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Regulation of Coagulation Factors by MicroRNAs:Role in Interindividual Variability and Implications for Hemostatic Disorders.

Regulación de los Factores de la Coagulación a través de MicroRNA: Papel en la Variabilidad Interindividual y su Implicación en Trastornos Hemostáticos.

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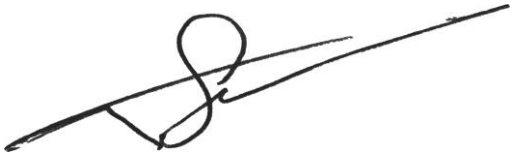

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A mis padres

Caminante, no hay camino,

se hace camino al andar.

- Antonio Machado -

List of Abbreviations

Symbol	Description
AGO	Argonaute
cDNA	Complementary deoxyribonucleic acid
CDS	Coding Region
CLIP	cross-linking immunoprecipitation
crasiRNA	Centromere associated RNA
DMEM/F-12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
DMEN-glutaMAX	Dulbecco's modified Eagle medium with glutamine
DNA	Deoxyribonucleic acid
HCT-DK	Human colon carcinoma cell lines-Dicer knockout
HepG2	Human hepatocellular liver carcinoma cell line
HMWK	High molecular weight kininogen
HNF4α	Hepatic nuclear factor 4 alfa
LETS	The Leiden Thrombophilia Study
lncRNAs	Long non-coding RNAs
MEGA	Multiple Environmental and Genetic Assessment
MEM	Minimum Essential Media
miRISC	miRNA-induced silencing complex
miRNA	microRNA
ncRNA	non-coding RNA
P-bodies	processing bodies
PAI-1	Plasminogen activator inhibitor-1
PASRs	Promoter-associated short RNAs
piRNA	PIWI interacting RNA
PLC/PRF/5	Primary hepatocellular carcinoma cell line
Pol II	RNA polymerase II
Pre-miRNAs	precursor miRNAs
Pri-miRNAs	primary miRNAs
PubMed	US National Library of Medicine
rasiRNA	Repeat associated small interfering RNA
RIII	ribonuclease III

RNA	ribonucleic acid
RT	Reverse transcription
saRNA	Small activating RNA
sdRNA	SnoRNA-derived RNA
SD	Standart desviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	small interfering RNA
SNP	Single nucleotide polymorphism
SPSS	Statistical Package for the Social Sciences
TF	Tissue Factor
TFPI	Tissue Factor pathway inhibitor
TiRNA	Transcription initiation RNAs
TRBP	transactivation-responsive RNA-binding protein
tRFs	tRNA derived RNA fragments
UTR	Untranslated regions
vWF	von Willebrand factor
XPO5	exportin 5

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INTRODUCTION

1. Fundamentals in hemostatic system

The hemostatic system, a highly conserved machinery from zebrafish to human [1, 2], is maintained by a delicate equilibrium between pro- and anticoagulant factors. This balance of opposing forces is a complex physiological system where platelet aggregation, coagulation, and fibrinolysis are closely interconnected and integrated in perfect harmony, responding to a high effectiveness and always restricting their effect to the site of vascular damage. By this way, it allows for physiological response to hemorrhage while simultaneously avoids development of pathologic thrombi.

For teaching purposes, hemostasis is categorized as either a primary or secondary process. When a vessel is injured, primary hemostasis involves the response of the vascular system and platelets while secondary hemostasis involves the response of the coagulation system to repair the wounded vessel. Hemostasis is required to control bleeding from large wounds and actually, primary and secondary hemostasis are spatial and temporally overlapping processes. Whereas the outcome of primary hemostasis is the formation of the platelet plug, the outcome of secondary hemostasis is the formation of a thrombus [3].

The coagulation system gathers several factors that interact to form a fibrin clot. The fibrin clot reinforces the platelet plug formed during primary hemostasis [3]. In the classic waterfall scheme of the coagulation (Figure 1), thrombin and fibrin formation proceed through a series of sequential reactions involving activation of plasma proteases by limited proteolysis [4]. The cascade model describes two converging pathways that are initiated either by exposure of blood to a damaged vessel wall (the extrinsic pathway or tissue factor pathway) or by blood-borne components of the vascular system (the intrinsic pathway or contact activation pathway).

Briefly, the tissue factor pathway, which is essential for fibrin formation at a site of injury following vascular damage, is initiated when the plasma protease FVIIa forms a complex with tissue factor (TF), which is ubiquitously expressed in subendothelial vascular cells. Activity of FVIIa/TF complexes is tightly regulated

by the TF pathway inhibitor (TFPI), which rapidly inactivates FVIIa/TF enzyme complexes on membranes [5]. Therefore, additional stimuli for propagating fibrin formation to support the formation of a three-dimensional thrombus are required.

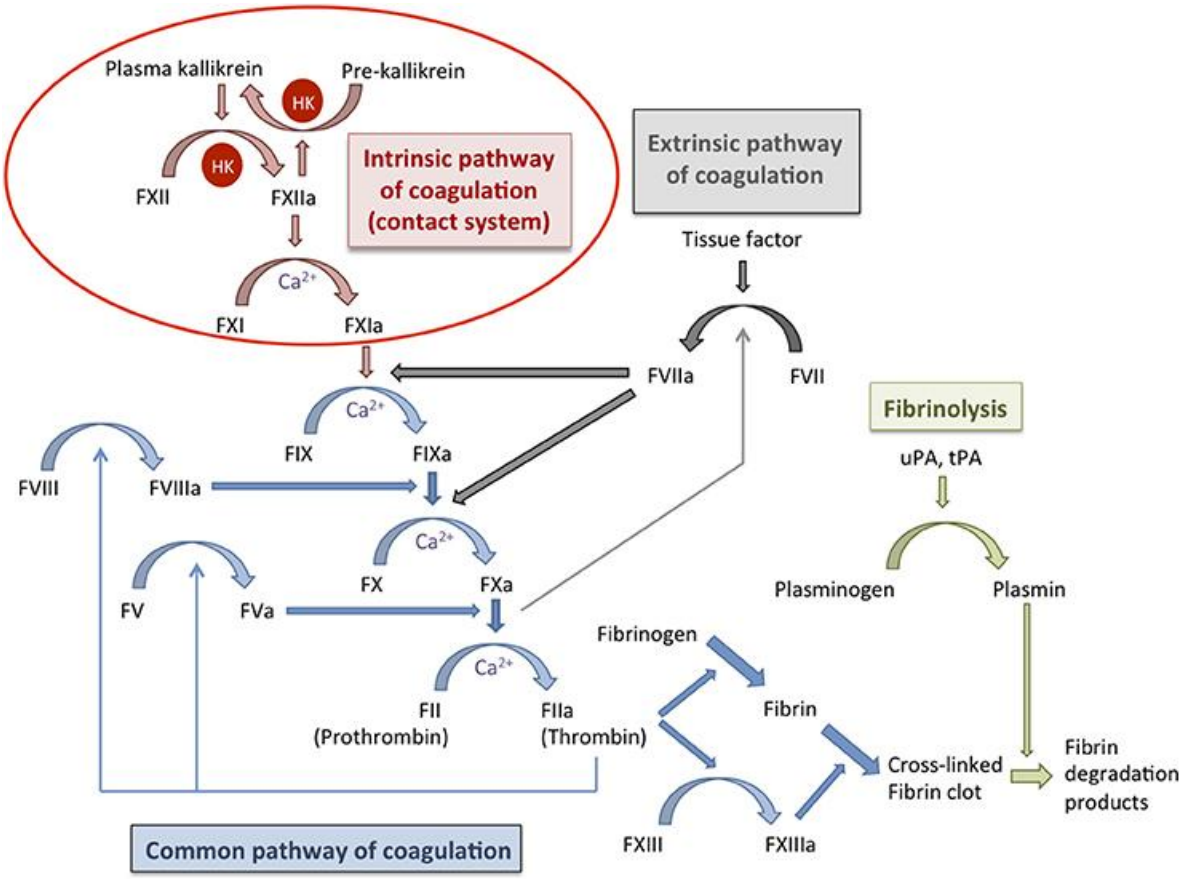

Figure 1| An overview of the coagulation cascade. The coagulation cascade of secondary hemostasis has two pathways that lead to fibrin formation. These are the contact activation pathway (formerly known as the intrinsic pathway), and the tissue factor pathway (formerly known as the extrinsic pathway). The pathways are a series of enzymatic conversions, turning inactive proenzymes into activated enzymes and culminating in the formation of thrombin. Thrombin then converts the soluble plasma protein fibrinogen into the insoluble fibrous protein fibrin. (Original source: Loof TG, Deicke C and Medina E (2014). The role of coagulation/fibrinolysis during *Streptococcus pyogenes* infection. *Front. Cell. Infect. Microbiol*. **4**:128. doi: 10.3389/fcimb.2014.00128.).

The contact activation pathway is initiated when FXII comes into contact with negatively charged surfaces, in a reaction involving high molecular weight kininogen and plasma kallikrein. FXII activation is triggered by negatively charged polyanions. In reactions that are propagated by platelets, activated FXII (FXIIa) activates its substrate FXI. The common point in both pathways is the activation of FX to FXa. FXa activates prothrombin (FII) to thrombin (FIIa). Thrombin, in turns, converts fibrinogen to fibrin.

1.1. Primary hemostasis

Primary hemostasis refers to platelet aggregation and platelet plug formation. There are normally between 150 and 400 billion platelets per liter of blood in a healthy adult, produced by megakaryocytes in the bone marrow. In the event of injury to a blood vessel, platelets recognize the perturbation of the endothelial cells lining the blood vessels or the exposed underlying fibrous matrix. They rapidly adhere to the site of injury and to each other, become activated, secrete the contents of intracellular storage organelles and aggregate to form thrombi, which subsequently undergo contraction and consolidation to prevent blood loss and promote wound healing. Activated platelets also express surface phospholipids that promote localized coagulation, leading to thrombin generation and fibrin formation [6]. They also recruit leukocytes as an early step in innate immunity and inflammation, beyond their role in primary hemostasis [7].

1.2. Coagulation factors

Our knowledge of the blood coagulation system has expanded tremendously in the last 60 years [8].The unified nomenclature of coagulation factors was required by the mid-1950s because many of them had been named independently by several groups of workers who studied different properties and who initially thought that they had discovered different factors. An International Committee was, therefore, established in 1954 with the aim of harmonizing the nomenclature of the various factors (Table 1). The protein factors are designated by Roman numerals according to their sequence of discovery and not by their point of interaction in the coagulation cascade. The first 4 of the 12 originally identified factors are not referred to by their Roman numeral but by their common names, i.e., fibrinogen, prothrombin, tissue factor and calcium. The more recently discovered clotting factors (e.g. prekallikrein and high-molecular-weight kininogen) have not been assigned Roman numerals. Factors V and VIII are also referred to as the labile factors because their coagulant activity is not durable in stored blood.

Most of clotting factors are precursors of proteolytic enzymes known as zymogens that circulate in an inactive form. Suffixing letter “a” to the Roman numeral depicts the activation of each zymogen. With the exception of TF,

calcium, most of the procoagulants and anticoagulants factors are synthesized in the liver. Several of the protein coagulation factors (FII, FVII, FIX and FX) undergo a posttranslational modification (vitamin K dependent Ɣ carboxylation of glutamic acid residues) that enables them to bind calcium and other divalent cations and participate in coagulation cascade [9].

Table 1| Nomenclature of coagulation factors and function:

Coagulation factor number	Coagulation factor name	Function
I	Fibrinogen	Clot formation
II	Prothrombin	Activation of FI, FV, FVII, FVIII, FXI, FXIII, protein C, platelets
III	TF	Cofactor of FVIIa
IV	Calcium	Facilitates coagulation factor binding to phospholipids
V	Proaccelerin	Co-factor of FX-prothrombinase complex
VII	Stable factor, proconvertin	Activates FIX and FX
VIII	Antihemophilic factor A	Co-factor of FIX-tenase complex
IX	Antihemophilic factor B (Christmas factor)	Activates FX: Forms tenase complex with FVIII
X	Stuart-Prower factor	Prothrombinase complex with FV: Activates FII
XI	Plasma thromboplastin antecedent	Activates FIX
XII	Hageman factor	Activates FXI, FVII, and prekallikrein
XIII	Laki-Lorand factor	Crosslinks fibrin
XIV	Prekallikerin (F. Fletcher)	Serine protease zymogen
XV	HMWK-(F.Fitzgerald)	Co-factor
XVI	VWF	Binds to FVIII, mediates platelet adhesion
XVII	Antithrombin	Inhibits FIIa, FXa, and other proteases
XVIII	Heparin cofactor II	Inhibits FIIa
XIX	Protein C	Inactivates FVa and FVIIIa
XX	Protein S	Cofactor for activated protein C
TFPI	Tissue Factor Pathway Inhibitor	Binds FXa, forms a quaternary structure with TF/FVIIa to inhibit TF/FVIIa activity

1.3. Variability of the coagulation factors

The levels of these factors are likely to affect each other in numerous ways with both positive and negative influences [10].

Exceed thresholds of coagulation factors may disturb the fragile balance of hemostatic system, therefore leading to thrombotic or hemorrhagic disorders. In addition, plasma levels of coagulation factors have a substantial inter-individual variability in healthy population, as shown in Table 2. Reference ranges may vary with age, gender, race, pregnancy, diet, use of prescribed or herbal drugs, and stress [11, 12].

Table 2|Coagulation reference ranges

Normal value references	
Male %	42.5
Age (years)	44.8 (14.6–72.3)
D-dimer (ng mL^{-1})	74.9 (4.0–1608.9)
Fibrinogen(g L^{-1})	3.3 (1.7–6.3)
Prothrombin	103.8 (63.0–153.0)
TF (pg ml^{-1})	177 ± 37
FV	131.2 (47.0–277.0)
FVII	110.5 (41.0–171.0)
FVIII	121.8 (49.0–232.0)
FIX	102.9 (52.0–188.0)
FX	103.5 (49.0–163.0)
FXI	97.1 (55.9–203.4)
FXII	105.6 (18.0–191.0)
FXIII A	107.6 (45.0–203.0)
FXIII B	100.1 (47.0–176.0)
Prekallikerin	99.7 (50–193)
vWF antigen	98 (48–199)[a], 127 (66–245)[b]
vWF activity	81 (39–167)[a], 110 (62–195)[b]
Antithrombin	99.1 (63.0–125.0)
Heparin cofactor II (μM)	~1.2 ± 0.4
Protein C	62 – 126
Protein S	45 – 176
TFPI activity (ng mL^{-1})	49.4 ± 22.6
Free TFPI antigen (ng mL^{-1})	11.2 ± 3.05

All values are mean (range) except D-dimer [median (range)], sex (% male) and TFPI activity, TFPI antigen, TF antigen, and heparin cofactor II (Mean ± SD). All coagulation factors are expressed in U dL^{-1}. [a]type O group (ABO Blood Type), [b]non-type O group (ABO Blood Type). All ranges are found in the following normal population-based studies: [11, 13-17]

There are several coagulation factors that deserve detailed mention for having an important role in the coagulation cascade and in several hemostatic disorders (Table 1). Among them, FXI deserves a special attention in this dissertation.

2. Factor XI

Coagulation FXI was discovered nearly 50 years ago; however, its intricate role in hemostasis and thrombosis was not revealed until the past decade and in the recent years significant progress has been made on understanding its role in coagulation and anticipating and treating thrombosis by using FXI as a potential target [18].

As briefly mentioned before, FXI is a plasma serine protease zymogen with a key role in bridging the initiation phase and the amplification phase of blood

coagulation *in vivo*. FXI deficiency usually does not lead to spontaneous bleeding, but is associated with increased risk of bleeding with hemostatic challenges, while the severity of bleeding correlates poorly with the plasma level of FXI [19, 20].

2.1. Structure & synthesis

Human plasma FXI is primarily synthesized by hepatocytes. FXI can be also expressed outside of the liver in tissues such as renal tubules and pancreatic islet cells [21]. The existence of megakaryocyte-synthesized platelet FXI remains controversial and although platelet FXI contributes in less than 1% of total circulating FXI clotting activity its contribution to hemostasis is unclear [22]. However, a study has identified apolipoprotein E receptor 2 (ApoER2, LRP8), a member of the LDL receptor family, as a platelet receptor for FXI [23].

The approximately 23kb human *F11* gene contains 15 exons and is located in chromosome 4 (4q35). This gene has 7 transcripts (splice variants). Functional human *F11* mRNA has 3278 bp (Figure 2). FXI protein is a 160-kDa dimer comprised of identical subunits of 607 amino acids. Each subunit contains four 90- or 91-amino-acid repeats named apple domains (A1 to A4 from the N-terminus, heavy chain) and a C-terminal catalytic domain (light chain).

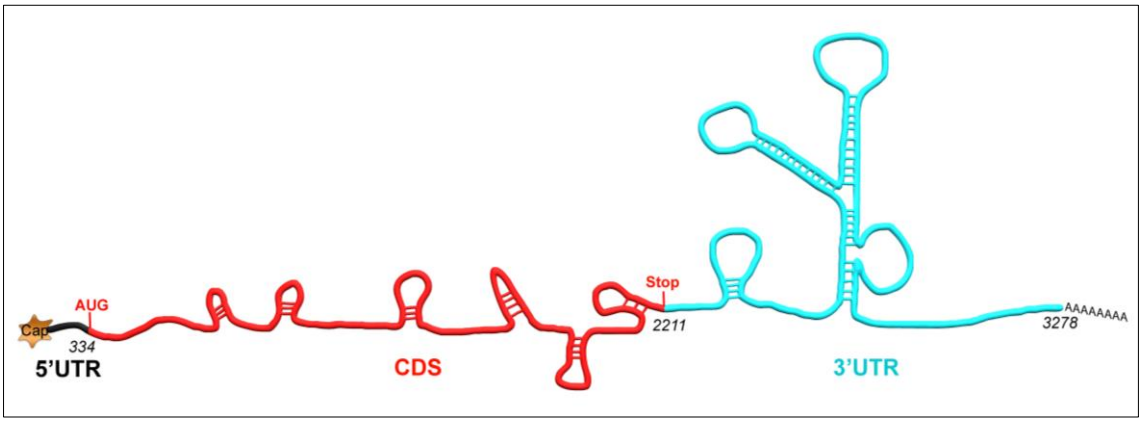

Figure 2| Homo sapiens coagulation F11 mRNA structure. NCBI Reference Sequence: NM_000128.3. (Including 5'UTR, CDS and 3'UTR)

2.2. Molecular pathology of FXI deficiency (hemorrhagic disorders)

FXI deficiency was first described as hemophilia C by Rosenthal et al. in 1953 [24]. Typically inherited as an autosomal recessive injury related hemorrhagic disorder, FXI deficiency also may behave as a dominant trait in some families. It occurs with equal frequency in men and women. Homozygotes and compound heterozygotes usually have FXI levels of less than 20 U/dL (severe deficiency); heterozygotes commonly reach up to 20 to 60 U/dL (moderate deficiency) or 61 to 80 U/dL (mild deficiency). FXI deficiency is rare, observed with a frequency of approximately 1/10,000 worldwide (http://www.factorxi.org). Although inherited FXI deficiency has been found in almost all ethnic groups, it has a relatively higher frequency in the Ashkenazi Jews, French Basques, and in English patients from the north west of England [25-27]. In the past two decades, more than 220 mutations in the FXI gene have been reported in patients with FXI deficiency, of which 7 showed a founder effect. Apart from the mutations that are common in these discrete populations, the mutational spectrum is wide and heterogeneous in other ethnic groups [28]. Recently, one novel and seven recurrent mutations have been identified in Chinese FXI deficient patients [29], whose signature of founder effect in the Chinese population and its potential relationship with other Asian population remains to be established. In addition, two single nucleotide polymorphisms (SNPs) in *F11* gene (rs2289252 and rs2036914) were independently associated with high levels of plasmatic FXI and deep venous thrombosis [30].

2.3. *F11* as a new target to prevent thrombosis

It is noteworthy that hereditary FXI deficiency in humans yields mild bleeding tendency and, in turns, certain protective effects from thrombotic diseases [31]. On the other hand, a high level of FXI has been associated with thrombotic events [31, 32]. Therefore, inhibition of *F11* has been proposed as a novel approach in the development of new antithrombotic target to achieve an improved benefit-risk ratio. Several groups have developed strategies to target this molecule for protecting from thrombosis in animal models of arterial or vein

thrombosis: knock-out (KO) mice, antisense oligonucleotide knock down of *F11* expression, neutralizing antibodies, and peptidomimetic inhibitors [33].

All these strategies have showed that reducing FXI levels attenuates thrombosis without causing bleeding in mice. In humans, it has been very recently published that reducing FXI levels by using an antisense oligonucleotide that targets *F11* in patients undergoing elective primary unilateral total knee arthroplasty, was an effective method in the prevention of postoperative venous thrombosis, and appeared to be safe with respect to the risk of bleeding [18].

3. Hemostatic disorders

The first medical description of the clinical and inherited features of hemostasis can be dated back more than 1000 years. Since then, continuous and revolutionary scientific developments have contributed to decode this essential physiologic puzzling process that is finely regulated. Imbalances in hemostasis can trigger abnormalities that may contribute to bleeding and/or thrombosis. Besides the essential role of hemostasis in protecting vascular integrity and maintaining normal blood flow, accumulating data suggest lately two-way relationship between hemostasis and inflammation, underscoring the role of both systems in many complex diseases, including atherothrombosis, cardiovascular, and liver diseases. Interestingly, numerous studies in animals have also documented that hemostasis is closely linked to the pathophysiology of atherogenesis and cardiovascular diseases [34, 35]. Indeed the hemostatic profile of patients with liver diseases is frequently different from that of healthy individuals [36]. The liver is the primary site for synthesis of most procoagulant and anticoagulant proteins; therefore, the coagulopathy of liver disease is complex. It has long been assumed that patients with liver disease have a natural bleeding tendency and are protected from thrombosis. This assumption is false; the average patient with liver disease is actually in a state of "rebalanced hemostasis" that can relatively easily be tipped toward both bleeding and thrombosis [36].

Hemostatic disorders are probably multifactorial; genetic, environmental, and acquired factors act jointly for defining the threshold for the individual thrombotic

or hemorrhagic risk. These factors may be able to misbalance the hemostatic equilibrium resulting in hemostatic disorders. Better characterized genetic defects in natural anticoagulant systems are FV Leiden, the prothrombin G20210A variation (PT20210A), high levels of clotting FVIII, FIX, and FXI, and antiphospholipid antibodies, all of them associated with an increased tendency toward venous thrombosis [37]. Among the genetic elements that drive the synthesis of coagulation factors, a hereditary component has been described for several of them although the heritable basis for high or low levels of factors remains unknown [11]. Interestingly, it was also described that common regulatory genes coordinate simultaneously the expression of several clotting factor genes which would allow to categorize individuals in those with high or low levels of coagulation factors [11, 38].

Despite growing insight into identification of such genetic factors, including genome wide association studies, up to 30% of thrombosis are idiopathic. Therefore more research is needed to completely clarify the pathophysiology of human hemostasis. Then, new trigger mechanisms and novel elements able to affect the hemostatic system should be identified to the development of new and better treatments to reduce the incidence of thrombosis.

Moreover, common risk factors for venous thrombosis fail to improve prediction models for thrombosis and so compromise our ability to tailor prophylactic treatment [37].

4. Non-coding RNAs

The central dogma of biology that information flows basically from DNA to RNA to protein is nowadays opened to new dimensions. Recent studies have shown that although most of the human genome gets transcribed much of such transcripts do not code for proteins. This implies that the non-coding transcripts or non-coding RNAs (ncRNAs), which have been found occupying a major portion of the genomic space, are also final functional forms. As a consequence, proteins are not the only functional molecules in the cell, as previously thought, but also ncRNAs are regulatory RNAs that contribute by diverse mechanisms to gene expression and regulation (Figure 3) [39].

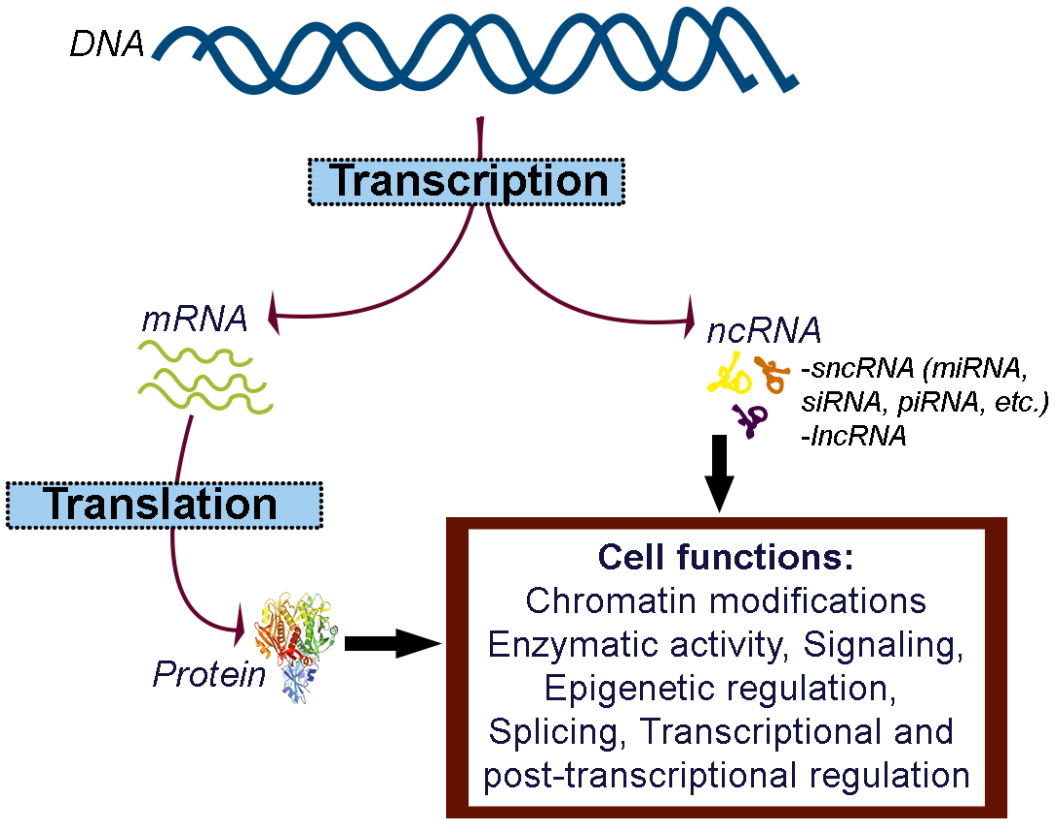

Figure 3| Recent and novel diverse functions for ncRNAs. NcRNAs can be as functional and efficient as proteins

A large heterogeneity between ncRNA has been described (Table 3). In an attempt to classify them, two major categories have been established according to their average sizes: 1) the small non-coding RNAs (sncRNAs) and 2) the long non-coding RNAs (lncRNAs). The sncRNAs include microRNAs (miRNAs), small interfering RNAs (siRNAs), PIWI interacting RNAs (piRNAs), and Alu RNAs among others. The other classes of abundant ncRNAs, the lncRNAs, are > 200 nucleotides in length. (Table 3)

With the improved methods to explore the transcriptome, important advances in identifying and understanding ncRNAs have been developed in recent years. The research described in this thesis is focused in miRNA function thus, in the following sections concepts and special challenges in miRNA profiling are discussed.

Table 3| Different non-coding RNAs [39]

Type of RNA	Length (nt)	Origin	Function
miRNAs	20-22	Processed by Drosha from endogenously coded pre-miRNAs	Repression by degradation of target mRNAs and translational inhibition
Mirtrons	~22	Derived from introns by a Drosha-independent pathway	Function similar to miRNAs
siRNA	20-22	Centromeric, telomeric, and other repetitive sequence	Post-transcriptional gene silencing via RNA interference mechanism
piRNA	26-31	Transcribed from piRNA clusters. They are lined up end-to-end and originate from the same strand	Post-transcriptional gene silencing of retrotransposons in germ line
rasiRNA	26-31	Formed from annealed transcripts of transposable elements	Sub-class of piRNAs, involved in maintenance of heterochromatin, regulation of transcription from repetitive sequences
tRFs	15-25	Derived from processing of tRNAs	Translational repression; control of silencing activities of miRNA and siRNA
sdRNA	17-30	Small nucleolar RNAs are processed to give sdRNAs	Putative role in gene silencing
crasiRNA	~40	Transcribed from satellite repeats in centromere	Maintenance of centromere integrity and repressive histone marks in that region
AluRNA	~300	Transcribed from Alu-retroposons	Cause repression by forming transcriptionally inert complexes with RNA pol II
TiRNA	17-18	Transcribed from sequences immediately downstream to transcription start sites of many actively transcribed genes	Hypothesized to position nucleosomes and cause gene activation
saRNA	~19	Synthetic small dsRNA exogenously injected into cells. Endogenous sources yet unknown	Activation by mediating loss of lysine-9 methylation on histone 3 at the target sites
PASRs	<200	Transcribed from 5'UTRs of genes	Proposed to mediate both activation and repression of genes
AAGAG NuMat RNA	~3kb	AAGAG satellite repeats	Structural maintenance of nuclear matrix
lncRNAs	>200	Transcribed from their respective genes	Act by diverse mechanisms to both repress and activate target gene expression

4.1. miRNAs

Since their discovery, miRNAs have provided a new perspective and area of investigation on their role in the regulation of gene expression. [40]. MiRNAs are abundant regulatory RNAs involved in the regulation of many key biological processes. They are conserved post-transcriptional regulators of gene expression that are integral to almost all known biological processes, including cell growth, proliferation and differentiation, as well as metabolism and apoptosis [41].

For instance, miRNAs are predicted to constitute approximately 3% of human genes and may contribute to the post-transcriptional regulation of more than 60% of total human genes [42, 43].

4.1.1. The miRNA revolution

More than two decades ago, the existence of miRNAs was completely unknown [44]. In 1993, the importance of miRNAs began to be revealed when Ambros and his colleagues discovered a single-stranded non-protein-coding regulatory RNA molecule in *C. elegans*; lin-4, which acts by negatively regulating the level of LIN-14 protein, creating a temporal decrease in LIN-14 protein starting in the first larval stage. Authors hypothesized that lin-4 regulated LIN-14 gene, in part through Watson base pairing, revealing the first miRNA and mRNA target interaction [45]. Not until eleven years later the second miRNA in *C. elegans* and the first one in humans were discovered [46]. Two years later, Calin *et al.* showed the correlation between miRNA abundance and human disease, particularly in B-cell leukemia [47].

Since then, several breakthroughs leaded to a miRNA revolution that unequivocally established miRNAs as important molecules in the control of post-transcriptional regulation in both physiological and pathological processes (Figure 4).

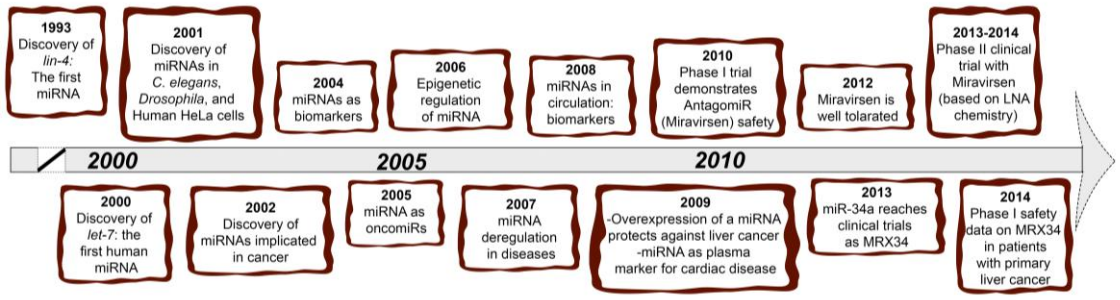

Figure 4| Historical point of view of the key discoveries made in the miRNA field.

Moreover, miRNAs represent a rapidly growing research area, the number of miRNA-related publications is increasing exponentially every year, reaching more than 42,860 available papers until 2015 in PubMed (Figure 5). These numbers highlight that miRNA regulation is still a very active and productive research field.

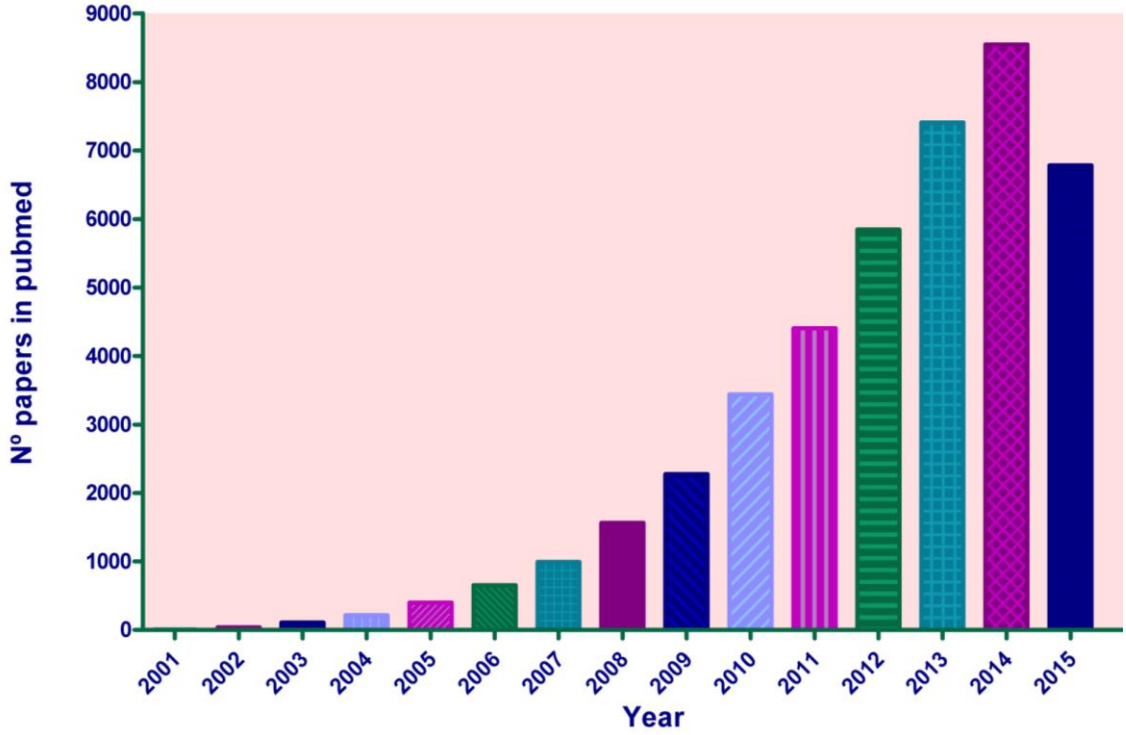

Figure 5| Number of articles in PubMed: Representing graph of number of entries (articles) in PubMed published every year (http://www.ncbi.nlm.nih.gov/pubmed)

4.1.2. Structure and biogenesis of miRNA

MiRNAs are short endogenous RNAs that regulate protein expression from targeted genes by pairing to sites in the 3′UTR [48]. A complete list and details about the nomenclature of the miRNA can be reviewed at miRBase Sequence Database - Release 21 from June 2014 (http://www.mirbase.org/) [49]. To date, miRBase database has catalogued 35,828 mature miRNA products in 223 species; among these 2,588 corresponding to human mature miRNAs sequences.

MiRNA biogenesis pathway in animals can be divided into two steps (Figure 6). Initially, miRNAs are transcribed by RNA polymerase II as primary miRNAs (pri-miRNAs) with hundreds to thousands of nucleotides in length. A ribonuclease III (RNase III) enzyme (Drosha) cleaves the flanks of pri-miRNAs to liberate ~70 nucleotide stem-loop structures, called precursor miRNAs (pre-miRNAs). Pre-miRNA hairpins are exported from the nucleus by Exportin-5. In the cytoplasm, the pre-miRNAs are processed into ~22 nucleotide duplex miRNAs (miR-3p/miR-5p) by the RNase III enzyme Dicer. Next, one or both strands of the miRNA duplex are incorporated into the protein complex RISC (RNA-induced

silencing complex) to function as a guide, directing the silencing of target mRNA. When complementarities between the 3'UTR mRNA and the miRNA are perfect, the target mRNA is degraded; however if the complementarities are partial (non-perfect pairing), the translation of the target mRNA may also be repressed (Figure 6) [50-54].

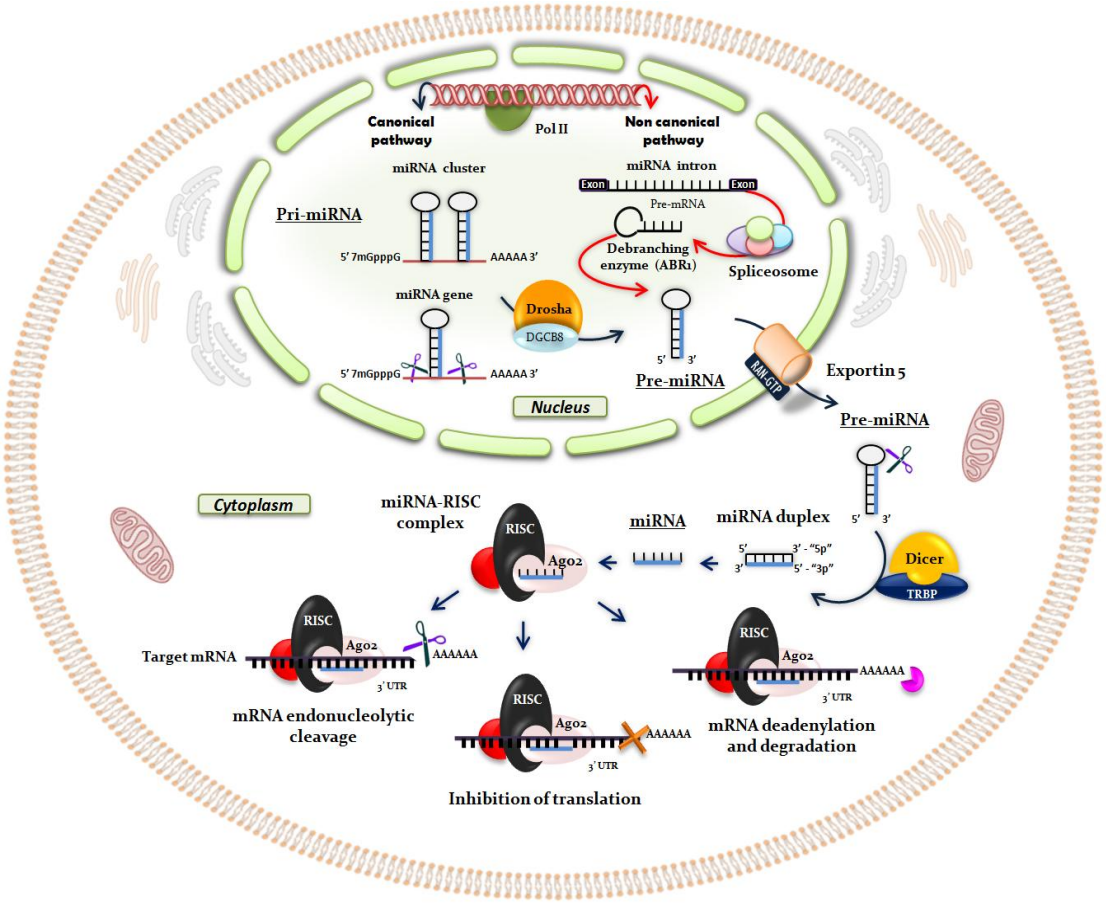

Figure 6| MiRNA biogenesis and mechanisms of target regulation. Reprinted with permission of John Wiley & Sons, Inc. from Journal of Thrombosis and Haemostasis [55], copyright (2015).

4.1.3. Mechanisms of action of miRNAs

In plants, miRNAs can perfectly base pair with targets and induce their cleavage and degradation by an RNAi-like mechanism. Conversely in animals, the majority of miRNAs only form imperfect duplexes with their targets.

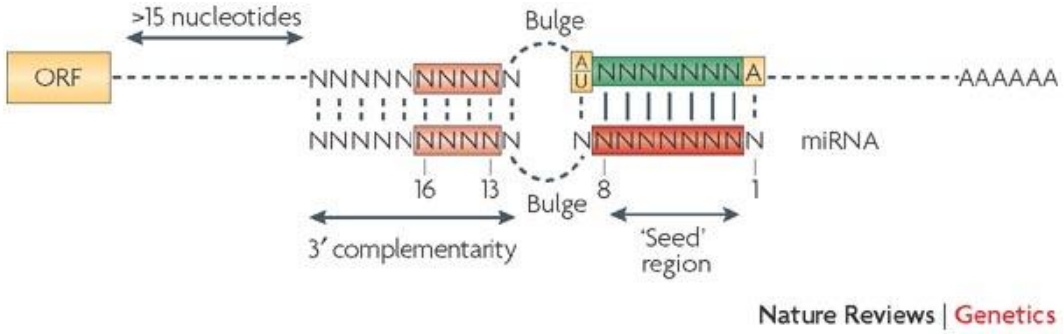

Figure 7| MiRNAs interact with their mRNA targets by base pairing. Reprinted by permission from Nature Publishing Group: [Nature Reviews (Genetic)] [56], copyright (2009).

Both experimental and bioinformatics analyses helped to identify and to characterize the canonical miRNA-target interaction in animals and that most of the *in silico* miR-algorithms apply for their predictions (Figure 7). Briefly, the key essential interactions [56] can be set as follows:

a) The 'seed' region is a common motif that usually represents 2-8 nucleotides of base pairing between miRNA and target (shown in dark red and green in Figure 7). In order to improve this site efficiency, an A residue across position 1 of the miRNA and an A or U across position 9 is found (shown in yellow in Figure 7).

b) There is a central bulge that is also present in the region of miRNA-mRNA duplex that helps to preclude the Argonaute 2 (AGO2)-mediated cleavage of mRNA.

c) Residues 13-16 of the miRNA (shown in orange in Figure 7) are also important especially when seed region binding is insufficient as it stabilizes the whole interaction [56].

Nevertheless, more recently a variety of non-canonical miRNA-target interactions have been described in different species demonstrating that the interaction of miRNAs with their target is much more complex and heterogeneous than what was expected few years ago [57]. Thus, animal miRNAs could: i) bind imperfectly to their targets but having a perfect pairing in the seed region (Figure 8b) or ii) in the absence of perfect seed pairing, the extensive pairing of the 3′ end of the miRNA to the target sequence plays a role to compensate (Figure 8c). MiRNA could also regulate genes joining to middle sequences of target sites (Figure 8d). In addition, the regulation of mRNAs through coding regions (Figure 8e) has also been described [57].

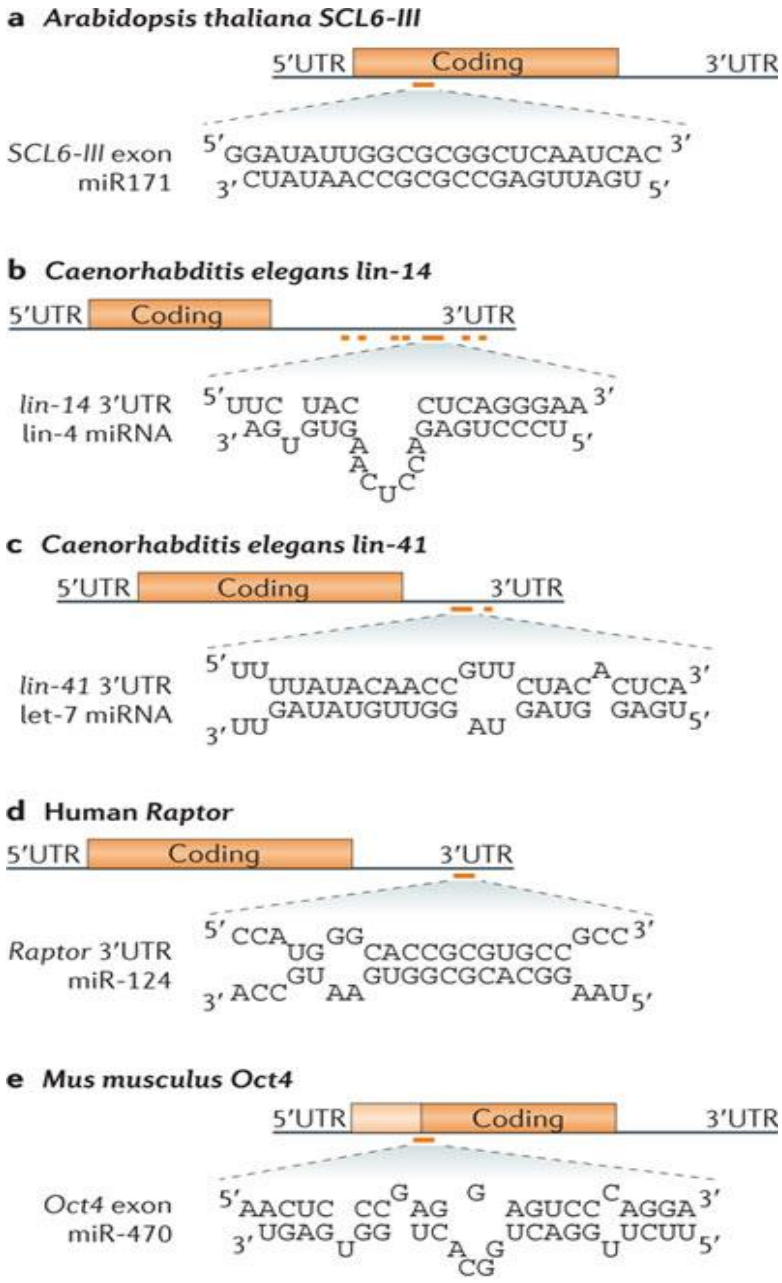

Figure 8| Examples of functional miRNA target sites. Different degrees of base pairing mediate target recognition by miRNAs. a) Only in plants miRNAs could bind perfectly base pair with targets. b), c), d), e) different non-canonical interactions in animals. Reprinted by permission from Nature Publishing Group: [57], copyright (2015).

Indeed the greater part of miRNA functional studies are related to miRNAs that bind to the 3'UTR of their target transcripts, however the current dogma about miRNA function is lately changing providing new evidence for different sites of action such as the 5'-UTR [58], coding region [59] or promoter [60] of genes (Figure 9).

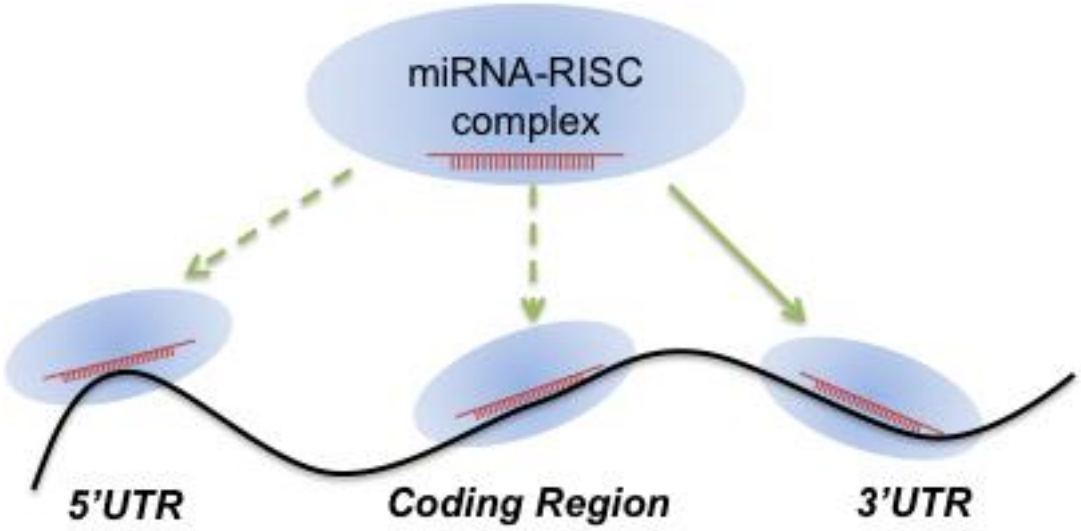

Figure 9| MiRNA target sites in either 3'UTR, coding region or 5'UTR of genes.

It is worth mentioning that one miRNA may regulate many genes as its targets (even hundreds), at the same time as one gene may be targeted by many miRNAs (Figure 10), indicating that relationships between miRNAs and their targets may not be one-to-one [57-59].

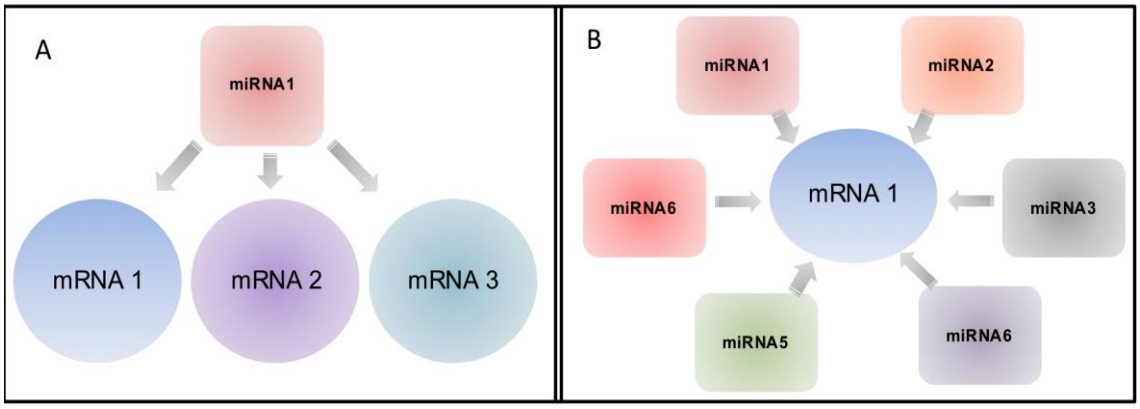

Figure 10| Widespread regulation between miRNAs and mRNA targets.

4.1.4. MiRNA binding sites in the coding region of mRNAs

While it is well established that miRNAs bind to 3'UTRs to destabilize the target mRNA being 8mers/canonical sites the most effective sites, a recent work suggests, as mentioned before, that miRNA binding to the CDS also inhibits translation. The regulatory function of binding sites located in the CDS remains more indefinable and a non-canonical mechanism of action has been proposed (Figure 11) [57, 59, 61].

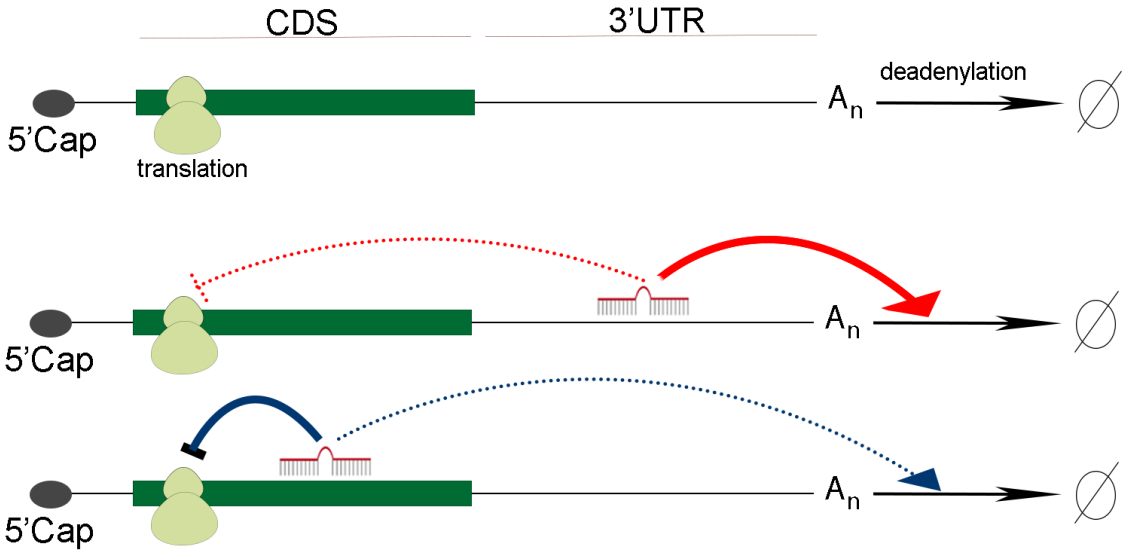

Figure 11| Representation of possible miRNAs binding effects on the 3'UTR or CDS of mRNA. (A_n): poly-A tail. mRNA deadenylation occurs mainly when miRNAs bind to the 3'UTR while binding to CDS primarily inhibits translation. Solid lines represent the strong preference of each miRNA to target the 3' UTR (sold red line) or the CDS (solid blue line), while dotted lines represent the less preference to target.

MiRNA binding to 3'UTRs leads to changes in protein abundance mainly by mRNA deadenylation while binding to sites in the CDS mainly represses the translation with little impact on the polyA tail of the target mRNA (Figure 11). Additional work would be helpful to better characterize the magnitude and the time-scale of gene regulation by CDS sites.

4.1.5. Tools for identifying potential miRNA targets

Approaches to identification of potential targets of miRNA could be diverse; forward genetics where the study starts with a phenotype and moves toward the identification of a miRNA related to such phenotype [62] or reverse genetics where the study starts with a miRNA and assays the effect of the genes disruption [63].

Since most miRNA researches begin with the identification of the miRNA, an important approach is to predict miRNAs targets by *in silico* algorithms. Current prediction methods are diverse and open access (Table 4), both in approach and performance to facilitate the analysis of miRNAs and their target prediction [50].

Table 4| Tools for predicting mammalian miRNA targets

Tool	Criteria for Prediction and Ranking	Website URL	Reference
Site Conservation Considered			
TargetScan	Stringent seed pairing, site number, site type, site context (which includes factors that influence site accessibility); option of ranking by likelihood of preferential conservation rather than site context	http://targetscan.org	Friedman et al., 2008
PicTar	Stringent seed pairing for at least one of the sites for the miRNA, site number, overall predicted pairing stability	http://pictar.mdc-berlin.de	Lall et al., 2006
ElMMo	Stringent seed pairing, site number, likelihood of preferential conservation	http://www.mirz.unibas.ch/ElMMo2	Gaidatzis et al., 2007
mirSVR	Moderately stringent seed pairing, site number, pairing to most of the miRNA	http://www.microrna.org	Betel et al., 2008
miRBase Targets	Moderately stringent seed pairing, site number, overall pairing	http://www.mirbase.org	Griffiths-Jones et al., 2008
PITA Top	Moderately stringent seed pairing, site number, overall predicted pairing stability, predicted site accessibility	http://genie.weizmann.ac.il/pubs/	Kertesz et al., 2007
Site Conservation Not Considered			
TargetScan	Stringent seed pairing, site number, site type, site context (which includes factors that influence site accessibility)	http://targetscan.org	Agarwal et al., 2015
PITA All	Moderately stringent seed pairing, site number, overall predicted pairing stability, predicted site accessibility	http://genie.weizmann.ac.il/pubs	Kertesz et al., 2007
RNA22	Moderately stringent seed pairing, matches to sequence patterns generated from miRNA set, overall predicted pairing and predicted pairing stability	http://cbcsrv.watson.ibm.com	Miranda et al., 2006

As aforementioned, most plants miRNAs have nearly perfect complementarity with their target, which makes the target identification much easier. Nevertheless in animals the identification of target sequences is hard by standard sequence comparison as miRNAs complementarity to target sequences is not perfect [64]. Currently, there are several web servers that contain predicted targets for miRNAs across many species. Probably the most

common and updated software used is miRSVR software (http://www.microrna.org) [65, 66]. miRSVR algorithm identifies potential binding sites and then checks the potential target site in the 3'UTR to see whether the sites are conserved in orthologous transcripts. The TargetScan algorithm identifies mRNAs with conserved pairing to the 5' region of the miRNA and assesses the quantity and quality of these complementary sites (http://www.targetscan.org) [62]. Another algorithm, PicTar, predicts miRNA targets in vertebrates and Drosophila species and has the ability to predict targets for single miRNAs and for combinations of miRNAs (http://pictar.mdc-berlin.de) [63]. Overall, these programs have allowed for the creation of target databases that may potentially facilitate functional studies for miRNAs.

The main disadvantage of theses algorithms is that given the size of seed regions (2-7 nucleotides), a high number of matches may emerge from each search. showing that ~70% of predictions may be false [67]. Despite that predictions of algorithms are based on different principles such as base-pairing pattern, evolutionary conservation, secondary structure and nucleotide composition, they are still lacking sensitivity and specificity. Thus many degrees of false positives and false negatives are present, not only because most algorithms have a poor overlap and produce extensively discordant predictions that are difficult to evaluate but also because miRNAs can follow other rules of binding uncovered by theses algorithms [non-canonical, (Figure 8)]. Combining the results of several algorithms could be a good practice to perform a reliable *in silico* study. Another disadvantage is that most of algorithms are unable to predict possible interactions that may occur outside the 3'UTR, such as 5'UTR or CDS, except RNA22.

In order to resolve these obstacles, biochemical approaches are also recently used to identify endogenous target sites by several techniques such as immunopurification of miRNA ribonucleoprotein (miRNP) complexes, sequencing those that co-immunoprecipitate with miRISC factors using techniques such as CLIP coupled with high-throughput sequencing (CLIP-seq) or high-throughput sequencing together with CLIP (HITS-CLIP). The most successful and extensive applications of CLIP are identifying miRNA targets. The first report of this type of works is from 2009, describing the analysis of an

Ago2-miRNA-mRNA ternary complex in mouse brain using HITS-CLIP [68]. Moreover, Aleksandra *et al.* reported over 18,000 miRNA-target interactions, and found that approximately 60% of seed interactions are non-canonical [69]. Today, there are non-canonical targets identified by taking advantage of CLIP data. CLIP and its variants are accurate, and serve as a high-throughput method to decode RNA-protein interactions, with excellent application prospect in studies of RNAs [70]. Recently databases with CLIP results are available to complement *in silico* data [71]. This can be coupled with a genome-wide scale through transcriptome profiling using microarrays or RNA sequencing (RNA-seq) and through proteomic approaches, such as stable isotype labelling with amino acids in cell culture (SILAC) followed by quantitative mass spectrometry to identify miRNA-mRNA interactions. Overall, these approaches complement *in silico* approaches to be more accurate to miRNA target identification and also to reveal uncommon miRNA-target interactions [57].

The development of precise and fast assays for miRNA target identification and verification will play a significant role in the study of miRNA functions and the biological processes in which they are involved [44, 72-74].

4.1.6. The impact of 3'UTR variants

Gene expression differences due to inherited factors may be caused by variations in enhancer or promoter binding sites, variations in epigenetic regulation impacting methylation or chromatin modifications, variations in expression of trans-acting factors or differences in regulation by miRNAs. Single nucleotide polymorphisms (SNPs) falling specifically in the 3′UTR of genes may interfere with mRNA stability and translation through effects on polyadenylation and regulatory protein-mRNA and miRNA-mRNA interactions [63, 75].

It has been proposed [76, 77] that the direct binding of miRNAs to regulatory regions can be altered by the existence of genetic variants (called miR-SNPs) localized within mRNA regulatory regions or mature miRNAs and can modulate the miRNA–mRNA interaction creating or destroying miRNA binding sites.

Then, a miR-SNP may provoke the following possible situations (Figure 12):

a) Creation of a new miRNA binding site due to the presence of a miR-SNP in the 3’UTR mRNA region (either near or within the binding site) or

to a miR-SNP located in the miRNA sequence (Figure 12A). These miR-SNPs may inhibit the expression of the new created target.

b) Deletion of a miRNA binding site due to the presence of a miR-SNP in the 3'UTR of an mRNA sequence (either near or within the binding site) or in the miRNA mature sequence (Figure 12B). These miR-SNPs may increase target expression. Importantly, miR-SNPs located in 3'UTR outside the miRNA binding region may also alter mRNA-RISC interaction [78].

c) Modification of the levels of miRNA expression due to a miR-SNP in or close to the MIRNA gene. These miR-SNPs may either inhibit or increase target expression depending if miRNA expression levels are positive or negatively affected.

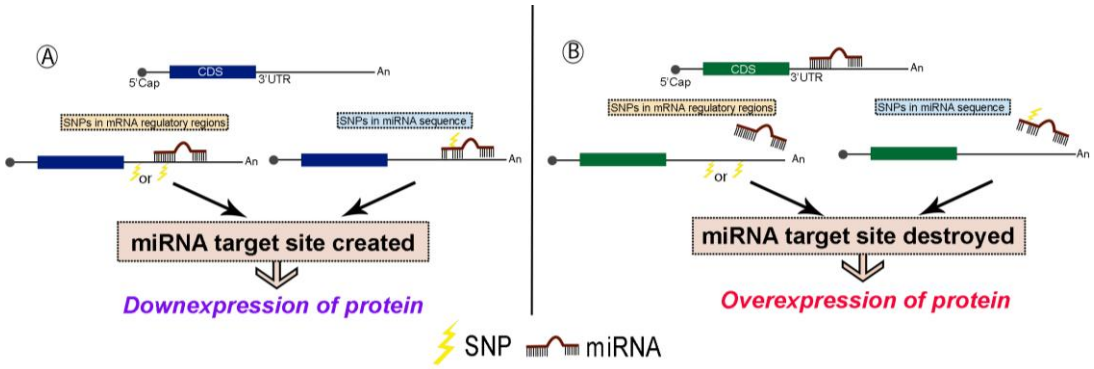

Figure 12| Diagram of interaction between miRNAs and genes to regulate gene/protein expression. A and B: Mutation/polymorphism in the 3′-untranslated region (UTR) of a gene may modify the affinity of a miRNA for the 3′-UTR of the gene and, therefore, alter the protein expression.

Variants in regulatory regions are predicted to play an important role in disease susceptibility of common diseases. SNPs mapping to miRNA binding sites have been shown to disrupt the ability of miRNAs to target genes resulting in differential mRNA and protein expression. Preliminary studies in humans have identified variations in the 3′UTR of genes that appear to affect cancer risk by disrupting normal miRNA binding [79]. One such variant in the *KRAS2* gene increases the risk for lung and ovarian cancer by changing the ability of miRNA *let-7* to bind [80]. Recently, it has been suggested that a functional variant in *IL1A* 3' UTR located within miRNA-122 binding site affects individual susceptibility to human papillomavirus associated oral squamous cell carcinoma [81]. Moreover, in neurodegenerative disorders such as Alzheimer's disease, it has also been shown that specific variants in the 3'UTR mRNA of amyloid

precursor protein may affect the miRNA binding and as consequence, the amyloid protein function [82]. Current studies suggest that miRNA-mRNA interaction could be affected by multiple genetic SNPs (for review see [77]) .

4.1.7. miRNA roles beyond gene expression control

Beside the role that miRNAs execute as endogenous regulatory element (Figure 13), a plethora of recent works have shown that miRNAs may have many other functions outside the tissues where they are transcribed.

a. miRNAs as biomarkers: miRNA detection in body fluids is emerging in the field of biomarkers. Thus, the discovery of miRNAs in body fluids opens up the possibility of using them as non-invasive biomarkers in the detection of several pathologies including cancer, cardiovascular diseases and more recently, in venous thrombosis [83-85]. As well, this possibility is being spread to as predictors of therapy response in clinical trials [86].
b. Exogenous regulatory element: miRNA could also be transported in plasma and delivered to recipient cells by high density lipoproteins, exosomes, microparticles or bound to Ago2 [87]. For example, different studies have shown that miRNAs from platelets may have a real impact in the function of endothelial cells and thus in pathologies where endothelium plays an important role such as thrombosis, angiogenesis, atherosclerosis, etc. [88, 89].
c. Novel therapy: more specific regulation of miRNA activity can be achieved using miRNA mimics or anti-miRs such as locked nucleic acids (LNAs), antagomirs, and miR sponges, which bind and thereby functionally block specific miRNAs [90]. Although most miRNA therapeutics are still in preclinical development, two have currently reached clinical trials: one (LNA)–modified antisense oligonucleotide targeting liver-expressed miR-122 (miravirsen; phase 2 clinical trial) for treatment of hepatitis C virus infection [91] and one miRNA mimic, miR-34a (MRX34; phase 1 clinical trial) to inhibit cancer cell proliferation and tumor growth [92, 93].

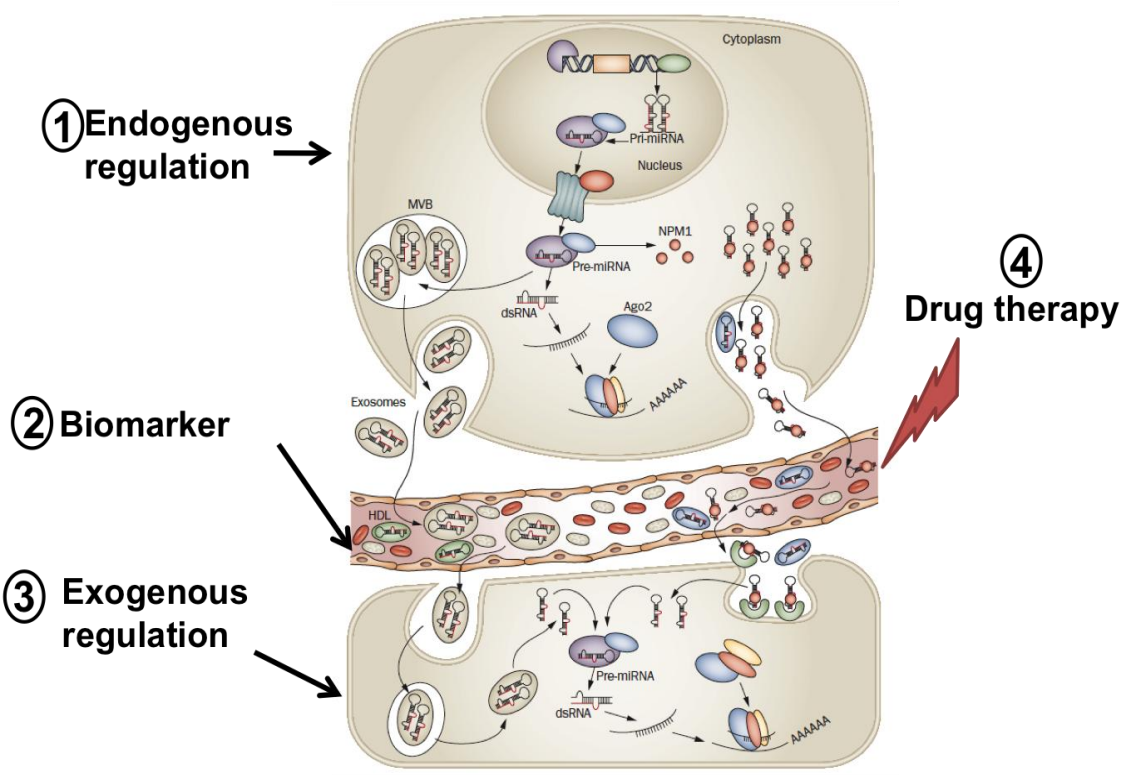

Figure 13| Biogenesis and different mechanism of action of miRNAs. MiRNA could be considered as 1) endogenous regulatory elements, 2) biomarkers in vessel wall 3) exogenous regulatory elements or 4) novel therapy. Modified and reprinted by permission from Nature Publishing Group: [Nature Reviews Clinical Oncology] [92], copyright (2011).

4.1.8. Regulation of hemostasis by miRNAs

Anucleate platelets are now assumed to contain transcripts that might relate to other physiological or pathological circumstances, be released into the circulation, participate in protein formation, and engage in horizontal RNA transfer to other vascular cells [84].

These platelet transcripts include miRNAs that seem to participate in vascular homeostasis, inflammation, and platelet function. In addition, levels of platelet miRNAs in the circulation are associated with the presence or extent of cardiovascular diseases, such as atrial fibrillation and peripheral vascular disease. Emerging data suggest mechanistic roles for platelet-derived miRNAs in hemostasis, thrombosis, and diseases such as unstable coronary syndromes [84]. In addition, miRNAs are emerging as significant regulators of mRNA complexity of several coagulation factors [55]. To date only few recent studies have demonstrated that key haemostatic proteins such as PAI-1 [94-97],

fibrinogen [98], TF [99-101], antithrombin [102], protein S [103] or VWF [104] may be regulated by miRNA, probably just reflecting the tip of the iceberg concerning regulation of hemostasis by miRNA (For review see [55]) (Figure 14).

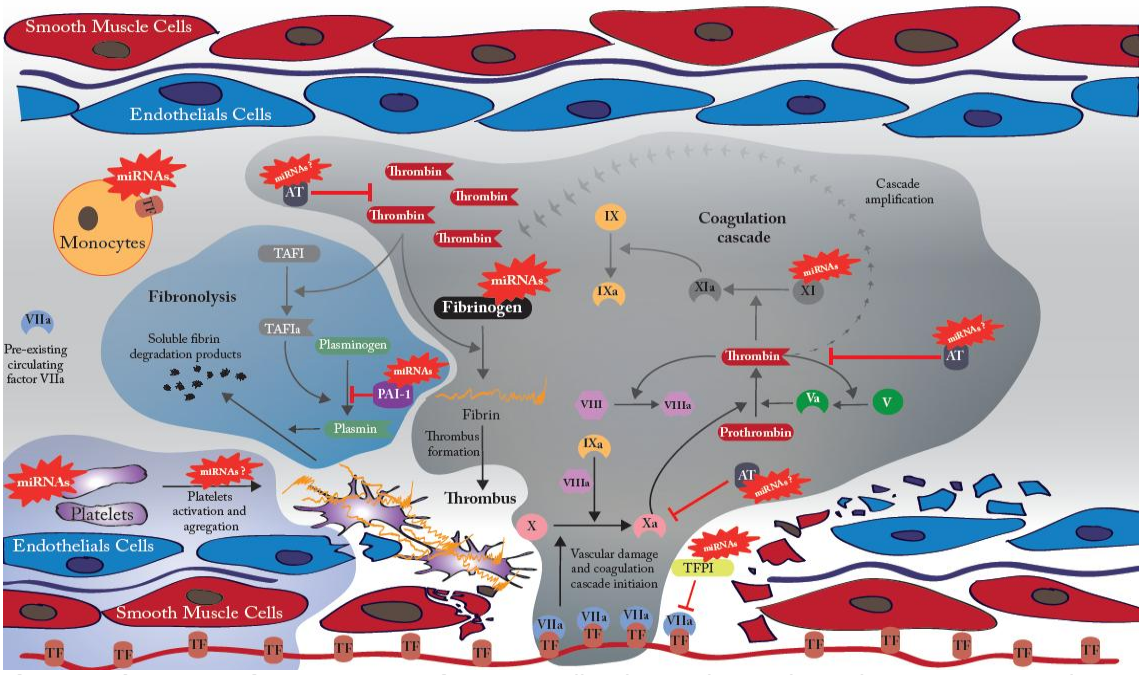

Figure 14|Hemostatic system & miRNA. Studies have shown that miRNAs exert regulatory mechanisms in different elements of hemostasis. Reprinted with permission of John Wiley & Sons, Inc. from Journal of Thrombosis and Haemostasis [55], copyright (2015).

Thus, until now up to seven coagulation factor genes have been described to be targets of miRNAs (Table 3). The more intuitive and better-described mechanism of action of miRNA on these hemostatic proteins is a CIS-suppressive-regulation on target genes. However, indirect regulation of hemostasis by miRNAs is still an unexploited research field.

Table 5|miRNAs related to coagulation factors:

Hemostatic factor	miRNA	Reference
Fibrinogen	miR-409-3p and miR-29 family	[98]
Antithrombin	miR-18a	[99-101, 105]
TF	miR-17-92 cluster, miR-223	[99-101, 105]
PAI-1	miR-421, miR-30c, miR-143/-145, miRNA-181b, miR-449a/b	[94-97]
Protein S	miR-494	[103]
vWF	miR-24	[104]
TFPI	miR-27a/b, miR-494	*Arroyo et al. 2015; Omar Ali et al. 2015 (submitted)*

5. The hepatocyte nuclear factor-4-alpha (HNF4α) and coagulation

Hepatocyte-nuclear-factor 4 alpha (HNF4α, gene symbol *HNF4A*) also known as NR2A1, is a member of the super-family of nuclear receptors. Since its discovery and characterization in the early 90's, HNF4α, essential for liver homeostasis, has been widely associated with the transcriptional regulation of genes specifically implicated in lipid metabolism, glucose metabolism, differentiation, morphogenesis and coagulation [106]. HNF4α is one of the most highly conserved nuclear receptors and is found in every animal organism examined thus far, from sponge to man. It is expressed in liver (hepatocytes), kidney (proximal tubules), small intestine, colon and pancreas (beta cells), although hepatocytes remain one of the highest expressing cell types [106, 107].

HNF4α is at the center of a complex network of transcriptional control involving other HNFs and NRs [108] and has been directly linked to several human diseases including diabetes, hemophilia, and hepatitis B viral infections [109] (Figure 15). Mutations in the *HNF4A* coding region and promoter are directly linked to Maturity Onset Diabetes of the Young 1.

Ongoing works as well as background information on HNF4α and an on-line tool to predict HNF4 binding sites in any DNA sequence can be looked up HNF4 Motif Finder (http://www.sladeklab.ucr.edu/HNF4.shtml).

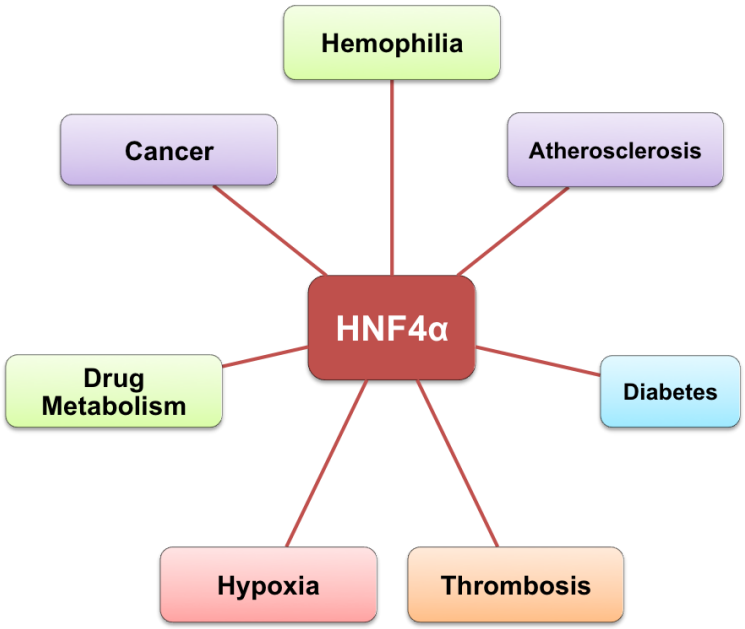

Figure 15| HNF4α and human disease. HNF4α is linked to several human diseases via mutations in its binding sites (hemophilia, diabetes) or in the *HNF4A* gene itself (diabetes). It is also indirectly linked to many human diseases via the target genes it regulates

5.1. HNF4α and coagulation

Since HNF4α was first identified as a factor that bound DNA response elements in the promoter region of liver-specific genes, many target genes were quickly identified using classical promoter deletion techniques. These genes encode many of the proteins involved in intermediary metabolism, as well as those involved in blood maintenance, such as *F8*, *F9*, *F10*, etc. (Table 6).

Full-body *Hnf4a* knockout mouse is embryonic lethal which has demonstrated that HNF4α is essential for development [110]. In turns, gene targeting using short interfering RNA (siHNF4A) confirmed the impact of HNF4α in regulating hepatic coagulation transcription. Thus, *in vitro* and *in vivo* studies confirmed that hepatic HNF4α is critical for regulating several procoagulant, anticoagulant as well as fibrinolytic genes in the liver. From these data, the HNF4α importance in blood coagulation homeostasis is confirmed [111, 112].

Table 6| Hemostatic factors regulated by HNF4α

Symbol	Full Gene Name	References
F2	Prothrombin	*Ceelie et al. 2003*
F7	Coagulation F VII	*Erdmann et al. 1995, Arbini et al. 1997*
F8	Coagulation F VIII	*Figueiredo and Brownlee 1995*
F9	Coagulation F IX	*Reijnen et al. 1992, Naka and Brownlee 1996*
F10	Coagulation FX	*Miao et al. 1992*
F11	Coagulation F XI	*Tarumi et al. 2002*
F12	Coagulation F XII	*Farsetti et al. 1998*
PROS1	Protein S	*Hall et al. 2006*
PROZ	Protein Z	*Sugawara et al. 2007*
SERPINC1	Antithrombin	*Tremp et al. 1995, Fernández-Rachubinski et al. 1996*

In addition, the regulation of *HNF4A* gene has been well studied, mainly at a transcriptional level. It has been shown that *HNF4A* gene activation requires the action of *HNF1A* and *HNF6* on the proximal promoter of *HNF4A*, which communicates via a looping with a distant enhancer bound by *HNF1A*, *HNF3B,* and *C/EBPA* [113]. Moreover, several studies have identified that *HNF4A* expression is also regulated post-transcriptionally by miRNAs. The miRNA dependent regulation not only affects HNF4α expression but also downstream HNF4α target genes, which may have important consequences on the liver phenotype [59, 113, 114].

OBJECTIVES

Dysregulated hemostatic activity contributes to thrombotic or hemorrhagic diseases. Such dysregulation, which disturbs the fragile balance of the hemostatic system, implies that a safety threshold has been exceeded, therefore leading to thrombotic or hemorrhagic disorders. There are different genetic and acquired factors that have been identified that contribute to break the hemostatic equilibrium. However, the molecular mechanisms that trigger the high percentage of thrombotic diseases are still unknown. In the past decade, miRNAs have been recognized as critical regulators of gene expression. They are able to reduce gene expression mostly through decreasing mRNA translation or increasing mRNA degradation. Recent *in vitro* studies demonstrated their role in regulating levels of hemostatic factors. Genetic variations in the miRNA binding site of a target gene can affect protein expression.

In the present thesis, I have addressed three points of investigation in the thrombosis field that have been reported as independent Chapters with a common nexus, the role of miRNA as new regulators of the hemostatic system.

Chapter I: *Regulation of coagulation FXI expression by miRNAs in the human liver.*

The study of FXI regulation raises special interest because recent findings point toward contact pathway as not having an important function *in vivo*, based on that FXII deficiency in humans and animals, is not associated with impaired hemostasis. In contrast, patients lacking FXI report a mild trauma-induced bleeding disorder. So, it has been hypothesized that FXI plays a critical role in the development of pathological thrombus formation, while having limited (or no effect) on physiologic hemostasis.

In this framework, the objective in this first Chapter was:

To investigate the potential relevance of miRNAs as new elements that may modulate FXI in liver.

Chapter II: *Identification of coagulation gene 3'UTR variants as new risk factors for venous thrombosis.*

3'UTR regions are essential regulatory regions able to drive transcription efficiency by different mechanisms: poly(A) site efficiency and also through miRNA binding. Common genetic variations in the miRNA binding site within 3'UTR regions of a target gene can therefore affect the effect of miRNAs on protein regulation. Indeed, an effect of polymorphisms in miRNA binding sites has been found on the risk of diseases such as Parkinson's disease, colorectal cancer, and childhood asthma. So, alterations of miRNA target sites may have a relevant effect in the expression of proteins regulated by these miRNAs. This possibility has not been investigated in the thrombosis framework. Functional consequences of such alterations of miRNA target sites may be (i) differences of plasma levels and (ii) an increase in the risk of suffering thrombosis.

The objective for this Chapter was:

To identify 3'UTR variants in coagulation genes that influence coagulation factor levels and deep vein thrombosis risk through miRNA modulation.

Chapter III: *Indirect regulation of hemostatic proteins by miR-24 and miR-34a through Hepatic Nuclear Factor 4α.*

The notion that common regulatory genes coordinate simultaneously the expression of several genes is an attractive point to be explored in the hemostatic system. Clustering the hemostatic genes under the control of transcriptional units would allow categorizing individuals with high or low levels of coagulation factors. A good candidate among these common regulatory genes is HNF4α. The hypothesis of miRNAs repressing common transcription factors, then working as TRANS-regulatory elements for some genes, remains to be explored in hemostasis.

Then, my objective in this point was:

To thoroughly gain a deeper insight into the physiological modulator role of miRNAs in the expression of coagulation factors regulated by HNF4α.

CHAPTERS

I. Regulation of coagulation FXI expression by miRNAs in the human liver

1. Introduction

Although coagulation FXI was discovered nearly 50 years ago [24], its role in pathophysiological conditions is still not fully understood. A wide range of FXI plasma levels has been found in the healthy population [11]. The available functional data on FXI function are confusing, probably reflecting the fact that FXI might be involved not only in hemostasis but also in pathologic processes as inflammation or innate immunity [115, 116]. Epidemiological and animal model studies have associated FXI levels with the risk of thrombotic disease (for review see [19, 117]) or septic survival advantage [118]. On the other hand, FXI deficiency does not usually lead to spontaneous bleeding, but it is associated with an increased risk of bleeding when the hemostatic system is challenged [19, 20]. Moreover, FXI inhibition has been proposed as a novel approach to developing new anti-thrombotic therapies to achieve an improved benefit-risk ratio [18, 119].

In this framework, several groups have been engaged in an intensive study of the influence of genetic and environmental factors on FXI plasma levels in an attempt to understand whether the heterogeneous values found in the healthy population confer a pro- or anti-thrombotic phenotype. Although some of these studies have identified the involvement of common single nucleotide polymorphisms in the structural *F11* gene and alterations in other genes that might indirectly regulate plasma levels of this factor [30, 120, 121], the molecular mechanisms of FXI regulation are still largely unknown.

During the last four years, several groups including ours, have evaluated the role of miRNAs in the regulation of hemostasis [55]. In the current study, we investigated the potential relevance of miRNAs aiming to discover new elements that may modulate FXI in the liver. This study demonstrates that FXI expression in the human liver is directly regulated by a specific miRNA, miR-181a-5p, opening up new prospects in a better understanding of the pathophysiology of hemostatic diseases where FXI is involved and in the development of miRNA-based therapeutic technologies.

2. Materials and Methods

2.1. Cell line and tissue samples

HepG2 (American Type Culture Collection, Manassas, VA), PLC/PRF/5 (kind gift of Fernando Corrales, CIMA, Pamplona, Spain. Original commercial source: American Type Culture Collection, Manassas, VA), and human colon cancer cell line deficient for Dicer (HCT-DK) (kind gift of Dr. Renata Baserga, Department of Cancer Biology, Thomas Jefferson University) [122] were conventionally cultured. Briefly, HepG2 and PLC/PRF/5 were cultured in DMEM (Life Technologies, Madrid, Spain) and HCT-DK in McCoy's 5A (Sigma-Aldrich, Madrid, Spain). All media were supplemented with 0.1mM non-essential amino acids, 2mM Glutamax I, and with 10% fetal calf serum (Life Technologies, Madrid, Spain). Cells were grown at 37°C under 5% CO_2. Liver samples from Caucasian donors (n = 114) were kindly provided by the Biobanc CIBERehd [123] (La Fe Hospital, Valencia, Spain) (n = 19) and by St. Jude Children's Research Hospital Liver Resource (Liver Tissue Procurement and Distribution System (NIH Contract #N01-DK-9-2310) and the Cooperative Human Tissue Network) [122](n = 95) (Table 1). All donors gave a written informed consent that was recorded following the procedures of each Biobank. Human liver studies were further approved by Local Ethics Committee from Hospital Universitario Morales Meseguer in Murcia (#ESTU-19/12).

Table 1| Characteristics of liver donors

Characteristics	Liver donors (n=114)
Age (years)	
Mean	51.0
Median	53.0
Range	2-87
Gender (%M)	55

2.2. MiRNA array and *in silico* identification of miRNA binding sites in *F11* mRNA

To identify miRNAs expressed in healthy liver, we performed expression arrays including 1,898 human mature miRNAs (LCSciences, Houston, TX; Sanger mirBase Release 18.0 and 19.0) using total RNA extracted from 4 healthy human liver samples (Appendix 1; Table S1). Raw miRNA microarray data are

available in public archives under corresponding author name (GEO accession: GSE61219). For the determination of mature hepatic miRNAs potentially regulating human *F11* 3′UTR, we used four miRNA prediction algorithms.

2.3. Cell transfection

To validate *in silico* experiments, we performed transfection assays in cell lines mentioned above. Briefly, cells were seeded twenty four hours before transfection in complete medium without antibiotics and transfected with 100 nM of chemically modified double-stranded RNAs that mimic endogenous miRNAs (mimic), 100 nM miRNA inhibitors (miRCURY LNA microRNA Inhibitor from Exiqon, Vedbaek, Denmark) or 100nM of non-specific scrambled negative control (SCR) from Life Technologies (Madrid, Spain) as previously described [123]. Transfection efficiency was >90% (Figure 1). After 48 h, supernatants and cells were collected for subsequent mRNA and protein analyses.

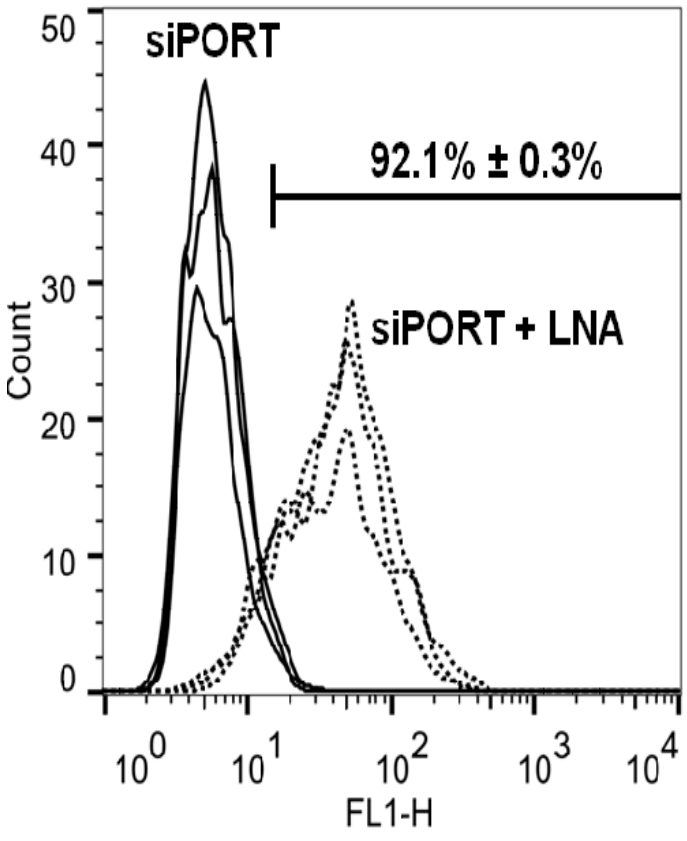

Figure 1| miRNA inhibitor transfection efficiency. miRCURY LNA microRNA Inhibitor Negative Control (100 nM) labeled with fluorescein (Exiqon, Vadbaek, Denmark) were transfected into PLC/PRF/5 cells with siPORT™ NeoFX™ (Life TechnologiesTM, Madrid, Spain), following manufacturer's instructions. After 6 hours transfection, cells were harvested and washed with PBS. Flow cytometry was performed using a BD FACSCalibur™ flow cytometer (BD Biosciences, Madrid, Spain) and samples were run through the flow cytometer until 2,000 events were collected. The mean ± SD of transfection efficiency for three replicates was 92.1%±0.3%. X-axis represents the intensity of fluorescence for FL1 channel in log scale and Y-axis the numbers of cells. We definetransfection efficiency as a percentage of cells positive for FL1 (dotted lines); taken as background signal the non-LNA transfected cells signal (solid lines).

2.4. MiRNA and mRNA expression levels

Total RNA was isolated from both fresh livers and transfected cells using TRIzol® Reagent (Life Technologies, Madrid, Spain). RNA (400 ng) and SuperScript III First-Strand Synthesis System (Life Technologies, Madrid, Spain) were used for reverse transcription (RT) reactions. *F11* and *ACTB* (as endogenous reference control) genes expression were quantified by qRT-PCR (Hs01030011_m1 and Hs99999903_m1, respectively, from Life Technologies, Madrid, Spain). Commercial assays for miR-181a-5p, miR-23a-3p, miR-16-5p, miR-195-5p, miR-494, and U6 snRNA (endogenous reference control) (Life Technologies, Madrid, Spain) were used to quantify expression levels of miRNAs in human cell lines and/or hepatocytes.

2.5. Western blot

Proteins from the lysate of transfected HepG2 cells (60 µg) or liver (50 µg) were blotted and immunostained with anti-human FXI polyclonal antibody (Enzyme Research Laboratories, Swansea, UK) and anti-human β-actin monoclonal antibody (Sigma-Aldrich, Madrid, Spain). Additionally, we collected and lyophilized 500 µL supernatants from HepG2 and PLC/PRF/5 using CentriVap Concentrator (Labconco, Kansas, MO) and 24 µL were blotted and immunostained with anti-human FXI polyclonal antibody. FXI and β-actin were immunodetected with the appropriate secondary antibody labeled with peroxidase (GE Healthcare, Barcelona, Spain). Detection was performed using ECL Prime Western Blotting Detection Kit (GE Healthcare, Barcelona, Spain) and ImageQuant LAS 4000 Imager (GE Healthcare, Barcelona, Spain). Densitometric analysis was performed with ImageJ software (http://imagej.nih.gov/ij/). Data were expressed as changes relative to the values of the cells transfected with SCR, taken as 100%.

2.6. Luciferase reporter assay

In order to test if the regulation of *F11* by miRNAs is done through a direct interaction between both molecules, we performed luciferase reporter assays, as described below.

2.6.1. Plasmid construction

PCR product (1,060 bp) containing the F11 3′UTR from human genomic DNA (NM_000128), obtained using primers *F11*-3′UTR_F and *F11*-3′UTR_R, was cloned into the pCR 2.1 vector (Life Technologies, Madrid, Spain) (Table 2). Positive clones were digested with SpeI and MluI (New England Biolabs, Ipswich, MA) and the insert was subcloned into luciferase reporter plasmid pMIR-REPORT (Life Technologies, Madrid, Spain) previously digested with SpeI and MluI. Insertion of *F11* 3′UTR was checked by sequencing (ABI3130 XL, Life Technologies Corporation, Carlsbad, CA). All sequence analyses and alignments were performed with the SeqmanPro program (Lasergene version 7.1, DNASTAR, Madison, WI).

Table 2| Primers used in the study

Primer Name	Sequence (5'-3')
F11-3'UTR_R	ACGCGTCACTTGATGAATTGTATAGTTG
F11-3'UTR_F	GGATTCTGGAGAAAACTCAAGC
del_181_S	GTAGACACGAGCTAAGAGGAAGATAACAGAATTTC
del_181_AS	GAAATTCTGTTATCTTCCTCTTAGCTCGTGTCTAC

To generate mutations in the predicted target site for miR-181a-5p, seven nucleotides (TGAATGT) located in the seed sequence were deleted using the QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA).

Using RNAHybrid [124], *in silico* prediction of miR-181a-5p binding to the mutated sequence showed a 33% increase in the minimum free energy value (Figure 2) indicating that miR-181a-5p: *F11* mRNA interaction was completely suppressed. Sequencing was performed to check for the deletion of the seed sequence. The primers used (del_181_AS and del_181_S) are detailed in Table 2.

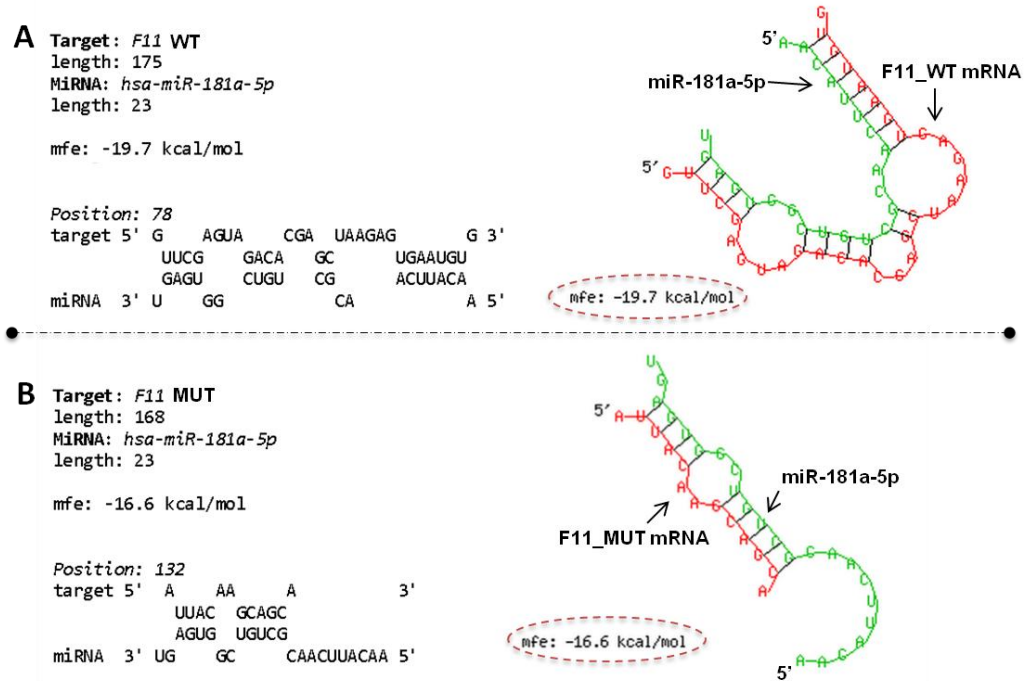

Figure 2| Version: RNAHybrid *in silico* prediction of miR-181a-5p analysis to: A) F11 wild type (F11 WT) and B) F11 mutated where the seed region is deleted. mfe: minimum free energy.

2.6.2. Luciferase vector transfection

HCT-DK cells, that do not express miR-181a-5p, were seeded at a density of 80,000 cells/well in 24-well plates with complete McCoy's 5A supplemented with 10% fetal calf serum without antibiotics. The following day, cells were co-transfected with miR-181a-5p (both pMIR-REPORT plasmids -1000 ng/well-wildtype or mutated for the miR-181a-5p seed site) or SCR precursor, and 100 ng/well of renilla luciferase control plasmid (pRL-TK; Promega, Madison, WI) using Lipofectamine LTX Reagent (Life Technologies, Madrid, Spain) according to the manufacturer's instructions. Luciferase assays were performed as previously described [123].The enzymatic activities of renilla and firefly luciferases were quantified in a Synergy 2 luminometer (Biotek, Winooski, VT). Each combination of pMIR-REPORT (wild-type and mutated 3′UTR) and pRL-TK was tested in triplicate in five independent experiments. Firefly luciferase activity was normalized to renilla luciferase activity for each transfected well. The normalized data were expressed as changes relative to the data of the cells transfected with 100 nM miR-181a-5p mimic, SCR was taken as 100%.

2.7. Statistical Analysis

Comparisons between groups were performed by the unpaired t-test. Data are given as mean ± SD. Linear regression tests were performed; β regression coefficient (r) and r^2 were calculated. Results were considered statistically significant for $p<0.05$. Analyses were carried out using Statistical Package for Social Science (version 21.0; SPSS, Chicago, IL).

3. Results

3.1. A microarray and *in silico* target search yielded four miRNAs that could potentially bind to *F11* mRNA

In order to select miRNAs with the potential to bind to *F11* mRNA, two criteria were established (i) the miRNA expression cut-off in liver had to be >500 arbitrary units (au) (see array in Table S1 in Appendix 1) and (ii) the miRNA binding had to be anticipated in 4 or more of the prediction algorithms of miRNA targets used (n = 8). Such filtering allowed the selection of four miRNAs: miR-181a-5p (liver expression= 1233 au; 6 prediction algorithms), miR-23a-3p (liver expression= 6052 au; 5 prediction algorithms), miR-16-5p (liver expression= 3513 au; 4 prediction algorithms), and miR-195-5p (liver expression= 3046 au; 4 prediction algorithms) (see Table S1 in Appendix I and Table 3).

Table 3| *In silico* prediction results.

miRNA	RNAhybrid[A]	TargetScan[B]	miRSVR[C]	miRWALK					TPA	Liv. E
				DIANAmT	*miRDB*	*PITA*	*RNA22*	*PICTAR5*		
miR-181a	1 (-19.70)	1 (-0.05)	1 (-0.36)	1	1	1	—	—	6	1233
miR-23a	1 (-14.70)	1 (-0.07)	1 (-0.67)	1	—	1	—	—	5	6052
miR-16	1 (-15.00)	1 (-0.13)	1 (-0.13)	1	—	—	—	—	4	3513
miR-195	1 (-8.00)	1 (-0.13)	1 (-0.14)	1	—	—	—	—	4	3046
miR-494	—	—	1 (-0.03)	—	—	1	—	—	2	1395

The presence of the selected miRNA in the target prediction tool is recognized in the table by number "1".
[A]: RNA hybrid minimum free energy (MFE; kcal/mol) [124], [B]: TargetScan (context + score) [62], [C]: miRSVR score [65].
TPA: Total Prediction Algorithms. **Liv. E**: Liver Expression (arbitrary units)

Additionally, a negative control miRNA (miR-494; see Table 3), which was not predicted to bind to *F11* mRNA (only 2 prediction algorithms) and with a liver expression >500 au, was also investigated (Table 3).

Putative binding sites for miRNAs are shown in Figure 3. Whereas miR-181a-5p and miR-23a-3p bind to two closely located sites, miR-16-5p and miR-195-5p share the same binding site located ~200 bp downstream of the miR-23a-3p seed (Figure 3).

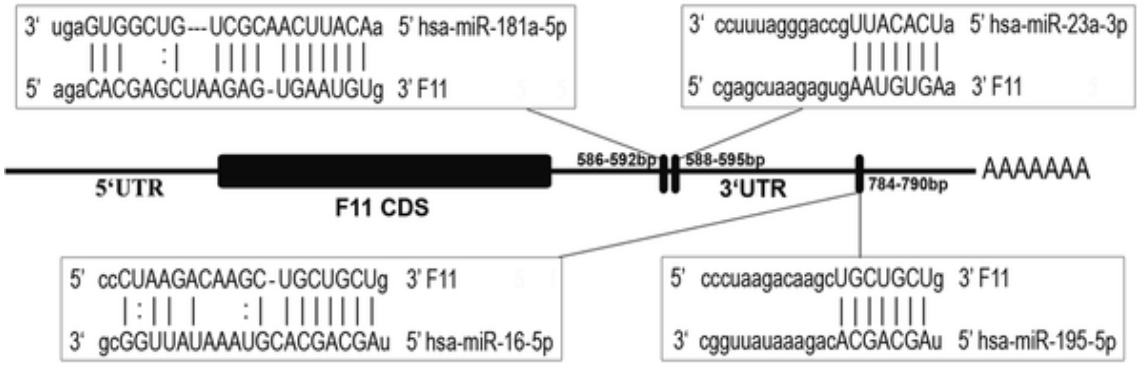

Figure 3| Schematic representation of predicted target sites of miRNAs in *F11* 3′UTR. The predicted binding sites of miR-181a-5p, miR-23a-3p, miR-16-5p, and miR-195-5p are indicated in the *F11* 3′UTR (1060 bp). The start- and end-positions of the complementary sequence between *F11* and miRNAs are indicated. Complementarities between the seed region (7 nucleotides) of miRNAs and 3′UTR of *F11* mRNA target site are shown in parentheses. MiR-16-5p and miR-195-5p share the same binding site. MiRNA:mRNA interactions are represented by upper-case letters (provided by mirSVR algorithm).

3.2. *In vitro* studies suggested miR-181a-5p as a direct inhibitor of FXI expression

To test which miRNAs may inhibit FXI expression, we employed HepG2 cells, expressing lower levels of these miRNAs than the liver (Figure 4A). Transfection of HepG2 cells with the different miRNA mimics showed that only miR-181a-5p mimic provoked a significant reduction of endogenous *F11* mRNA levels of almost 30% (100% *vs*. 71±9%; p= 0.03; N= 3) compared with SCR transfection (Figure 5A).

No inhibition was found when transfecting HepG2 with the other selected miRNAs or with miR-494 (Figure 5A). In fact, miR-181a-5p caused a significant decrease (~30%) in the levels of extracellular FXI (100% *vs*. 71±7%; p= 0.04; N = 3) (Figure 5B). In addition, when the intracellular levels of FXI in cells transfected with miR-181a-5p were evaluated an almost 50% decrease was observed compared with cells transfected with SCR (100% *vs.* 53±16%; p= 0.02; N= 3) (Figure 5C).

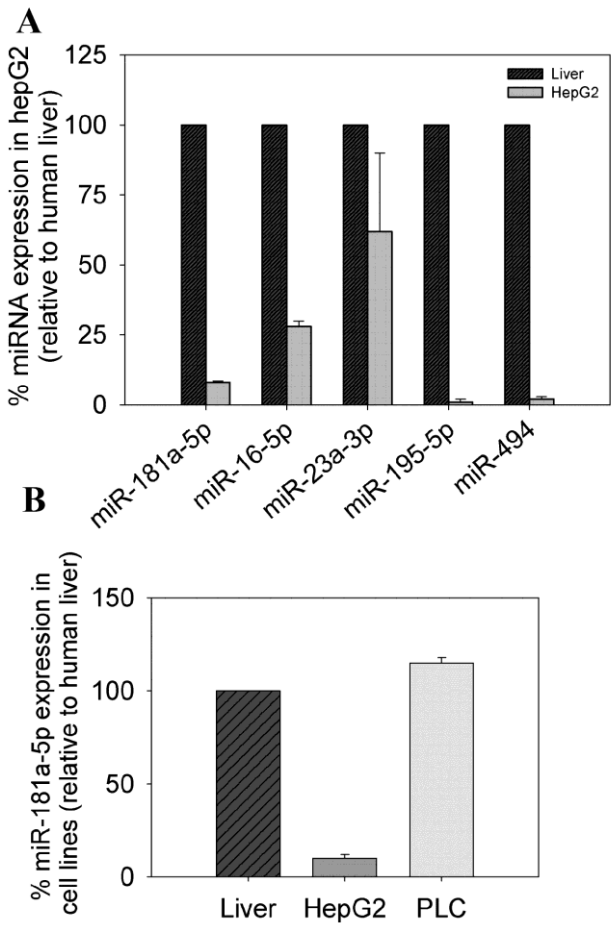

Figure 4| Levels of miRNAs in human liver and cell lines. Levels of miRNAs were Quantified by qRT-PCR. (A) Levels of miRNAs in HepG2 relative to human liver. (B) Levels of miR181a-5p in HepG2 and PLC/PRF/5 relative to human liver. Results are represented as mean ± SD of three replicates from two independent experiments

Next, we tested the effect of a miR-181a-5p inhibitor in another hepatic cell line where miR-181a-5p expression levels were similar to those reported in the liver (Figure 4B), PLC/PRF/5 hepatic cell line. Our results indicated that inhibition of miR-181a-5p increased both the levels of *F11* mRNA (100% *vs.* 121±3%; p= 0.006; N = 3) (Figure 5D) and of extracellular FXI (100% *vs.*116±6%; p= 0.038; N= 3) (Figure 5E) further supporting a physiological regulation of FXI by miR-181a-5p.

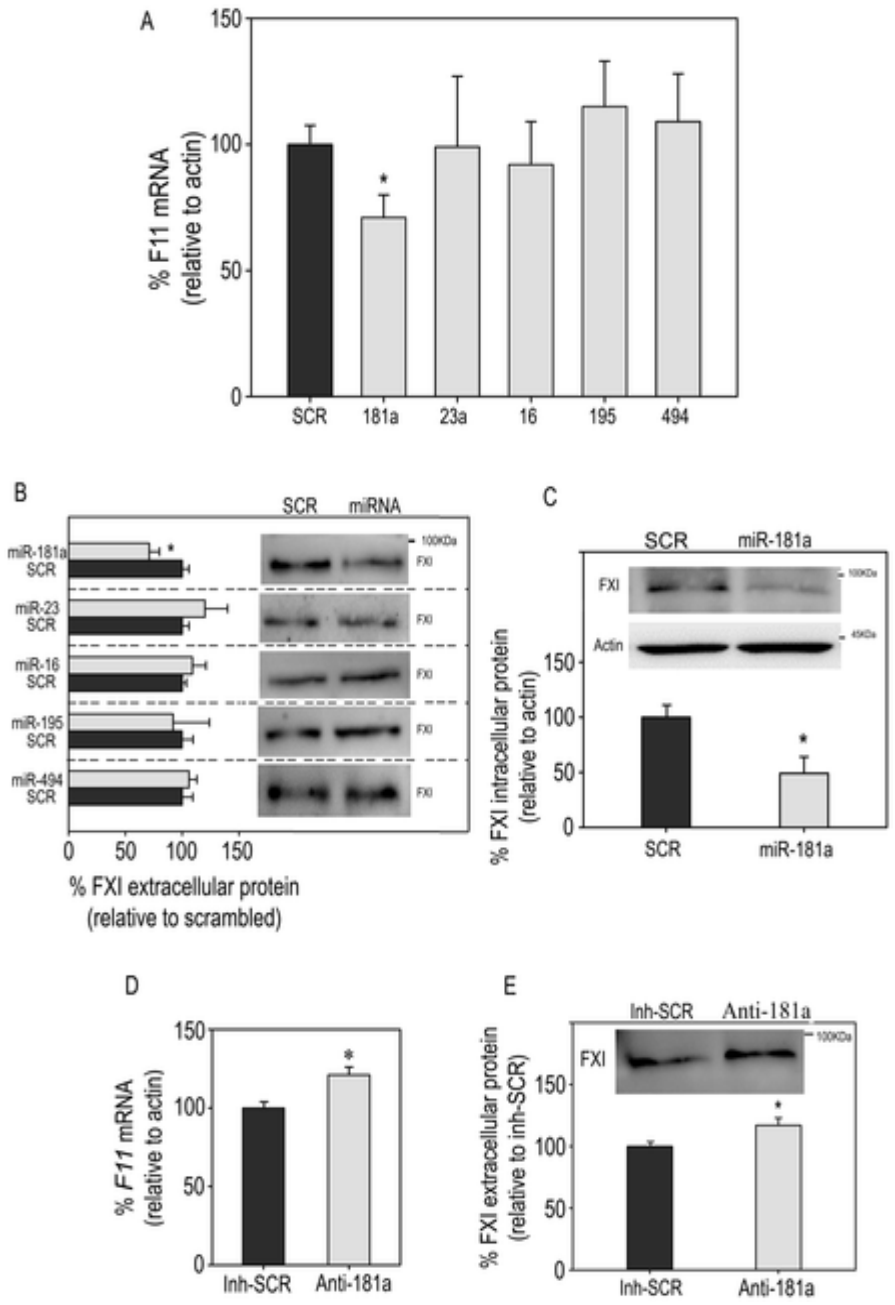

Figure 5| Effect of miRNAs on FXI expression. HepG2 cells were transfected with 100 nM mimic precursors miR-181a-5p (181a), miR-23a-3p (23a), miR-16-5p (16), miR-195-5p (195), miR-494 (494) or SCR. Protein lysate, total RNA and extracellular media were obtained after 48 h incubation and analyzed. (A) qRT-PCR analysis of *F11* mRNA expression. (B) Densitometric analysis of FXI extracellular protein expression with a representative Western blot. (C) Densitometric analysis of FXI intracellular protein expression with a representative Western blot in cells transfected with SCR and 181a. (D) qRT-PCR analysis of *F11* mRNA expression in PLC/PRF/5 cells transfected with 100 nM miR-181a-5p inhibitor or SCR inhibitor. (E) Densitometric analysis of FXI extracellular protein expression with a representative Western blot in PLC/PRF/5 cells transfected with miR-181a-5p inhibitor (anti-181a) or SCR inhibitor (Inh-SCR). Results are represented as mean ± SD of three replicates from three independent experiments. The normalized data were expressed as changes relative to the data of the cells transfected with SCR or SCR inhibitor and set as 100%. *P<0.05. Student's t-test was calculated in mimic *vs*. SCR.

Whether the lower levels of *F11* mRNA observed in transfected HepG2 were due to an indirect effect of miR-181a-5p or to mRNA decay was further investigated. Co-transfection of HCT-DK cells with a luciferase reporter vector containing the 3′UTR of *F11* and miR-181a-5p showed a significant decrease of ~30% of the luciferase activity in comparison with SCR (100±17% *vs*. 71±27%; p= 0.04; N= 3). This inhibition was not observed when using a mutated vector, which lacked the binding site of miR-181a-5p (Figure 6).

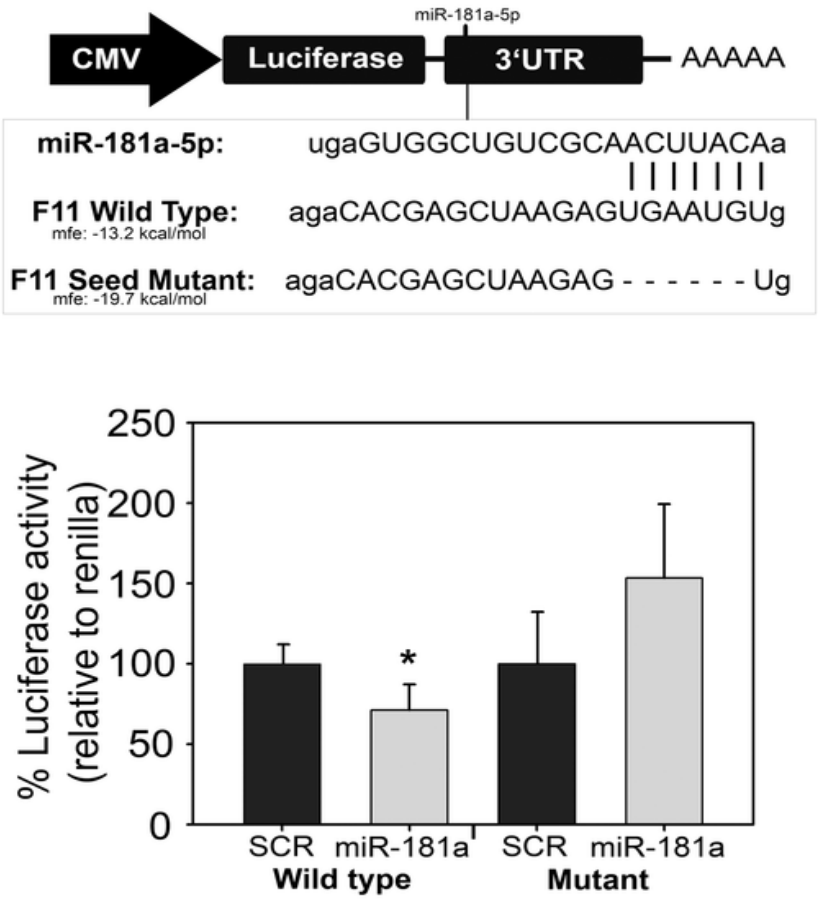

Figure 6| Luciferase reporter assays. Schematic diagram of the luciferase reporter plasmids including *F11* WT 3′UTR or *F11* mutant 3′UTR in which the seven nucleotides forming the seed region of miR-181a-5p were deleted. HCT-DK cells, that do not express miR-181a-5p nor other Dicer-dependent miRNAs that may interfere in miRNA overexpression experiments, were transfected with either *F11* WT 3′UTR or *F11* mutant 3′UTR along with 100 nM miR-181a-5p precursor. A SCR precursor was used as control. Luciferase activities were normalized to renilla activities. Results are represented as mean ± SD of three replicates from three independent experiments. The normalized data were expressed as changes relative to the data of the cells transfected with SCR and set as 100%. *P<0.05. Student's t-test was calculated in mimic vs. SCR.

3.3. *F11* mRNA levels were inversely correlated with miR-181a-5p in human livers

In order to establish the potential physiological significance of the above results, we measured *F11* mRNA and miR-181a-5p levels in samples from a cohort of healthy livers that had been used for liver transplant. Using a linear regression model, *F11* mRNA levels were found to be inversely and significantly related to miR-181a-5p levels ($r = -0.184$; $p<0.05$) (Figure 7A). To confirm the specificity of this result, we compared levels of *F11* mRNA with those of miR-494 (that showed no *in silico* or *in vitro* effect on FXI expression) and we found no association between these two molecules ($r = -0.033$; $p = 0.727$) (Figure 7B).

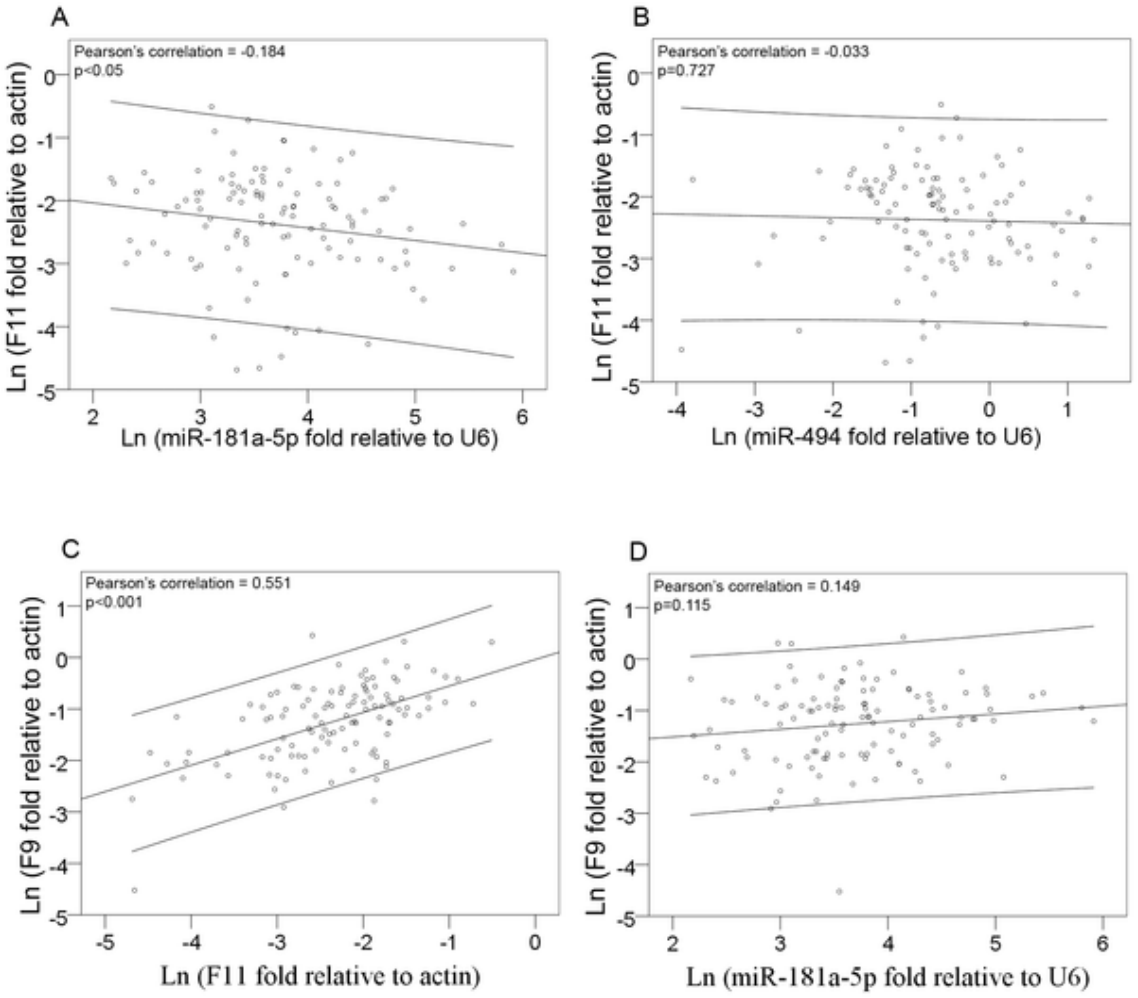

Figure 7| *Ex vivo* expression of *F11* mRNA and mature miRNAs. Linear regression analysis between endogenous mature miRNAs (miR-181a-5p and miR-494) levels and *F11* mRNA (A & B respectively). (C) Linear regression between *F11* and *F9* mRNAs and (D) between miR-181a-5p and *F9* mRNA. qRT-PCR were performed in total RNA purified from healthy livers (n= 104). Statistical significance was taken as $p<0.05$. The results are presented as Ln fold change with respect to the normalization standard.

Previous studies have shown that FXI plasma antigenic levels are correlated with those of FIX [11]. In this study, we observed a strong correlation between *F9* and *F11* mRNA levels (Figure 7C), which supported the described correlation in plasma. Aiming to further confirm the specificity of *F11* mRNA: miR-181a-5p interaction, we investigated a potential correlation between miR-181a-5p and *F9* mRNA levels. As expected, our results demonstrated that miR-181a-5p had no influence on *F9* mRNA levels in healthy livers (p= 0.115) (Figure 7D).

Next, we investigated the dynamic range of expression of *F11* mRNA in hepatocytes, finding that it was larger than that described for plasma levels of FXI [32]. More specifically, there was a three-fold difference in *F11* mRNA values between the 75th and 25th percentiles (Figure 8A). Interestingly, we also observed a wide range of expression of miR-181a-5p among individuals. In fact, miR-181a-5p expression in liver samples with *F11* mRNA levels below 15th percentile was two-fold higher than in the samples above the 85th percentile (Figure 8B).

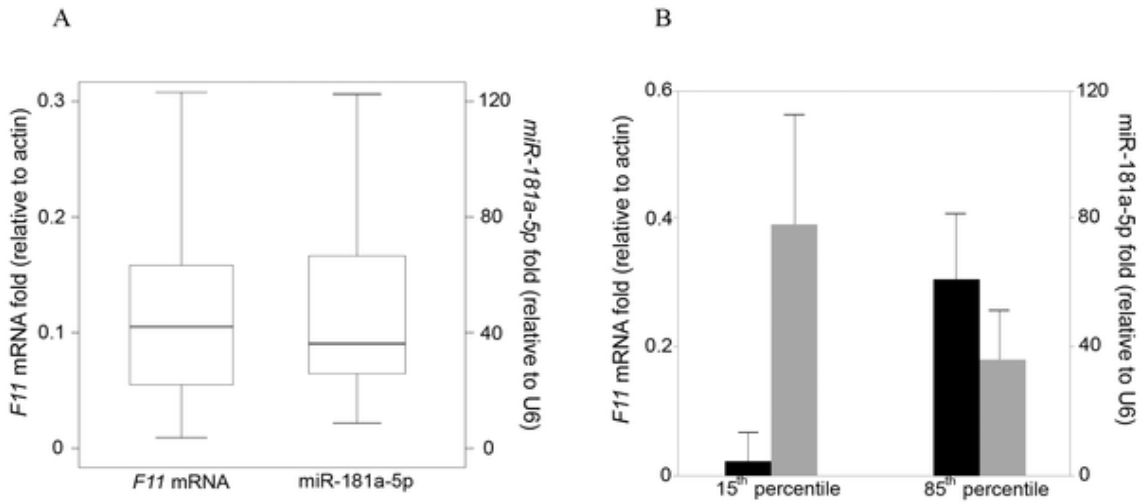

Figure 8| Expression range of miR-181a-5p and *F11* mRNA in healthy livers. (A) Box plot of *F11* mRNA and miR-181a-5p levels in livers. The upper and lower bars are the 90th and 10th percentiles, respectively. (B) Levels of miR-181a-5p (black) in livers corresponding to 15th and 85th percentiles of *F11* mRNA expression (grey), mean ± SD.

4. Discussion

It has been consistently reported that miRNAs may regulate hemostatic proteins such as fibrinogen, tissue factor, PAI-1 or antithrombin, while variations in the levels of miRNAs or in the efficacy of the miRNA: mRNA interaction might have an impact on the development of thrombotic diseases [55]. In the present study, we provide new evidence that miRNAs fine-regulates the expression of FXI in human hepatocytes, which in turn opens an alternative regulation pathway that may be exploited for future studies.

The *in silico* results were the starting point in our work. Bearing in mind that approximately 70% of all *in silico* predictions are thought to be false-positive [67], this encouraged us to more accurately establish filters to select the candidate miRNAs to be studied. Our filters led to the selection of 4 candidate miRNAs with potential to bind to *F11* mRNA and, even then, we observed 75% false positives. The effective prediction of an miRNA:mRNA pair in animal systems is still a challenge because of the complexity of this interaction [125]. Future studies to unravel these issues will probably help identify a more selective *in silico* search method for additional miRNAs, which target FXI and other haemostatic factors.

In a second step consisting in performing an *in vitro* validation, only miR-181a-5p was potentially a direct regulator of *F11* expression, which enabled it to induce a significant decrease in both extra- and intracellular FXI and *F11* mRNA levels. As expected, and in accord with other studies describing that the effect of a given miRNA on its target is generally modest [67, 126, 127] and that it is the total of various miRNA interactions that would determine the effect as a whole, we found that the effect of miR-181a-5p on FXI expression was mild. Future studies will determinate additional miRNAs that may act in conjunction with miR-181a-5p to regulate FXI expression. In this sense, we found that a neutral miRNA (miR-494) neither interacted nor correlated with *F11* mRNA (Figure 7B). Moreover, the lack of correlation found between miR-181a-5p and *F9* mRNA further supported our hypothesis that miR-181a-5p has a specific effect on FXI expression.

Next, we investigated the physiological relevance of FXI regulation by miR-181a-5p. Given our inability to test this hypothesis *in vivo*, we performed *ex vivo* analysis in livers from healthy donors that had been used for transplant. Our data showed that levels of miR-181a-5p and *F11* mRNA were correlated in human livers (Pearson's coefficient= −0.184, $p<0.05$). In fact, the *in vitro* study demonstrated that the decrease of both target *F11* mRNA and protein levels was proportional and therefore we speculate that the *ex vivo* correlation may be extrapolated. Together with the fact that protein levels are determined by additional tightly modulating processes including protein degradation rates, the final effect of a miRNA on an mRNA is very difficult to predict [128]. In this sense, we found a three-fold difference in *F11* mRNA values between the 75th and 25th percentiles in healthy livers, whereas only a 1.3-fold difference in plasma between the same percentiles has previously been found [32]. Therefore, translational, post-translational, and degradation processes, together with the regulation of the secretory pathway (involving both miRNAs and target genes), may act to adjust the final amount of FXI in plasma.

Many observations of miRNA-mediated regulation in mammalian cells considered them as fine-tuners of gene expression [129]. Indeed, miRNA expression can be regulated by the same genetic alterations that modulate protein coding genes [130] as well as by environmental factors. Specifically, miR-181a-5p has been shown to be regulated by dopamine [131] and TGF-β [132]. Our data showed a surprising heterogeneity of miR181a-5p expression in human liver samples (Figure 7A). We observed a 2.5-fold difference in miRNA values between the 75th and 25th percentiles, which reached more than 12-fold when the 90th and 10th percentiles were compared. Interestingly, recently Mendell and Olson suggested that miRNA deficiency or overexpression may have an important impact under pathophysiological stress conditions [133] while, in physiological conditions, miRNAs may only play a modest role in regulating their target.

Overall, our *in vitro* and *ex vivo* results establish miRNAs as new modulators of FXI, opening up new prospects for the regulation of FXI by miRNAs that deserves further attention and confirmation. However, our *in vitro* experimental

conditions did not allow us to test the effect of miR-181a-5p on functional activity of FXI. Additional studies are necessary to fully understand this new FXI regulation and to test the possibility that it is regulated by other miRNAs in an indirect way. It would also be useful to further investigate the association of miR-181a-5p expression with the development of thromboembolic disease. In this sense, FXI is seen as a potential therapeutic target since its inhibition prevents thrombosis without bleeding episodes [134]. Indeed, the use of several miRNA-based therapies in the liver is under investigation. The most advanced is the use of anti-miR-122 in the treatment of hepatitis C [135]. On the other hand, it has already been shown that antisense oligonucleotides inhibit FXI expression *in vivo* using a drug antisense in patients with total knee arthroplasty [18]. Therefore, the characterization of miR-181a-5p that regulates FXI expression may be seen as an opportunity to start envisaging the potential use of miRNA precursors as an anti-thrombotic drug.

II. Identification of coagulation gene 3'UTR variants as new risk factors for venous thrombosis

1. Introduction

Venous thrombosis is a common disease with an incidence of about 1 to 3 new cases per 1000 individuals per year in Western Countries [11, 12]. Potential major complications are death from a pulmonary embolism and development of a disabling post-thrombotic syndrome [136]. The susceptibility for venous thrombosis involves multiple genetic and environmental risk factors [137].

Genetic variation accounts for a large proportion of the risk of venous thrombosis [138] as well as a large proportion of the variation in plasma levels of coagulation factors [139]. In recent years, many genetic variants have been identified in coagulation-related genes with an effect on coagulation factor levels or venous thrombosis, but most of the associations were weak or could not be replicated [140]. A search for novel genetic risk factors for venous thrombosis therefore remains warranted.

In the past decade, miRNAs have been recognized as critical regulators of gene expression [141]. They mostly lower gene expression by decreasing mRNA translation or increasing mRNA degradation. Recent *in vitro* studies demonstrated their role in regulating levels of hemostatic factors [55].

Genetic variations in the miRNA binding site of a target gene can affect the effect of miRNAs on protein regulation [142] We therefore set out to study the association between miRNA target site variants in coagulation genes and venous thrombosis.

In this report on miRNA and venous thrombosis, we sequenced the 3'UTRs of genes coding for key hemostatic factors in subjects from two case-control studies with extreme phenotype levels of the traits of interest, i.e. high or low levels of coagulation factors. Associated variants were subsequently genotyped in the complete sample of 4485 cases and 4889 controls and associated with coagulation factor plasma levels and venous thrombosis risk.

2. Material and Methods

2.1. Study subjects

Study subjects were included in two case-control studies: the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA) study and the Leiden Thrombophilia Study (LETS). The design of the MEGA study has been previously described [143]. In brief, the MEGA study included over 5000 consecutive patients aged 18-70 years with a first deep venous thrombosis or pulmonary embolism from six anticoagulation clinics in the Netherlands between March 1999 and September 2004. Partners of participating patients were invited as control individuals. In addition, control subjects from the same geographical region were recruited by a random digit dialing method from January 2002 until September 2004. Controls were between 18 and 70 years old, had no history of venous thrombosis and were frequency-matched to patients with regard to age and sex. In LETS, as described earlier [144], 474 patients (age 18-70 years) were included with a first objectively confirmed deep venous thrombosis from three anticoagulation clinics in the Netherlands between January 1988 and December 1992. Participants had no overt malignancy. As control subjects, 474 friends or partners of patients with the same sex and approximately the same age (within 5 years) were included in the study. LETS and MEGA participants provided informed consent. Both studies were approved by the Medical Ethics Committee of the Leiden University Medical Center, Leiden, The Netherlands.

For the identification of 3'UTR variants, we selected thrombosis cases and controls with levels below the 2.5th and above the 97.5th percentile (of the distribution in individuals without thrombosis) of prothrombin, FIX and FXI, fibrinogen, protein C, protein S, antithrombin and TFPI. Selecting individuals with extreme phenotypes increases the likelihood of detecting trait-related sequence variations [145]. For all factors (except TFPI), we selected individuals from the MEGA study and excluded MEGA individuals with low quality DNA or plasma samples, on anticoagulant therapy, with chronic diseases (cancer, liver disease, paralysis, kidney disease and diabetes), or women pregnant or on birth

control pills or hormone replacement therapy at the time of the blood draw. In addition, for vitamin-K dependent factors, we excluded those with levels above the 90th percentile or below the 10th percentile of at least four vitamin-K dependent factors to exclude a dietary vitamin K deficiency. For fibrinogen we excluded activity levels above the 90th percentile (3.96 g/L). For TFPI, which was not measured in MEGA, we selected LETS individuals and excluded individuals on anticoagulant therapy, pregnant women and women using oral birth control pills or hormone replacement therapy at the time of the blood draw. See Table 1 for the final selection of MEGA and LETS subjects per coagulation factor.

Table 1| Number of sequenced individuals with extreme coagulation factor levels

Factor*	Total N	Extremely low levels		Extremely high levels	
		N	Range	N	Range
Prothrombin activity	88	37	65-86	51	**137-455**
FIX antigen	96	39	52-73	57	**140-201**
FXI activity	96	40	36-66	56	**143-195**
Fibrinogen activity	96	36	1.37-2.27	60	**4.67-8.63**
Protein C activity	96	57	37-82	39	**160-194**
Protein S antigen	96	55	54-74	41	**144-188**
Antithrombin activity	93	45	49-79	48	**131-147**
TFPI free antigen	76	41	34-62	35	**142-275**

*Activity levels were in % of normal (100% equals 1 IU/mL), except for fibrinogen (g/L). Antigen levels were measured in U/dL (FIX, protein S) or ng/mL (TFPI).

2.2. DNA Analysis and selection of variants for further genotyping in MEGA

A detailed description of blood collection and DNA isolation in MEGA and LETS has been published previously [143-146]. The 3'UTR sequences of coagulation factor genes were PCR amplified. For *FGA*, *FGG* and *TFPI* alternative transcript 3'UTRs were also sequenced as they did not overlap the sequence of the most abundant transcript. Thermal cycling conditions were: 7 min 94° C, 36 cycles of 94° C 30 s, 63° C 45 s, 72° C 1 min; 72°C 10 min. PCR products were sequenced according to the manufacturer's instructions using Big Dye terminator and AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) and analyzed on an ABI Applied Biosystems 3730 DNA analyzer. Thermal cycling conditions were: 1 min 94°C, 25 cycles of 94°C 10 s,

50°C 5 s, 60°C 4 min. The reference sequence was obtained from Ensembl release 56.

The PolyPhred program was used to identify variants [147]. Individuals harboring variants that could not be unambiguously called were re-sequenced. For *TFPI*, we did not succeed in sequencing the 3'UTR from nucleotide 1367 to nucleotide 1465.

Variants were considered candidates for further genotyping when they showed a difference in frequency between highest and lowest level groups of the coagulation factor of interest with a nominal p-value <0.05. These variants were genotyped in all available DNA samples from the MEGA study, i.e. samples from 4485 cases and 4889 controls. Genotyping was performed using KBiosciences competitive allele-specific PCR SNP genotyping assays (KASPar, KBioscience Ltd., Hoddesdon, UK) for three *F11* variants (rs4253429, rs4253430 and rs1062547). Primers and assay conditions used for genotyping are available on request. Fluorescence endpoint reading was done on an ABI 7900 HT (Applied Biosystems/ Applera, Foster City, CA, USA) by a technician who was unaware of the status of the sample (high/low value, case/control) and data were entered in a dedicated database in a blinded manner.

2.3. Coagulation factor assays

In MEGA, activity levels of prothrombin and FXI were measured with a mechanical clot detection method. Activity levels of antithrombin and protein C were determined using chromogenic assays on a STA-R coagulation analyzer following the instructions of the manufacturer (Diagnostica Stago, Asnières, France). In addition, also on the STA-R analyzer, fibrinogen activity was measured according to the method of Clauss and von Willebrand factor antigen levels were measured with the immuno-turbidimetric method using the STA liatest kit (rabbit anti-human von Willebrand factor antibodies). FIX antigen levels and total protein S levels were measured in MEGA and TFPI-free antigen levels in LETS by an enzyme-linked immunosorbent assay (Diagnostica Stago).

2.4. In silico prediction of miRNA binding sites

For each identified variant, the following miRNA resources were searched for a potential influence on miRNA binding:

- TargetScan (release 5.2: http://www.targetscan.org) [62]
- miRSVR (http://www.microrna.org/microrna/home.do) [65]
- PolymiRTS (http://compbio.uthsc.edu/miRSNP/) [148]
- Patrocles (http://www.patrocles.org/Patrocles.htm) [149]

Sequence alignments of the potential miRNA target sites in the *F11* 3′-UTR with the miRNA sequence were conducted using RNAhybrid webtool [124] (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/).

2.5. Luciferase assays

To validate *in silico* results, we performed transfection assays in human colon cancer cell line HCT-116 deficient for Dicer (HCT-DK) conventionally cultured[122]. Briefly, cells were seeded twenty four hours before transfection in complete medium without antibiotics and transfected with 100 nM of chemically modified double-stranded RNAs that mimic endogenous miRNAs (mimic) or non-specific scrambled control (SCR) from Life Technologies™ (Madrid, Spain) as previously described [123]. After 48 h, supernatants and cells were collected for subsequent mRNA and protein analyses.

A 1060 bp PCR product containing the *F11* 3'UTR from human genomic DNA (NM_000128), obtained using primers *F11*-3'UTR_F and *F11*-3'UTR_R, was cloned into the pCR 2.1 vector (Life Technologies™). Positive clones were digested with Spe I and Mlu I (New England Biolabs, Ipswich, MA) and the insert was subcloned into the luciferase reporter plasmid pMIR-REPORT™ (Life Technologies™) previously digested with Spe I and Mlu I. Insertion of *F11* 3'UTR was checked by sequencing (ABI3130 XL, Life Technologies Corporation, Carlsbad, CA). All sequence analyses and alignments were performed with the SeqmanPro program (Lasergene version 7.1, DNASTAR, Madison, WI). SNPs rs4253430 and rs1062547 were generated using the QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara,

CA). Sequencing was performed to check for the presence of the SNPs. Primers used in these procedures are detailed in Table 2.

Table 2| Primers used in the study

Oligonucleotide Name	Sequence (5'-3')
F11-3'UTR_R	ACGCGTCACTTGATGAATTGTATAGTTG
F11-3'UTR_F	GGATTCTGGAGAAAACTCAAGC
F11_rs4253430_S	AAGATTACTATATAGGCAGATATACCAGAAAATAACCAAGTAGTGG
F11_rs4253430_AS	CCACTACTTGGTTATTTTCTGGTATATCTGCCTATATAGTAATCTT
F11_rs1062547_S	CCTATGTGAACACATTTCTTTTGTAAAGAAAGAATTTGATTGCATTTAATGGC
F11_rs1062547_AS	GCCATTAAATGCAATCAAATTCTTTCTTTACAAAAGAAATGTGTTCACATAGG

HCT-DK cells, that do not express miR-544 nor miR-513a-3p, were seeded at a density of 80,000 cells/well in 24-well plates with complete DMEM supplemented with 10 % fetal calf serum without antibiotics. The following day, cells were co-transfected with miR-544 or miR-513a-3p *vs* SCR precursor and wild type or 3'UTR variant pMIR-REPORT plasmids -1000 ng/well-, and 100 ng/well of renilla luciferase control plasmid (pRL-TK; Promega, Madison, WI) using Lipofectamine LTX (Life Technologies™,) according to the manufacturer's instructions. Luciferase assays were performed as previously described [123].The enzymatic activities of renilla and firefly luciferases were quantified in a Synergy 2 luminometer (Biotek, Winooski, VT). Each combination of pMIR-REPORT (wild-type and 3'UTR variants) and pRL-TK was tested in triplicate in three independent experiments. Firefly luciferase activity was normalized to renilla luciferase activity for each transfected well. The normalized data were expressed as changes relative to the data of the cells transfected with miR-544 or miR-513a-3p mimics, SCR was taken as 100 %.

2.6. Statistical analysis

A workflow of the complete study is provided in Figure 1. Differences in genotype distributions between individuals with extremely high or low levels were quantified with Pearson's χ^2-test. *F9* gene variants, which lie on the X-chromosome, were analyzed in men and women separately. For variants genotyped in 4485 MEGA cases and 4889 MEGA controls, we tested for departure from Hardy-Weinberg equilibrium (HWE) in MEGA control individuals using the Pearson's χ^2-test.

For all genotyped variants, we also determined the effect on venous thrombosis risk in 4485 cases and 4889 controls by performing logistic regression with case status as outcome and genotypes as independent variable with adjustment for age and, additionally, for intermediate plasma levels of interest.

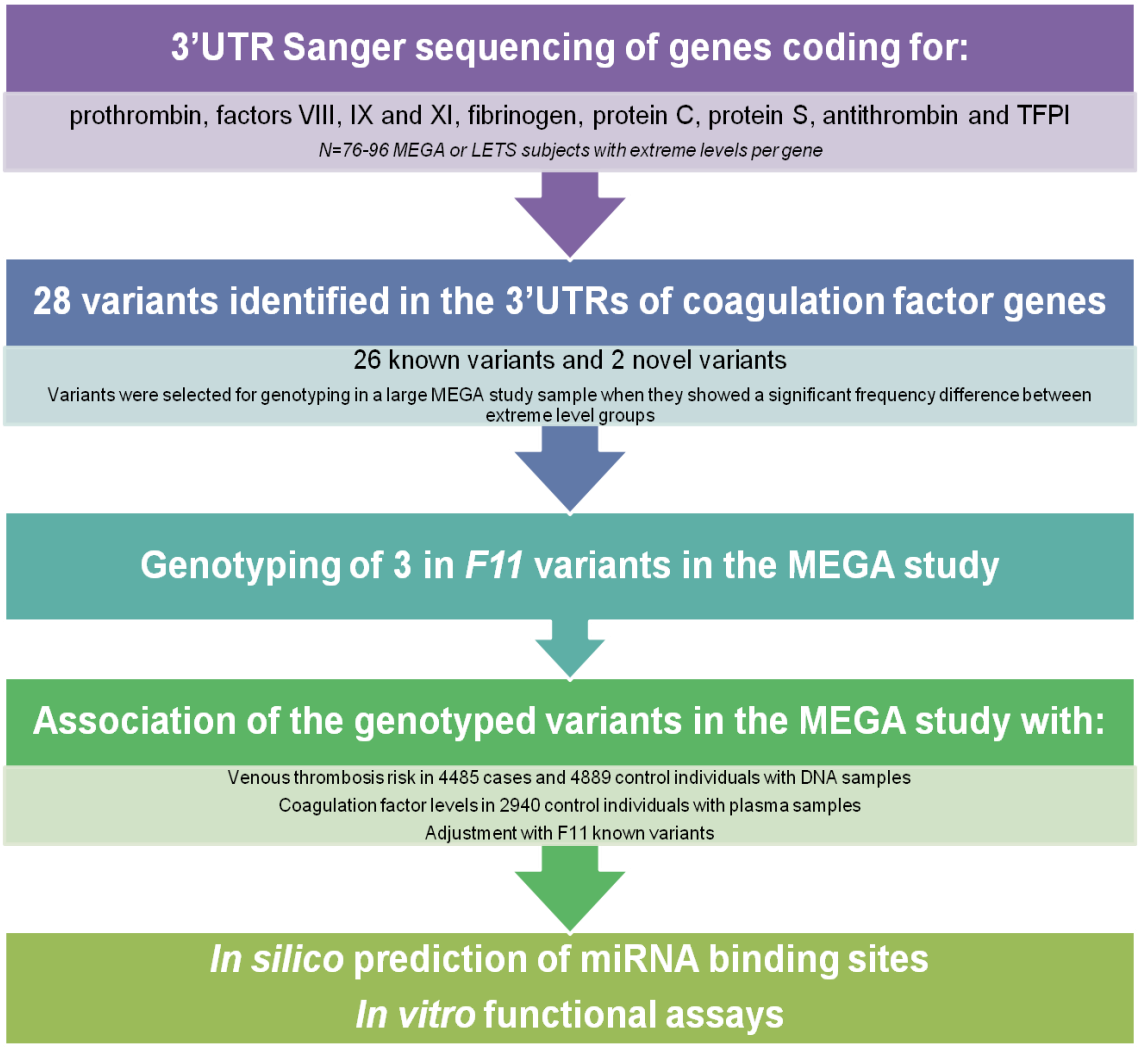

Figure 1| A workflow diagram of the complete study

In the 2940 MEGA control individuals with available plasma samples, we determined and compared covariate-adjusted mean levels of FXI for each genotyped *F11* variant genotype by running a general linear model with protein levels as dependent variables, the variant of interest as fixed factor, and age as covariate. Furthermore, for all genotyped variants, we assessed the linkage with other known variants associated with the risk of venous thrombosis and

adjusted the associations with coagulation factor levels and the risk of venous thrombosis for these variants where applicable. All analyses were performed using SPSS statistical software (version 20.0; SPSS, Chicago, IL, USA).

3. Results

Sequencing of the 3'UTR-regions of the nine genes in individuals with extreme low or high protein levels (76-96 individuals per gene) led to the identification of 26 variants with a range of one to six variants per gene (Table 3).

Table 3| Gene variants identified in extreme phenotype groups

Gene	Variant	major/minor allele*		Minor allele carriers, N/total N (%)		P#
				Extreme low levels	Extreme high levels	
F2	rs1799963	G/A		0/33 (0)	15/45 (33)	**<0.001**
F9	rs440051	G/A	*Men*	3/17 (18)	11/29 (38)	**0.15**
			Women	8/19 (42)	9/26 (35)	**0.76**
	Novel (799 bp)	A/T	*Men*	1/17 (6)	0/29 (0)	**0.19**
			Women	1/19 (5)	1/26 (4)	**0.82**
	rs35599944	(GT)5/(GT)6	*Men*	2/17 (12)	11/28 (39)	**0.05**
			Women	3/15 (20)	7/20 (35)	**0.33**
F11	rs4253429	A/G		17/40 (43)	4/56 (7)	**<0.001**
	rs4253430	G/C		27/40 (68)	21/56 (38)	**0.004**
	rs4253865	G/A		7/40 (18)	3/56 (5)	**0.13**
	rs1062547	A/T		27/40 (68)	21/56 (38)	**0.004**
	rs186377697	A/T		2/40 (5)	1/56 (2)	**0.37**
	rs4253431	G/A		10/39 (26)	16/56 (29)	**0.93**
FGA	rs182736373	C/T		0/36 (0)	1/60 (2)	**0.44**
FGA**	rs148317511	-/28 bp ins##		16/36(44)	28/60 (47)	**0.75**
	rs2070022	C/T		13/36(36)	17/59(29)	**0.46**
FGB	rs2227421	A/C		21/36 (58)	31/59 (53)	**0.17**
FGG	rs187316301	T/G		1/35 (3)	0/58 (0)	**0.20**
	rs183036893	G/A		1/35 (3)	0/58 (0)	**0.20**
FGG**	rs1049636	T/C		27/36 (75)	35/59 (59)	**0.17**
PROS1	rs9681204	G/T		15/53 (28)	8/41 (20)	**0.48**
	rs137965257	G/A		1/54 (2)	0/41 (0)	**0.38**
	Novel (1103 bp)	A/G		1/54 (2)	0/40 (0)	**0.39**
TFPI	rs8176629	G/A		2/41 (5)	3/35 (9)	**0.52**
	rs13392310	A/T		25/41 (61)	21/35 (60)	**0.19**
	rs145571146	A/T		1/41 (2)	0/34 (0)	**0.36**
	rs72904378	C/A		5/41 (12)	3/34 (9)	**0.64**
	rs4667166	T/A		31/41 (76)	23/35 (66)	**0.62**
	rs7595810	T/C		2/41 (5)	3/35 (9)	**0.52**

*According to dbSNP. **3'UTR of a common alternative transcript of the gene. #Pearson's χ^2-test. ##GAAGTGGGAATGGGAGCACTCTGTCTTC.

The frequency of the 26 identified SNPs in individuals with extreme protein values is shown in Table 2. From this analysis, five common SNPs were considered candidates for further analysis as their prevalence differed between individuals with high and low levels of the coagulation factor of interest (Table 2; *F2* rs1799963, *F11* rs4253429, *F11* rs4253430 and *F11* rs1062547). The well-known prothrombin G20210A polymorphism (*F2* rs1799963) has already been genotyped in the MEGA study [143]. The remaining three SNPs had not previously been studied in relation to thrombosis and were genotyped in all 4485 cases and 4889 controls with available DNA samples from the MEGA study. *F11* SNPs genotypes were in HWE in all control individuals.

Table 4| Effect of *F11* 3'UTR SNPs on the risk of venous thrombosis in MEGA

Variant	Genotype	N cases (%)	N controls (%)	Odds ratio (95% CI)*	Odds ratio (95% CI) independent functional variants#
rs4253429	AA	3228	3308	Ref	**Ref**
	AG	1088	1375	0.81 (0.74-0.89)	**1.07 (0.85-1.34)**
	GG	113	152	0.77 (0.60-0.98)	**1.08 (0.79-1.48)**
rs4253430	GG	1968	1907	Ref	**Ref**
	GC	1973	2252	0.85 (0.78-0.93)	**1.20 (0.80-1.80)**
	CC	484	669	0.70 (0.61-0.80)	**1.35 (0.91-2.01)**
rs1062547	AA	1978	1902	Ref	**Ref**
	AT	1959	2266	0.83 (0.76-0.91)	**1.20 (0.80-1.80)**
	TT	488	667	0.70 (0.62-0.80)	**1.35 (0.91-2.01)**

Abbreviations: CI=confidence interval; Ref=reference category.
*Adjusted for age and sex, # in wildtype carriers of rs2289252/rs2036914.

When assessing the risk of venous thrombosis, the three *F11* SNPs also showed a reduction in thrombosis risk ranging from 15% to 19% for heterozygous carriers and from 23% to 30% for homozygous carriers (Table 4). SNP rs4253430 and rs1062547 were in strong LD, with over 98% of the cases and controls carrying both variants. When we included rs4253429 and rs4253430 in one logistic model, the risk reduction remained significant only for rs4253430 with an OR of 0.89 (95% CI 0.80-0.98) for heterozygotes and an OR of 0.74 (95% CI 0.63-0.87) for homozygotes.

Li *et al* previously reported on genetic variants associated with the risk of venous thrombosis, i.e., FXI rs2036914 and rs2289252 [30]. When assessing the linkage between these known variants and the variants described in this

study, it was clear that the FXI variants were in partial, inverse linkage (0.4 ≤r^2<0.5) with the known variants. When we subsequently adjust the analysis for the known FXI variants, the association with the risk of venous thrombosis was inversed, due to the inverse linkage with the known variants, and there was a mild increased risk of venous thrombosis associated with FXI rs4253430, i.e., when using wildtype carriers as a reference category: OR heterozygotes: 1.2 (95% CI: 0.8-1.8); OR homozygotes: 1.4 (95% CI: 0.9-2.0).

Table 5| Effect of *F11* 3'UTR SNPs on plasma levels in MEGA

Variant	Genotype	N controls with available plasma	Mean levels FXI activity**	Mean levels independent functional variants
rs4253429	AA	2011	101.7	**91.0**
	AG	849	97.4#	**92.2**
	GG	77	91.9#	**91.5**
rs4253430	GG	1188	103.0	**84.3**
	GC	1377	99.4#	**91.9#**
	CC	372	94.4#	**92.8#**
rs1062547	AA	1185	103.0	**84.3**
	AT	1381	99.3#	**91.9#**
	TT	371	94.4#	**92.8#**

*% of normal, adjusted for age and sex; # Significant compared to the reference allele (A for *F11* rs4253429 and *F11* rs1062547; G for *F11* rs4253430) with p<0.001

Subsequently, we assessed the effect of the described variants on the levels of the coagulation factor levels. For the three *F11* SNPs, in the crude analysis, there was a clear, graded association between genotype and plasma FXI levels, with about a 4-5 IU/mL difference in levels per variant allele (Table 5).

When restricting the analysis to wildtype carriers of FXI rs2036914 and rs2289252, only FXI rs4253430 and rs1062547 remained associated with FXI levels, i.e.: wildtype carriers: 84.3 IU/mL, heterozygotes: 91.9 IU/mL, homozygotes: 92.8 IU/mL with mean differences of 7.6 UI/mL (95% CI: 2.4-12.7) and 8.5 IU/mL (95% CI: 3.4-13.6) for heterozygotes and homozygotes, respectively, when using wildtype carriers as a reference category (Table 5).

Next, we searched *in silico* miRNAs binding sites that were eliminated or created by these SNPs using different miRNA resources. These 26 SNPs mainly predicted to delete miRNA binding sites (27 sites predicted), while only one variant was predicted to potentially create a new miRNA binding site (Table 6).

Table 6| Potential miRNA interactions affected by SNPs

Gene	variant	miRNA (deleted sites)	miRNA (created sites)
F2	rs1799963	-	-
F9	rs440051	-	-
	Novel (799 bp)	-	-
	rs35599944	-	-
F11	rs4253429	miR-137[#]	-
	rs4253430	miR-544[##]	-
	rs4253865	miR-2355[#], miR-1975[#], miR-93-5p[#], miR-4286[##]	-
	rs1062547	miR-513a-3p[#]	-
	rs186377697	miR-889[##]	-
	rs4253431	miR-622[#]	-
FGA	rs182736373	miR-3173-3p[##]	-
FGA*	rs148317511	miR186[#], miR-434[##], miR-3133[#]	-
	rs2070022	miR-4476[#]	-
FGB	rs2227421	miR-29b-1-5p[**], miR-759[#], miR-924[#], miR-4294[#], miR-627[#]	-
FGG	rs187316301	-	-
	rs183036893	-	-
FGG*	rs1049636	-	-
PROS1	rs9681204	miR-26b-5p[##]	-
	rs137965257	mir-375[##]	-
	Novel (1103 bp)	-	-
TFPI	rs8176629	-	-
	rs13392310	miR-200a[##], miR-200b[##], miR-429[##]	-
	rs145571146	-	-
	rs72904378	miR-605[##], miR-2355[#]	-
	rs4667166	miR-4302[##]	-
	rs7595810	-	miR-7[†]

[*]3'UTR of a common alternative transcript of the gene. [**]Predicted by Targetscan, miRSVR, and Patrocles algorithms, [#]Predicted by Targetscan or miRSVR algorithms, [##]Predicted by Targetscan and miRSVR algorithms, [†]Predicted by PolymiRTS

MiR-544 and miR-513a-3p are predicted to bind to the 3'UTR of *F11* (Figure 2A), and the two functional *F11* SNPs rs4253430 and rs1062547 may destroy miRNA:*F11* mRNA interaction respectively.

Using RNAHybrid program [124] for prediction of secondary structure and minimum free energy, we showed a 20% decrease in the minimum free energy value of miR-544 in a wild type sequence compared to the mutated (rs4253430), while apparently miR-513-3p:F11 mRNA interaction was not affected by rs1062547 variant (Figure 2B)

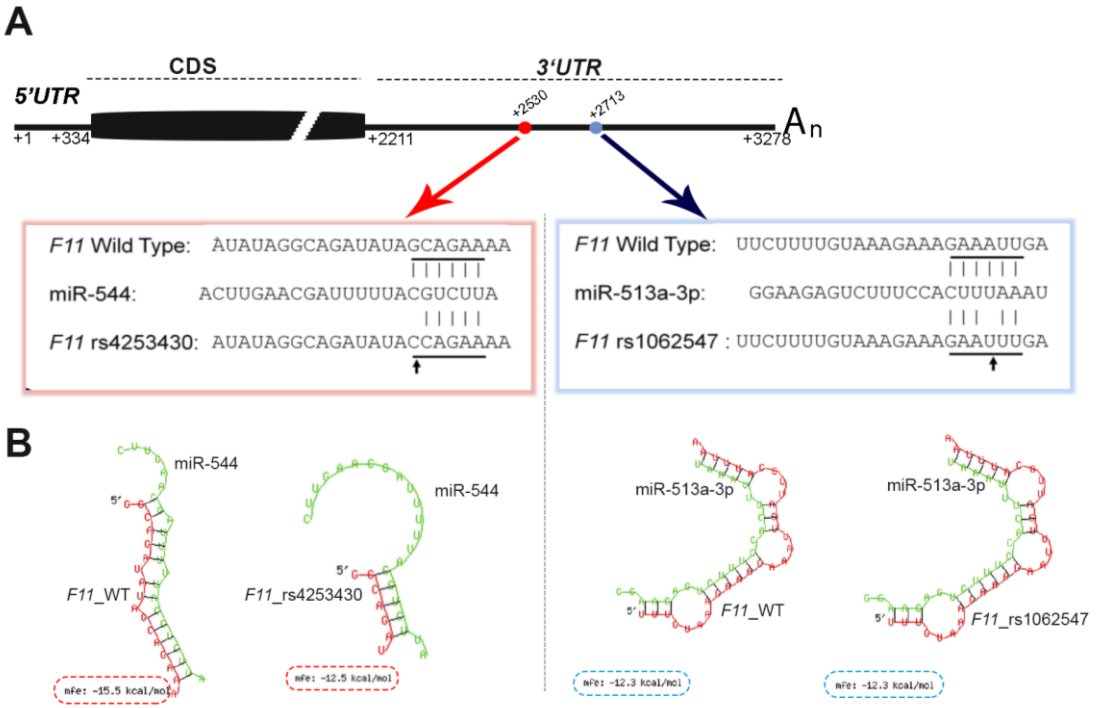

Figure 2| A) Schematic representation of miR-544 and miR-513-3p target sites in either *F11* 3′UTR Wild type (WT) or variants (rs4253430 and rs1062547, respectively). Within boxes, complementarities between the seed region (6 nucleotides) of miRNAs and 3′UTR of *F11* mRNA target site are underlined and bold arrows represent each of the variants. B) Secondary structure prediction using RNAHybrid webtool of miR-544 and miR-513-3p to either *F11* WT or variants; rs4253430 and rs1062547, respectively.

In order to test the effect of the two functional *F11* SNPs, rs4253430 and rs1062547, on miRNA binding, we performed reporter assays. Thus, co-transfection of HCT-DK cells with a luciferase reporter vector containing the 3'UTR of *F11* and miR-544 showed a significant decrease of ~35% of the luciferase activity in comparison with SCR (100 % *vs*. 46 ± 9%, p= 0.029). This inhibition was not observed when using a mutated vector with the rs4253430 polymorphism (Figure 3A). No effect was seen for miR-513-3p in presence or absence of rs1062547 (Figure 3B).

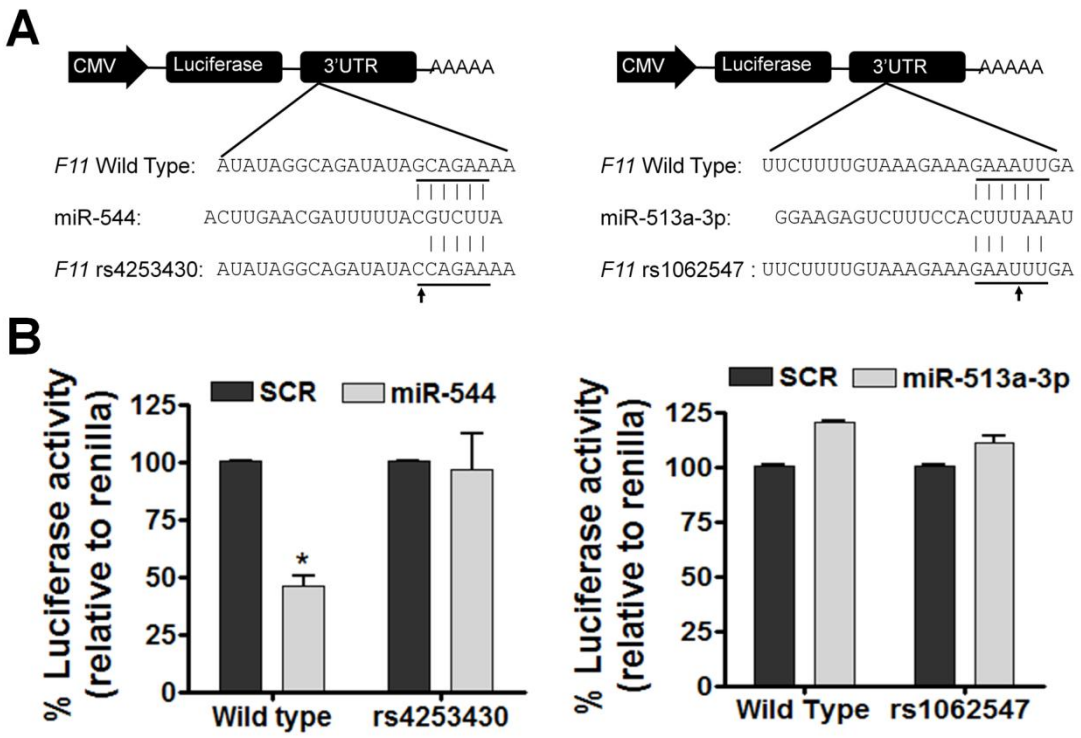

Figure 3| Luciferase reporter assays. (A) Constructs pRL-CMV of fulllength F11 3'UTR containing luciferase gene and 3'UTR of *F11* with different SNPs; rs4253430 and rs1062547 (G>C and A>U substitution respectively). Predict effect of SNPs rs4253430 and rs1062547 on miR-544 and miR-513-3p recognition respectively. (B) Luciferase reporter assays to measure F11 3'UTR either wild type or with variant rs4253430/rs1062547 with the absence or presence of miR-544/miR-513a-3p respectively. HCT-DK cells, that do not express miR-544 or miR-513a-3p nor other Dicer-dependent miRNAs that may interfere in miRNA overexpression experiments, were transfected with either *F11* WT or *F11* variants rs4253430/rs1062547 along with 100 nM miR-544/miR-513-3p mimic respectively. A SCR precursor was used as control. Luciferase activities were normalized to renilla activities. Results are represented as mean ± SD of three replicates from three independent experiments. The normalized data were expressed as changes relative to the data of the cells transfected with SCR and set as 100%. *P<0.05. Student's t-test was calculated in mimic *vs*. SCR.

4. Discussion

We sequenced the 3'UTRs of genes coding for prothrombin, FIX and FXI, fibrinogen (*FGA*, *FGB* and *FGG)*, protein C, protein S, antithrombin and TFPI using an extreme phenotype approach. Selecting individuals with extreme phenotypes increases the likelihood of detecting trait-related sequence variations [145]. The sequencing led to the identification of 28 3'UTR variants of which five common variants were considered candidates for further analysis as their prevalence differed between highest and lowest level groups (*F2* rs1799963, *F11* rs4253429, *F11* rs4253430 and *F11* rs1062547). Subsequently, these variants, except the established *F2* rs1799963 SNP

(prothrombin G20210A) [143], were associated with coagulation factor levels and risk in all MEGA patients and controls with available DNA.

Six common *F11* 3'UTR variants were identified, of which three variants showed a significant difference between extreme level groups (rs4253429, rs4253430 and rs1062547). After adjustment for the effect of well-known variants in the FXI gene, we showed than FXI rs4253430 was associated with a mild increase in the risk of venous thrombosis and with increased levels of FXI, albeit still in normal range. In accordance with two other *F11* SNPs investigated in LETS and MEGA [30], the association between *F11* rs4253430 and the risk of venous thrombosis was at least partly explained by its effect on FXI activity levels.

In the 3'UTRs of *F9*, *FGA*, *FGB*, *FGG*, *PROS1* and *TFPI*, we found several known variants that we did not genotype in a larger sample of the MEGA study because we found only 1 carrier in one of the extreme level groups or because SNPs did not significantly differ in frequency between extreme phenotypes. It is therefore possible if not likely that we missed several variants with a smaller effect size. Given that we demonstrate that our approach resulted in several associated SNPs, a more comprehensive follow-up may be warranted. However, earlier studies found no significant effect of *F9* rs440051 and *F9* rs35599944 on FIX levels [150, 151], no effect on fibrinogen levels of haplotypes harboring *FGA* rs2070022, *FGB* rs2227421 or *FGG* rs1049636 [146], or of *FGA* rs148317511 [152], and an opposite effect of *PROS1* rs9681204 on protein S levels [153].

3'UTR regions play a key regulatory role by different mechanisms: poly(A) site efficiency [154] and also through miRNA binding [50]. Using *in silico* tools we evaluated the potential consequences on miRNA binding of the variations identified in the present study. In the *F2* 3'UTR, we only identified the prothrombin G20210A polymorphism, which is believed to be a more efficient poly(A) site than 20210G, leading to increased mRNA and protein expression [155] and probably does not influence miRNA binding. However, *in silico* predictions revealed that several SNPs located in the 3'UTR are able to modify the binding of miRNAs, whether deleting canonical binding sites or creating new binding sites. Actually, an effect of variants in miRNA target sites has been

found on the risk of diseases such as Parkinson's disease, colorectal cancer and childhood asthma [156]. So, alterations of potential miRNA binding sites may have a relevant effect in the expression of the target protein by affecting plasma levels and increasing the risk of thrombosis. On the other hand, rs7595810 located in *TFPI* mRNA may create a new binding site for miR-7, which potentially may reduce levels of this protein in plasma.

Indeed, we showed in this report that miR-544 may affect *F11* expression and that the presence of rs4253430 dramatically modified the interaction between miR-544 and its target. However, these results have a limitation since miR-544 is not expressed in liver (Figure 4), so physiologically this miRNA cannot be responsible for the phenotype observed with this SNP. Despite this limitation, our *in vitro* results clearly demonstrated that rs4253430 might mechanistically provoke an alteration in the interaction with miRNAs (Figure 2A).

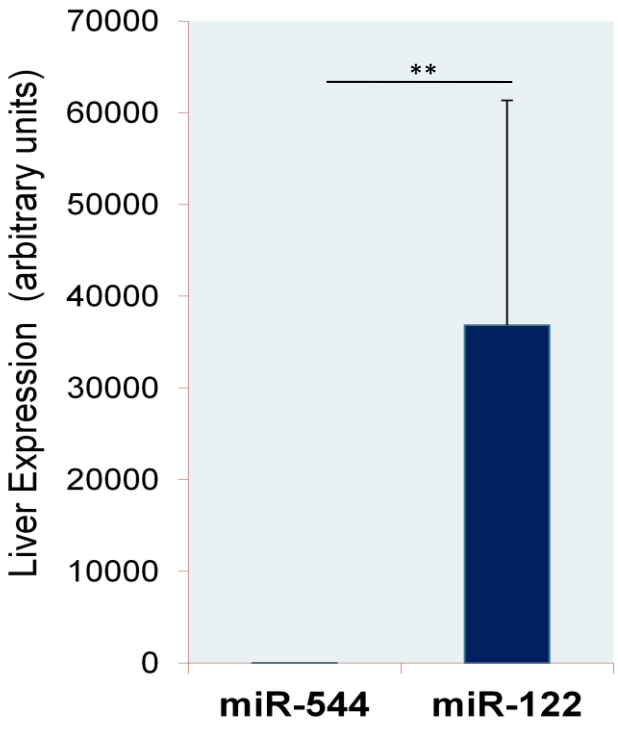

Figure 4: MiRNA expression of miR-544 compared to miR-122 (the most highly expressed miRNA in human liver) in a total of 5 human liver-microarrays expressed in arbitrary units. **:p<0.001

At present, there are many pitfalls and limitations in the *in silico* predictions and it may be that the available algorithms do not predict yet any binding in *F11* 3'UTR at rs4253430. On the other hand, massive sequencing technology is continuously discovering new mature miRNAs, thus some miRNAs not yet characterized may bind to *F11* at the site of this SNP. Indeed, these two hypotheses are extensive to rs1062547, where the fact that miR-513-3p did not bind to *F11* 3'UTR does not mean that another miRNA, yet to be characterized, may do so at this position. Yet, things get even more complicated since SNPs located in the mRNA target, outside of the binding site with its regulatory miRNA, may also play a relevant role in the interaction between both molecules. Mishra *et al* demonstrated that SNPs located several nucleotides downstream of the mRNA:miRNA binding site can inhibit mRNA interaction (Mishra PNAS) with RISC protein content. Thus, strategies considering this additional potential pitfall are also essential for the discovery of functional miR-SNPs involving a miRNA-related mechanism.

Importantly, Starikova *et al* have recently shown that a series of plasma miRNAs are differentially expressed in patients with venous thromboembolism in comparison with healthy controls strongly suggesting a role for miRNAs in venous thrombosis [85].

In conclusion, we identified a 3'UTR variant, *F11* rs4253430, with a significant effect on levels and venous thrombosis risk in 4485 cases and 4889 controls from the MEGA study. *In vitro* confirmations of predicted effects of miRSNPs on miRNA binding are highly recommended, but are complicated by the large number of false positives (>70%) generated by miRNA target prediction resources [67, 149]. Rather than confirming current predicted effects for these two variants from miRNA resources, we recommend future studies to screen large panels of miRNAs for an effect on FXI levels.

III. Indirect regulation of hemostatic proteins by miR-24 and miR-34a through HNF4α

1. Introduction

High levels of coagulation factors may disturb the fragile balance of hemostatic system then leading to thrombotic events. However, coagulation factors have a substantial inter-individual variability in healthy human plasma [157, 158] so that genetic, *e*nvironmental and acquired factors act jointly for defining the threshold for the individual thrombotic risk [157, 159-161]. Among the genetic elements that drive the synthesis of coagulation factors a hereditary component has been described for several of them although the heritable basis for high or low levels of factors remains unknown [11]. Interestingly, common regulatory genes coordinate simultaneously the expression of several clotting factor genes which would allow to categorize individuals in those with high or low levels of coagulation factors [158, 160].

A good candidate among these common regulatory genes is Hepatocyte Nuclear Factor 4 α (HNF4α, NR2A1, gene symbol *HNF4A*), a member of the nuclear receptor superfamily, essential for liver homeostasis and linked to several diseases including diabetes, hemophilia, atherosclerosis, and hepatitis [109]. HNF4α has been linked to the expression of a large number of coagulation genes such as prothrombin [162], FVII [163, 164], FVIII [165], FIX [166, 167], FX [168], FXI [169], FXII [170], protein S [171], protein Z [172] and antithrombin [173, 174]. The full-body *Hnf4a* knock-out mouse is embryonic lethal [110] and gene targeting using short interfering RNA (siHNF4A) confirmed the impact of HNF4α in regulating hepatic coagulation transcription [111, 112].

Several inductors and repressors that weave a complex regulatory net participate in the expression of *HNF4A* gene [175, 176]. In addition, *HNF4A* is also post-transcriptionally regulated since miRNA [113] have been described to be involved in its expression. Thus, Takagi et al. were the first describing *in vitro* the regulation of HNF4α by miR-24 and miR-34a [59].

The more intuitive and better described mechanism of action of miRNAs is as CIS-suppressive-regulatory elements. Thus, up to seven coagulation factor genes have been described to be targets of miRNAs (see Introduction). However, the hypothesis that miRNAs repress common transcription factors

then working as TRANS-regulatory elements for some genes remains to be explored.

Here, we aimed to thoroughly gain a deeper insight into the physiological modulator role of miRNA in the expression of downstream coagulation targets of HNF4α.

2. Material and methods

2.1. Cell culture and tissue samples

HepG2 (American Type Culture Collection, Manassas, VA) were conventionally cultured. Briefly, HepG2 were cultured in MEM (Life Technologies, Madrid, Spain). Medium was supplemented with 0.1mM non-essential amino acids, 2 mM Glutamax I, and with 10% fetal calf serum (Life Technologies, Madrid, Spain). Cells were grown at 37ºC under 5% CO_2.

Liver samples from Caucasian donors older than 18 years of age (n=104) were kindly provided by the Research Center of Experimental Pathology Department of La Fe Hospital and CIBERehd (Valencia, Spain) (n=17) and by St. Jude Children's Research Hospital Liver Resource (Liver Tissue Procurement and Distribution System (NIH Contract #N01-DK-9-2310) and the Cooperative Human Tissue Network) (n=87) [177]. All donors gave a written informed consent that was recorded following the procedures of each Biobank. Human liver studies were further approved by Local Ethics Committee from Hospital Universitario Morales Meseguer in Murcia (#ESTU-19/12).

2.2. Murine hepatocyte primary culture

Hepatocytes were isolated from 20-25 g C57BL/6J mice using a modified version protocol from Wu et al [178]. Mice were anesthetized with an intraperitoneal injection of a ketamine/xylazine mixture. A 24G clear cannula was inserted into the posterior vena cava and secured with a ligature. A second ligature was placed around the anterior vena cava, between the liver and the heart, and the portal vein was severed, allowing outflow of solution. The liver was then perfused at 37°C with oxygenized HBSS (in mM: 137 NaCl, 5.4 KCl,

0.8 $MgSO_4.7H_2O$, 0.3 $NaHPO_4.2H_2O$, 0.44 KH_2PO_4, 26 $NaHCO_3$, pH 7.4) 3 min at 5 mL/min and 5 min at 7 mL/min. The perfusion solution was then changed to HBSS supplemented with 4 mM $CaCl_2$ and containing 0.12% collagenase (Sigma-Aldrich, Madrid, Spain) for 5 min at 5 mL/min. The liver was additionally incubated with HBSS with 0.12% collagenase for 15 min, filtered through a cell strainer (100 µm from Becton Dickinson, Madrid, Spain). Hepatocytes had a viability >95% (trypan blue exclusion), readily attached to six-well plates pre-coated with 50 µg/mL collagen from Stemcell (Grenoble, France), and showed a characteristic cell morphology in culture. Primary hepatocytes were seeded at 250,000/well and cultured with DMEM/F12 media supplemented with 10% FCS, penicillin and streptomycin.

2.3. Cell transfection:

2.3.1. HepG2 transfection

Briefly, HepG2 cells were seeded twenty four hours before transfection in complete MEM medium supplemented with 10% fetal bovine serum at 37°C in a humidified incubator with 5% CO_2 without antibiotics and transfected with 100 nM of chemically modified double-stranded RNAs that mimic endogenous miRNAs or SCR (Life Technologies, Madrid, Spain) using PepMute transfection reagent from SignaGen laboratories; 100 nM of miRNA inhibitors or inhibitor control (miRCURY LNA microRNA Inhibitor from Exiqon, Vedbaek, Denmark) using RNAimax from life technologies PepMute transfection reagent from SignaGen Laboratories. Transfection efficiency assessed by FACS analysis was >80%. After 48 h, cells were collected for subsequent mRNA and protein analyses.

2.3.2. Primary mouse hepatocytes transfection

Primary hepatocytes were maintained in DMEM/F12 supplemented with 10% fetal bovine serum, at 37°C, in a humidified incubator with 5% CO_2. Cells were pre-cultured for 24h in complete medium supplemented with antibiotics, Insulin Transferrin Selenium solution (ITS), dexamethasone (Sigma-Aldrich, Madrid, Spain) and transfected at 40-60% confluence with 100 nM of either precursor

molecules for miR-24, miR-34a, and SCR (Applied Biosystems, Madrid, Spain) or the corresponding inhibitors of miR-24, miR-34a and inhibitor control (miRCURY LNA microRNA Inhibitor from Exiqon, Vedbaek, Denmark) by using Lipofectamine® 2000 Transfection Reagent (Life Technologies, Madrid, Spain). The cells were collected 48h after transfection and total RNA and lysates were extracted. Transfection efficiency assessed by FACS analysis was >80%.

2.4. RNA isolation and real-time RT-PCR

Total RNA was isolated using RNAzol®RT Reagent (Molecular Research Center, Inc. Cincinnati, OH). The RNA concentration and 260/280 ratio were determined by using NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE)**.** Retrotranscription reactions were performed using 100 ng of total RNA for each sample according to the manufacturer instructions (Life Technologies, Madrid, Spain).

Quantitative real-time PCR using TaqMan® Gene Expression Assays (Applied Biosystems, Madrid, Spain) and gene-specific primers/probes (Table S2 in Appendix I), was performed on a LightCycler® 480 Real-Time PCR System (Roche Applied Science, Barcelona, Spain). *F11* and *F9* were previously quantified in our previous study [179] and their mRNA expression data were used for analyses in this study. Data were analyzed using the comparative threshold cycle method ($2^{-\Delta Ct}$ method) with β-actin as an endogenous reference control for quantification and normalization. Commercial assays for miR-24, miR-34a, and U6 snRNA as an endogenous control (Applied Biosystems) were used to quantify expression levels of miRNAs.

2.5. Total protein extraction and western blot assay

Transfected HepG2 and murine hepatocytes cells were washed twice with phosphate-buffered saline (PBS). They were then lysed in RIPA buffer [150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 1 mM phenylmethylsulfonyl fluoride, pH 7.0] on ice for 20 min. The lysates were centrifuged at 12,000×*g* for 5 min at 4°C and the supernatants were collected for analysis of the protein

concentration using a Bicinchoninic Acid (BCA) Protein Assay (Sigma-Aldrich, Madrid, Spain). These lysates (each 20 µg) were blotted and immunostained with different monoclonal antibodies: anti-HNF4α (ab92378; Abcam, Madrid, Spain) and anti-human β-actin (Sigma-Aldrich, Madrid, Spain) in human hepatocytes; anti-HNF-4α (C-19: sc-6556; Santa Cruz Biotechnology, Inc., CA, US) and anti-GAPDH (ab128915; Abcam, Madrid, Spain) in murine hepatocytes. HNF4α, β-actin, and GAPDH were immunodetected with the appropriate secondary antibody labeled with peroxidase (GE Healthcare, Barcelona, Spain). Detection was developed using ECL Prime Western Blotting Detection Kit (GE Healthcare, Barcelona, Spain) and ImageQuant LAS 4000 Imager (GE Healthcare, Barcelona, Spain). Densitometric analysis was performed with ImageJ software (http://rsb.info.nih.gov/ij/).

Data were expressed as changes relative to the values of the cells transfected with SCR, taken as 100%.

2.6. Statistical Analysis

Statistical differences between groups were calculated by non-parametric Mann-Whitney U test using GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, US). A p-value <0.05 was considered to be statistically significant. Correlations were observed with Pearson's correlation of coefficient and analyses were carried out using Statistical Package for Social Science (version 21.0; SPSS, Chicago, IL).

3. Results

3.1. *HNF4A* correlates with coagulation factor expression levels in human liver

To verify whether the described effects of *HNF4A* on the expression of coagulation factors play a role in liver, we quantified mRNA levels of *HNF4A* and 10 genes involved in coagulation (*F5*, *F8*, *F9*, *F10*, *F11*, *F12, SERPINC1*, *PROC, PROZ,* and *PROS1)* in 104 human liver samples. We observed a widespread interindividual variation in expression levels of analyzed hepatic transcripts. As shown, the wider variability of expression was found for *F9* (from

14% up to 400%) and *SERPINC1* (from 6% up to 640%) whereas *PROC* and *PROZ* had the lower range of variability (Figure 2).

Thus, overall the distribution by percentiles according with the expression of analyzed factors showed up to 3-fold differences among percentile 25th and 75th (Figure 2). Similarly, a wide variability was also observed for both miR-24 and miR-34a, as shown in Figure 1.

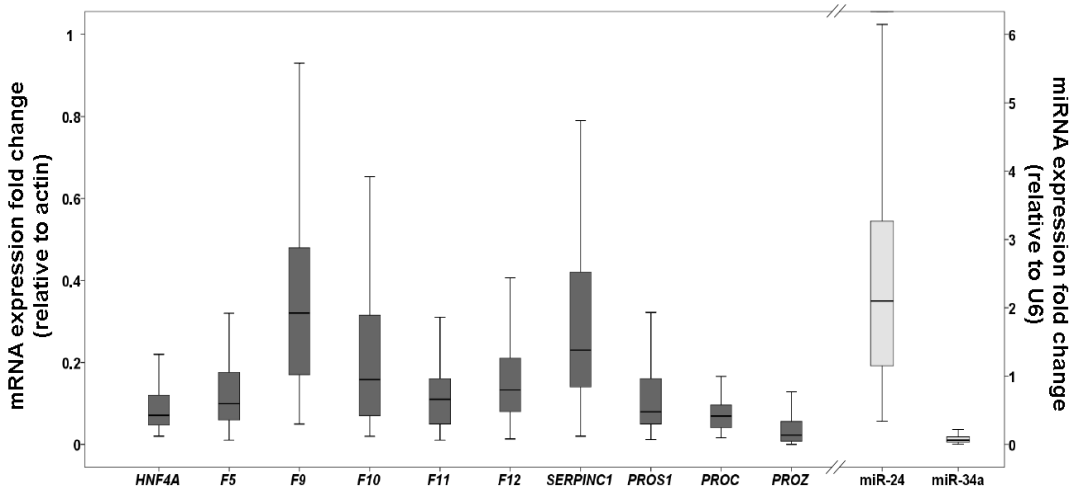

Figure 1| A) Hepatic expression profiles of mRNAs and miRNAs in human liver samples. Box plots indicate first and third quartiles of expression; the bold line in the box represents the median value; the whiskers represent the range.

Next, we analyzed the correlation between *HNF4A* expression and its hepatic downstream targets involved in coagulation in human livers. We found that those samples with higher coagulation factor expressions had also higher *HNF4A* expression (all $p<0.0001$; Figure 2).

These results suggest that differences in *HNF4A* expression might explain, at least in part, inter-individual variations seen in the expression of coagulation factors in human liver.

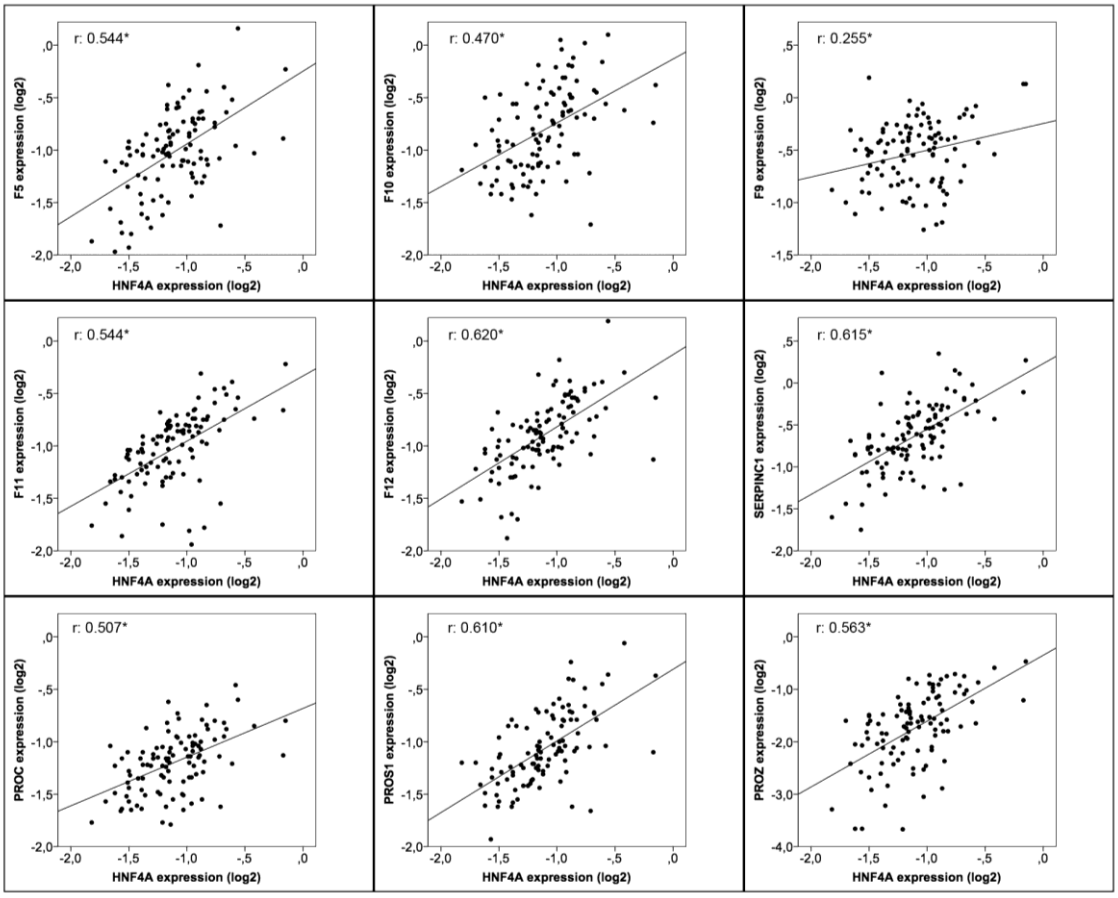

Figure 2| Correlation of mRNA expression levels of pro- and anti-coagulant factors with hepatic transcription factor *HNF4A*. Positive and significant correlations between procoagulant (F5, F9, F10, F11, and F12), anticoagulant (SERPINC1, PROC, PROS1, and PROZ) factors and HNF4A mRNA expression. qRT-PCR was performed in total RNA purified from healthy livers (n = 104). Each data point represents an individual liver tissue sample, and a correlation coefficient (r) is shown. The results are presented as Log2 fold change with respect to the normalization standard. The asterisk indicates a statistically significant difference ($p < 0.0001$).

3.2. miRNA selection

To study the possible indirect effect of miRNAs on coagulation factor expression, we selected two miRNAs, miR-24 and miR-34a, previously pointed as direct inhibitors of *HNF4α* [59, 113]. Both miRNAs bind to several sites in human *HNF4A* as described in Figure 3. While miR-34a interacts in three different sites located within *HNF4A* 3'UTR, miR-24a mostly inhibits HNF4α expression by binding to sites located within the coding region [59].

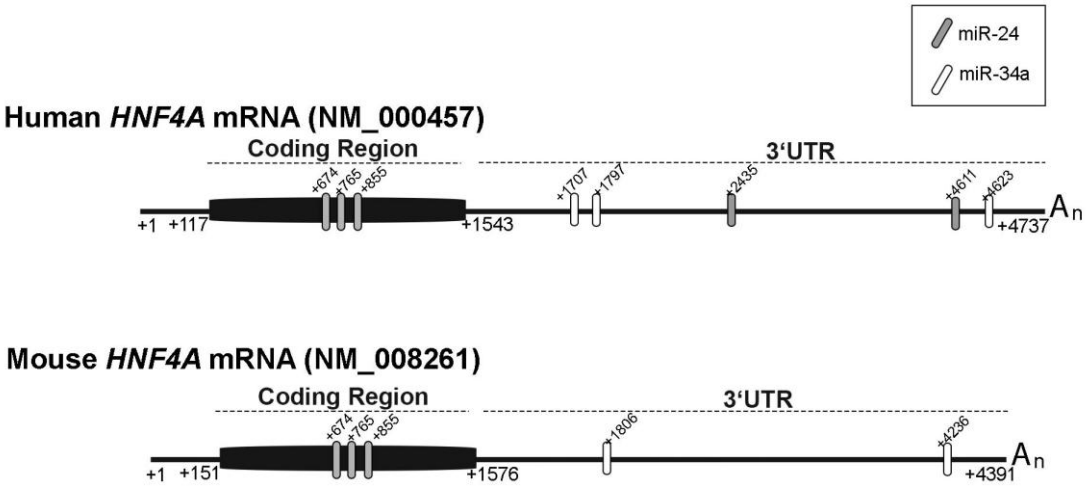

Figure 3| Schematic diagrams of human and mouse mRNA of *HNF4A*: *HNF4A* human and mouse *HNF4A* a highly conserved, miR-24 and miR-34 do not share the same binding sites in both species. Thus, miR-24 shares binding sites in the coding region of *HNF4A* in both species but it has one more within 3´UTR only in human gene. In turns, miR-34 binds to different seed regions in the 3'UTR of human or mouse.

3.3. *In vitro* study in HepG2 cells

3.3.1. MiR-24 regulates coagulation factors by targeting *HNF4A*

The functional role of miR-24 in regulating HNF4α downstream targets was tested in HepG2 cells by transfecting with miRNA mimics. As expected, results from western blot analysis using whole cell lysates from HepG2 confirmed a 45% decrease of HNF4α mediated by miR-24 (p=0.01) (Figure 4A) and a 25% decrease in mRNA levels (Figure 4B). However, HepG2 cells transfected with miR-24 inhibitor did not overexpress *HNF4A* (Figure 4B). Overall, these results suggest that miR-24 regulates HNF4α expression in hepatic cells.

To investigate whether the decrease of HNF4α was accompanied by a decrease of coagulation factors, we determined mRNA levels by qRT-PCR analysis. HepG2 transfection with miR-24 caused a decrease in mRNA of all selected factors although such reduction was only statistically significant for *F10*, *F12*, *PROS1, and SERPINC1* (Figure 4C).

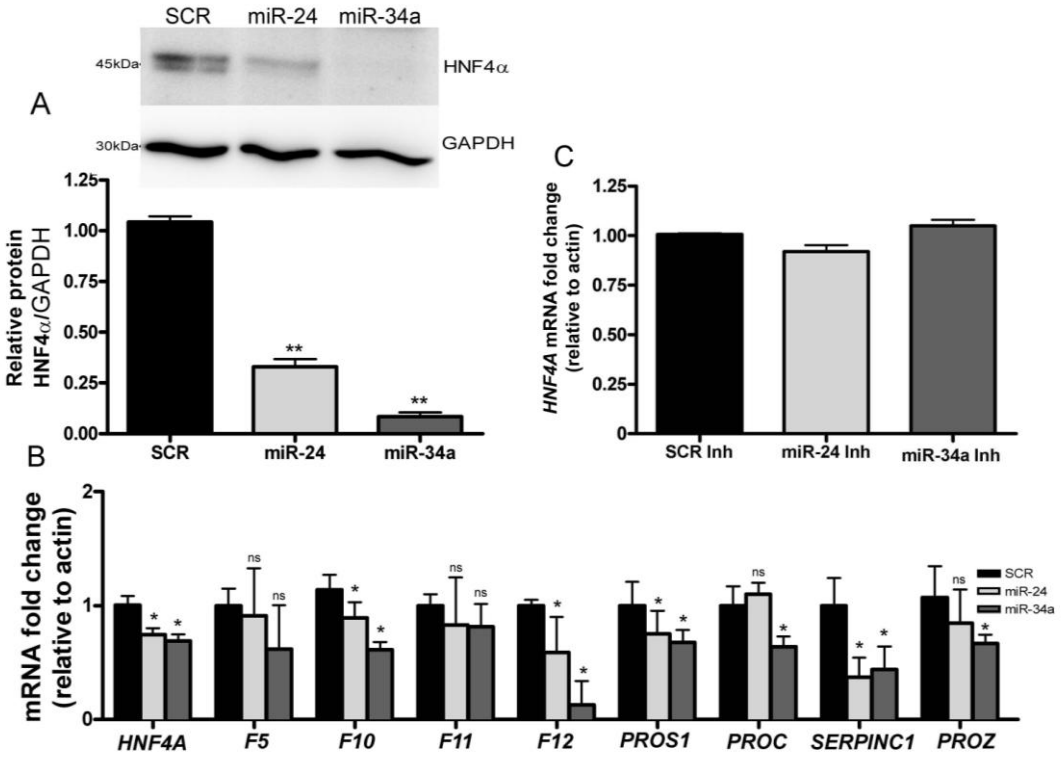

Figure 4| Effect of miRNAs on *HNF4A* and coagulation factors expression in HepG2. HepG2 cells were transfected with 100 nM mimic precursors of miR-24, miR-34a or SCR. Protein lysate and total RNA were obtained after 48 h incubation and analyzed. A) Densitometric analysis of HNF4α protein expression transfected with SCR, miR-24, and miR-34a with a representative western blot. B) qRT-PCR analysis of HNF4A and coagulation factors mRNA expression. The asterisk indicates a statistically significant difference ($P < 0.05$) and "ns" indicates a statistically non-significant difference ($P > 0.05$). C) miRNA inhibitor effect on *HNF4A* expression with 100 nM miR-24, miR-34a inhibitor or SCR inhibitor. Results are mean ± SD of three independent experiments performed in triplicate.

3.3.2. MiR-34a regulates coagulation factors by targeting *HNF4A*

The functional role of miR-34a on HNF4α was tested in HepG2 cells. Similarly to that seen for miR-24, western blot analysis of lysates from HepG2 showed a significant decrease of HNF4α (Figure 4A) and HNF4A mRNA (Figure 4B), as previously described [59, 113]., The use of miR-34 inhibitors did not increase *HNF4A* mRNA (Figure 4C).

We next investigated the consequences of HNF4α inhibition by miR-34a on coagulation factors. For this, mRNA levels of selected factors were tested by qRT-PCR. As shown in Figure 4B, miR-34a induced a decrease in the expression of all tested factors. The transcript decrease was significant for *F10*, *F12*, *PROS1, PROC, SERPINC1,* and *PROZ*, (Figure 4C).

Overall, these results confirmed the role of miR-24 and miR-34a in regulating *HNF4α* expression and showed a new trans-mechanism of regulation of several coagulation factors by miRNA through HNF4α.

3.4. Inverse correlation between *HNF4A* and both miR-24 and miR-34a expression levels in human livers

To verify the impact of miRNA variations on *HNF4A* and because there remains substantial unexplained variability in the expression of *HNF4A* and its downstream coagulation genes targets, we analyzed *ex vivo*, miRNA-mRNA correlations in human liver samples. We found an inverse correlation between *HNF4A* and both miR-24 and miR-34a hepatic transcript levels (r= -0.170; p= 0.08 and r= -0.228; p<0.05; respectively) (Figure 5).

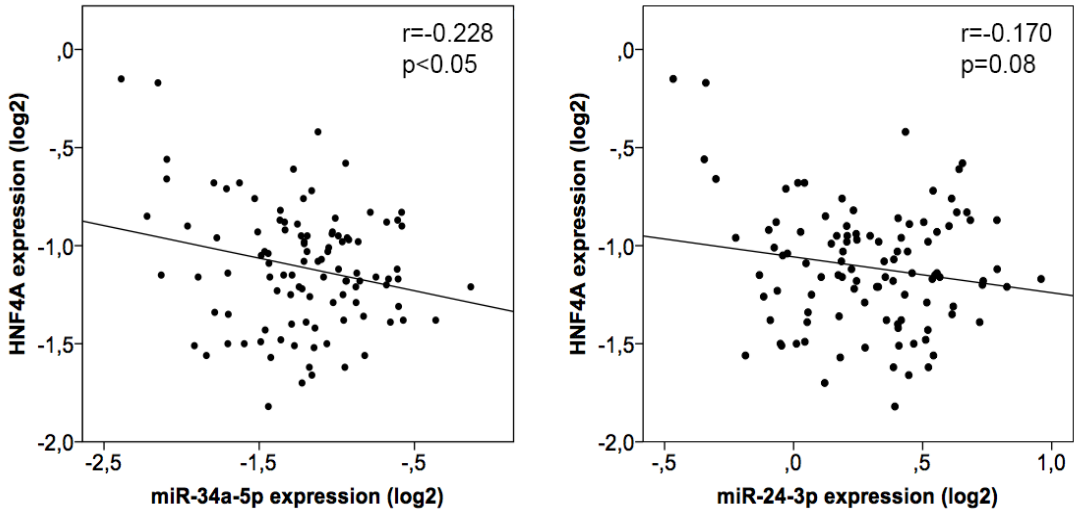

Figure 5| Linear regression analysis between endogenous mature miRNAs (miR-34a and miR-24) levels and *HNF4A* mRNA (A & B respectively). qRT-PCR were performed in total RNA purified from healthy livers (n = 104). Statistical significance was taken as p<0.05. The results are presented as Log2 fold change with respect to the normalization standard.

Interestingly, liver samples with extreme miRNA levels (percentiles 10th and 90th) showed inverse and in some cases significant levels of *HNF4A* and coagulation factors transcripts (Figure 6). As shown in Figure 6A, liver samples with lower expression of miR-24 had significantly higher expression of *HNF4A, F9, F11, PROS1 and PROZ. In* turn, liver samples with lower levels of miR-34a

also had the following higher coagulation factors levels: *HNF4α*, *F9*, *PROS1* and *PROZ* (Figure 6B).

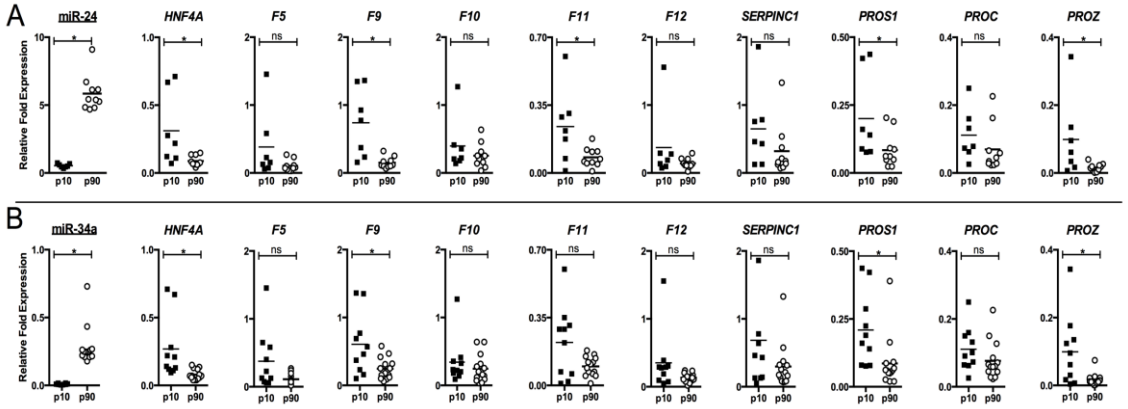

Figure 6| Expression range of miR-24 (A) and miR-34a (B) with *HNF4A* mRNA and its downstream targets in healthy livers. Dot plot diagram of *HNF4A* mRNA, selected coagulation factors and miR-24 (A) or miR-34a (B) levels in livers. P10 and p90 represent 10th and 90th percentiles of miR-24 (A) and miR-34a (B), respectively. Each data point represents an individual liver tissue sample. The results are presented as fold change with respect to the normalization standard. The asterisk indicates a statistically significant difference ($P < 0.05$) and "ns" indicates a statistically non-significant difference ($P > 0.05$).

3.5. Effect of miRNAs in coagulation factor expression through murine *Hnf4a* modulation

In order to investigate whether miR-24 and miR-34a also exerted a regulation on murine *Hnf4a* and, on selected coagulation factors, primary hepatocytes from mice were transfected with both miRNAs.

In silico studies showed that human miR-24 binding sites in the coding regions are conserved in murine *Hnf4a* although no studies have demonstrated that HNF4α inhibition occurs (Figure 2). On the other hand, human miR-34 binding regions are not conserved in mouse, but murine *Hnf4a* also displays two miR-34a binding sites located in the 3'UTR region (Figure 2).

As shown in Figure 6, transfection with miR-24 decreased *Hnf4a* mRNA up to 40%. On the other hand, the expression of HNF4α was reduced by 75% when cells were transfected with miR-34a respect to SCR (Figure 7A).

With regard to the consequences that HNF4α regulation by these miRNA might have on coagulation proteins, we found identical results for both miRNA. Thus, although all tested factor decreased when murine hepatocytes were transfected

with both miRNA, such differences reached statistical significance in *F9*, *F12, PROS1, PROC,* and *SERPINC1* (Figure 7B).

Transfection of primary murine hepatocytes with miRNA inhibitors of either miR-24 or miR-34a did not overexpress *Hnf4a* mRNA (Figure 7C). These results replicated those found when HepG2 cells were transfected with anti-miR (see above).

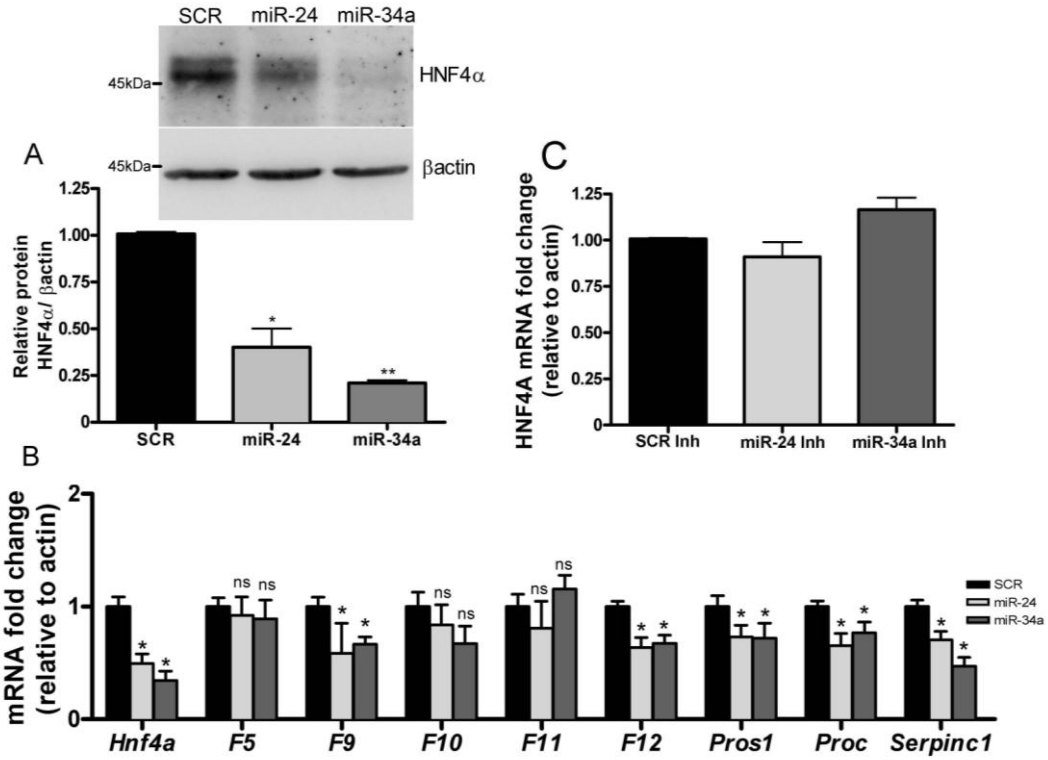

Figure 7| Widespread effect of miRNAs on *HNF4A* and coagulation factors expression in murine hepatocytes. A) Densitometric analysis of HNF4α protein expression with a representative western blot in murine hepatocytes transfected with 100 nM mimic precursors of miR-24, miR-34a or SCR. Protein lysate and total RNA were obtained after 48 h incubation and analyzed. B) qRT-PCR analysis of *Hnf4a* and coagulation factors mRNA expression. The asterisk indicates a statistically significant difference ($P < 0.05$) and "ns" indicates a statistically non-significant difference ($P > 0.05$). C) miRNA inhibitor effect on *Hnf4a* expression with 100 nM miR-24, miR-34a inhibitor or SCR inhibitor. Results are expressed in mean ± SD from three independent eexperiments performed in triplicate.

4. Discussion

Previous data from our group showed a noticeable variability in transcript levels of *F11* and *F9* in human healthy livers [179] suggesting that a common genetic regulator is behind specific clustering patterns for human hepatic coagulation factors. However, molecular basis underlying the wide coagulation factors variability in normal population probably involve a combination of factors. Given

their crucial role in controlling mRNAs processing, transcriptional factors are ideal candidates to investigate. Moreover, the tandem of some transcription factors and miRNA is an essential network for several biological processes, and its identification has elucidated some of its functions in inflammation [180] and drug metabolism [181] but it still remains to be described in coagulation. Therefore, we aimed to investigate the contribution of miRNAs as novel participants in the variable expression of coagulation factors as well as to investigate the role of HNF4α in this regulatory loop.

Our first interesting finding was a positive correlation between *HNF4A* and mRNAs of several coagulation genes. To our knowledge these are the first data describing transcriptional relationship between *HNF4A* and a large number of hepatic coagulation factors in the human liver.

The bibliographic review for experimentally-validated miRNA:*HNF4A* mRNA studies, conducted us to select miR-24 and miR-34a as indirect regulators candidates of coagulation factors [59, 113]. Our *in vitro* results showed that miR-24 and miR-34a had a significant impact on the expression of coagulation factors in both human (Figure 4B) and mouse (Figure 7B) hepatocytes. Similarly, both miRNAs inhibited HNF4α expression in human and in murine hepatocytes, suggesting a TRANS-regulation of several coagulation factors under miRNA control as a novel mechanism in hemostasis.

Given the direct effect of miRNA mimic on down-regulating *HNF4A* and their targets, it would be expected that miRNA inhibitors of either miR-24 or miR-34a up-regulate *HNF4A* expression. However, in our hands miRNA inhibitors (anti-miR-24 and anti-miR-34a) did not increase *HNF4A* expression neither in HepG2 (Figure 4C) nor in murine hepatocytes (Figure 7C). Nevertheless, we still are confident of our results as none of the previous validated studies reported an effect of anti-miR-24 and/or anti-miR-34a on *HNF4A* [59, 113]. A possible explanation for these apparently contradictory findings has been proposed by Androsavich *et al* [182]. They suggested that the absence of effect of a specific miRNA inhibitor is due to the fact that most genes are probably regulated by multiple miRNAs in which case it is more than likely the existence of alternative

miRNAs able to offset the function of a given miRNA [182]. Indeed, it has been reported that HNF4α is predicted to be targeted by up to 350 miRNAs [114].

Additionally, we investigated whether the integrated regulatory association of miR-24, miR-34a, and *HNF4A* observed *in vitro* could also take place in human healthy livers. We first tested the relationship between *HNF4A* mRNA and both miRNA levels confirming that liver samples with higher *HNF4A* transcript are those with the lower miRNAs levels, and *vice versa*. The idea that miR-24 and miR-34a regulation of gene expression is mediating as a significant mechanism contributing to variation in gene expression has been previously documented. Thus, Lamba *et al* [181] demonstrated that miR-34a had significant negative correlation with expression levels of multiple hepatic transcription factors (including HNF4α) and that it was involved in significant mediation of the association observed between *CYP2C19* and HNF4α [181]. Then, our results while confirming the miR-34a/*HNF4A* interaction, also showed, for first time, a key regulatory role of miR-34 on hepatic coagulation genes in humans.

In turns, we also confirmed here a regulatory connection between miR-24 and *HNF4A*, as did Hatziapostolou *et al* [180] in samples from 12 healthy livers. Our series of human liver samples extended to 104, provides additional consequences for miR-24/*HNF4A* interaction, as it had repercussions on the levels of coagulation factors transcripts. Of note, liver samples with extreme expression of miR-24 levels (percentiles 10^{th} and 90^{th}) were, in the opposite, those with utmost *HNF4A* and coagulation factors expression. Similar findings can be inferred from data reported by Hatziapostolou *et al* [180]. Thus, these authors described that transient inhibition of *HNF4A* in an hepatocellular carcinoma model drives a feedback loop circuit through several inflammatory miRNAs, miR-24 among them [180]. Moreover, our *in vitro* data supported a lower inhibitory effect for miR-24 in comparison with miR-34a, which might explain that only statistically significant values are found for liver samples from extreme percentiles. Alternatively, the sample size might be insufficient to reach statistical power.

Finally, our results show that murine HNF4α can also be regulated by miR-24 and miR-34a. These experiments were initially performed to validate a model in

which we may demonstrate for the first time that a miRNA dysregulation can have an effect on plasma coagulation factors levels. Additional *in vivo* studies are currently underway to demonstrate that an over- or downexpression of these two miRNAs can have an effect in hemostasis and in thrombosis.

In summary, our results would spread out, for first time, a key regulatory role of miR-24 and miR-34a to the inter-individual variability observed in expression of coagulation factors genes in humans. Thus, in both circuits *HNF4A* would work as a key switchboard. It remains to be clarified to what extent genetic variations in miRNA that change HNF4α activity may affect the expression of hepatic coagulation factors, and to what extent this may affect the development of thrombotic or hemorrhagic disorders.

CONCLUSIONS

This doctoral thesis provided evidences pointing towards miRNAs as regulators of the coagulation factors, with special mention for FXI, achieving the following conclusions:

1. Human liver expresses more than 280 miRNAs based on microarray studies. *In silico* predictions suggest that 4 miRNAs may bind to *F11* mRNA. However, only miR-181a-5p inhibits FXI by decreasing levels of both *F11* mRNA and protein expression, underlying the limitations of *in silico* available approaches.
2. The expression of *F11* mRNA in human liver is inversely correlated with the expression of miR-181a-5p, demonstrating for the first time that *F11* expression may be regulated by miRNAs. This new regulatory mechanism of *F11* expression could partially explain the interindividual variability of plasma FXI found in population.
3. Under the scope of this thesis, we have identified the first 3'UTR variant within *F11,* rs4253430, with a significant effect on protein levels. In addition, miR-544 affects *F11* expression and the presence of miR-SNP rs4253430 dramatically modified the interaction between this miRNA and its target. Therefore, we provided a possible mechanism for explaining FXI plasma levels that needs further demonstration.
4. *Ex vivo* correlation studies on human livers showed a wide interindividual variability of coagulation factors expression and a strong association between them, with features that are probably a direct consequence of a common and main regulatory element, HNF4α. We describe for the first time that *HNF4A* plays a role in clustering coagulation factors expression in human liver.
5. We identified that while miR-24 and miR-34a regulate the expression of HNF4α, a series of coagulation factors in hepatocytes are also modulated. This emphasizes the potentiality of the TRANS-effect of miRNA on coagulation factors through a transcription factor, HNF4α.

In summary, the cumulative results in the present thesis identify, and in some cases clarify, diverse mechanisms implicating miRNAs in the control of hemostasis. Further studies will help in linking these gene-silencing processes with pathologic processes as well as translating them into practical application.

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SUMMARY / RESUMEN

Introducción

El sistema hemostático es un sistema complejo altamente conservado que se rige por un delicado equilibrio entre elementos procoagulantes y anticoagulantes. Este sistema aglutina diferentes procesos tales como la hemostasia primaria, la coagulación y la fibrinolisis, dirigidos a responder ante un daño vascular de forma precisa y autorregulada, sincronizados espacio-temporalmente. La desregulación de la hemostasia perturbaría el umbral de seguridad y el frágil equilibrio del sistema hemostático contribuyendo al desarrollo de trastornos hemostáticos.

Hasta la fecha, se han identificado diferentes factores genéticos y adquiridos que contribuyen a romper el equilibrio hemostático. Estos factores desencadenantes afectan a los principales efectores procoagulantes del sistema hemostático, proteínas con actividad proteasa controladas por diferentes tipos de inhibidores, que incluyen varias serpinas. Estas proteínas se sintetizan mayoritariamente en el hígado *y* están en la sangre bien como precursores inactivos de enzimas proteolíticas bien como cofactores. Sus niveles plasmáticos presentan una gran variabilidad interindividual en la población sana, sin que se conozca con exactitud el significado fisiológico ó patológico de esta variabilidad.

El FXI de la coagulación, que juega un papel importante en la vía intrínseca de la coagulación, tiene una especial mención en la presente tesis. Los niveles plasmáticos de FXI son también enormemente heterogéneos en la población sana. Además, los niveles plasmáticos elevados de FXI se asocian con el riesgo de sufrir enfermedad tromboembólica. En los últimos años varios grupos han estudiado intensamente la influencia de factores genéticos y ambientales sobre los niveles plasmáticos de FXI. Estos estudios han descartado la participación sola y exclusiva de variaciones genéticas comunes (polimorfismos ó SNP) en el gen del *F11*, así como alteraciones en otros genes que pudieran regular indirectamente los niveles plasmáticos de este factor. Por tanto, a día de hoy se desconocen en gran medida los factores genéticos y/o ambientales que regulan la expresión de FXI y que son responsables tanto de la variabilidad

interindividual como de la susceptibilidad de sufrir enfermedad trombótica, inherente a niveles más elevados de este factor de la coagulación.

En la última década, los miRNA han sido reconocidos como reguladores críticos de la expresión génica a través de su interacción con los 3'UTR de sus dianas. Estas pequeñas moléculas de RNA son capaces de reducir la expresión de genes principalmente a través de la disminución de la traducción del mRNA ó del aumento de su degradación. Se ha descrito su implicación en la regulación post-transcripcional de diversos procesos biológicos que incluyen el crecimiento celular, proliferación y diferenciación, así como el metabolismo y la apoptosis. Estudios recientes describen que aparte de la función que tienen los miRNA sobre el 3'UTR de sus dianas, también existen mecanismos funcionales no canónicos, en los cuales los miRNA ejercen su función sobre otras regiones tales como el 5'UTR o la región codificante, cambiado así el dogma actual sobre la función de los miRNA. La búsqueda de miRNA como potenciales candidatos reguladores se realiza principalmente mediante estudios *in silico* con algoritmos de predicción. Estos algoritmos presentan un alto grado de falsos positivos y negativos dado que solo tienen en cuenta interacciones canónicas principalmente localizadas en el 3'UTR. Existen técnicas bioquímicas que podrían acoplarse a estas predicciones con el fin de obtener resultados más precisos y reducir los falsos positivos y negativos. El desarrollo de estos ensayos para la identificación de potenciales dianas de miRNA supondrá un enorme avance en el estudio de las funciones de los miRNA en los procesos biológicos en los que están involucrados.

Se ha propuesto que la unión directa de miRNA a regiones reguladoras puede verse alterada por la existencia de variantes genéticas comunes (llamadas miR-SNP). Estos miR-SNP se podrían localizar en las regiones reguladoras de mRNA o en los miRNA maduros lo que podría crear ó destruir la interacción miRNA-mRNA preexistente con la secuencia original. Además, los miR-SNP localizados en el sitio de unión de un miRNA a un mRNA diana pueden afectar la expresión de proteínas.

En los últimos años, estudios *in vitro* demuestran el papel de los miRNA en la regulación de diferentes elementos de la hemostasia tales como PAI-1,

fibrinógeno, FT, antitrombina, proteína S, VWF ó TFPI. Sobre estas proteínas los miRNA ejercen una regulación CIS (directa), quedando por evaluar las regulaciones tipo TRANS (indirectas), mediadas por ejemplo a través de factores de transcripción y con un efecto global sobre la coagulación. De hecho, se desconoce en gran medida el papel de los miRNA en el desarrollo de patología trombótica.

El factor nuclear 4α del hepatocito (HNF4α) es un factor clave en la transcripción de muchas proteínas hepáticas. HNF4α se une a la región promotora de diversos genes de expresión hepática, entre ellos aquellos involucrados en la hemostasia, como *F2*, *F7*, *F8*, *F9*, *F10*, *F11*, *F12*, *PROS1*, *PROZ* y *SERPINC1*. En modelo murino, tanto el silenciamiento de *HNF4A* como su supresión reducen la expresión de diferentes factores hemostáticos, revelando la importancia de HNF4α en la coagulación. Por otra parte, estudios *in vitro* han demostrado que la expresión de *HNF4A* se regula por miR-34a y miR-24 (que se unen al extremo 3'UTR y a la zona codificante del gen conservada en modelo murino, respectivamente). Sin embargo, se desconocen las consecuencias de la regulación de *HNF4A* por estos miRNA sobre los factores hemostáticos.

En la presente tesis, se han estudiado tres aspectos relacionados con la investigación en el campo de la trombosis divididos en tres capítulos independientes con un nexo común; el papel de los miRNA como nuevos reguladores del sistema hemostático.

Objetivos

1. Evaluar la regulación de la expresión de FXI por miRNA *in silico*, *in vitro* y *ex vivo* en hígado humano (Capítulo I).
2. Identificar SNP en el 3'UTR de factores de coagulación que desestabilicen la unión de miRNA con consecuencias tanto en los niveles plasmáticos de estos factores así como en el riesgo de sufrir trombosis venosa profunda (Capítulo II).
3. Estudiar el papel indirecto de miR-34a y miR-24 sobre la regulación de las proteínas hemostáticas hepáticas a través de *HNF4A* (Capítulo III).

Resultados

Capítulo I: Regulación de la expresión de FXI de la coagulación por miR-181a-5p en hígado humano

La predicción *in silico* identificó cuatro miRNA como potenciales candidatos a regular la expresión de FXI. Tras la validación *in vitro*, sólo miR-181a-5p causó una reducción tanto en los niveles de mRNA endógeno de *F11*, como en los niveles de proteína intracelular y extracelular. Además, la transfección de un inhibidor de miR-181-5p en células hepáticas aumentó tanto los niveles de mRNA de *F11* como de proteína extracelular. Los ensayos de luciferasa demostraron una interacción directa entre miR-181-5p y el 3'UTR de *F11*. Con el fin de validar estos resultados *ex vivo*, se analizó la relación entre los niveles de mRNA de *F11* y los de miR-181a-5p en tejido hepático sano (n=114). Los niveles de mRNA de *F11* se correlacionaron de forma inversa y significativa con los niveles de miR-181a-5p. Estos datos demuestran que la expresión FXI está regulada directamente por miR-181a-5p en hígado humano. Futuros estudios revelarán las posibles consecuencias de la desregulación de miR-181a-5p en patologías que involucran al FXI.

Capítulo II: Identificación de variantes polimórficas en los factores de coagulación potencialmente regulados por miRNA

En dos estudios caso-control, se secuenciaron los 3'UTR de los genes que codifican los siguientes factores hemostátcos: FII, FVIII, FIX, FXI, fibrinógeno, proteína C, proteína S, antitrombina y TFPI en sujetos con niveles altos (percentil 97.5) o bajos (percentil 2.5) de estos factores. Se identificaron en total 26 variantes, 4 de ellas se encontraban con más frecuencia en alguno de los grupos de los extremos (*F2*: rs1799963 y *F11*: rs4253429, rs4253430 y rs1062547). En un total de 4485 casos/4889 controles, se genotiparon estos SNP. Como se ha descrito previamente, rs1799963 se asoció significativamente con el riesgo de TVP, mientras que no encontramos asociación con los tres localizados en el F11. Estos 3 SNP de *F11* mostraron un alto desequilibrio de ligamiento (LD) con las variantes funcionales descritas rs2289252 y rs2036914. El análisis en controles wildtype para estas variantes

conocidas de *F11* mostró que rs1062547 y rs4253430 (LD> 98%) se asociaban con un aumento significativo de la actividad FXI en plasma en los heterocigotos (7.6 UI/mL) y homocigotos (8.5 UI/mL). Se evaluaron aquellos SNP que pudieran perturbar la unión de miRNA *in silico* e *in vitro* (ensayo de luciferasa). Los estudios de predicción *in silico* revelaron que la presencia de estos SNP podrían interferir en los sitios de unión de 2 miRNA (rs1062547 con miR-513a-5p y rs4253430 con miR-544). Los estudios de reporter mostraron que sólo miR-544 disminuía significativamente la actividad luciferasa en comparación con un control cuando se co-transfectaban células que no expresan miRNA (HCT-116 deficientes en Dicer) con un vector de luciferasa con el 3'UTR del F11 insertado y precursores sintéticos de miR-544. Esta inhibición no se observó cuando se utilizó un vector mutado con rs4253430. Las dos variantes en el 3'UTR del *F11* tienen un efecto sobre los niveles plasmáticos de FXI, que a su vez podrían estar influidos por la interacción de miR-544. Estos resultados avalan que los miRNA son potentes candidatos a desempeñar un papel en trastornos de la hemostasia tales como la trombosis.

Capítulo III: Regulación indirecta de la expresión de proteínas hemostáticas por miR-24 y miR-34a a través de la modulación de HNF4α

En 104 muestras de hígado humano sano se objetivaron variaciones interindividuales en los niveles de expresión de los factores de coagulación seleccionados (*F5*, *F9*, *F10*, *F11*, *F12*, *SERPINC1*, *PROC*, *PROZ* y *PROS1*) y de *HNF4A*, con diferencias entre los percentiles 25 y 75 de alrededor de 3 veces. Del mismo modo, se observó una amplia variabilidad en los niveles de expresión de miR-24 y miR-34a en estas muestras. Se encontraron correlaciones positivas y muy significativas entre los nieles de expresión de *HNF4A* y sus dianas hepáticas involucradas en la coagulación. Además, las muestras de hígados con niveles extremos de miRNA (percentiles 10 y 90) mostraron asociaciones inversas con los niveles de expresión de factores de coagulación. Estos resultados sugieren que las diferencias en la expresión de *HNF4A* pueden explicar, al menos en parte, las variaciones individuales observadas en la expresión de factores de coagulación en el hígado humano. Se seleccionaron 2 miRNAs; miR-24 y miR-34a, que han sido descritos previamente como reguladores de HNF4α. Ambos miRNA se unen a varios

sitios en *HNF4A* humano. Mientras que miR-34a interactúa en tres sitios diferentes situados en el 3'UTR de *HNF4A*, miR-24a inhibe la expresión de HNF4α mediante la unión a la región codificante. La transfección de HepG2 con miR-34a y miR-24 provocó una reducción respecto al control de la mayoría de los genes de coagulación seleccionados. MiR-24 redujo significativamente los niveles de *F10*, *F12*, *PROS1* y *PROC* mientras que miR-34a, además de los anteriores, redujo los niveles de *SERPINC1* y *PROZ*. Adicionalmente, en tejido hepático sano se observaron correlaciones negativas entre los niveles de *HNF4A*, miR-24 y miR-34a.

Estudios *in silico* demostraron que los sitios de unión de miR-24 a la región codificante están conservados en ratón. Por otro lado, miR-34a se une al 3'UTR del *Hnf4a* murino. La transfección con miR-24 y miR-34a redujo los niveles del HNF4α (proteína y mRNA) y los niveles de expresión de diferentes factores de coagulación en hepatocitos murinos primarios.

Estos resultados sugieren que pequeñas variaciones en los niveles de miR-24 y miR-34a podrían contribuir a cambios significativos de diferentes factores de coagulación. El papel de la variación de estos dos miRNA en patologías hemostáticas está por caracterizar.

Conclusiones

En su conjunto, los resultados obtenidos en los diferentes capítulos de esta tesis doctoral avalan la hipótesis planteada al inicio de la misma. Así, mediante aproximaciones *in silico*, *in vitro* e *ex vivo*, he constatado que ciertos miRNA funcionan como reguladores de la expresión de diversos factores hemostáticos. Las consecuencias patológicas de esta nueva regulación quedan por ser exploradas.

De forma más específica, las conclusiones derivadas de cada capítulo son las siguientes:

Capítulo I:

Según las predicciones *in silico,* de los más de 280 miRNA que se expresan en hígado humano sólo 4 de ellos pueden unirse al mRNA de *F11*. Pero además,

los resultados obtenidos de ensayos *in vitro* mostraron que sólo miR-181a-5p inhibe eficazmente la expresión de FXI.

Estos datos demuestran por primera vez que la expresión de FXI podría estar regulada directamente por miR-181a-5p en hígados humanos.

Capítulo II:

La secuenciación del 3´UTR del *F11* en un estudio caso/control de trombosis venosa permitió identificar 2 nuevas variantes polimórficas con efecto sobre los niveles plasmáticos de FXI. Los estudios in vitro realizados en esta tesis demuestran que el efecto funcional de una de ellas, miR-SNP rs4253430, consiste en dificultar la interacción del RNAm del F11 y *m*iR-544. Estos resultados demuestran que variaciones genéticas comunes en el gen de FXI, diana de miR-544, tienen consecuencias sobre los niveles de FXI.

Capítulo III:

Estudios de correlación *ex vivo* en hígados humanos sanos mostraron una gran variabilidad interindividual en la expresión de factores de coagulación y una fuerte asociación entre ellos y con un elemento regulador común, HNF4α. Así, se describe por primera vez que HNF4α juega un papel de modulador común de la expresión de factores de coagulación en hígados humanos sanos.

Además, los resultados obtenidos muestran que miR-24 y miR-34a regulan no sólo la expresión de *HNF4A*, sino también la de una serie de factores de coagulación en hepatocitos. Estos datos describen por vez primera un efecto en *TRANS* de miR-24 y miR-34a en la regulación de múltiples proteínas hemostáticas hepáticas a través de HNF4α.

APPENDIX I

Table S1: MiRNA expression in human liver. MiRNA arrays (n=4) using Sanger miRBase Release 18.0 and 19.0 revealed a significant expression of 219 miRNAs (significant signal intensities considered with p-values < 0.05). The sample signal median from the three independent arrays for each miRNA is shown.

Index	Human miRNA	Sample Signal (arbitrary units)	Index	Human miRNA	Sample Signal (arbitrary units)	Index	Human miRNA	Sample Signal (arbitrary units)
1	*miR-122-5p*	*28,636*	74	*miR-4530*	*1,001*	147	miR-374b-5p	227
2	*miR-26a-5p*	*14,457*	75	*miR-3607-5p*	*976*	148	miR-99b-5p	226
3	*let-7f-5p*	*10,867*	76	*miR-22-5p*	*966*	149	miR-378f	226
4	*let-7a-5p*	*10,749*	77	*miR-1973*	*962*	150	miR-378c	217
5	*miR-30b-5p*	*9,297*	78	*miR-4281*	*933*	151	miR-4532	215
6	*let-7d-5p*	*8,559*	79	*miR-378e*	*907*	152	miR-193a-3p	215
7	*miR-23b-3p*	*8,276*	80	*miR-320c*	*903*	153	miR-3591-3p	210
8	*miR-4443*	*8,147*	81	*miR-4324*	*891*	154	miR-1275	208
9	*miR-3960*	*7,402*	82	*miR-19b-3p*	*888*	155	miR-130b-3p	207
10	*miR-192-5p*	*7,183*	83	*miR-5001-5p*	*873*	156	miR-5100	202
11	*miR-3665*	*6,889*	84	*miR-4472*	*813*	157	miR-4284	201
12	*miR-1273g-3p*	*6,692*	85	*miR-378d*	*739*	158	miR-128	193
13	*miR-214-3p*	*6,628*	86	*miR-25-3p*	*729*	159	miR-30b-3p	191
14	*miR-30c-5p*	*6,448*	87	*miR-27a-3p*	*718*	160	miR-151a-3p	190
15	*let-7c*	*6,292*	88	*miR-466*	*712*	161	miR-193a-5p	185
16	***miR-23a-3p***	***6,052***	89	*miR-320d*	*669*	162	miR-3609	178
17	*miR-26b-5p*	*5,925*	90	*miR-320e*	*664*	163	miR-30c-1-3p	175
18	*miR-194-5p*	*5,007*	91	*miR-5096*	*664*	164	miR-331-3p	173
19	*let-7g-5p*	*4,837*	92	*miR-191-5p*	*651*	165	miR-4485	172
20	*miR-4787-5p*	*4,637*	93	*miR-17-5p*	*643*	166	miR-4286	172
21	*miR-451a*	*3,993*	94	*miR-139-5p*	*619*	167	miR-4534	162
22	*miR-125b-5p*	*3,952*	95	*miR-1246*	*617*	168	miR-4778-5p	158
23	*let-7b-5p*	*3,866*	96	*miR-4508*	*610*	169	miR-101-3p	155
24	*miR-148a-3p*	*3,747*	97	*miR-4734*	*608*	170	miR-340-5p	151
25	*miR-1260b*	*3,548*	98	*miR-3178*	*605*	171	miR-3195	151
26	*miR-145-5p*	*3,522*	99	*miR-320b*	*602*	172	miR-4505	150
27	***miR-16-5p***	***3,513***	100	*miR-4459*	*595*	173	miR-142-5p	145
28	*miR-24-3p*	*3,487*	101	*miR-29c-3p*	*593*	174	miR-375	139
29	*miR-29a-3p*	*3,419*	102	*miR-4466*	*580*	175	miR-3607-3p	138
30	*miR-146a-5p*	*3,212*	103	*miR-152*	*579*	176	miR-29c-5p	127
31	*miR-92a-3p*	*3,147*	104	*miR-106a-5p*	*544*	177	miR-197-3p	120
32	***miR-195-5p***	***3,046***	105	*miR-130a-3p*	*542*	178	miR-4484	120
33	*miR-1915-3p*	*2,943*	106	*miR-378g*	*536*	179	miR-1268b	115
34	*miR-221-3p*	*2,885*	107	miR-223-3p	499	180	miR-193b-3p	112
35	*miR-146b-5p*	*2,845*	108	miR-22-3p	487	181	miR-3676-5p	104
36	*miR-30a-5p*	*2,845*	109	miR-10a-5p	486	182	miR-181b-5p	102
37	*miR-638*	*2,830*	110	miR-20a-5p	483	183	miR-192-3p	99
38	*miR-99a-5p*	*2,598*	111	miR-376c	479	184	miR-200a-3p	93
39	*miR-4516*	*2,580*	112	miR-148b-3p	478	185	miR-497-5p	90
40	*miR-103a-3p*	*2,514*	113	miR-361-5p	467	186	miR-454-3p	80
41	*miR-215*	*2,418*	114	miR-126-3p	466	187	miR-484	80
42	*miR-122-3p*	*2,357*	115	miR-29b-3p	458	188	miR-127-3p	79
43	*miR-199a-3p*	*2,320*	116	miR-4454	456	189	miR-200b-3p	78
44	*miR-30d-5p*	*2,221*	117	miR-425-5p	445	190	miR-345-5p	76
45	*miR-107*	*2,193*	118	miR-423-5p	438	191	miR-377-3p	72
46	*miR-30e-5p*	*2,148*	119	miR-335-5p	422	192	miR-1307-3p	71
47	*miR-143-3p*	*2,130*	120	miR-186-5p	400	193	miR-532-5p	71
48	*let-7i-5p*	*2,062*	121	miR-155-5p	399	194	miR-4492	69

Index	Human miRNA	Sample Signal (arbitrary units)	Index	Human miRNA	Sample Signal (arbitrary units)	Index	Human miRNA	Sample Signal (arbitrary units)
49	*miR-27b-3p*	*1,769*	122	miR-98	377	195	miR-885-3p	69
50	*miR-100-5p*	*1,736*	123	miR-3940-5p	369	196	miR-148a-5p	67
51	*miR-125a-5p*	*1,643*	124	miR-4463	366	197	miR-487b	67
52	*miR-574-3p*	*1,556*	125	miR-644b-3p	357	198	miR-1469	66
53	*miR-3656*	*1,555*	126	miR-4298	349	199	miR-378a-5p	64
54	*miR-151b*	*1,547*	127	miR-4447	342	200	miR-4521	64
55	*miR-151a-5p*	*1,527*	128	miR-2861	338	201	miR-362-5p	59
56	*miR-494*	*1,395*	129	miR-483-5p	326	202	miR-324-5p	57
57	*miR-455-3p*	*1,382*	130	miR-34a-5p	315	203	miR-7-1-3p	55
58	*miR-23c*	*1,379*	131	miR-378i	307	204	miR-203	53
59	*miR-150-5p*	*1,308*	132	miR-15a-5p	294	205	miR-145-3p	53
60	*let-7e-5p*	*1,264*	133	miR-4739	291	206	miR-361-3p	49
61	*miR-92b-3p*	*1,249*	134	miR-149-3p	278	207	miR-500a-3p	46
62	***miR-181a-5p***	***1,233***	135	miR-424-5p	278	208	miR-1182	46
63	*miR-3196*	*1,222*	136	miR-4707-5p	253	209	miR-20b-5p	37
64	*miR-378a-3p*	*1,204*	137	miR-222-3p	251	210	miR-425-3p	33
65	*miR-885-5p*	*1,203*	138	miR-28-5p	249	211	miR-210	32
66	*miR-15b-5p*	*1,147*	139	miR-574-5p	249	212	miR-455-5p	32
67	*miR-4497*	*1,144*	140	miR-505-3p	246	213	miR-660-5p	29
68	*miR-140-3p*	*1,135*	141	miR-3141	245	214	miR-17-3p	21
69	*miR-4488*	*1,098*	142	miR-185-5p	239	215	miR-500b	21
70	*miR-342-3p*	*1,083*	143	miR-93-5p	237	216	miR-362-3p	18
71	*miR-320a*	*1,078*	144	miR-762	235	217	miR-18a-5p	18
72	*miR-199a-5p*	*1,073*	145	miR-28-3p	231	218	miR-363-3p	15
73	*miR-21-5p*	*1,065*	146	miR-106b-5p	228	219	miR-18b-5p	10

In bold are shown the selected miRNAs with an expression > 500 arbitrary units. In italics are shown the miRNAs with an expression > 500 arbitrary units.

Table S2: TaqMan® probes used for real time quantitative PCR of human and mouse genes

		TaqMan® probe
Human symbol Gene	*F5*	Hs00914120_m1
	F8	Hs00252034_m1
	F10	Hs00984443_m1
	F12	Hs00166821_m1
	SERPINC1	Hs00166654_m1
	PROC	Hs00165584_m1
	PROZ	Hs00187370_m1
	PROS1	Hs00165590_m1
	ACTB	Hs99999903_m1
	HNF4A	Hs00230853_m1
Mouse symbol Gene	*F5*	Mm00484202_m1
	F9	Mm01302526_m1
	F11	Mm01194987_m1
	F10	Mm00484177_m1
	F12	Mm00491349_m1
	Serpinc1	Mm00445573_m1
	Proc	Mm00435966_m1
	Pros1	Mm01343426_m1
	Actb	Mm00607939_m1
	Hnf4a	Mm01247712_m1

APPENDIX II

Diffusion of scientific results related with the thesis

Indirect regulation of hemostatic proteins by mir-24 and mir-34a through hepatic nuclear factor-4α. **Salloum-Asfar S,** Arroyo AB, García-Barberá N, Teruel-Montoya R, Luengo-Gil G, Vicente V, González-Conejero R, and Martínez C. Manuscript in preparation

Identification of coagulation gene 3'UTR variants as new risk factors for venous thrombosis. van Hylckama Vlieg A, Martínez C, Vossen C Y, **Salloum-Asfar S**, Hugoline G. de Haan, Teruel-Montoya R, Corral J, Reitsma P H,. Rosendaal F R. Manuscript in preparation

Regulation of coagulation factor XI expression by microRNAs in the human liver. **Salloum-Asfar S**, Teruel-Montoya R, Arroyo AB, García-Barberá N, Chaudhry A, Schuetz E, Luengo-Gil G, Vicente V, González-Conejero R, Martínez C. PLoS One. 2014 Nov 7;9(11):e111713.

APPENDIX III

Diffusion of scientific results unrelated with the thesis

Role of platelets, neutrophils, and factor xii in spontaneous venous thrombosis in mice. Heestermans M, **Salloum-Asfar S**, Salvatori D, Laghmani EH, Luken B M, Zeerleder SS, Spronk H MH, Wagenaar G TM, Pieter H Reitsma, and van Vlijmen B JM. Submitted to *Blood. 2015.*

Peritoneal fluid modifies the microRNA expression profile in endometrial and endometriotic cells from women with endometriosis. Braza-Boïls A, **Salloum-Asfar S**, Marí-Alexandre J, Arroyo AB, González-Conejero R, Barceló-Molina M, García-Oms J, Vicente V, Estellés A, Gilabert-Estellés J, Martínez C. Hum Reprod. 2015 Oct;30(10):2292-302.

The immediate and late effects of thyroid hormone (triiodothyronine) on murine coagulation gene transcription. **Salloum-Asfar S**, Boelen A, Reitsma PH, van Vlijmen BJ. PLoS One. 2015 May 26;10(5):e0127469.

Prognostic role of MIR146A polymorphisms for cardiovascular events in atrial fibrillation. Roldán V, Arroyo AB, **Salloum-Asfar S**, Manzano-Fernández S, García-Barberá N, Marín F, Vicente V, González-Conejero R, Martínez C. Thromb Haemost. 2014 Oct;112(4):781-8.

Control of post-translational modifications in antithrombin during murine post-natal development by miR-200a. Teruel R, Martínez-Martínez I, Guerrero JA, González-Conejero R, de la Morena-Barrio ME, **Salloum-Asfar S**, Arroyo AB, Águila S, García-Barberá N, Miñano A, Vicente V, Corral J, Martínez C. J Biomed Sci. 2013 May 16;20:29.

miR-133a regulates vitamin K 2,3-epoxide reductase complex subunit 1 (VKORC1), a key protein in the vitamin K cycle. Pérez-Andreu V, Teruel R, Corral J, Roldán V, García-Barberá N, **Salloum-Asfar S**, Gómez-Lechón MJ, Bourgeois S, Deloukas P, Wadelius M, Vicente V, González-Conejero R, Martínez C. Mol Med. 2013 Jan 22;18:1466-72.