardiovascular disorders increases markedly with age, starting in the late 40s, with a dramatic increase occurring at 60 years of age[5]. They account for approximately 32% of all deaths worldwide, underscoring the need of illuminating underlying mechanisms and devising therapeutic interventions to treat and prevent cardiovascular disorders[6].

The immune system and the hemostatic system are closely linked[7] and their responses tend to reinforce each other[8, 9]. Activation of coagulation and fibrin deposition in response to inflammation is well known. This led to the emergence of the concept of immunothrombosis, a defense mechanism in which inflammatory cells participate in thrombotic processes, and thrombosis in turn acts as an intravascular effector of innate immunity by limiting the spread of invading pathogens[10]. However, a derailed hemostatic response can lead to a situation where coagulation, fibrin deposition and thrombosis contribute to disease, as evidenced by the propagation and exacerbation of atherosclerotic plaques[11]. Another example is the systemic activation of coagulation combined with microvascular failure resulting from the systemic inflammatory response to severe infection or sepsis, which eventually contributes to multiple organ dysfunction, such as in septicemia[3] or COVID-19[12].

The multifaceted and intricate link between hemostasis and inflammation involves crosstalk between both systems at multiple levels[3, 7-11], including coordinated changes in gene expression in megakaryocytes, immune cells, the vessel wall and/or the liver. A notable example is the acute phase response, in which central hemostatic components such as fibrinogen[13, 14], Von Willebrand factor[15, 16] and factor VIII[17-21] are induced in response to inflammatory signals. Such changes in gene expression are primarily regulated at the level of transcription, and the transcriptional regulation of hemostasis-related genes in physiological and pathological conditions has been well studied[22-27].

In the present review we focus on emerging concepts of posttranscriptional mechanisms underlying the control of hemostasis and its crosstalk to other systems. In doing so, we discuss examples of the complexity of the transcriptome architecture arising from the use of alternative transcription start sites, exons and polyadenylation sites, as well as gene regulation by non-coding RNAs (miRNAs, lncRNAs, circRNAs), RNA-binding proteins and mechanisms of RNA modification. Remarkably, many of these regulatory principles also play an important functional role in tuning the immune system[28-32], suggesting conserved regulatory links between both systems. Finally, we also illustrate the emerging therapeutic opportunities on the cusp of a new era of targeted therapeutic approaches[33], exemplified by the recent introduction of novel RNA therapeutics in the hemostatic system[34].

Role of splicing regulation in the hemostatic system

With the completion of the human genome project in 2003, it became apparent that the human genome comprises around 22.000 protein-coding genes, far less than actually required for the functional complexity in higher eukaryotes[35]. On the other hand, next generation RNA sequencing and particularly the recently introduced long-read sequencing technologies[36, 37] are uncovering a perplexingly complex transcriptome architecture that arises from the use of alternative transcription start sites, exons and polyadenylation sites[38, 39]. The combinatorial use of such elements considerably expands genomic information and is subject to dynamic spatial and temporal modulation during development and adaptation (Figure 1).

Pre-mRNA splicing, *i.e.* the accurate removal of introns and ligation of exons, is a pivotal step in the co- and posttranscriptional regulation of gene expression[40]. Depending on how the exon/intron structure of the pre-mRNA is decoded by the spliceosome, the same primary transcript may be processed into different mature mRNAs (alternative splicing), encoding different isoforms of the same protein. In fact, the recognition of exon/intron boundaries in the pre-mRNA is critically dependent on the engagement of nearby splicing enhancer and silencer sequences by trans-acting proteins (splicing factors) whose availability varies in different cell types and disease states. As a consequence, splicing patterns are typically regulated in a tissue-specific manner and may change according to the developmental stage or in response to pathological processes. Moreover, they can be disrupted by genetic variants that weaken (or strengthen) the consensus sequences recognized by the spliceosome on the pre-mRNA. This is a well-known mechanism of disease in mendelian disorders[41], but it is increasingly appreciated that much of the genetic variation associated with complex traits also acts by altering splicing patterns[42, 43]

Like most human genes[44], many genes encoding proteins of the hemostatic system are alternatively spliced[45-57]. This often results in isoforms with distinct structural and functional

characteristics, as exemplified by two major components of the extrinsic coagulation pathway (Figure 2).

Tissue factor (TF), the main trigger of blood coagulation, acts as cofactor of the circulating serine protease factor VIIa (FVIIa) and comes in two isoforms: as membrane-bound (full-length) protein and as a shorter, alternatively spliced variant that is secreted in soluble form (Figure 2)[58]. The two isoforms are identical at the N-terminal end, but the soluble form, which arises from exon 5 skipping, lacks the transmembrane and cytoplasmic domains, and has a completely different C-terminal sequence[58]. Just as full-length TF, alternatively spliced TF is produced by a variety of cell types[58, 59], is induced by pro-inflammatory stimuli[59, 60] and enhances factor X (FX) activation by FVIIa, albeit less potently than full-length TF[58]. However, while membrane-bound TF is essential for normal hemostasis, elevated intravascular levels of TF have been proposed to contribute to venous as well as arterial thrombosis[61]. Despite conflicting data, it has been suggested that soluble TF, which is most likely dispensable for normal hemostasis, may represent a preferential target for antithrombotic therapy than full-length TF, due to a lower risk of bleeding[62].

Tissue factor pathway inhibitor (TFPI) is a glycoprotein that functions as an inhibitor of coagulation and of TF-dependent signaling[63]. The *TFPI* gene encodes two main splicing isoforms that are generated by the alternative inclusion of exon 8 (TFPIβ) or exons 9-10 (TFPIα) in the mature mRNA (Figure 2). Both isoforms are expressed in endothelial cells, but TFPIα is also found in plasma, platelets and the extracellular matrix[64]. Structurally, TFPIα comprises an acidic N-terminus, three Kunitz domains and a basic C-terminus, whereas TFPIβ lacks the third Kunitz domain and the basic C-terminus, which are replaced by a glycosylphosphatidylinositol-anchor that tethers the protein to the cell membrane[65]. Both TFPI isoforms inhibit TF/FVIIa and FXa with their Kunitz-1 and Kunitz-2 domains, respectively, but TFPIα has additional properties by virtue of its Kunitz-3 domain (which binds protein S) and basic C-terminus (which binds FV/FV-short). Binding to protein S and FV/FV-short prevents the clearance of plasma TFPIα from the circulation[51, 66, 67] and promotes its association with biological membranes, enhancing its anti-FXa activity[68-70]. Moreover, the interaction with FV/FV-short allows TFPIα to inhibit FV activation[71] and early prothrombinase activity[72, 73], while TFPIβ lacks these anticoagulant functions.

These and other[51, 74] examples illustrate how alternative splicing can change the structural and hence functional properties of central components in the hemostatic system[75]. Extracellular signals, such as pro-inflammatory cytokines, can modify global patterns of alternative splicing[76] and it will be interesting to explore how this plays out in different (disease) contexts, including COVID-19[77]. Moreover, since alternative splicing is pervasive and there are increasingly new therapeutic means to (re)direct splicing[78, 79], modulation of alternative splicing may become relevant for the therapeutic manipulation of the hemostatic system. In particular, many studies

support the utility of antisense oligonucleotides (ASOs) to mask specific splicing signals on the pre-mRNA and thus prevent the recognition of these sequences by spliceosomal components, thereby re-directing splicing[80]. Alternatively, *ad hoc* engineered U1snRNA can be employed to promote the usage of donor splice sites that are naturally weak or have been disrupted by mutation[81].

Apart from diversifying the transcriptome and proteome, alternative splicing has been proposed to contribute to the overall regulation of gene expression through its coupling with nonsense mediated decay (NMD), a surveillance pathway that degrades mRNAs containing premature stop codons. In fact, it has been observed that up to one third of all human transcripts are normally spliced into non-viable mRNAs that are substrates for NMD. This phenomenon, known as "regulated unproductive splicing and translation" (RUST), has been interpreted as a mechanism for the post-transcriptional temporal and spatial fine-tuning of gene expression[82]. Evidence that this control mechanism may apply within the realm of hemostasis has been provided for the *F11* gene, encoding coagulation factor XI[47]. Interestingly, targeting non-productive splicing by antisense oligonucleotides can be exploited for the upregulation of gene expression from wild-type or hypomorphic alleles in disease states[83].

Role of polyadenylation in the hemostatic system

In addition to capping and splicing, almost all eukaryotic transcripts undergo further processing at the RNA 3'-end (Figure 1). For most genes, this involves endonucleolytic cleavage and non-templated polyadenylation (CPA) before the mature RNA can be exported to the cytoplasm[84]. As CPA controls almost all genes, regulation of CPA has evolved as an important layer of gene expression regulation. CPA is carried out by a multi-subunit complex involving over 80 trans-acting proteins organized in four core protein subcomplexes[85]. The recruitment of these multimeric complexes to dedicated, but largely poorly conserved, RNA sequence elements[86] ensures that 3'-end processing of the nascent transcript occurs timely and at the right position[87, 88]. Perturbations of this process - due to mutations in RNA sequence elements or defects in the RNA processing machinery - have drastic consequences, as exemplified by numerous diseases[89, 90].

The common thrombophilia mutation in the prothrombin (*F2*) gene (*F2* G20210A) is a prime example of how mutations in noncoding regions can become pathogenic[84]. This mutation affects the last nucleotide of the 3'-untranslated region (UTR), where the pre-mRNA is cleaved and polyadenylated. As a result of the mutation, the efficiency of endonucleolytic cleavage is increased, leading to more prothrombin mRNA and protein expression. Although this mutation merely increases the amount of the precursor of a central hemostatic component (i.e., thrombin), it already shifts the balance of the hemostatic system toward a procoagulant condition[91-93]. Consequently, the expression of *F2* must be tightly controlled: even small

changes (1.5- to 1.7-fold) in gene expression due to mutations at this and other nearby positions (*F2* C20209T and *F2* G20221T)[93, 94] can result in clinically relevant thrombophilia[94-97].

Compared to other genes, the architecture of sequence determinants directing 3'-end processing in *F2* is unconventional[96]. It consists of weak signals, which explains the unusual susceptibility to thrombophilic gain-of-function mutations[94, 97]. At the same time, this configuration allows for mechanisms that enhance processing and thereby upregulate *F2* expression when needed[98]. This is achieved through complex, mutually exclusive binding of suppressive and stimulatory RNA binding proteins (RBPs), and is regulated by activation of p38 MAPK (Figure 3)[99]. After phosphorylation by p38 MAPK, inhibitory RBPs (FBP2, FBP3) can no longer bind to the processing sites in the *F2* pre-mRNA, allowing 3'-end processing to proceed. Thus, virtually all types of 'environmental' conditions that lead to activation of p38 MAPK[100, 101] can induce *F2* expression.

Inflammatory conditions are known to trigger *F2* expression[102-107]. Consistently, the mechanism described here was found to account for the induction of *F2* expression under inflammatory conditions, including septicemia[99, 108]. While this may contribute to the initial onset and undesirable propagation of hemostatic perturbances during septicemia, such mechanisms may also play a compensatory role[3]. After an initial hypercoagulable state, septicemia is often followed by a hemorrhagic phase, in part due to consumption of procoagulant components[109]. Such conditions of increased turnover and demand require mechanisms to restore the hemostatic balance and stockpile hemostatic components[110].

In addition to the critical function in hemostasis, the role of thrombin in angiogenesis[111] suggests that regulatory mechanisms have evolved a sensor for low oxygen pressure. This could explain why *F2* is overexpressed due to ischemic events[112] or in the tumor micromilieu[99]. Consistent with its role in oxygen pressure sensing[100, 101], activation of p38 MAPK also drives *F2* overexpression in the tumor microenvironment. This activates protease-activated receptors (PARs) that induce genes with a role in angiogenesis and tumor dissemination[99].

Thus, regulated 3′-end processing emerged as an important mechanism of gene regulation in the control of the hemostatic system. While such mechanisms are desirable under physiological conditions (to replenish the amount of blood coagulation factors under high turnover, see above), they can be 'hijacked' under pathological conditions (such as inflammation or cancer), thereby leading to a thrombophilic state[108, 113]. Since prothrombin is expressed in a wide variety of organs and cells[108], this type of regulation may become relevant to numerous other thrombin-mediated diseases[113]. However, it also appears that tissue-specific mechanisms can be used to selectively target deleterious prothrombin expression without altering essential prothrombin expression in the liver[108].

Targeted interference with cleavage and polyadenylation is increasingly perceived as an important therapeutic means. This involves either redirection of aberrant RNA processing (through ASOs, U1snRNP interference or trans-splicing) or the elimination of faulty transcripts[89] to prevent the fatal consequences of aberrant 3'-end processing[114, 115]. Perturbations of 3'-end processing can, for example, act as nongenomic oncogenic drivers of tumorigenesis[115], but they also play important roles in inflammatory conditions[116]. Deciphering the underlying mechanisms is of paramount importance for establishing targets with therapeutic selectivity and specificity.

RNA-protein interactome studies[117] and transcriptome-wide profiling of polyadenylation[118] are thus central to defining new therapeutic targets, their specificity and downstream consequences[119]. Since most miRNA binding sites are localized in the 3'-UTR, when and where a pre-mRNA is polyadenylated has a critical impact on the regulatory properties of the resulting mRNA molecule (see below). A significant proportion of genetic variants in 3'-UTRs, often dismissed as 'non-functional' polymorphisms, are therefore likely to disrupt important regulatory mechanisms, ultimately leading to pathologies including a dysbalanced hemostatic system[89]. This is supported by the thrombophilia variants discovered in the *F2* gene. However, this also extends to other coagulation factor 3'-UTR variants that affect, for example, miRNA regulation[120, 121].

Role of microRNAs in the hemostatic system

MicroRNAs (miRNAs) are small single-stranded non-coding RNAs (17-25 nucleotides in length) that post-transcriptionally down-regulate target gene expression by RNA silencing[122]. After transcription, miRNAs are processed in the nucleus by the microprocessor complex consisting of Drosha and DGCR8 to produce a pre-miRNA[123]. After export to the cytoplasm and further processing by Dicer[124], the mature miRNA duplex is incorporated into the RNA-induced silencing complex (RISC)[125]. This complex is guided by miRNA base pairing to a target gene mRNA resulting in translational inhibition and/or transcript degradation[126]. Generally, miRNAs target mRNAs via the 3'-UTR. In a few cases, miRNAs can also carry out their inhibitory function by binding to the coding region or the 5'-UTR of target mRNAs[127].

Over 2600 human miRNAs have been identified[128], regulating the majority of human genes[129]. Thus almost every biological process is modulated through miRNAs[130]. Although miRNAs generally fine-tune gene expression[131], they can also function as master regulators[132]. For example, multiple miRNAs can cooperatively silence a single gene to gain regulatory specificity, with the targeting of particular network hub genes enabling the regulation of entire pathways[133]. In addition, a single miRNA can target multiple genes, allowing broad regulation of molecular networks[127]. Perturbations of miRNA expression are observed in most disorders,

with some of them even causally contributing to the development and progression of disease[130].

A growing number of studies document a contribution of miRNAs to the regulation of hemostatic[134-138] and thrombotic[121, 135, 137-140] functions. miRNAs directly regulate multiple hemostatic factors through interactions with the 3'-UTR (Table 1). Additionally, miRNAs can tune hemostatic factors indirectly, for example fibrinogen via interleukin-6-mediated signaling[141], factor IX by repressing NMD[142], plasminogen activator inhibitor 1 (PAI-1) via SMAD2 signaling[143] and CXCL12 to reduce inflammatory response and thrombosis, altering the expression of multiple factors including TF, PAI-1 and VWF[144].

Further evidence implicating miRNAs in the hemostatic system comes from the important roles that miRNAs play in the development of bleeding disorders and thrombosis. Blood miRNA levels are associated with hemostatic perturbations, suggesting their potential use as prognostic or diagnostic tools in VTE[145] and beyond[146]. These include aberrant coagulation in sepsis[147], venous thromboembolism[140, 148-158], trauma-induced coagulopathy[159], atherosclerosis[160-164], coronary artery disease[165-167], ischemic stroke[168, 169] and autoimmune inflammatory conditions such as systemic lupus erythematosus (SLE)[170-172].

Recently, using an unbiased systematic search based on a biophysical miRNA interaction study coupled to high-throughput sequencing, the Atlas of the Hemostatic miRNA Targetome was released[135]. This screening identified more than 1500 miRNA/3'-UTR interactions with potential function in the hemostatic system from nearly 4500 miRNA/3'-UTR biophysical interactions[135]. A proof-of-concept, rigorous filtering combined with loss-of-function studies (limited to 96 of the 1500 miRNA/3'-UTR interactions with a potential function) identified dozens of miRNAs targeting 27 hemostasis-associated gene 3′-UTRs globally or in a gene-specific manner (Figure 4). This highlights the global importance of miRNAs in controlling the hemostatic system and suggests that many more functional miRNAs will be discovered in this system.

The unbiased view on miRNAs regulating the hemostatic system also sheds light on hitherto functionally poorly characterized connections between different physiological systems and diseases. These include the link between tumor formation and hemostatic perturbations[135], or the intricate relationship between the hemostatic system and inflammatory processes (Table 1). For example, miR-181 family members that target the 3'-UTR of *F11* mRNA[135] are involved in several aspects of hemostasis, including vascular inflammation[152, 173-175] and platelet activation[176]. Another example is miR-24 which controls the expression of VWF[177]. Here, hyperglycemia-induced repression of miR-24 increases VWF expression and secretion in diabetes mellitus, linking metabolic dysfunction to a miRNA-mediated mechanism of hemostatic deregulation.

On the other hand, polymorphisms affecting miRNA binding sites in hemostatic genes can be associated with disease. For example, deletion of the miR-759 binding site of *FGA* is associated with susceptibility to chronic thromboembolic pulmonary hypertension[178], and SNPs in the 3′-UTR of the *F2*, *F8* and *F11* genes are associated with increased activity levels of these hemostatic components[120, 179-182].

The importance of miRNAs in hemostasis is further corroborated by their role in platelet biology[136]. Here miRNAs modulate the expression of target mRNAs important for hemostatic and thrombotic function[183-187]. For example, miRNA levels are altered in platelets from patients with essential thrombocythemia and this in turn is associated with elevated platelet counts and an increased risk of thromboembolic events[188]. Additionally altered miRNA expression is often observed in atherosclerotic plaques[189] (and refs therein).

In light of the functional importance of miRNAs in the hemostatic system[135] and the increasingly recognized role of miRNA therapeutics[190] currently conquering the cardiovascular system[191], it is tempting to turn this knowledge into new therapeutics (see targeting section below). In support of this, miRNA treatment has been demonstrated to result in therapeutic response in thrombosis and hemostasis. In murine models of venous thrombosis, overexpression of miRNAs contributes to thrombus resolution[193], reduces thrombogenesis[194], enhances endothelial progenitor cell migration and tubulogenic activity[195], angiogenesis and thrombosis recanalization[196]. Furthermore, the use of antagomirs (i.e., molecules that silence miRNAs) has been shown to block miR-19b-3p-mediated silencing of *SERPINC1* (antithrombin), resulting in increased antithrombin expression and activity *in vivo*[135]. This documents the in-principle druggability of the hemostatic system in a miRNA-directed manner and opens opportunities to target other hemostatic components such as coagulation FXI[138].

Other means of posttranscriptional regulation of the hemostatic system

RNA binding proteins beyond their function in the biogenesis of mRNAs

In addition to co- and posttranscriptional processing, much of the fate of RNAs from synthesis to decay depends on RNA-binding proteins[197]. RBPs regulate RNA localization, transport, translation, stabilization and degradation of bound RNA molecules. In fact, much of the rapid adjustment of gene expression in inflammation and the immune system[30-32] is executed via modulation of RNA stability and decay. The same is likely to apply to the hemostatic system as well, and the adaptation of prothrombin expression (Figure 3) may be a prototype for analogous occurrences[198]. It is interesting to note that even in apparently non-polar cells such as hepatocytes, the major source of most hemostatic components, localization of transcripts and thus protein output critically depends on UTR-RBP interactions[199]. This suggests that dynamic changes of 5’ and 3’-UTR structures of mRNAs, due to the use of alternative transcription start sites and alternative splicing/polyadenylation, may have a critical impact on

protein output and ultimately function. This is corroborated, for example, by the role of 5'-UTR variants that alter upstream open reading frames in cardiovascular disorders (CVD)[200].

RNA modification and networks of competitive RNA-RBP binding

As soon as the nascent RNA molecules emerge from the RNA polymerase during transcription, they are instantly decorated with RBPs. While this ensures that co-transcriptional processing takes place effectively and at the right position, RBP loading also prevents the hybridization of the nascent RNA molecule with the DNA strand. This helps to avoid the formation of reactive RNA:DNA hybrids (so called R-loops)[201, 202], which can lead to genomic instability[203, 204]. Most importantly, binding of RBPs and non-coding RNAs to (pre-)mRNAs can occur in a complex, sometimes mutually exclusive manner, thereby determining the posttranscriptional fate of mRNAs selectively[84] or in a global manner[205, 206]. This is supported by the observation that the density of RBP and miRNA binding to the UTRs of coagulation factor mRNAs is very high[138], and that numerous RBP and miRNA binding sites are in close proximity (Figure 5).

Hence, there must be mechanisms that coordinate the binding of such molecules. Although not yet studied in great detail, it is likely that modifications of both RNAs[207] and RBPs[208] can result in remodeling of the 3'-UTR-RBP architecture and thereby change the fate of RNAs encoding coagulation factors under inflammatory conditions. In support of this notion, posttranslational modifications of RBPs have been shown to change the fate of mRNAs encoding central hemostatic components (Figure 3)[99]. But also variations in N^6-methyladenosine (m^6A), the most prevalent RNA modification with a wide biological impact[209, 210], have been documented in various RNA transcripts in vascular tissues of septic rats[211]. Additionally, there is growing evidence that m^6A modification is closely related to the development and progression of CVD, including cardiac hypertrophy, heart failure, ischemic heart disease and pulmonary hypertension[212, 213]. It is tempting to explore if therapeutic modulation of the cellular m^6A machinery (for example in COVID-19 [214]) might be useful in preserving vascular integrity and function in sepsis and/or CVD. Interestingly, the fat mass and obesity-associated protein (FTO), one of the few m^6A erasers, has emerged as an important pharmaceutical target in many pathophysiological conditions[209]. As many more RNA modifications are currently being discovered[215], this holds great potential for systematically uncovering their importance in human diseases and defining novel therapeutic avenues.

Long non-coding RNAs and circRNAs

Despite the unexpectedly small number of protein-coding genes identified by the human genome project, RNA sequencing has shown that up to 85% of the human genome is transcribed[216]. This led to the identification of a large number of non-coding RNA molecules with regulatory functions[217]. In contrast to small non-coding RNAs (such a miRNAs, snoRNAs

or piRNAs), long-noncoding (lnc)RNAs are around 200 nucleotides or more[218] and often undergo alternative splicing, which further expands their repertoire. LncRNAs can bind to DNA, mRNAs, miRNAs and proteins depending on sequence and secondary structure, thereby modulating gene expression under physiological and pathological conditions[219]. Their modes of action include epigenetic, transcriptional and post-transcriptional mechanisms. Accordingly, this new class of ncRNAs is increasingly taking center stage in the modulation of the cardiovascular system. As an example, lncRNA H19 is involved in the pathogenesis of atherosclerosis[220]. The expression of lncRNA H19 is significantly increased in patients with ischemic stroke compared to healthy controls[221]. Genome-wide association studies have identified SNPs in the lncRNA ANRIL associated with CVD, such as coronary atherosclerosis and cardiac infarction[222, 223], while variants in lncRNA ZFAS1 are associated with susceptibility to ischemic stroke[224]. Recently, a transcriptome wide association study on VTE also revealed further lncRNA hits (RP11-747H7.3, RP4-737E23.2)[225], corroborating their function in CVD.

Unlike miRNAs or proteins, lncRNA function cannot currently be simply inferred from sequence or structure, and the diversity of lncRNAs described to date precludes simple generalizations[219]. In the context of the hemostatic system, this hitherto poorly explored area deserves attention. This is also supported by the role lncRNAs have in platelets[226, 227], although their role is still under active investigation. In analogy to the central regulatory function of non-coding RNAs in the immune system and because of the resulting therapeutic implications[228], it will be important to better understand the pathophysiological dimension of this class of regulators in thrombosis and its connection to inflammation.

Circular RNAs (circRNAs) are another class of endogenous non-coding regulatory biomolecules. They are prevalent and arise from a non-canonical splicing event called 'backsplicing' [229]. They exert important biological functions by acting as miRNA or protein sponges, by regulating protein function or by being translated[230]. As such, circRNAs regulate a plethora of biological functions including ROS formation and cardiovascular metabolic inflammation[231]. Accordingly, perturbations of these process(es) can become pathogenic and result in CVD. For example, a haplotype on 9p21 that protects against coronary artery disease has been shown to be associated with the abundance of circRNA ANRIL, which in turn regulates ribosomal RNA maturation, conferring atheroprotection[232]. Accordingly, *circANRIL* has been proposed as a potential therapeutic target for the treatment of atherosclerosis. The in-principle therapeutic utility of circRNA is also supported by recent preclinical observations demonstrating their use, for example, to attenuate cell apoptosis in cerebral ischemia-reperfusion[233]. Finally, circulating circRNA may have diagnostic potential and serve as biomarkers for acute ischemic stroke[234] and even help distinguish different etiologies (i.e., atherothrombotic, cardiothrombotic vs undetermined stroke)[235].

What comes next? Alternative polyadenylation and 3'-UTR diversity as central regulatory hubs

Much of the posttranscriptional regulation of the hemostatic system depends on players that determine the fate of RNAs encoding the respective hemostatic components. The different layers of regulation are largely inter-dependent, as alternative splicing and polyadenylation are coupled to each other[84] and thereby determine not only the final open reading frame, but also the 3'-UTR sequence and hence the susceptibility of the mature mRNA to posttranscriptional control by RBPs and ncRNAs.

Since much of the posttranscriptional regulation of gene expression takes place at the level of the 3'-UTR, to which RBPs and ncRNAs are abundantly recruited, the 3'-UTR architecture has an important regulatory function (Figure 6)[84]. Diversification of the transcriptome at the 3'-end by alternative polyadenylation (APA) has recently emerged as a pervasive and evolutionarily conserved layer of gene expression control[236] (Figure 1), which affects more than 70% of all genes. APA considerably expands the diversity of the transcriptome 3'-end, affecting protein output, isoform composition and protein localization[237].

APA is globally regulated in various conditions, including developmental and adaptive programs[89]. It is thus likely that APA also tunes the hemostatic system, as exemplified by alternative processing of TF and TFPI, where alternative splicing also generates different 3'-UTRs (Figure 2). In addition, a recent large scale RNAi screen based on the depletion of more than 170 putative APA regulators revealed how individual regulators affect the APA landscape[115], including the resulting impact on gene ontologies[119]. Several significantly enriched GO terms suggest a critical function of UTR structures in inflammatory processes and innate and adaptive immunity[119]. APA affects key components broadly involved in inflammation and blood coagulation (Table 2). This is consistent with findings that APA is a critical component in the control of inflammatory processes[116, 238, 239] (including COVID-19[240]), that typically result in shorter mRNA isoforms (Figure 6).

Strikingly, several hemostatic components have alternative transcripts that differ not only in their exon composition but also in their 3'-UTR structure (see NCBI Ref seq). These include essential components of the protein C pathway (i.e., protein C and protein S) with established functions at the interface of coagulation and inflammation[241]. For the protein C cofactor protein S, 3'-UTR dynamics are already documented[119], which appear to be regulated by specific RBPs (RNPS1) or other components (CDKN2D). This points to a regulatory function of APA at the interface of the hemostatic and the immune system. Due to the pervasive regulatory function of APA in various processes[119] (with perturbations leading to numerous diseases[89]), it is plausible that much of this diversity in the hemostatic system is regulated in response to inflammatory signals. This is illustrated by inflammation-triggered alternative processing of the *FGG* mRNA[242], resulting in gamma prime (γ') fibrinogen[74]. γ' fibrinogen is the fibrinogen fraction

that contains the γ' chain, which arises when the *FGG* mRNA is polyadenylated at an alternative polyadenylation signal, resulting in a polypeptide with a unique 20-amino acid extension encoded by intron 9 [74]. Thanks to the strongly negatively charged C-terminus of the γ' chain, fibrinogen γ' can bind with high affinity to thrombin exosite II, decreasing thrombin activity on several substrates (antithrombin I activity)[243]. As a consequence, low γ' fibrinogen levels have been associated with an increased risk of venous thrombosis[74, 244], while a potential role in CVD[245] and ischemic stroke[246] is under debate[247]. This highlights how seemingly subtle changes through alterations of APA and 3'-UTR diversity can have most significant functional effects in the hemostatic system. It also serves as an example illustrating the complex interdependency of posttranscriptional processing of RNA molecules and hence functional output.

Interrogating system-wide posttranscriptional gene regulation[38, 39] and transcriptome 3'-end diversity[118, 119], combined with unbiased RNA interactome studies[117, 135] and strategies to disentangle the functional significance of genomic perturbations in non-coding elements[248], therefore holds great potential to unravel novel layers of coupling of the hemostatic system with inflammatory processes. This could also open entirely new therapeutic perspectives[89] to combat medical threats centering around thromboinflammation such as sepsis, which is still the leading cause of death in the Western world and in critically ill patients worldwide[1].

Targeting post-transcriptional regulation of the hemostatic system

The multiple layers of posttranscriptional control of gene expression offer various opportunities and targets for therapeutic intervention. For example, RNA-based therapeutics can be used not only to re-direct splicing[80] and polyadenylation[249], but also to silence an mRNA or to prevent its interaction with other RNAs or RBPs[250, 251].

Compared to 'conventional' small therapeutic molecules, RNA-based therapeutics such as ASOs, siRNAs and miRNAs offer the advantage of being able to act on 'non-druggable' targets (i.e., proteins that lack enzymatic function or whose conformation is inaccessible to traditional drug molecules), as they can be designed to affect virtually any gene of interest[192].

ASOs are relatively short, chemically modified single-stranded nucleic acids that selectively pair to specific regions of mRNA resulting in endonucleolytic cleavage and degradation[250]. Currently, more than 60 ASO therapies are in or have completed phase I/II trials, with a substantial number of antithrombotic ASO therapeutics currently under development[138].

The recent introduction of ASOs down-regulating FXI expression exemplifies the potential of such therapeutics to modulate the hemostatic system *via* post-transcriptional mechanisms[34]. This phase II study in patients undergoing knee surgery revealed that the FXI-targeting ASO effectively protects patients against venous thrombosis with a relatively limited risk of bleeding. However, this proof-of-concept trial was too small to assess the effect on other thrombotic end

points**.** Other genes that are being explored as potential targets for antithrombotic therapy using silencing ASOs are FII, FVII, FXII, prekallikrein, plasmin activator inhibitor, thrombopoetin and FMO3[138]. A possible concern is that changes in platelet counts were observed in non-human primates treated with ASOs[252], which has been attributed to peripheral clearance[253] and could potentially impact hemostasis.

miRNA therapeutics represent another highly versatile therapeutic means in the context of the hemostatic system[138]. MiRNA mimics may be employed to silence pro-coagulant genes to treat thrombosis (or alternatively, anticoagulant genes to treat bleeding). Conversely, antagomirs or target site blockers can be used to relieve silencing of anticoagulant genes to treat thrombosis. Moreover, some miRNAs target several hemostatic components at the same time (Figure 4), and silencing of such miRNAs can be intentionally used to control several hemostatic components. On the other hand, undesired pleiotropy is one of the conceptual downsides of therapeutic miRNA targeting.

MiRNA therapeutics are currently at an early stage of development and not yet applicable in the clinical setting[254]. In preclinical studies, several miRNA mimics and antagomirs have been shown to reduce thrombus formation[138] or increase the antithrombin activity in vivo[135]. One of the biggest challenges in the clinical development of miRNA-based therapeutics is the identification of key miRNA candidates and targets, their specificity and effect size. There is currently a relatively small number of experimentally validated miRNA:mRNA interactions, making knowledge of the miRNA targetome in the hemostatic system a major trove for future targeted therapeutics[135].

ASOs and most siRNAs exhibit perfect complementary to their targets, which usually results in degradation of the target mRNA[255]. In contrast, partial base-pairing of miRNAs prevents the cleavage activity of RISC, predominately causing translational repression, and only in some cases deadenylation, decapping and finally mRNA degradation[256]. Although the proportion of mRNA target degradation varies widely[257], a number of targets are almost exclusively repressed at the level of translation[258]. How much each mechanism contributes to down-regulation depends on characteristics, such as seed-flanking nucleotides, of the individual miRNA–mRNA pair[259].

In the context of the hemostatic system, it is interesting to note that miRNA regulation of transcripts encoding secretory proteins results almost exclusively in translational repression, because miRNA translational repression is stronger for mRNAs translated at the endoplasmic-reticulum compared to free cytosolic ribosomes[258]. Thus, miRNA-mediated therapeutic targeting without degradation of the target mRNAs preserves physiological cell intrinsic regulatory mechanisms carried out by 3'-UTRs and their binding partners (such as RBPs, miRNAs, lncRNAs, circRNA or miRNA sponges). This allows for 'compensatory' on-demand

adjustments of protein output even in the presence of the miRNA therapeutic, and thus may represent a conceptual advantage of miRNA therapeutics over ASO-based approaches[138].

While RNA therapeutic approaches have been used in the development of new drugs and clinical trials are underway[260], there are still concerns and challenges to be overcome. These include, but are not limited to, off-target effects[261], triggering innate immune responses[262], stability of the therapeutic RNA molecule and design of optimal delivery systems for disease-specific release with minimal toxicity[190].

Finally, there are increasingly strategies to modulate other facets of the RNA biogenesis. This concerns the targeted interference with splicing[80] or with cleavage and polyadenylation[249], involving either redirection of aberrant RNA processing (through ASOs, U1snRNP interference or trans-splicing) or the elimination of aberrant transcripts[79, 89]. The characterization of the transcriptome dynamics thus becomes the next milestone to exploit the untapped therapeutic opportunities arising from the increasingly available RNA therapeutics.

Summary

Besides transcriptional control, posttranscriptional regulation of gene expression is taking center stage in the modulation of the hemostatic system. The highly regulated use of alternative transcription start sites, exons and polyadenylation sites makes the transcriptome highly dynamic in time, space and in response to pathological processes. Additional posttranscriptional regulation by non-coding RNAs, RNA-binding proteins and RNA modification mechanisms further modulate the functional output of numerous biological processes, including the hemostatic system. Many of these regulatory principles also play an important functional role in tuning the immune system[28-32], suggesting conserved regulatory links between both systems. It will be critical to characterize these links to identify rational targets for the emerging repertoire of RNA therapeutics to effectively combat the dangerous alliance of the hemostatic and the immune system.

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Figure

Figure 1

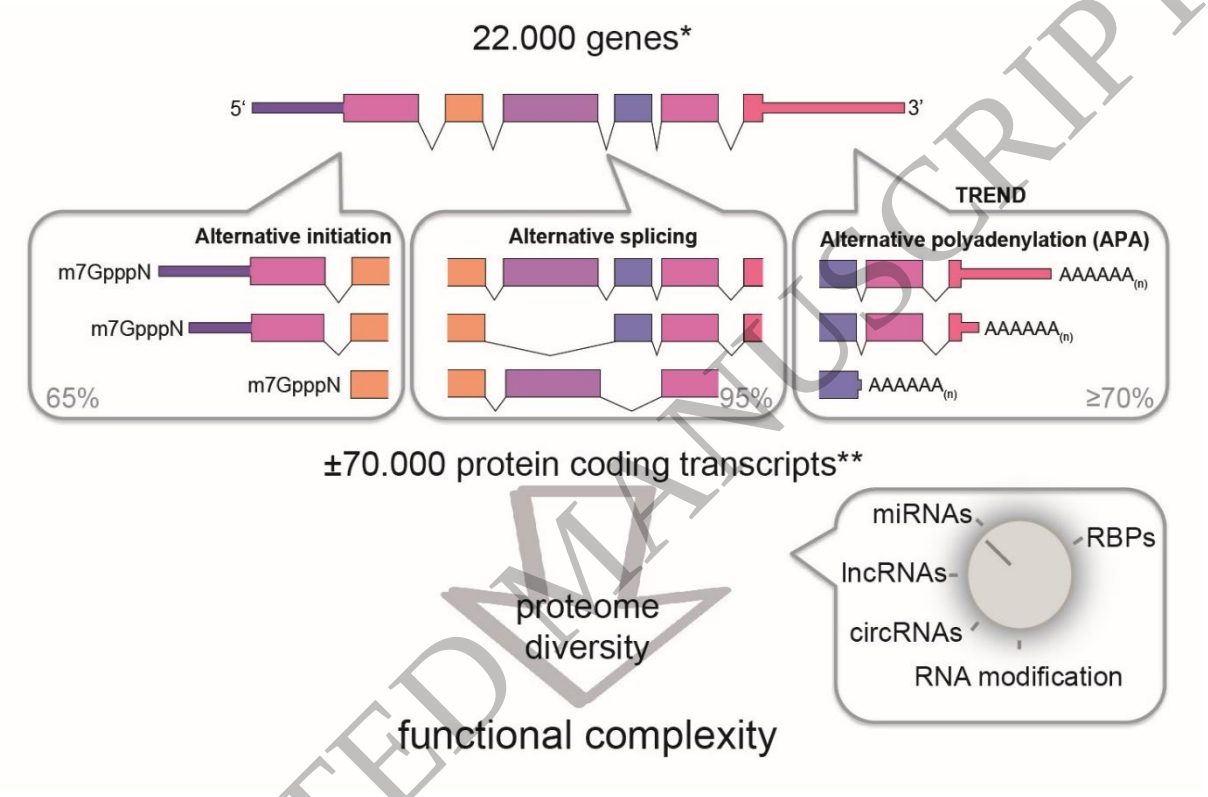

Figure 1. The functional complexity encoded by approximately 22.000 genes is substantially diversified by co- and posttranscriptional mechanisms involving alternative transcription initiation, alternative splicing and alternative polyadenylation (APA). Regulation by non-coding RNAs such as micro (mi)RNAs, long-non-coding (lnc)RNAs, circular (circ)RNAs, as well as RNA-binding proteins (RBPs) and RNA modifications, further tunes the functional output of the transcriptome. Modulation of the biogenesis and the posttranscriptional fate of RNAs (RNA localization, transport, translation, stability or decay, RNA modifications) are emerging therapeutic principles (further details see text; *[263], **[264]).

Figure 2

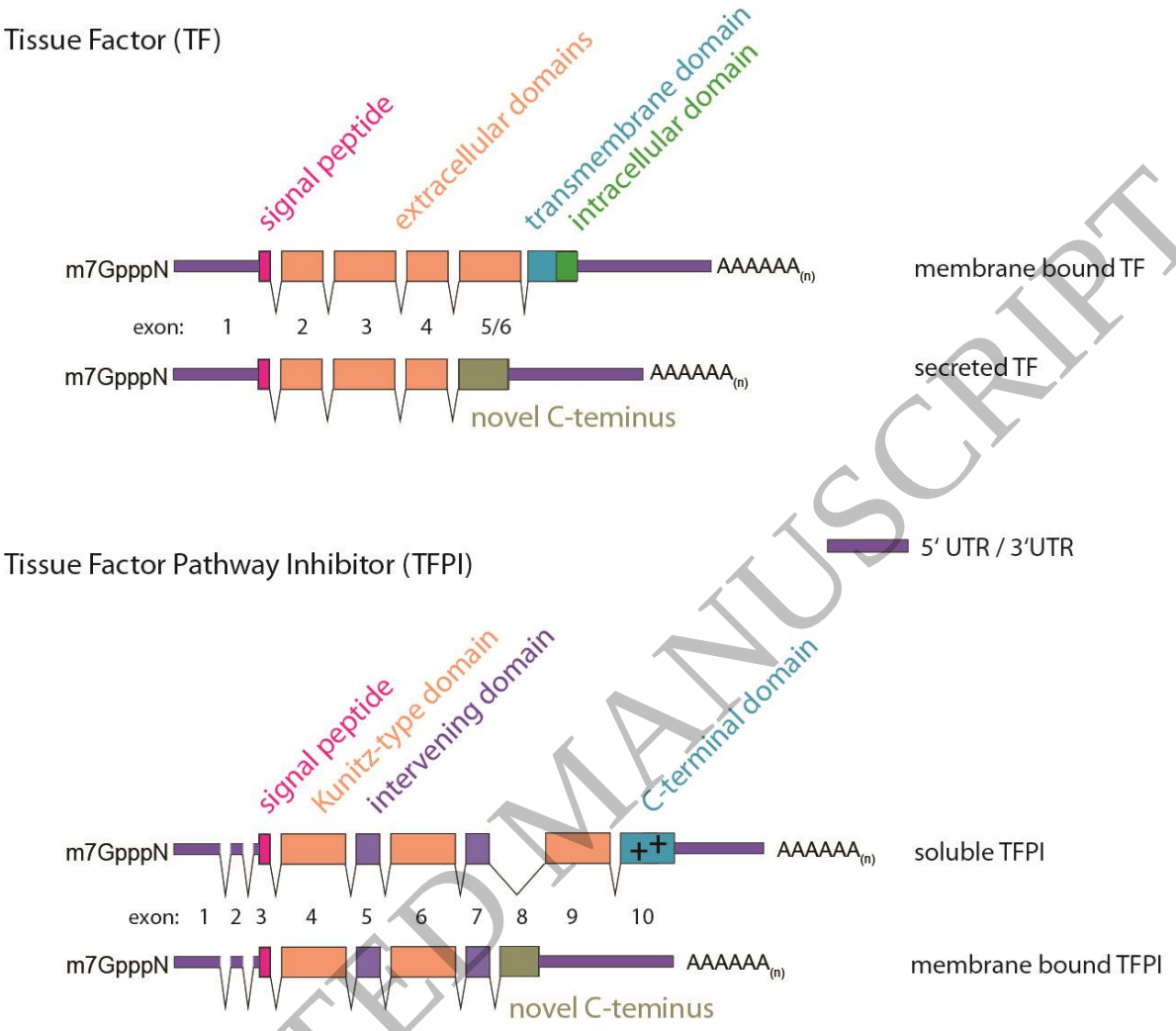

Figure 2. Alternative splicing in components of the hemostatic system, resulting in distinct structural and biochemical characteristics. Of note, the 5'UTR of TFPI contains several non-coding exons (not to scale), a regulatory feature found in many genes [265, 266].

Figure 3

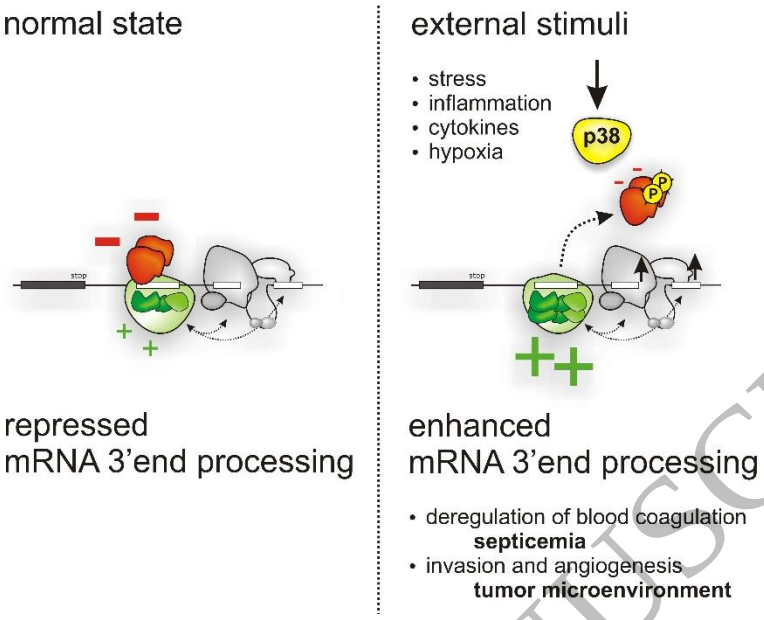

Figure 3. Modulated 3'end processing as a principle to rapidly adjust protein output. Example shown for the prothrombin (*F2*) gene, where mutually exclusive binding of inhibitory (red) and stimulatory (green) RNA-binding proteins modulates cleavage and polyadenylation of the *F2* pre-mRNA. Upon induction of p38 MAPK, the abundance of cleavage and polyadenylation (CPA) factors (grey) is induced, and the inhibitory proteins (FBP2 and FBP3, shown in red) are phosphorylated. This impairs RNA binding of these proteins, and allows for binding of stimulatory components (green), which eventually enhances RNA maturation and protein output (modified from [99]).

Figure 4

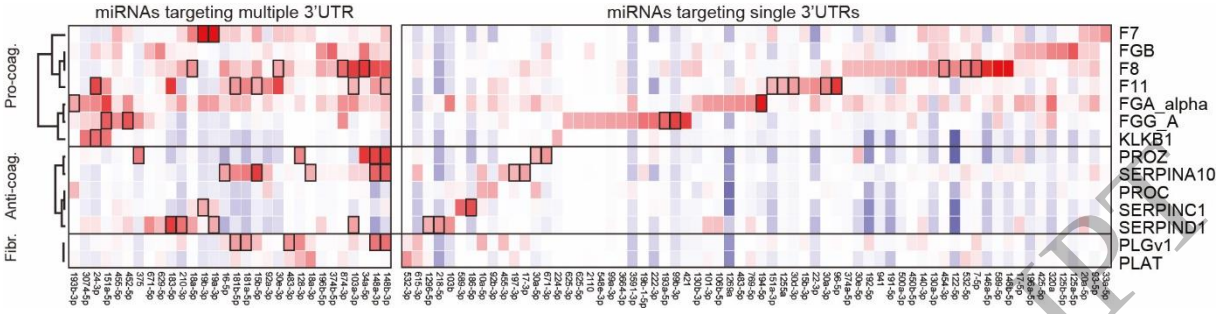

Figure 4. Snapshot on the human hemostatic miRNA targetome (for the full miRNA atlas see [135], Table S4). Heatmap of miRNA/3′UTR interactions (only highly stringent interactions are depicted). Results of miTRAP assays were divided into three functional categories of procoagulant, anticoagulant and fibrinolytic components, and for miRNAs targeting multiple 3′UTRs each category subjected to unsupervised hierarchical clustering as indicated by tree on the left (modified from [135]). For further information of miRNA-mediated regulation of hemostatic components see Table 1.

Figure 5

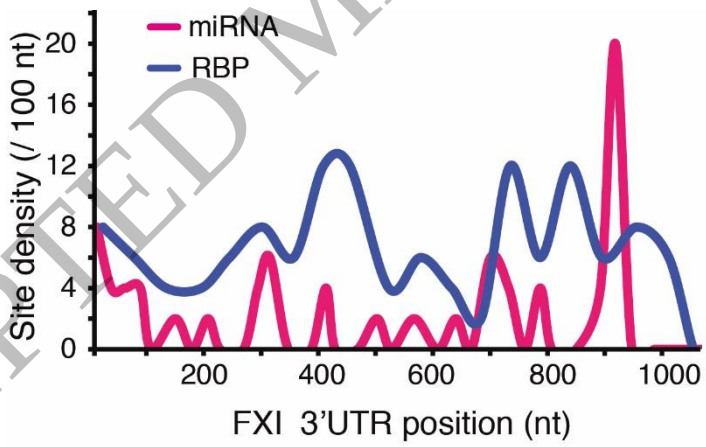

Figure 5. FXI 3'-UTR interactome. The graph depicts the density of sites for miRNA and RNA-binding proteins (RBPs) across the FXI 3'-UTR (based on 125 FXI 3'-UTR/miRNA interactions identified by miTRAP/RNA-seq[135] with 41 mapped to the FXI 3'-UTR using miRWalk target site prediction, and 392 FXI 3'-UTR/RBP interactions identified by miTRAP/MS and of which 66 are mapped to the FXI 3'-UTR using RBPDB target site prediction. Site density calculated by number of sites present in 50 nt windows over length of the FXI 3'-UTR).

Figure 6

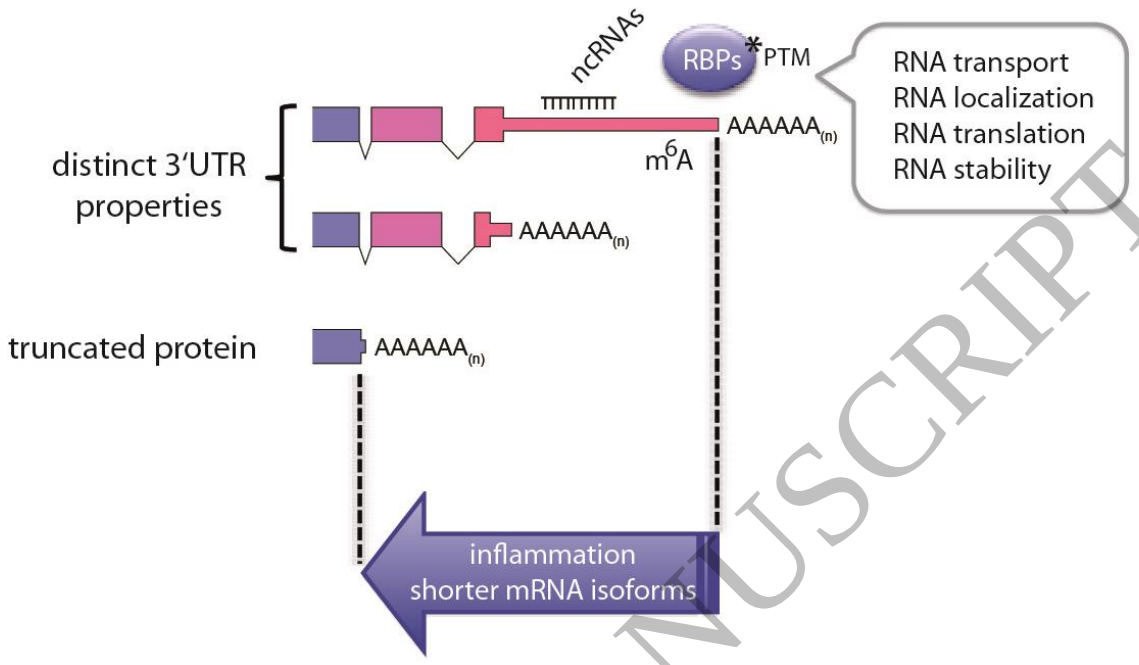

Figure 6. Alternative polyadenylation is a pervasive gene regulatory mechanism that results in mRNA isoforms with different 3'-ends. This can result in mRNA isoforms encoding truncated proteins or in mRNA isoforms with distinct 3'-UTR properties altering RNA transport, localization, translation, and/or stability (through binding to non-coding RNAs (such as miRNAs, lncRNAs, ceRNA), through binding to RNA binding proteins (RBPs) and/or through complex, sometimes mutually exclusive, interactions of RNA motifs with RBPs and/or ncRNAs. Of note, modifications of RNAs (such as "m^6A") or posttranslational modifications (PTMs) of RBPs introduce further layers of modulation). Inflammatory conditions tend to result in the generation of shorter mRNA isoforms (either lacking elements of 3'-UTR regulation or resulting in truncated proteins; [119, 116]). Alternative polyadenylation affects numerous genes involved in blood coagulation and inflammation (Table 2).

Table 1. Hemostatic components under miRNA control and relation to thromboinflammation. For full Hemostatic miRNA Targetome Atlas see [135].

Procoagulant		Main miRNAs (functionally validated)	Ref.
fibrinogen alpha	*FGA*	miR-193b-3p [135] miR-194-5p [135] miR-759 [267]	135, 267
fibrinogen beta	*FGB*	miR-409-3p (miR-29 family)	268
fibrinogen gamma	*FGG*	miR-99b-3p miR-193a-5p	135
coagulation factor III, tissue factor	*F3*	miR-19b • Anti-thrombotic protector in patients with unstable angina [269] miR-19b, miR-20a • Down-regulation contributes to a hypercoagulable state in SLE and APS [270] miR-126 • Reduces thrombogenicity in diabetes mellitus [271] miR-145 • Impedes thrombus formation in venous thrombosis [194] miR-223 • Partially blocks TNF-α-induced increase of TF activity in endothelial cells [272] miR-365a-3p • Interacts with TF 3'-UTR to modulate TF-initiated thrombin generation [273]	194, 269-272, 274
coagulation factor VII	*F7*	miR-19a-3p miR-19b-3p	135
coagulation factor VIII	*F8*	miR-7-5p [135] miR 454-3p [135] miR-532-5p [135] miR-1246 [275]	135, 275
coagulation factor XI	*F11*	miR-15b-5p [135] • Biomarker for PAD [276] • Influences platelet reactivity and clopidogrel response [277] miR-24-3p [135] • Biomarker for acute cerebral infarction, arteriosclerosis obliterans, atherosclerosis and severe trauma [278-281] miR-30a-3p [135] • Biomarker for AMI and ischemic stroke [282, 283] miR-30d-3p [135] miR-96-5p [135] • Biomarker for DVT and DIC [284, 285] miR-103a-3p [135] • Involved in atherosclerosis and vascular inflammation by suppression of KLF4 [286] • Biomarker for VTE [287] miR-145-5p [288]	

			• Biomarker for CAD, AMI, stroke, long-term outcome [289-296] • Impedes thrombus formation in atherosclerosis by targeting tissue factor and influencing platelet reactivity [277, 297] miR-148b-3p [135] miR-151a-3p [135] miR-181a-5p [298, 299] • Biomarker for AMI and PAD [276, 300] miR-181b-5p [135] miR-191b-5p [298] miR-544a [302] miR-1255a [135] • Biomarker for stroke [303]	
	(pre)kallikrein	*KLKB1*	miR-24-3p	135
	Von Willebrand factor	*VWF*	miR-24	177, 304
	ADAM metallopeptidase with thrombospondin type 1 motif 13	*ADAMTS13*	miR-525-5p	305
Anticoagulant				
	tissue factor pathway inhibitor	*TFPI*	miR-27a/b miR-494 miR-27a/b-3p	306, 307
	antithrombin	*SERPINC1*	miR-19b-3p miR-186-5p	135
	protein C	*PROC*	miR-494 let-7 family	135
	protein S	*PROS1*	miR-494	308
	protein Z	*PROZ*	miR-30a-5p miR-128-3p miR-148a-3p miR-148b-3p miR-375 miR-671-3p	135
	protein Z-dependent protease inhibitor	*SERPINA10*	miR-15b-5p miR-16-5p miR-17-3p miR-197-3p	135
	heparin cofactor 2	*SERPIND1*	miR-183-5p miR-210-3p miR-218-5p miR-1296-5p	135
Fibrinolytic				
	plasminogen	*PLG*	miR-148a-3p miR-148b-3p miR-181a-5p miR-181b-5p miR-483-3p	135
	tissue-type plasminogen activator	*PLAT*	miR-340	309
	plasminogen activator inhibitor	*SERPINE1*	miR-30c • Biomarker for inflammatory and thrombotic disorders[310]	310-312

			miR-421 • Biomarker for inflammatory and thrombotic disorders[310] miR-301a	

Table 2. Alternative polyadenylation regulates components involved in blood coagulation and inflammation. Each column depicts genes belonging to the GO term "blood coagulation", "regulation of inflammation" and "complement" that are affected by alternative polyadenylation (APA) upon depletion of central APA regulators (CPSF6, NUDT21, PCF11). Data obtained from TREND-DB[119]; for further APA affected genes and -effectors: http://shiny.imbei.uni-mainz.de:3838/trend-db/.

	regulated by **CPSF6**-dependent APA		
affedcted GO term	blood coagulation	regulation of inflammation	complement
affected genes	ARRB1	DDX3X	C7
	CBX5	DROSHA	CD59
	CD59	LDLR	
	GATA2	LYN	
	GNA11	MACIR	
	GNA12	NDFIP1	
	GNA13	PBK	
	GNB1	PDCD4	
	GNG2	PRCP	
	H3-3B	SETD6	
	LMAN1	SMAD3	
	LYN	STMP1	
	MAPK1	SYT11	
	PRCP	VPS35	
	PRKAR1A		
	PRKAR2B		
	RAB27A		
	RAC1		
	RAD51C		
	STXBP1		
	YWHAZ		

	regulated by **NUDT21**-dependent APA		
	blood coagulation	regulation of inflammation	complement
	ARRB1	ATM	C7
	CAPZB	CD47	
	CBX5	HSPD1	
	GATA2	ISL1	
	GATA4	LYN	
	GGCX	MACIR	
	GNA11	MCPH1	
	GNA12	NDFIP1	
	GNA13	PDCD4	
	GNB1	SETD6	
	GNG2	SMAD3	
	H3-3B	SOD1	
	HPS5	SYT11	
	LMAN1	TREX1	
	LYN	VPS35	
	PHF21A		
	PRCP		
	PRKAR1A		
	RAB27A		
	RAC1		
	STXBP1		

	regulated by **PCF11**-dependent APA		
	blood coagulation	regulation of inflammation	complement
	ACTG1	ABHD12	HSP90AB1
	ARRB1	DROSHA	RAB27A
	GNA12	GPS2	
	GNB1	NDFIP1	
	GNG2	NEAT1	
	H3-3B	NT5E	
	IRF2	PRCP	
	PRCP	STMP1	
	PRKAR1A	VPS35	
	PRKAR2B		
	RAB27A		
	VAV2		
	VPS45		

Platelet degranulation
Thrombin/G-Protein coupled receptor signaling
Complement regulation
Positive regulation of secretion by cell
Regulation of inflammatory response/cytokine production
Angiotensin conversion