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Molecular bases of the modulation of coagulation factor levels

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Contents

Abbreviation

vWF	von Willebrand Factor
GPIb-V-IX	Glicoprotein Ib-V-IX
II	Prothrombin
IIa	Thrombin
TF	Tissue Factor
FV	Factor V
FVa	Activated FV
FVII	Factor VII
FVIIa	Activated Factor VII
FVIII	Factor VIII
FVIIIa	Activated Factor VIII
FIX	Factor IX
FIXa	Activated Factor IX
FX	Factor X
FXa	Activated Factor X
FXI	Factor XI
FXIa	Activate Factor XI
MPs	Microparticles
PAR	Protease-Activated Receptor
TAFI	Thrombin Activatable Fibrinolysis Inhibitor
TFPI	Tissue Factor Pathway Inhibitor
AT	Antithrombin
PC	Protein C
APC	Activated Protein C
TM	Thrombomodulin
PS	Protein S

Chapter 1

General Introduction

1.1 Hemostasis

Hemostasis is a dynamic process whereby blood is maintained fluid under normal conditions, but is allowed to clot in case of trauma. It is a tightly regulated mechanism involving various cellular and molecular components. Blood coagulation is part of this important host defense mechanism.
In the resting state endothelial cells, that tile the walls of the blood vessels, inhibit the platelet adherence and thus the activation of the blood coagulation. Moreover, the synthesis of prostacyclin and heparin-like substances, and the presence of protein complexes (thrombin-thrombomodulin), leading to generation of anticoagulant proteins (activated protein C), prevent clot formation in normal blood vessels [1].
After vessel injury, damaged endothelial cells expose negatively charged phospholipids and release procoagulant proteins [2], platelets adhere to macromolecules in subendothelial tissues and aggregate to form the primary hemostatic plug that temporary blocks blood loss.
The interaction between platelets and the damaged endothelium requires von Willebrand Factor (vWF), a large multimeric plasma protein. vWF acts as a bridge by binding to exposed collagen in the sub-endothelium and Glicoprotein Ib-V-IX (GPIb-V-IX), a specific receptor on platelet surface [1]. Platelets activation triggers specific morphologic and biochemical alterations on their membrane surface; phospholipid composition changes, resulting in negatively charged phopshatidylserine exposure on the outer leaflet [3, 4]. Platelet α-granules release fibrinogen, Factor V (FV), vWF and other proteins involved in hemostasis; δ-granules secrete Calcium ions and ADP. Glycoprotein IIb-IIIa (G-IIb-IIIa) expression on platelet membrane surface mediates interaction with fibrinogen, vWF, fibronectin and platelet aggregation [1, 9].
Later, as wound healing occurs, fibrin clot are broken down and removed [5].

1.2 Blood coagualtion

In a classical view coagulation is represented as a "cascade"or "waterfall"model, divided into two pathways: an "intrinsic pathway", so named because all the components are present in blood and an "extrinsic pathway", in which the subendothelial cell membrane protein tissue factor (TF) is required in addition to circulating components. The initiation of both pathways resulted in activation of Factor X (FX) and the eventual generation of a fibrin clot through a common pathway [6]. Although these concepts represented a significant advance in the understanding of coagulation and served for many years as a useful model, more recent clinical and experimental observations [7] explain how the cascade/waterfall hypothesis does not fully and completely reflect the events of hemostasis in vivo [8].
A cell-based model of coagulation explain, in a more physiological way, how coagulation cascade evolves in consequence of a vascular injury, underlaying the roles of cellular elements. Several cells play different roles in the coagulation process, due to their procoagulant and anticoagulant properties. Blood platelets and TF-bearing Microparticles (MPs) play a major role in supporting procoagulant reactions, supplying negatively charged phospholipid essential for the correct assembly of molecular complexes. Vascular endothelial cells play a key role in maintaining the anticoagulant properties of the vasculature.
Formation of an impermeable platelet and fibrin plug at the site of vessel injury is essential, but it is also required that procoagulant substances activated in this process remain localized on damaged site.
According to this model, coagulation pathway proceeds as a sequence of events localized on the site of vessel injury.

1.2.1 Initiation phase

The process of blood coagulation starts by the exposure of TF-expressing cells to flowing blood (Fig.1.1). TF is expressed constitutively on cells such as smooth muscle cells and fibroblasts but not on resting endothelium; it is also expressed in several other districts and constitutes an hemostatic envelope normally not in contact with blood [9]. Disruption of the endothelium or activation of endothelial cells or monocytes results in the exposure of TF on blood flow [10]. Stronger evidence suggests that TF circulates in blood exposed on the surface of MPs; that derive from various cell types : white blood cells, endothelial cells, and platelets, and might play important roles in development of pathological hemostasis (thrombosis) opposing to normal clotting [11].

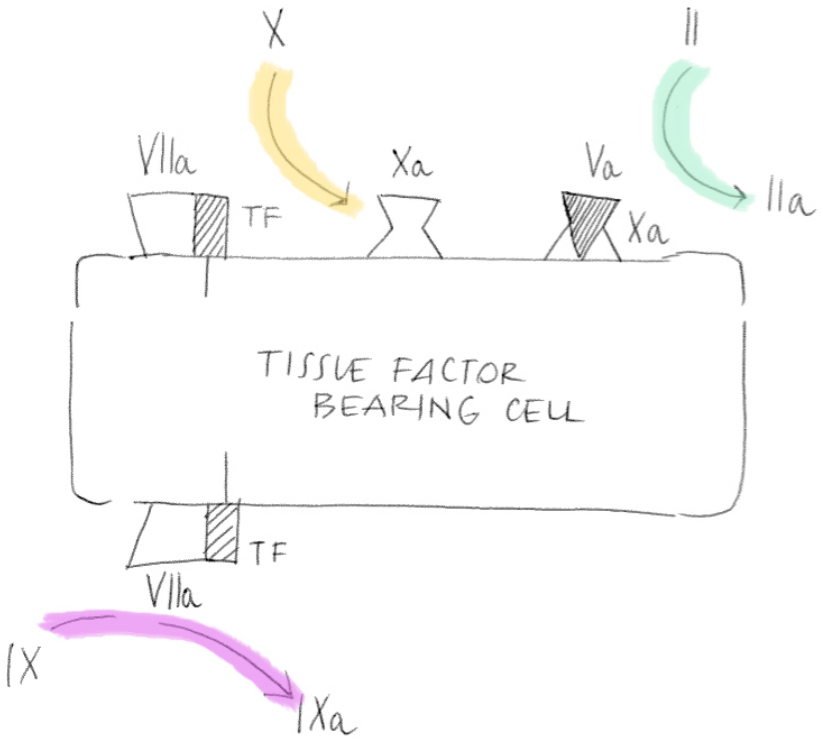

Figure 1.1: Factor VIIa bound to TF activates both factor X and factor IX. Factor Xa formed by factor VIIa/TF binds to factor Va on that cell and converts a small amount of prothrombin to thrombin

Exposed TF interacts with Factor VII/activated Factor VII (FVII/FVIIa) and the new-formed complex TF/FVIIa activates small amounts of FX and Factor IX (FIX). Activated FX (FXa) associates with its cofactor, activated Factor V (FVa), and forms the so called "prothrombinase-complex"on the surface of the TF-bearing cells [12], and leads the activation of small quantity of circulating Prothrombin (II) into Thrombin (IIa).

The adhesion process partially activates the platelets and promotes secretion of partially activated FV from their α-granules [13].

Zymogen FV can also be activated by FXa [14] or by noncoagulant proteases [15].

Low level activity of the TF pathway probably occurs at all times in the extravascular space. The coagulation proteins leave the vasculature, percolate through the tissues, and are found in the lymph roughly in proportion to their molecular size [16]. Thus, FVII is probably bound to extravascular TF

even in the absence of an injury[12], and the extravascular FX and FIX can be activated as they pass through the tissues. This idea is consistent with the finding that low levels of the activation peptides from coagulation factors are present in the blood of normal individuals [17]. This process does not lead to clot formation under normal circumstances, because the really large components of the coagulation process, platelets and Factor VIII (FVIII)/vWF complex, are kept sequestered in the vascular space. Coagulation only proceeds when damage to the vasculature allows platelets and Factor VIII/vWF exposure into the extravascular tissues.

1.2.2 Amplification phase

The small amount of thrombin generated on the TF-bearing cell in the initiation phase, has several important functions; one of that is activation of platelets. Although platelets have already adhered at the site of injury and become partially activated, the addition of thrombin can induce a higher level of procoagulant activity than adhesive interactions alone [18]. As a result platelets release partially activated forms of FV onto their surfaces.

Another function of thrombin formed during the initiation phase is the activation of the cofactors FV and FVIII on the activated platelet surface. In this process, the FVIII/vWF complex is dissociated, permitting vWF to mediate additional platelet adhesion and aggregation at the site of injury (Fig. 1.2).

Thrombin also activates Factor XI (FXI), activated by the prekallekrein/kininogen/Factor XII cascade in the classic "intrinsic parhway", which acts as a "booster"of thrombin generation on the platelet surface [19, 20]. This finding also strengthen the hypothesis that the intrinsic mechanism gives no contribute to *in vivo* coagulation process.
By the end of the amplification phase, the "stage"is set for large-scale thrombin generation in the propagation phase.

1.2.3 Propagation phase

The propagation phase of clot formation occurs on the surface of activated platelets. First, activated-Factor IX (FIXa) generated during the initiation phase can now bind to its cofactor, activated-Factor VIII (FVIIIa), on the platelet surface, assembling in the so called "tenase-complex".
Second, additional FIXa can be supplied by platelet-bound activated-FactorXI (XIa).
Third, because FXa cannot move effectively from the TF-bearing cell to the activated platelet, FXa must be provided directly on the platelet surface by

the FIXa/FVIIIa complex.
Fourth, the FXa rapidly associates with FVa bound to the platelet during the amplification phase, producing a burst of thrombin generation of sufficient magnitude to clot fibrinogen [9, 21, 22]. Hence more than 95% of the total amount of thrombin production takes place after initial clot forma-

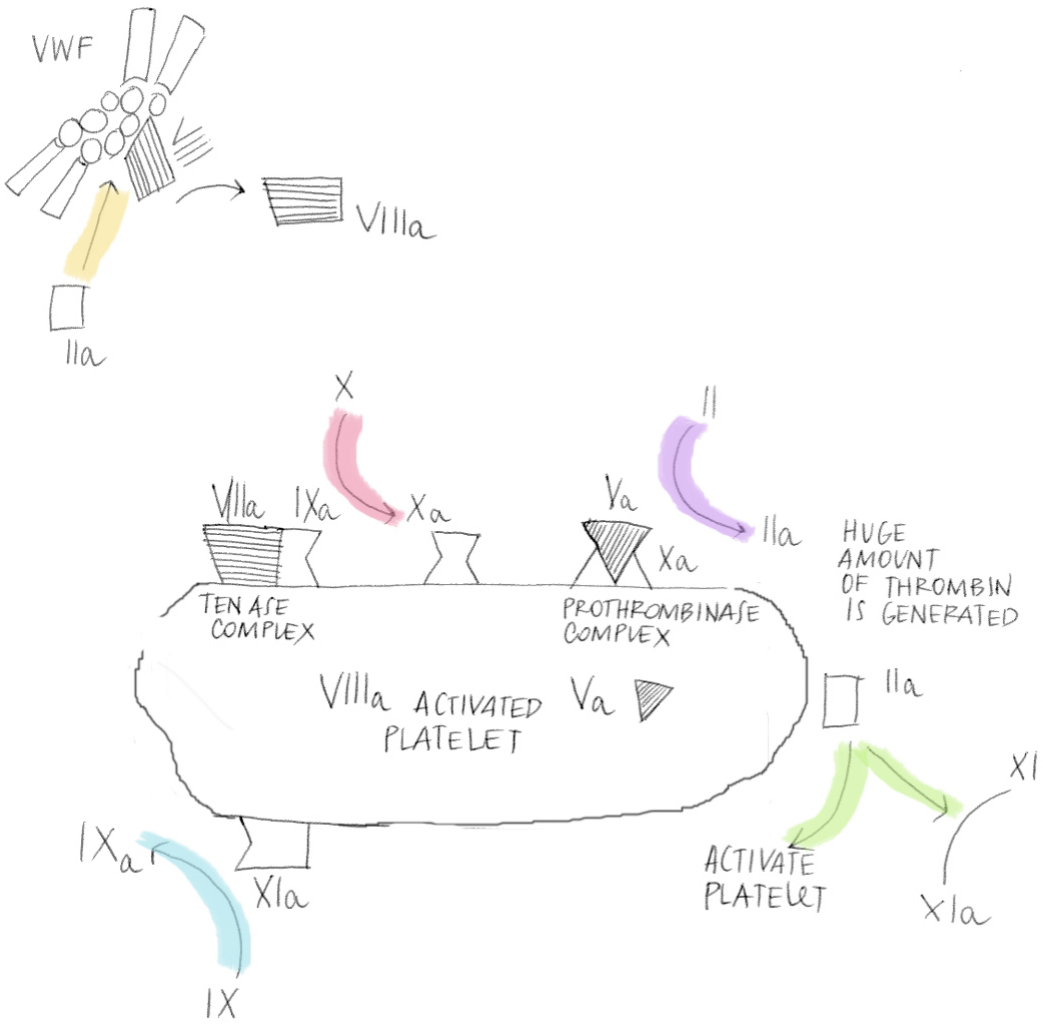

Figure 1.2: On the surface of an activated platelet, factor IXa formed on the TF-bearing cell can incorporate into a Xase complex. Addition factor IXa is formed by plateletbound factor XIa. Factor Xa formed on the platelet surface is channeled into IIase complexes, leading to a burst of thrombin generation. Because factor XI is activated on the platelet surface by thrombin, PK, HK, and factor XII are not required for thrombin generation in this model.

tion, during propagation phase [23]. This excess of thrombin has been proposed to play an important role in stabilizing the clot by: activating Factor XIII (FXIII), the fibrin stabilizing factor [24]; cleaving the platelet protease-activated receptor-4 (PAR-4), that contribute to full activation of human platelets [25]; activating thrombin activatable fibrinolysis inhibitor (TAFI) [26]. TAFI is a carboxypeptidase that removes terminal lysine residues from fibrin, thereby removing potential binding sites for fibrinolytic enzymes and enhancing clot resistance to fibrinolysis [27]. Greater levels of thrombin activity are needed to activate TAFI than to form a fibrin clot. Failure in TAFI activation is thought to contribute significantly to the bleeding tendency in hemophilia [28].

1.2.4 Termination phase

Once a fibrin platelet clot is formed over a damaged area, the clotting process must be limited to avoid thrombotic occlusion in other normal areas of the vasculature [12].
The TF/FVIIa activity is inhibited by the Kunitz type inhibitor, Tissue Factor Pathway Inhibitor (TFPI) [29–31], secreted by endothelium. TFPI binds to FXa forming a quaternary complex with TF/FVIIa that quickly limits coagulation [35].
The serine protease inhibitor Antithrombin (AT)[32] neutralize enzymes of the coagulation system; its physiological role is to protect the circulation from free enzymes and limit the coagulation process to sites of vascular injury. Circulating AT is a relatively inefficient serpin, but its activity is stimulated by heparin and presumably by heparin-like molecules such as sulfated glycosaminoglycans that are synthesized and expressed by endothelial cells [34]. The increasing efficiency of AT by heparin is the molecular basis for the use of heparin as a therapeutic anticoagulant. Antithrombin is the major thrombin-inactivating protein [33].
TFPI and AT inhibit coagulation acting directly against proteins and macromolecular complexes, instead another mechanism act in an indirect way switch off coagulation.
The protein C (PC) anticoagulant system inhibits the procoagulant functions of FVIIIa and FVa, the cofactors in the tenase and prothrombinase complexes respectively [36, 37].
The key component in the system is protein C, a vitamin K-dependent zymogen (proenzyme), activated by thrombin bound to the membrane protein thrombomodulin (TM) on the surface of intact endothelial cells. Activated PC (APC) cleaves a few peptide bonds in each of the phospholipid membrane bound cofactors FVa and FVIIIa. APC can also cleave the intact form of

FV, converting FV to an anticoagulant cofactor of APC in the inactivation of FVIIIa in the tenase complex [9].
APC activity is enhanced by another vitamin Kdependent inhibitory cofactor, protein S (PS). In human plasma, about 30% of protein S circulates as free protein; the remaining is bound to the complement regulatory protein C4b-binding protein. Only the free form of PS functions as a cofactor to APC.

1.3 Clotting factors

Procoagulant and anticoagulant proteins are composed of multiple structural domains. Among the different proteins, these domains have both a high degree of structural and functional homology.

1.3.1 Signal peptide

Both procoagulant and anticoagulant proteins found in plasma are initially synthesised with a signal peptide. This usually highly hydrophobic short peptide, needed for translocation of the growing polypeptide chain into the endoplasmatic reticulum, is cleaved off prior to secretion.

1.3.2 Propeptide/γ-Carboxyglutamic Acid-Rich Domain

All vitamin K-dependent proteins, prothrombin, FVII, FIX, FX, PC PS, contain a γ-Carboxylation recognition site located on the propeptide domain between the signal peptide and the γ-Carboxyglutamic Acid-Rich domain (Gla domain). This region directs, in a Vitamin-K dependent mechanism, γ-Carboxylation of the γ-Carboxyglutamic acid residues located in the adjacent, approximately 40 AA residues long Gla domain. Gla domain is crucial for the Ca^{2+} mediated binding of these proteins with negatively charged membranes. After carboxylation the propeptide is cleaved off.

1.3.3 Epidermal Growth Factor Domain

Several procoagulant and anticoagulant proteins contain two or more epidermal growth factor (EGF)-like domains. These domains are about 43 to 50 amino acid residues in length and their structure is determined by three typical disulfide-bonds. The function of EGF-like domains on many clotting proteins, although not fully understood, appears to be involved in the formation of protein complexes. The EGF-like domains in FVII are important for the binding to TF. The second EGF-like domain of Factor IX contains a binding site for activated FVIII. The second EGF-like domain of protein C has been shown to be involved in the binding of protein S. The TM's binding sites for thrombin and PC are located on the fifth and the fourth EGF-like domains, respectively.

1.3.4 Kringle Domain

Kringle domains are about 100 amino acid residues in length and characterised by a pattern of three disulfide bonds. These domains are likely to be involved in protein complex formation. Only two procoagulant proteins, prothrombin and FXII contain kringle domains. The second kringle of prothrombin probably contains the main binding site for activated factor V.

1.3.5 Catalytic Domain: Serine Protease Module

The catalytic domain of all procoagulant enzymes have an active site and an internal core that is nearly identical to that of trypsin. Conversion of an inactive proenzyme to an active enzyme depends on limited proteolysis and for some proteins the cleavage of so-called activation peptides.
The enzyme activity of all clotting proteases depends on a serine, an aspartic acid and a histidine within the active site, which is characteristic for all serine proteases. The molecular surfaces surrounding the active site are responsible to specifically define the enzyme substrate [38].

1.4 Clotting factors levels

Within the vast majority of the healthy population the range of pro and anticoagulant components varies significantly. Counting the only thrombin generation, the responsiveness for a given TF initiating dose in the healthy population is quite extraordinary different [39].
The basis for the heterogeneity of the procoagulant response in human population is probably a consequence of both genetic and environmental factors, which influence the status of the proteomic phenotype when evaluated in either a healthy or pathological state [40].
Recently has been estimate that there are 24500 human protein coding genes [45]; considering all molecular mechanisms that fold a protein from a primary mRNA transcript to the final physiologic function, e.g. alternative splicing and post translational modification, we might estimate a huger amount of primary transcript protein products. The initial peptide product and most of its biosynthetic modifications are dictated primarily by genetics, with each modification system under the direction of multiple genes. A variety of instructions are included in the genetic package for the primary product which target the protein through multiple, chemically driven assembly and transport processes which also incorporate additional post-translational modifications. The completed, modified, intracellular product must be further processed proteolytically at multiple sites prior to export. In addition, extracellular modifications, by both biologically directed processes and environmental accidents, may further alter the circulating product. As a consequence of all these modification events, the number of members of the phenotypic proteome will probably extend well over the million(s) range [40].
In addition other elements could influence the variation of circulating clotting proteins; it has been reported that even with equivalent genetic deficiencies, there is a wide range of expression of risk events, either thrombotic or hemorrhagic, suggesting that multiple genetic, acquired and environmental factors influence the possibility of an individual to undergo a clinically significant hemorrhagic or thrombotic event.
Concomitant presence of genetic defects in an individual, so called "gene-gene interaction", also influence the normal or predicted levels of clotting factor.
It is well established that plasma concentrations of several clotting factors, i.e. fibrinogen, FVII, FVIII, vWF, FIX, FXII, high molecular-weight kininogen, and prekallikrein, increase with progressing age in healthy humans [46]. Moreover, in elderly people there are seasonal variation in fibrinogen concentration and the highest plasma levels have been observed during the coldest months [47, 48]. However, high plasma levels of the coagulation activation

markers in older populations do not necessarily reflect a high risk of arterial or venous thrombosis [49].
Disease states, drug consumption, and also human behavior or attitude i.e. smoking, diet, sedentariness, could in such aspect influence clotting factor levels, conditioning the fragile hemostatic balance.
Hence, only a few information dealing with the meaning of the heterogeneity of the plasma vitamin K-dependent proteins or the influence of this heterogeneity on their biological performance with respect to activation, catalytic function or turnover is available.

1.4.1 "Normal"proteins levels

Various coagulation proteins circulate in blood at very different concentrations related to their specific roles in the blood coagulation system [56–59].

Table 1.1 Coagulation Factor Levels

Factor	Plasma concentration μg/mL
Fibrinogen	3000
Prothrombin	9
Factor V	10
Factor VII	0.5
Factor VIII	0.1
Factor IX	5
Factor X	8
Factor XI	5
Factor XII	30
Factor XIII	10
Protein C	4
Protein S (free)	10
Prekallikrein	50
High molecular weight kininogen	70
AntithrombinIII	290

Fibrinogen is the predominant clotting factor having a concentration that is approximately 30 000-fold higher than that of FVIII. The high level of fibrinogen is required for the formation of the fibrin clot, whereas the low concentration of FVIII is more than sufficient to support FIXa in the activation of FX. Taking into account other vitamin K-dependent proteins, FVII is the least abundant, FIX and FX have intermediate levels and prothrombin circulating at the highest concentration. Several studies conducted in knockout

mice may help in understanding relative importance of various coagulation factors in vivo [60].
The embryonic lethal phenotype, associated with TF deficiency, demonstrates the crucial importance of the TF pathway [61–63], also confirmed by the absence of natural TF deficiency in humans.
FVII deficient mice develop normally in utero but die shortly after birth due to severe bleeding [64]. The difference in severity between TF and FVII knockout animals suggests a role for TF during embryogenesis in fibrin formation.
Prothrombin and FV deficiency are associated with partial embryonic lethality and fatal haemorrhage [65–67]. In contrast, FIX and FVIII-deficient mice develop normally in utero but acquire haemophilia-like disease after birth [68, 69].
Fibrinogen-deficient mice have normal fetal development and suffer a moderate-to-severe bleeding phenotype similar to human fibrinogen deficiency [70]. This shows that thrombin generation is more important than fibrin deposition.
Mice deficient in TAFI have no pathologic phenotype, demonstrating that TAFI deficiency is fully compatible with life [71].
Hemostasis is based on anticoagulant protein too. No TFPI deficiency are described in humans, which may indicate that lack of TFPI is not compatible with life; TFPI knockout mice have a lethal phenotype suffering from uncontrolled activation of coagulation with consumption of coagulation factors [72].
Homozygous AT knockout mice have a lethal phenotype, demonstrating the importance of the protein for control of coagulation [73].
The protein C system is physiologically very important, which is clearly demonstrated by the severe thromboembolic disease associated with homozygous deficiency of protein C in both man and mice [74]. Mice lacking the protein C or TM genes are affected by a lethal phenotype, actually TM deficiency is particularly severe affecting embryogenesis even before the development of a functional cardiovascular system [75, 76].

Within healthy population, there is a wide range of hemostatic phenotype; as a result it is difficult to define the "edge"of normal/abnormal, or pathologic, clotting factors levels.
Since thrombin plays a central role in formation of the primary hemostatic plug, the total amount of thrombin formed and the rate of its generation could provide a good reflection of the potential coagulation activity; but an effective clot cannot be formed without adequate levels of procoagulant factors.
The levels of coagulation factors in plasma of normal individuals vary amply

(generally 50% to 150% of the level in normal pooled plasma), as evaluated in several papers [39–44]. This suggests that a wide range of factor levels is compatible with normal hemostatic function. However, even within the normal range, variations can affect the thrombin generation [41].

The effect of coagulation factors levels on thrombin generation's pattern is complex. For most of the coagulation factors, changing the level between 50% and 150% has little effect on the rate and pattern of thrombin generation [41, 50].

Decreasing level of factors VIII, IX, or XI to $<$ of 50% results in a modest decline in thrombin generation, with a dramatic decline only after levels fall below 10% to 20% of normal. Thus, the wide range of levels found in the normal population probably has little effect on thrombin generation and, therefore, little effect on hemostatic function. The pattern for FX is little different than the other factors. Again, changes between 50% and 150% have essentially no effect on thrombin generation. However, thrombin generation is maintained down to FX levels as low as 1% to 5% in *in vitro* experiments before falling off sharply [50, 51]. Thus, variation in FX levels probably contributes little to the pattern of thrombin generation unless combined with a deficiency of other factors.

The relationship between prothrombin levels and thrombin generation is different from the other coagulation factors, actually total amount of thrombin produced are proportional to the prothrombin level [41]. The rate of thrombin generation activity achieved during clot formation significantly affect the structure and stability of the resulting fibrin clot [52]. This means that any variation in prothrombin level is reflected in the pattern of thrombin generation and could have an effect on the hemostatic effectiveness of the resulting clot.

The relationship between prothrombin level and thrombin generation is achievable also for supernormal prothrombin concentrations: elevated plasma prothrombin results in an increase in the rate and amount of thrombin generation. This may be the reason that elevated levels of prothrombin are correlated with a risk of arterial and venous thrombosis [53].

FVIII and FXI levels above the normal range result in a modest increase in the rate of thrombin generation, and elevated levels of these factors have been reported to be associated with the risk of thrombosis [54, 55].

1.4.2 Inherited and acquired coagulation disorder

As already mentioned, the hemostatic Vitamin K-dependent factors of coagulation are encoded by genes with virtually identical exon/intron distributions [77], suggesting that they have evolved relatively recently from a common an-

cestor by a process of gene duplication and divergence [78]; in this context it is believable that both the genes and the proteins have common regulatory mechanisms.
It has been provided the direct quantification of the genetic and environmental correlations between the plasma levels of a group of closely related vitamin K-dependent hemostasis factors, i.e. prothrombin, factor VII, IX, X, PC and functional PS. This investigation indicates how, actually, a set of common genes affects for a large proportion the phenotypic variation in vitamin K-dependent proteins, and similarly how a set of common unmeasured environmental factors also appears to influence plasma levels of vitamin K-dependent proteins [79].
A major contributor to our understanding of the complex hemostatic process, however, has occurred because of individuals with hereditary deficiencies of molecular elements fundamental to the hemostatic pathway.

Bleeding disorders

Genetic defects causing bleeding disorders can affect different proteins in the coagulation process.
The most common forms of haemophilia are due to different types of genetic defects (deletions, insertions, point mutation) resulting in inherited deficiency of FVIII (haemophilia A) or FIX (haemophilia B). Other coagulation factor deficiencies are very rare [82, 83]. Haemophilias occur in severe, moderate and mild forms, corresponding plasma levels being $<1\%$ of the normal plasma concentration, 1-5% and 5-30% respectively [83]. In the most common human hemophilias, the linkages between the degree of pathology and the molecular severity of the mutations are also not always correlated [86]. These observations imply that the genetic/environmental background in which a gene deficiency is observed may produce very different phenotypic responses.
von Willebrands disease (vWD) is a relatively common bleeding disorder affecting both males and females [85]. vWF is important for platelet adhesion and for maintaining the normal level of FVIII [87, 88], thus patients with vWD could have a primary haemostasis defect, caused by deficient adhesion of platelets to exposed subendothelial collagen, or, associated to severe forms of vWD, a secondary deficiency of FVIII, as the vWF is a carrier of FVIII in blood.
Faulty platelet function or thrombocytopenia can cause bleeding defects but clinically important bleeding only occurs when the abnormalities are severe [80, 81]. The symptoms associated with the inherited platelet disorders rarely are severe haemorrhage. Inherited deficiency of GPIb-V-IX (Bernard-Soulier

syndrome) is characterized by thrombocytopenia, giant platelets and lack of vWF binding and affected individuals have bleeding tendency. Acquired platelet function defects can originate by different medication (e.g. aspirin) and by different chronic diseases such as renal failure or autoimmune disease. Combined deficiency of the vitamin K-dependent coagulation proteins is another very rare inherited cause of bleeding. This may be caused by genetic defects in one of the two known enzymes that are involved in the γ-carboxylation of the vitamin K-dependent coagulation proteins: the γ-carboxylase and the vitamin K epoxide reductase [89].
Acquired bleeding problems can also be related to deficiency of vitamin K, which is required in the biosynthesis of many of the coagulation proteins. Malabsorption of the lipidsoluble vitamin K results in deficient γ-carboxylation of the vitamin K-dependent coagulation proteins, which in severe cases may result in increased bleeding. Even more common is vitamin K deficiency due to excessive intake of vitamin K antagonists, e.g. warfarin, used as anticoagulant therapy.
Bleeding disease can be due to the development of autoantibodies against a coagulation factor, the most common is directed against FVIII [90–93]. These conditions mainly affect elderly people. The molecular mechanisms responsible for the development of the autoantibodies are poorly understood.
In disseminated intravascular coagulation (DIC), acquired bleeding is due to the consumption of platelets and coagulation factors due to widespread pathological proteolysis [93, 95]. In this condition, multiple proteolytic enzyme systems including the coagulation and fibrinolytic systems are activated causing microvascular thrombosis and major disturbances of the capillary circulation. DIC may complicate malignancy, traumatic injury, surgery or pregnancies, and is often caused by severe infections with septicaemia.

Thrombotic disorders

Epidemiological studies illustrate that significant thrombotic risk is associated with the inheritance of common mutations (polymorphisms) which influence functions or concentrations of coagulation proteins and cells [96]. But determining the global risk for thrombotic events (venous and arterial) is difficult because thrombosis is a multicausal disorder. The pathogenesis of thrombosis involves inherited and acquired risk factors. Most acquired risk factors are of short duration, like pregnancy, surgery, and immobilization; other are environmental factors, such as obesity [97, 98], oral contraception [99, 100], hormone replacement therapy [100], age [101], alcohol use [102] and potentially smoking [103]; in addition there are lifelong inherited factors. Thus, a thrombotic episode may appear to be induced by an acquired

risk factor when in fact the disease is due to a combination of genetic and acquired risk factors. The natural balance between pro- and anticoagulant forces is affected by most of the inherited risk factors for thrombosis.
The majority of the genetic risk factors impair the function of the protein C anticoagulant system. The most common, found in 20-40% of patients with thrombosis, is a single point mutation in the FV gene, which keeps full procoagulant capacity but causes the APC resistance phenotype (FV Leiden). This mutation impairs degradation of mutant FVa by APC, causing the loss of one APC cleavage sites in FVa. Furthermore impairs the degradation of FVIIIa, because mutant FV shows poor anticoagulant-cofactor activity for APC in the degradation of FVIIIa.
A single point mutation (G20210A) in the 3' untranslated region of the prothrombin gene is the second most common genetic risk factor for thrombosis (three- to five-fold increased risk) found in 68% of patients with thrombosis and in around 2% of healthy controls [104–106]. The prothrombin function is unaffected by the mutation but the levels of prothrombin in plasma are slightly increased as a result of the mutation, which may be the basis for the increased risk. Also other impairment in providing normal levels of FVIII [107], FIX [108], FXI [109] and fibrinogen [110] have been reported to be independent risk factors of venous thromboembolism (VTE). Mechanisms through which these modifications could influence thrombotic risk, however, are not completely understood. Most individuals with a single genetic risk factor, although they have a lifelong increased risk, will not suffer from thrombosis during their lives because the associated risk is relatively low. People affected by more than one risk factor, either genetic or acquired are at higher risk and it is now considered that venous thromboembolism is a typical multigenetic/multifactorial disease.
The antiphospholipid syndrome (lupus anticoagulant) is an acquired risk factor for both arterial and venous thrombosis [111–113]. Pregnant women with the antiphospholipid syndrome have an increased risk of spontaneous abortions.

1.5 Level's analysis

It is a well known problem in clinical hematology that traditional coagulation tests, such as the prothrombin time (PT) and activated partial thromboplastin time (APTT), do not assess the whole coagulation system. These tests use clot formation as their endpoint, which occurs when only around 5% of all physiologically relevant thrombin is formed [114, 115] and also are insensitive to prothrombotic states. Coagulation factor assays can identify specific deficiencies but these do not always closely correlate with the clinical phenotype. Hence neither the prothrombin time, nor the activated partial thromboplastin time, nor the level of the individual clotting factors can accurately predict the presence and severity of a bleeding tendency. The observation that thrombin generation varies up to 40-fold when measurements are done with individual coagulation factors at the extremes of the normal ranges in a synthetic plasma system [39], also demonstrate the limitations of the traditional tests.
Thrombin is the central enzyme in the coagulation cascade; estimation of an individuals potential to generate thrombin may correlate more closely with a hyper- or hypo-coagulable phenotype, compared to traditional coagulation tests. Measurement of an individuals capacity to generate thrombin, however, captures the end result of the interaction between proteases and their inhibitors and is therefore potentially more useful as a reflection of a thrombotic (high thrombin generation) or haemorrhagic (low thrombin generation) phenotype compared to conventional coagulation tests [116].
Controlled TF concentration used to trigger the assay may evidence different role of procoagulant proteins among coagulation cascade. The positive feedback activation of thrombin by the intrinsic pathway can only be demonstrated at low (<1 pmol/L) TF concentrations [117]. Furthermore the assay is sensitive to FVIII and FIX at TF concentrations up to 5 pmol/L [118]. Small differences in TF concentration can therefore cause significant differences in the thrombin generating potential of plasma. Low TF trigger concentration may exacerbate differences in thrombograms, the thrombin generation curve, of patients with the same pathology, e.g. hæmophilia; instead higher quantity could masks this result due to the decreased sensitivity of the system [119]. Conversely, thrombin generation measurements at low TF concentrations in hæmophilia are difficult because of the low signal obtained and it may not always be possible to obtain full curves [119], limiting the information gained from the measurements. Low TF concentrations are also needed to demonstrate the protein C independent effect of protein S on anticoagulant process [120].
Also FIXa could be used as trigger in order to measure the effect of FVIII

on thrombin generation. FIXa was found to be sensitive to differences in thrombin generation at very low FVIII concentrations [121]. Even if thrombin generation assay in common laboratory usage is not sensitive to the actions of the protein C pathway, *in vitro* sensitivity can be increased by adding truncated human recombinant thrombomodulin (sTM) [122], APC [123], or the snake venom Protac [124, 125]. The use of these agents makes the assay more sensitive to deficiencies in protein C (not detected if APC is used), protein S, FV R506Q (factor V Leiden) and conditions associated with acquired protein C resistance.

Thus thrombin generation measurements can be performed in several ways that have different sensitivities for various haemostatic or thrombotic defects. As small variations in pre-analytical variables can cause significant changes in the sensitivity for these defects, results cannot be interpreted without detailed information on how the assay was performed. Since thrombin is the central enzyme in the coagulation cascade, evaluating hypothetical thrombin generation based upon the individuals blood composition may correlate more closely with a hyper- or hypo-coagulable phenotype, compared to traditional coagulation tests. Finally, even when a single well standardized test is available it is unlikely that it will be suitable in all situations and separate modified tests designed for specific clinical situations may need to be developed.

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Chapter 2

Combined effect of hemostatic gene polymorphisms and the risk of myocardial infarction in patients with advanced coronary atherosclerosis.

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2.1 Abstract

Relative little attention has been devoted until now to the combined effects of gene polymorphisms of the hemostatic pathway as risk factors for Myocardial Infarction (MI), the main thrombotic complication of Coronary Artery Disease (CAD). The aim of this study was to evaluate the combined effect of ten common prothrombotic polymorphisms as a determinant of MI. **Methodology/Principal Findings** We studied a total of 804 subjects, 489 of whom with angiographically proven severe CAD, with or without MI (n= 307; n= 182; respectively). An additive model considering ten common polymorphisms [Prothrombin 20210G>A, PAI-1 4G/5G, Fibrinogen β-455G>A, FV Leiden and R2, FVII -402G>A and -323 del/ ins, Platelet ADP Receptor P2Y12 -744T>C, Platelet Glycoproteins Ia (873G>A), and IIIa (1565T>C)] was tested. The prevalence of MI increased linearly with an increasing number of unfavorable alleles (χ^2 for trend = 10.68; P= 0.001). In a multiple logistic regression model, the number of unfavorable alleles remained significantly associated with MI after adjustment for classical risk factors. As compared to subjects with 3-7 alleles, those with few ($\leq$2) alleles had a decreased MI risk (OR 0.34, 95%CIs 0.13-0.93), while those with more ($\geq$8) alleles had an increased MI risk (OR 2.49, 95%CIs 1.03-6.01). The number of procoagulant alleles correlated directly (r = 0.49, P= 0.006) with endogenous thrombin potential. **Conclusions** The combination of prothrombotic polymorphisms may help to predict MI in patients with advanced CAD.

2.2 Intoduction

Myocardial Infarction (MI), the leading complication of coronary atherosclerotic disease (CAD), generally occurs in the late stages of disease because of coronary thrombosis superimposed on a ruptured/unstable plaque [1]. In clinical practice it is well-known that, in spite of the documented presence of advanced CAD, only a subset of patients develops acute MI during their life-course [2]. The reasons for individual differences in susceptibility to MI are poorly understood. In principle, subjects with an increased tendency to form blood clots (i.e. with hypercoagulability) may be at increased risk, as observed for venous thrombosis. Lessons from animal models suggest that excessive thrombin generation may be particularly harmful during the later stages of atherosclerosis, when thrombotic complications often occur [3,4]. However, this is difficult to assess in clinical practice, since we lack a unique and reliable laboratory marker of hypercoagulability [5]. Moreover, functional tests evaluating concentration and/or function of blood coagu-

lation proteins are often subjected to multiple transient interferences, e.g. due to the use of antithrombotic and anticoagulant agents or the presence of concomitant inflammation. Genetic polymorphisms with a documented functional effect on blood coagulation proteins may represent a useful tool, by reflecting the individuals lifelong exposure to even a mild prothrombotic state. During the last decade, extensive studies on various individual polymorphisms as risk factors for CAD and MI have yielded largely inconclusive results [6-11]. These results reflect at least two critical issues: 1) the multifactorial and multistep pathogenesis of CAD, involving many different biochemical pathways and intermediate phenotypes (e.g. hyperlipidemia, diabetes, hypertension), each in turn under the control of many different genes; 2) the enormous heterogeneity of investigations in terms of study design, typology of patients included, and clinical endpoints [10]. There has also been relatively little attention devoted to assess the combined effect of genes, which might be anticipated by analogy to the well-known additive effects of conventional risk factors. Generally, individual polymorphisms confer a marginal to moderate CAD risk that becomes evident only across many thousands of individuals, as was recently demonstrated by meta-analysis for Factor V 1691 G>A (Factor V Leiden), prothrombin 20210 G>A, and PAI-1 -675 4G/5G [8]. This renders such polymorphisms unhelpful in assessing individuals risk clinically. On the other hand, the value of analyzing multiple alleles simultaneously for determining the risk is not well studied.

In this study we evaluated the combined effect of ten common genetic variants, with known modest effects on the hemostatic balance (listed in Table 2.1) [6,12-21], in modulating the risk of development of MI. Because of the relatively late occurrence of MI in the natural history of CAD, we focused on a selected population of high risk patients with angiographically documented, advanced CAD. A thrombin generation assay was also used in a subset of patients to explore the propensity to form blood clot as a function of the number of hemostatic polymorphisms.

2.3 Materials & Methods

2.3.1 Study population

This study was performed within the framework of the Verona Heart Project, a regional survey aimed to search for new risk factors for CAD and MI in subjects with objective angiographic documentation of their coronary vessels. Details about enrolment criteria have been described in detail elsewhere [35,36]. A total of 804 subjects, for whom complete analyses of 10 polymor-

Polymorphism	Chromosome location of gene	Effects on intermediate phenotype
FIBRINOGEN beta-chain –455 G>A [fibrinogen]	4q28	-455 AA genotype associated with fibrinogen concentrations that are 10% higher than GG genotype [12]
Factor VII A1/A2 [coagulation factor VII]	13q34	A2 associated with reduced factor VII concentrations [13]
Factor VII–402 G>A [coagulation factor VII]	13q34	-402A associated with increased factor VII concentrations [14]
Factor V Leiden (R506Q) [coagulation factor V]	1q23	506Q is a cause of activated protein C (APC) resistance [15]
Factor V R2 (6755 A>G) [coagulation factor V]	1q23	6755G associated with mild APC-resistance and impaired APC mediated factor VIII inactivation [16]
PROTHROMBIN 20210 G>A [precursor of thrombin]	11p11-q12	20210A associated with increased plasma prothrombin levels [17]
PAI-1–675 5G/4G [inhibitor of plasminogen activator]	7q21.3-q22.1	4G associated with increased plasma PAI-1 Levels [18]
GP IIIa Leu33Pro [platelet receptor for fibrinogen and von Willebrand factor]	17q21.32	33Pro might increase sensitivity to platelet aggregation [19]
GP Ia/IIa alfa2 873 G>A [platelet receptor for collagen]	5q23-q31	873A (in linkage with 807T polymorphism) might increase the receptor density [20]
P2RY12 H1/H2 (-744T>C) [platelet receptor for ADP]	3q24-q25	-744C, in absolute linkage disequilibrium with 3 others SNPs, marks the H2 haplotype, that is associated with maximal aggregation response to ADP [21]

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Table 2.1: Description of the haemostatic gene polymorphisms, analysed in this study, and their associated intermediate phenotype.

phisms of genes involved in hemostatic pathways were available, were included in the present study. Three-hundred fifteen subjects had completely normal coronary arteries, being submitted to coronary angiography for reasons other than CAD, mainly valvular heart disease (CAD-free group). These controls were also required to have neither history nor clinical or instrumental evidence of atherosclerosis in vascular districts beyond the coronary bed. Fourhundred eighty nine subjects had angiographically proven CAD (the majority of them being candidates for coronary artery bypass grafting) with objective documentation of presence/absence of MI. The disease severity was determined by counting the number of major epicardial coronary arteries (left anterior descending, circumflex, and right) affected with $\geq$1 significant stenosis ($\geq$50%). According to the hypothesis to be tested, subjects with non-advanced CAD (i.e. coronary stenosis <50%) were not included in the study. Cardiologists unaware that the patients were to be included in the study assessed the angiograms. Patients were classified into MI (n= 307) and non-MI (n= 182) subgroups on the basis of a thorough review of medical records including history, electrocardiogram, enzyme changes, and/or the typical sequelae of MI on ventricular angiography.

All participants came from the same geographical area (Northern Italy), with a similar socio-economic background. At enrolment, a complete clinical history was collected, including the assessment of cardiovascular risk factors such as obesity, smoking, hypertension and diabetes. The study was approved by the Ethic Committee of our Institution (Azienda Ospedaliera, Verona). A written informed consent was obtained from all the participants after a full explanation of the study.

2.3.2 Biochemical analysis

Samples of venous blood were drawn from each subject at enrolment, before coronary angiography and after an overnight fast. Serum lipids, as well as other CAD risk factors, including high-sensitivity C-reactive protein (hs-CRP) were determined as previously described [26].

2.3.3 Genetic analysis and nomenclature

Genomic DNA was extracted from whole blood samples by a phenol-chloroform procedure using the Puregene kit (Gentra Systems) according to the manufacturers protocol. The 10 genetic polymorphisms, selected on the basis of prior evidence of potential functionality in modulating the hemostatic pathway, are listed in Table 2.1. Seven out of ten polymorphisms (fibrinogen beta-chain - 455G>A, Factor VII A1/A2, Factor V Leiden, Prothrombin

20210 G>A, PAI-1 -675 5G/4G, GP IIIa Leu33Pro, GP Ia/IIa alfa2 873 G>A) were examined by a previously described and validated linear-array assays for candidate markers [37]. The accuracy of the linear-array genotyping system as compared with standard genotyping approaches reported elsewhere [35,36] was evaluated and the findings provide reassurance regarding the validity of the system used, as previously described [38]. The remaining three (Factor VII - 402 G>A, Factor V R2, P2RY12 H1/H2) were analyzed by previously described, standard genotyping approaches [36,39,40]. Genotype interpretation for each polymorphism was performed independently by two investigators and very few samples (<1%) with unclear result were re-genotyped.

2.3.4 Measurement of thrombin generation activity

This assay was performed in a subset of CAD patients on samples drawn at enrolment, in order to evaluate a possible functional counterpart of an increasing number of prothrombotic alleles in terms of propensity to form blood clot. Plasma sample were centrifuged at 23,000 g at 4°C for 1 hour before testing. Calibrated automated thrombin activity measurement was conducted according to Hemker et al. [41,42] in a microtiter plate fluorometer (Fluoroskan Ascent, ThermoLabsystems, Helsinki, Finland) using the Thrombinoscope software (Synapse BV, Maastricht, The Netherlands). The assay was carried out at 37°C essentially as previously reported [43]. Coagulation was triggered in platelet poor plasma by recalcification in the presence of 1 pM recombinant human tissue factor and 4 μM phospholipids. Thrombin generation was then evaluated overtime by exploiting a specific fluorogenic substrate (Z-Gly-Gly-Arg-AMC). Thrombin generation measurement was conducted in parallel in plasma samples after the addition of a thrombin calibrator provided by the manufacturer (Synapse BV). The software enables the estimate of the following parameters: a) the Lag Time of thrombin generation, b) the time to reach the maximum concentration of thrombin (time to Peak), c) the maximum concentration of thrombin (Peak), d) the total duration of thrombin generation activity (Start Tail), and e) the total amount of thrombin activity assessed as the area under the curve, i.e. the endogenous thrombin potential (ETP). All experiments were carried out in duplicate.

2.3.5 Statistics

Calculations were performed mainly with SPSS 13.0 statistical package (SPSS Inc., Chicago, IL). Distributions of continuous variables in groups were ex-

pressed as means±standard deviation. Logarithmic transformation was performed on skewed variables, for whom geometric mean with 95% confidence interval (CI) are given. Quantitative data were assessed using the Students t-test or by ANOVA with Tukeys post-hoc comparison of the means. Correlations between quantitative variables were assessed using Pearsons correlation test. Qualitative data were analyzed with the χ^2-test or the Fisher exact-test when indicated. Hardy-Weinberg equilibrium was tested for each genotype within each group by means of χ^2-test. A value of $P<0.05$ was considered statistically significant.

Within each group examined, the frequencies of the genotypes associated with each of the polymorphisms were compared by the χ^2- test, with the values predicted on the basis of the Hardy-Weinberg equilibrium. To assess the extent to which gene polymorphisms were associated with MI, odds ratios with 95% CIs were estimated by univariate logistic regression analysis. Adjustment for other variables (i.e. number of affected vessels, age, sex, smoking, BMI, LDL- and HDL-cholesterol) was performed by adding those covariates in a set of multiple logistic-regression models.

The existence of gene-gene interactions was first explored by a data mining technique similar to the Adaboost algorithm, and based on classification and regression trees (CART): the gradient boosting machine [44]. The statistical significance of the interactions found with this method was then estimated by the likelihood ratio test applied on two logistic models (with and without the interaction terms). After observing that no significant interaction was present, we focused on an additive model. On this basis, we attributed to each patient a prothrombotic score (PS), reflecting the sum of 10 concomitant unfavourable prothrombotic alleles, theoretically ranging from 0 (no prothrombotic allele present) to 20 (all the prothrombotic alleles present). The association between the prothrombotic score and MI was evaluated by χ^2 for linear trend analysis. The prothrombotic score was analysed by logistic regression both as continuous variable and as categorised variable. Odds ratios with 95% CIs were estimated by univariate logistic regression analysis and then by multiple logistic regression with adjustment for number of affected vessels, age, sex, smoking, BMI, LDL- and HDLcholesterol. The predictivity of our models was then evaluated by the receiver-operating-characteristics (ROC) curve, estimating the area under the curve (AUC).

2.4 Results

2.4.1 Haemostatic polymorphisms in the CAD group as a whole versus CAD-free subjects

Supplemental Table 1 (Table S1) shows the genotype frequencies for each of the 10 polymorphisms in CAD-free (n=315; males 66.0%; mean age 59.26±11.9 years) and in CAD subjects (n=489; males 83.6%; mean age 60.36±9.3 years). All alleles were in Hardy- Weinberg equilibrium. For each polymorphism there was no significant difference in genotype distribution between CAD and CAD-free groups. The distribution of the prothrombotic score (PS) in the whole study population (n=804) is shown in figure 2.1A. The score ranged from 0 (1 subject) to 10 prothrombotic alleles (7 subjects), with a median level of 5. Figure 2.1B shows the distribution of the PS in CAD-free and in CAD subjects. No association was found between the PS and CAD (P=0.889 by χ^2-test).

2.4.2 Individual haemostatic polymorphisms and MI risk in subjects with advanced CAD

Supplemental Table 2 (Table S2) shows the general characteristics of the CAD population divided in two groups on the basis of presence/absence of MI. As compared to CAD patients without MI, MI patients were significantly younger, more frequently males, had a higher degree of CAD in terms of number of diseased vessels, and lower HDL-cholesterol levels. No significant difference was found for the other variables. Table 2.2 shows the genotype frequencies of the 10 genetic variants in CAD patients with or without MI. Two polymorphisms, factor VII -402 G>A and fibrinogen β-chain -455 G>A, showed nominal association with MI at the univariate analysis. However, these associations were no longer significant after multiple logistic regression adjusted for sex, age, disease severity, smoking status, BMI, LDL- and HDLcholesterol (P =0.155 for factor VII -402 G/A and P= 0.998 for fibrinogen β-chain -455 G/A).

2.4.3 Combined effect of haemostatic polymorphisms and MI risk

No significant interaction was found by CART among polymorphisms in determining MI risk (all P for interaction >0.05). As shown in figure 2.1C, the proportion of CAD patients with MI increased progressively with increasing

number of unfavourable alleles (χ^2 for linear trend= 10.68; P= 0.001). In a multiple logistic regression model the prothrombotic score remained significantly associated with MI after adjustment for sex, age, degree of CAD, smoke, BMI, LDL- and HDL-cholesterol (OR for 1-point increase in prothrombotic score= 1.22 with 95%CI 1.06-1.39, P =0.004). Using the median of PS as cut-off, CAD patients with >5 alleles had a significantly increased risk of MI as compared to subjects with ≤5 alleles (OR 2.02 with 95%CI 1.27-3.21, P= 0.003, by multiple logistic regression). Using approximately the 5^{th} and the 95^{th} percentiles of PS distribution (i.e. 2 and 8, respectively), the study population could be classified into in 3 subgroups: a low-risk group with less than 3 unfavourable alleles (n= 26), an intermediate-risk group with 3 to 7 unfavourable alleles (n= 417), and a high-risk group with more than 7 unfavourable alleles (n= 46). The prevalence of MI among these groups increased progressively (38.5% in low-risk; 62.6% in intermediate- risk; and 78.3% in high-risk; P= 0.001 by χ^2 for linear trend), while they were similar for the other clinical and laboratory variables (data not shown). Considering the intermediate-risk group as the reference group, carriers of <3 alleles had a lower risk of MI, while carriers of >7 alleles had an increased risk (Figure 2.2). Comparing the two extreme groups, the subjects with >7 alleles had a remarkably higher MI risk (OR 7.28 with 95%CI 2.01-26.36, P= 0.002 adjusted by multiple logistic regression). The ROC curve for information provided by our polygenic approach for MI prediction in CAD patients is plotted in Supplemental Figure 1 (Figure S1). The AUC was 0.581 with a 95% CI from 0.530 to 0.632.

2.4.4 Combined effect of haemostatic polymorphisms and thrombin generation activity

To get insights on the pathophysiological effect of combined hemostatic alleles, we assessed the characteristics of thrombin generation activity curves as a function of the number of procoagulant alleles (i.e. fibrinogen β-chain -455 A, Prothrombin 20210 A, Factor V Leiden, Factor V R2, Factor VII A1, Factor VII -402 A and PAI-1 -675 4G). Since this assay pertains only to the coagulation pathway, the three platelet-related polymorphisms were not considered for this analysis. This assay was performed in a subset of 29 CAD patients (26 males and 3 females, 22 with and 7 without MI), selected among those without possible confounders (i.e. concomitant anticoagulant therapy or overt signs of inflammation, documented by hs-CRP,5 mg/l), in order to form three groups matched for age and sex representing the previously defined risk groups (low-risk: n= 9, 8 males and 1 female, mean age 53.7±8.5;

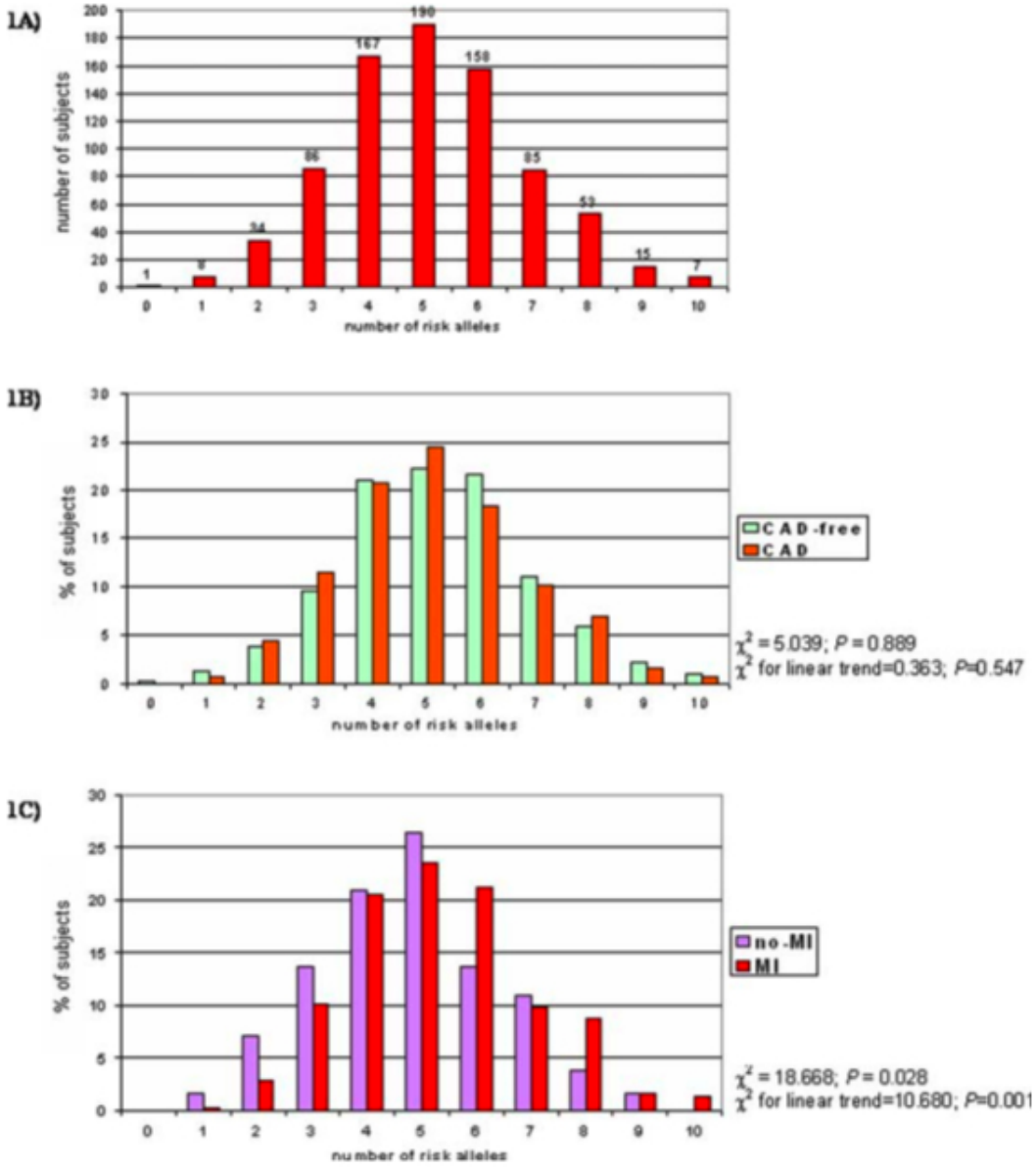

Figure 2.1: Study population (n = 804) stratified on the basis of number of risk alleles (1A). The distribution of number of risk alleles in CAD-free (n = 315) and in CAD patients (n = 489) (1B) and in CAD patients with (n = 307) or without a history of MI (n = 182) (1C).

intermediate-risk: n =10, 9 males and 1 female, mean age 57.8±7.4; high-risk: n =10, 9 males and 1 female, mean age 56.0±8.6). The number of procoagulant alleles was significantly associated with ETP and with Start Tail, but not with Lag Time, Peak or Time to Peak (Table 2.3). Similarly, subjects with a high number of procoagulant alleles (≥5) had significantly higher ETP values as compared to subjects with fewer alleles (Table 2.4). These two groups were similar not only for age and sex, but also for smoking, hypertension and diabetes (data not shown). Their median thrombin generation activity curves are showed in Figure 2.3.

2.5 Discussion

Evidence that a hypercoagulable state is associated with increased mortality has been provided by some recent studies [11,22]. To our knowledge, this is the first study that attempts to look at the impact of the combined effect of several common prothrombotic polymorphisms in the identification of CAD patients at different risk of developing MI. To put our results into perspective, we propose the following considerations.

2.5.1 Single haemostatic polymorphisms and MI risk

This study focused on relatively few genetic variants associated with defined biochemical alterations. While some of them (i.e. Factor V Leiden and prothrombin 20210 G>A) are established risk factors for venous thromboembolism, their association with arterial thrombosis is much less convincing [6,9]. Here too, despite some nominal significant P values, we found no consistent association when each polymorphism was considered individually. Indeed, CAD and MI are paradigms of complex disease, in which the effect of individual genes on the risk is anticipated to be weak [23,24]. Moreover, emphasizing the principle that "the highest the allele effect, the lowest the allele frequency"[25], it is plausible that genetic variants such those investigated in the present study, relatively frequent in the general population, could have at best only a mild effect on a potentially lethal phenotype like MI. Indeed, until now only a recent large meta-analysis including tens of thousands of patients has been able to detect a moderate but significant increase in the risk of coronary disease associated with either the Factor V Leiden mutation or the prothrombin 20210A variant [8].

	No MI (n = 182)	MI (n = 307)	P *
FIBRINOGEN beta-chain –455 G>A			
GG	65.4	59.9	0.028
GA	34.1	35.2	
AA	0.5	4.9	
Factor VII A1/A2			
A1A1	63.7	70.4	0.240
A1A2	31.3	26.7	
A2A2	5.0	2.9	
Factor VII–402 G>A			
GG	73.1	61.2	0.016
GA	23.1	35.5	
AA	3.8	3.3	
Factor V Leiden (R506Q)			
RR	96.7	96.8	0.725
RQ	3.3	2.9	
QQ	0	0.3	
Factor V R2 (6755 A>G)			
AA	83.5	82.1	0.917
AG	15.4	16.6	
GG	1.1	1.3	
Prothrombin 20210 G>A			
GG	96.7	94.1	0.204
GA	3.3	5.9	
AA	0	0	
PAI-1–675 5G/4G			
4G-4G	29.2	30.0	0.951
4G-5G	51.6	50.1	
5G-5G	19.2	19.9	
GP IIIa Leu33Pro			
Leu/Leu	75.8	69.7	0.210
Leu/Pro	23.7	28.3	
Pro/Pro	0.5	2.0	
GP Ia/IIa alfa2 873 G>A			
GG	41.2	37.8	0.740
GA	46.2	48.2	
AA	12.6	14.0	
P2RY12 H1/H2 (-744T>C)			
TT	77.5	73.6	0.234
TC	22.5	25.1	
CC	0	1.3	

*: by χ^2-test

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Table 2.2: Genotypes frequencies (%) of the CAD population, with or without MI.

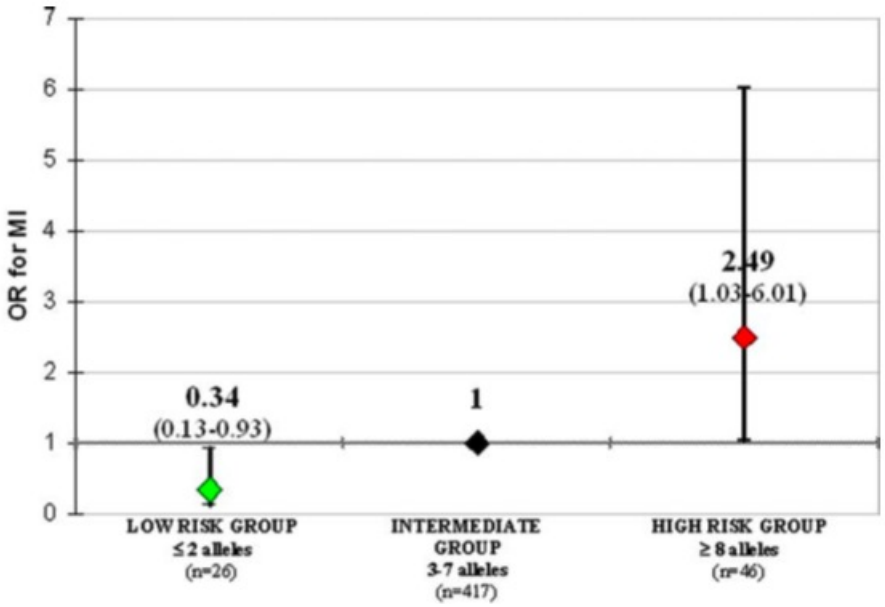

Figure 2.2: OR for MI in groups stratified on the basis of number of unfavourable alleles. The intermediate group (from 3 to 7 unfavourable prothrombotic alleles), representing the 85.3% of the whole population, is considered as reference group.

	Pearson correlation coefficient	*P*
Number of procoagulant alleles–ETP (nM×min)	0.494	0.006
Number of procoagulant alleles–Lagtime (min)	0.187	0.332
Number of procoagulant alleles–Peak (nM)	0.244	0.203
Number of procoagulant alleles–Time to Peak (min)	0.230	0.230
Number of procoagulant alleles–Start Tail (min)	0.396	0.033

Table 2.3: Correlations between number of procoagulant alleles and different characteristics of thrombin generation activity. The analysis was performed in a subgroup of CAD patients (n = 29) without anticoagulant therapies and without signs of overt inflammation. Procoagulant alleles were fibrinogen beta-chain -455 A, Prothrombin 20210 A, Factor V Leiden, Factor V R2, Factor VII A1, Factor VII -402 A, PAI-1 -675 4G.

	<5 alleles (n = 19)	≥5 alleles (n = 10)	$P^{\dagger}$
ETP (nM×min)	1,341±158	1,661±277	0.005
Lagtime (min)	6.49±0.79	6.89±1.33	0.326
Peak (nM)	191±37	221±43	0.058
Time to Peak (min)	10.08±0.87	10.64±1.59	0.224
Start Tail (min)	27.76±2.84	30.20±3.40	0.050

Table 2.4: Characteristics of thrombin activity generation curves in groups stratified on the basis of number of procoagulant alleles, with a threshold level at 5 alleles. The analysis was performed in a subgroup of CAD patients (n = 29) without anticoagulant therapies and without signs of overt inflammation. Procoagulant alleles were fibrinogen beta-chain -455 A, Prothrombin 20210 A, Factor V Leiden, Factor V R2, Factor VII A1, Factor VII -402 A, PAI-1 -675 4G. †: by t-test.

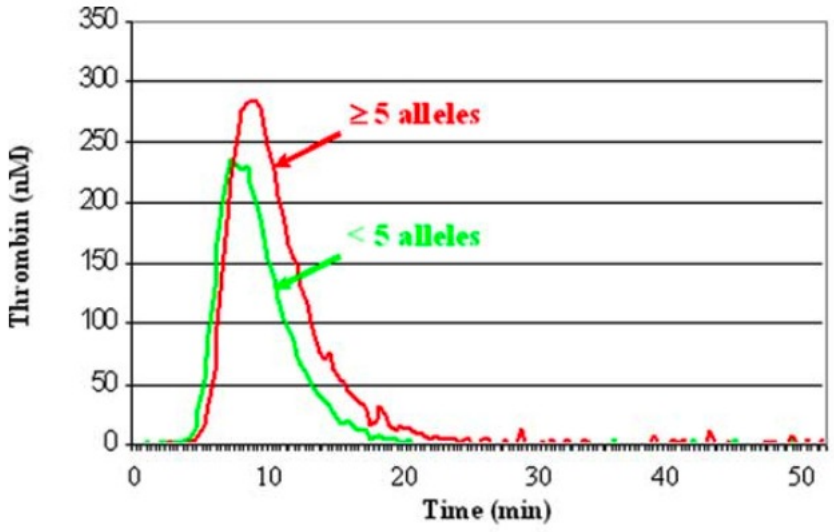

Figure 2.3: Comparison between the median thrombin generation activity curves in groups stratified on the basis of number of procoagulant alleles, with a threshold level at 5 alleles.

2.5.2 Combined effect of haemostatic polymorphisms and MI risk

Recently, a polygenic approach has been demonstrated to be a valid tool to identify subjects at risk for another complex trait such as type 2 diabetes [26]. A similar strategy was used in the present study, suggesting that in subjects with advanced CAD, an increasing number of prothrombotic alleles may confer a significant risk of developing MI. It is biologically plausible that the simultaneous presence of several genetic variations with modest but defined effects on the hemostatic process could influence the risk of the major thrombotic complication in a given CAD patient. Under certain stimuli, such as plaque erosion or rupture, this condition may predispose to sustained thrombin generation leading to the acute thrombotic event [2]. Accordingly, our in vitro functional studies showed an association between the number of procoagulant alleles and thrombin generation. The latter is known to be a highly variable and complex phenomenon modulated by the interplay of several factors, none of them with predominant influence, many of them under genetic control [27]. It is noteworthy that our clinical model focused on a homogeneous group of patients with angiographically proven advanced CAD. Elegant studies in animal models, i.e. Factor V Leiden mice crossbred with apolipoprotein E-deficient mice, indicates that unregulated thrombin generation is particularly harmful during the later stages of atherosclerosis. [3,4]. Conversely, a mild hypercoagulable state may be less meaningful in absence of underlying vulnerable atherosclerotic plaques. Our results may thus apply only to the specific clinical model of this study, and not to all CAD patients. While it is reasonable that genetically-induced excessive thrombin generation may be clinically relevant in subjects with extensive coronary plaques, this excess might be less influential in the atherogenetic process, where other genetic factors (i.e. those involved in modulation of lipid metabolism, antioxidant balance, and so on) may be prominent. This could explain why we found no association between the hemostatic polymorphisms and the CAD phenotype.

2.5.3 Study limitations and strengths

One strength of our study is the clear definition of phenotypes, allowing comparison of patients with angiographically proven, advanced CAD, with or without MI. The CAD population had a substantial burden of traditional risk factors and thus represented a typical patient population seen in clinical practice.

Our study has several limitations, including the relatively low number

of subjects and polymorphisms and a retrospective casecontrol design. In this setting the possible confounding of the survivor effect should also be taken into account. The prothrombotic score, calculated as the sum of prothrombotic alleles, is likely an oversimplification, since it standardized the contribution of each gene variant and does not allow distinguishing the possible different transmission models, as well as the different biological weight of the polymorphisms. Nevertheless, for complex traits the presence of additive effects of many genes is considered more likely than interactive effects [28,29], and additive models have been shown to perform well, even when the underlying model is unknown [30,31]. This study can be viewed as hypothesis-generating, shedding light into the potential usefulness of a polygenic approach in appropriate clinical contexts. Indeed, the predictive power of our additive genetic model was relatively low, yielding an area under the ROC curve (AUC) of 0.58. The AUC is a measure of the discriminatory power of a test, ranging from 0.5 for no discriminatory power to 1 for a perfect test [32]. We tested only ten polymorphisms here, and there is reason to believe that the predictive power of genetic information could be greater. Increasing technological resources with decreasing costs are likely to allow the inclusion, in models similar to that used in the present study, of other genetic variants reproducibly associated to functional consequences on coagulation factors, either newly identified (i.e. the long-anticipated genetic modulators of Factor VIII), or not included in this study (i.e. Factor XIII Val34Leu). With larger data sets, it may also be possible to capture gene-gene and gene-environmental factors.

2.5.4 Conclusions

Our data support the idea that, while individual genetic susceptibility variants are of limited clinical use, the combined information from a number of these variants can permit the identification of groups of people at high and low risk of developing a complex trait such asMI [33,34]. The polygenic model used in this study, considering the cumulative effect of hemostatic gene variants, was significantly associated to some in vitro measurements of thrombin generation. In the specific context of advanced CAD, similar approaches may be useful as surrogate markers of the propensity to form blood clots leading to MI. Further studies on larger samples are needed to confirm this intriguing working-hypothesis, as well as to improve predictive modelling.

2.6 Supporting Information

Table S1 Genotypes frequencies (%) in CAD-free and in CAD subjects.
Found at: doi:10.1371/journal.pone.0001523.s001 (0.08 MB DOC)

Table S2 Characteristics of the CAD population, with or without MI.
Found at: doi:10.1371/journal.pone.0001523.s002 (0.06 MB DOC)

Figure S1 ROC for the information provided by our polygenic model of prothrombotic alleles after fitting a logistic regression model.
Found at: doi:10.1371/journal.pone.0001523.s003 (0.05 MB DOC)

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Chapter 3

Vitamin K-induced modification of coagulation phenotype in VKORC1 homozygous deficiency.

Marchetti G, Caruso P, Lunghi B, Pinotti M, Lapecorella M, Napolitano M, Canella A, Mariani G, Bernardi F.

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3.1 Abstract

Background: Combined vitamin K-dependent clotting factor (VKCF) deficiency type 2 (VKCFD2) is a rare bleeding disorder caused by mutated vitamin K 2,3- epoxide reductase complex subunit 1 (VKORC1) gene. **Methods and results**: An Italian patient with moderate to severe bleeding tendency was genotyped, and found to be homozygous for the unique VKORC1 mutation (Arg98Trp) so far detected in VKCFD2. The activity levels of VKCFs were differentially reduced, and inversely related to the previously estimated affinity of procoagulant factor propeptides for the γ-carboxylase. The normal (factor IX) or reduced antigen levels (other VKCFs) produced a gradient in specific activities. Vitamin K supplementations resulted in reproducible, fast and sustained normalization of PT and APTT. At 24 h the activity/antigen ratios of VKCFs were close to normal, and activity levels were completely (factor VII and IX), virtually (prothrombin, factor X and protein C) or partially (protein S) restored. Thrombin generation assays showed a markedly shortened lag time. The time to peak observed at low tissue factor concentration, potentially mimicking the physiological trigger and able to highlight the effect of reduced protein S levels, was shorter than that in pooled normal plasma. At 72 h the thrombin generation times were normal, and the decrease in activity of procoagulant VKCFs was inversely related to their half-life in plasma. The improved coagulation phenotype permitted the uneventful clinical course after invasive diagnostic procedures. **Conclusions**: Modification of coagulation phenotypes in VKCFD2 after vitamin K supplementation was clinically beneficial, and provided valuable patterns of factor specific biosynthesis, half-life and decay.

3.2 Introduction

Impaired γ-carboxylation [1,2] produces multiple deficiency of vitamin K-dependent coagulation factors (VKCFD), a rare bleeding disorder inherited as an autosomal recessive trait [3]. The VKCFD type 2 (VKCFD2) is caused by defective regeneration of vitamin K hydroquinone by the vitamin K 2,3-epoxide reductase complex subunit 1 (VKORC1) [4]. Only one causative missense change (VKORC1 Arg98Trp) has been found in the homozygous condition in families of Lebanese and German origin [5,6].

In VKCFD2, the activity of the γ-glutamyl carboxylase enzyme, impaired by the limiting concentration of the vitamin K hydroquinone, can be transiently restored by vitamin K supplementation. These features make this condition an ideal model to investigate in vivo the temporal variations in levels

of vitamin K-dependent factors, driven by changes in γ-glutamyl carboxylation activity. Comparison of this natural condition with the pharmacological inhibition of the vitamin K cycle could provide valuable information to interpret observations obtained in plasma of patients on anticoagulant therapy [7-11].

The study of plasma phenotype variation in VKCFD2 would also contribute to validate, through in vivo observations, the cellular and molecular investigations [4,12-16] aimed at dissecting the mechanisms through which components of the vitamin K cycle [17] participate in the regulation of circulating factor level activity. Moreover, an extended analysis of levels after vitamin K supplementation in VKCFD2 would also provide information about the natural decay of vitamin K dependent clotting factors, of great interest for replacement therapy in inherited coagulation disorders [18].

As a thorough characterization of this deficiency by single factor parameters and by assays integrating the contribution of procoagulant and anticoagulant components has not been reported, we have investigated an VKCFD2 Italian patient over time after vitamin K supplementation.

3.3 Patients, materials and methods

3.3.1 Patient and family

The proposita, a 34-year-old woman, had experienced since childhood repeated nose and gum bleeds, menorrhagia, a severe post-partum hemorrhage at the age of 23 requiring blood transfusions, hemoperitoneum following ovarian cyst rupture, and rectal bleeding. In addition she presented with osteoporosis.

Laboratory testing of the patient's plasma revealed reduced activity levels of several procoagulant factors. Family members showed coagulation parameters in the normal range. Informed consent was obtained from the family members entering this study.

3.3.2 DNA analysis

The screening of the molecular defect responsible for the altered coagulation profile in the proposita was performed in both the γ-glutamyl-carboxylase (GGCX) and VKORC1 genes.

Direct scanning of exons and intron-exon boundaries of GGCX and VKORC1 genes were performed by PCR amplification followed by automated sequencing with the ABI Prism 377DNASequencer (PEApplied Biosystems, Foster

City, CA, USA). Specific primers for GGCX and VKORCI genes were derived from the Gene Bank database (references U65896 and AY587020, respectively). In the GGCX gene three single nucleotide changes, the g.1156G>C (intron 1), the g.9167T>C (exon 9), that predicts the Arg406Arg synonymous change, and the g.11665G>C (intron 14) were detected.

3.3.3 Coagulation laboratory assay

Plasma samples were withdrawn before (0 h) and after (at 4, 24, 28 and 72 h) intravenous administration of 10 mg vitamin K (KonakionRoche, Basel, Switzerland). Plasma samples before and after 24 hwere also collected during a second course of vitamin K application conducted 5 months later, before an esophagogastroduodenoscopy and colonoscopy with biopsy. Venous blood was drawn in sodium citrate (12.9 mmol L^{-1}) and immediately centrifuged at 2000 g for 20 min at 4°C. Plasma was separated, snap-frozen, and stored in aliquots at -80°C.

Factor clotting activities were assessed by conventional PT (factor (F) II, FVII, FX and PS) or APTT -based assays (FIX and PC) with the corresponding commercial factor-depleted plasma (HemosIL^{TM}; Instrumentation Laboratory, Milan, Italy). PT was also assayed in a FII, FVII, FIX and FXdepleted bovine plasma triggered with bovine thromboplastin (Pro-IL-Complex kit, HemosIL^{TM}). PC amidolytic activity was measured chromogenically (HemosIL^{TM} Protein C).

The total activity of the protein C pathway was evaluated by the APTT-based method Pro-C® Global (Dade Behring Diagnostics, Marburg, Germany). The normalized ratio (PCAT-NR) of the APTT determined in the presence and in the absence of activator Protac (PCAT:PCAT/0 ratio) was reported.

3.3.4 Antigen assays

Antigen levels were measured by ELISA using a mouse monoclonal anti-human FVII antibody, a sheep polyclonal anti human-FVIII antibody, a goat polyclonal anti-human FIX antibody (Affinity Biologicals Inc, Ancaster, Canada), a rabbit polyclonal anti-human FX antibody (DakoCytomation, Glostrup, Denmark) and a mousemonoclonal anti-human free PS antibody (Instrumentation Laboratory) directed to the C4bBP domain. Prothrombin levels were evaluated byWestern blot analysis as previously reported for FX [19]. Upon electrophoresis of diluted patient's plasma (1:70) on a Novex Tris-Glycine 4-20% PAA Gel (Invitrogen, Carlsbad, CA, USA), and

electroblotting onto nitrocellulose membranes (Schleicher & Schuell Microscience, Keene, NH, USA), prothrombin was probed by a sheep polyclonal anti-human prothrombin-HRP (Affinity, Carlsbad, CA, USA). Densitometric analysis of bands was conducted by the GS-700 instrument (BIORAD, Hercules, CA, USA).

3.3.5 FXa generation

Plasma FVII activity towards FX was assayed essentially as previously described [20]. Generation of activated FX (FXa) was monitored continuously by exploiting a specific fluorogenic substrate ($MeSO_2$-D-CHA-Gly-Arg-AMCAcOH, American Diagnostica, Greenwich, CT, USA). Fluorescence (360 nm excitation, 465 emission) was measured on Spectra- FluorPlus microplate reader (TECAN, Salzburg, Austria).

3.3.6 Thrombin generation

Platelet-poor plasma (PPP) samples were centrifuged at 23 000 g at 4°C for 1 h before testing. Calibrated automated thrombin activity measurement was conducted according to Hemker et al. [21] in an automated microtiter plate fluorometer (Fluoroskan Ascent; Thermo Labsystems, Helsinki, Finland) using the Thrombinoscope software (Synapse BV, Maastricht, The Netherlands). The assays were carried out at 37°C essentially as previously reported [22]. Coagulation was triggered in recalcified PPP under the following experimental conditions: 4 μM phospholipids (PLP, 20% phosphatidyl serine-80% phosphatidyl choline); 1 pM recombinant human tissue factor (TF)/4 μM PLP; 5 pM TF/4 μM PLP; 10 pM TF/ 8 μM PLP. The snake venom Protac (HemosIL^{TM}; Instrumentation Laboratory) was used in the presence of 3.5 pM TF/10 μM PLP. Thrombin generation was evaluated overtime by exploiting a specific fluorogenic substrate (Z-Gly- Gly-Arg-AMC) and was conducted in parallel in each plasma sample supplemented with a thrombin calibrator (Synapse BV). Experiments were carried out in duplicate or triplicate.

3.4 Results

3.4.1 DNA studies

Sequencing of the VKORC1 gene showed that the proposita was homozygous for the c.292C>T transition resulting in the amino acid change Arg98Trp.

The proposita's mother, two brothers and one sister were found to be carriers, as indicated by the AciI restriction analysis.

Frequent polymorphisms predicting variation in coagulation factor levels were also investigated. The proposita was found to be homozygous for the -323 ins10 in F7 gene [23], a condition associated with a remarkable reduction in FVII levels [24].

	Plasma sampling						
	0 h		4 h	24 h		28 h	72 h
APTT	33	39*	26	23	24*	23	26
PT-INR	3.18	3.46*	1.66	1.15	1.14*	1.11	1.79
PT†	107			37			
PCAT-NR	0.64			1.29			
FVII	14	10*	30	113	115*	117	19
FIX	26	21*	58	142	93*	108	67
FX	8	8*	30	68	79*	66	39
	31‡			77‡			64‡
FII	26	13*	42	67	84*	67	43
PC	36	34*	55	92	105*	89	48
PS	27	21*	36	54	46*	50	45

Table 3.1: Coagulation times and activity levels of vitamin K-dependent coagulation factors measured before (0 h) and after vitamin K treatment. *Values obtained before and after the second course of vitamin K administration. The APTT and PT† are expressed in seconds (normal range 2535 s and 1827 s, respectively). The PT† was measured in a FII, FVII, FIX and FX-depleted bovine plasma triggered with bovine thromboplastin. PCAT-NR, normalized ratio of the APTT determined in the presence and in the absence of the protein C activator Protac. Clotting activity (FVII, FIX, FX, FII) and amydolitic activity (PC) levels are reported as percentage of PNP (70120, normal range). Free protein S antigen is reported as percentage of PNP (53109, normal range). ‡FXa levels (% of PNP) measured at the peak of the FXa generation curves.

3.4.2 Coagulation studies

To get a comprehensive evaluation of the hemostatic profile and of its variations following vitamin K supplementation, APTT and PT were measured in the patient's plasma before and after intravenous supplementation of 10 mg vitamin K (Table 3.1). Before treatment the APTT was mildly prolonged and the PT-INR was remarkably increased. At 4 h a normal APTT and a remarkable decrease of the PT-INR were observed. The APTT was shorter

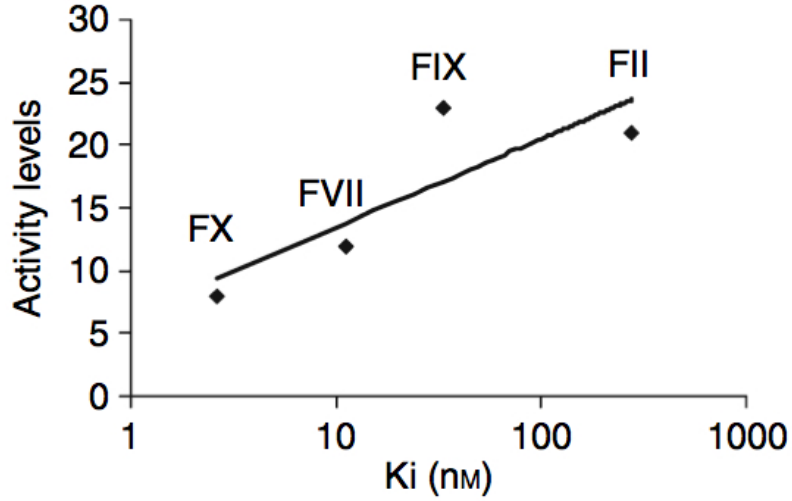

Figure 3.1: Baseline activity level of procoagulant factors and inhibition constants (Ki) of coagulation factor propeptides for c-carboxylase [25]. The mean activity levels measured in two independent plasma samples are reported. Logarithmic relation between variables ($R^2 = 0.70$).

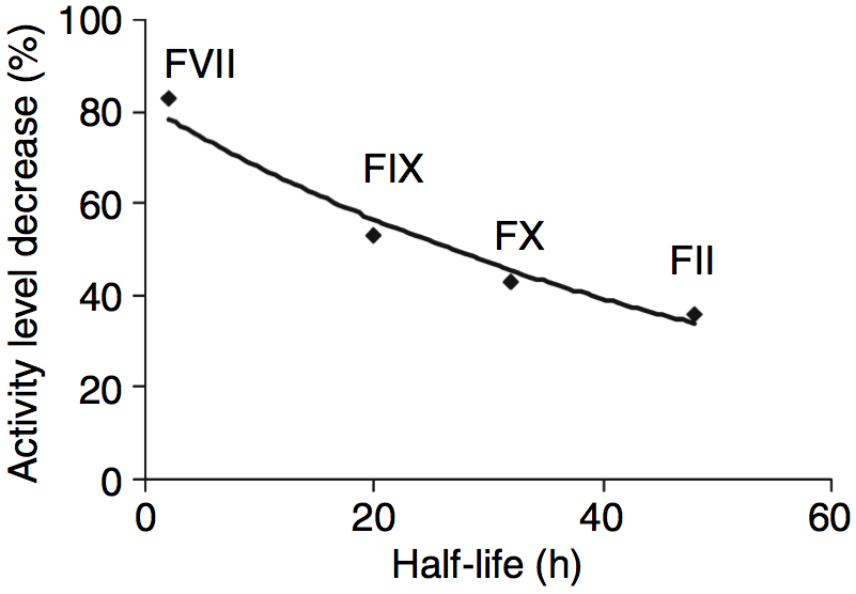

Figure 3.2: Decrease in activity levels (expressed as percentage) from peak to 72 h and half-life of procoagulant factors. The lowest values in the half-life range [26] were used ($R^2 = 0.97$, exponential curve).

than that of PNP (30 s) both at 24 and 28 h, and at the same time points PT was normalized. At 72 h the beneficial effects of vitamin K supplementation were noticeable, particularly for APTT.

Variations in PT and APTT were evaluated in the light of level variations of the specific vitamin K-dependent factors.

Procoagulant factors

The baseline activity levels, measured on different occasions (Table 3.1), were constant for FX and showed modest (FVII and FIX) or pronounced (prothrombin) variations.

As the differentially reduced activity values might reflect the residual carboxylase activity, we investigated their relationship with the binding affinities of carboxylase for the propeptide of the vitamin K-dependent procoagulant factors, previously estimated through peptide inhibition constants [25]. In two plasma samples obtained 5 months apart before vitamin K supplementation, mean activity levels showed a logarithmic relation ($R^2 = 0.70$) with the inhibition constants (Fig. 3.1). The best relation was found for FVII, FX and prothrombin levels ($R^2 = 0.99$), which indicated that their activity was

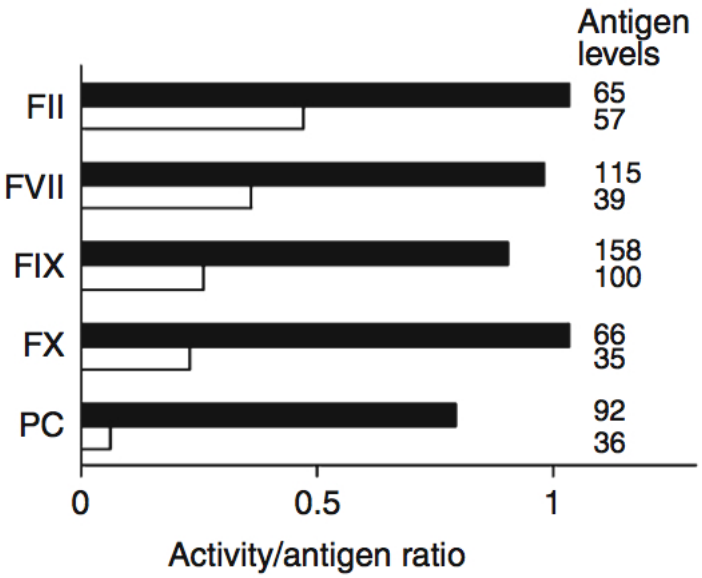

Figure 3.3: Activity/antigen ratios measured before (white column) and after vitaminK-treatment (black column). For PC the anticoagulant/amydolitic activity ratio is reported. The antigen levels are indicated. Prothrombin antigen levels were evaluated by densitometric analysis of Western blot.

inversely related with the affinity of the vitamin K-dependent carboxylase for coagulation factor propeptides.

For all factors an appreciable and reproducible (Table 3.1) increase in activity levels was measured after vitamin K supplementation. Plasma activity levels reached normal values for FVII and FIX, and borderline values for FX and prothrombin.

Levels higher than those at baseline were still detectable at 72 h, with the exception of FVII, which decreased at pretreatment levels (Table 3.1). The decrease in levels from the peak to 72 h was inversely related to (Fig. 3.2) the known half-life values of coagulation factors [26]. Particularly, the decrease showed the best fitting (exponential curve, $R^2 = 0.97$) with the lowest values of the half-life range. The R^2 using the highest half-life values was 0.75.

Defective VKOR activity appeared to affect activity and secretion of vitamin K-dependent factors to a different extent, and was responsible for the presence of dysfunctional molecules in plasma, as indicated by an excess of antigen levels (Fig. 3.3). FIX antigen levels were normal at baseline whereas those of FVII and FX were similarly reduced. At 24 h antigen levels were restored (FVII) or remarkably increased (FX).

The activity/antigen ratios at baseline and their variations induced by vitamin K supplementation after 24 h are summarized in Fig. 3.3. Before vitamin K administration, the unbalance between activity and antigen levels was remarkable with ratios of 0.2 (FX), 0.3 (FIX) and 0.5 (FVII and FII), which would strongly indicate the presence of partially carboxylated forms of vitamin K dependent clotting factors (PIVKA).

The overall dysfunctional effect of poorly carboxylated forms was indicated by PT assays conducted in a FII, FVII, FIX and FX-depleted bovine plasma triggered with bovine thromboplastin (Table 3.1). The PT observed in the patient (107 s, 5% of PNP) was more prolonged than those measured in plasma from patients on anticoagulant therapy (range 44-89 s, 7-28% of PNP).

Vitamin K administration substantially increased activity/antigen ratios of all procoagulant factors. The increase in amount of functional molecules was also indicated by the amelioration of parameters of PT and APTT assays (Table 3.1). However, the PT obtained in the bovine plasma (clotting time 37 s and 42% activity) was still far from normal (18-27 s).

As expected, the antigen level of FVIII (1.31 IU $mL^{-}1$), not requiring vitamin K for biosynthesis, did not change after vitamin K administration (1.33 IU $mL^{-}1$).

Anticoagulant factors

Repeated measurements indicated that the constant but defective amydolitic activity of PC at baseline was substantially improved by vitamin K supplementation (Table 3.1). Anticoagulant assays indicated a very ample variation in PC activity (from 2%to 73% of PNP).

Assuming that amidolytic activity parallels or is lower than antigen levels, the increase in PC specific activity (ratio from 0.06 to 0.8; Fig. 3.3) appears the most pronounced among the evaluated serine proteases.

Protein S free antigen levels at baseline (Table 3.1) were similar to those found in type I PS deficiency. Although levels were doubled at 24 h, they were only partially restored. This pattern was confirmed in the second course of vitamin K supplementation (Table 3.1).

At 24 h the PS anticoagulant function, evaluated in a PTbased assay, resulted in 58% of PNP, which roughly normalizes the activity/antigen ratio, as observed for vitamin Kdependent serine proteases.

The protein C pathway was further investigated through activation of endogenous protein C by the venom Protac in the APTT-based assay ProC global [27] (Table 3.1). The normalized ratio at baseline (NR = 0.64) was comparable with that of a heterozygous FV Leiden plasma (NR = 0.72), and was substantially increased by vitamin K supplementation (NR = 1.29).

3.4.3 Thrombin and FXa generation assays

The vitamin K-induced modification of thrombin generation parameters was evaluated under different conditions (Fig. 3.4). At all TF concentrations the lag-time and time to peak were both prolonged before treatment. In two plasma samples, obtained severalmonths apart and assayed at 1 pM TF, the lagtime ranged from 7.7 ± 0.01 to 9.8 ± 0.16 min and the time to peak from10.3 ± 0.01 to 12.8 ± 0.16 min. By comparison, the PNP lag-time (4.5 ± 0.5 and 4.6 ± 0.16 min) and time to peak (8.2 ± 1.17 and 8.6 ± 0.16 min) were constant. In the absence of TF thrombin generation was undetectable at 40 min (not shown).

At 24 h after vitamin K supplementation, the lag-time was similar to normal (Fig. 3.4).Whenmeasured at 1 pM TF (Fig. 3.4) the time to peak was reproducibly shorter (5.7 ± 0.33 and 6.6 ± 0.16 min) than in PNP.

Before treatment ETP values were clearly reduced at all TF concentrations (Fig. 3.4), ranging from 26% (1 pM TF) to 48% of PNP (10 pM TF). After treatment, the ETP was close to normal, ranging from 70% (1 pM TF) to 78% (10 pM TF) of PNP. Normal time parameters (lag-time 4.7 ± 0.01 min, time to peak 7.0 ± 0.01 min) and sustained ETP (61% of PNP) were

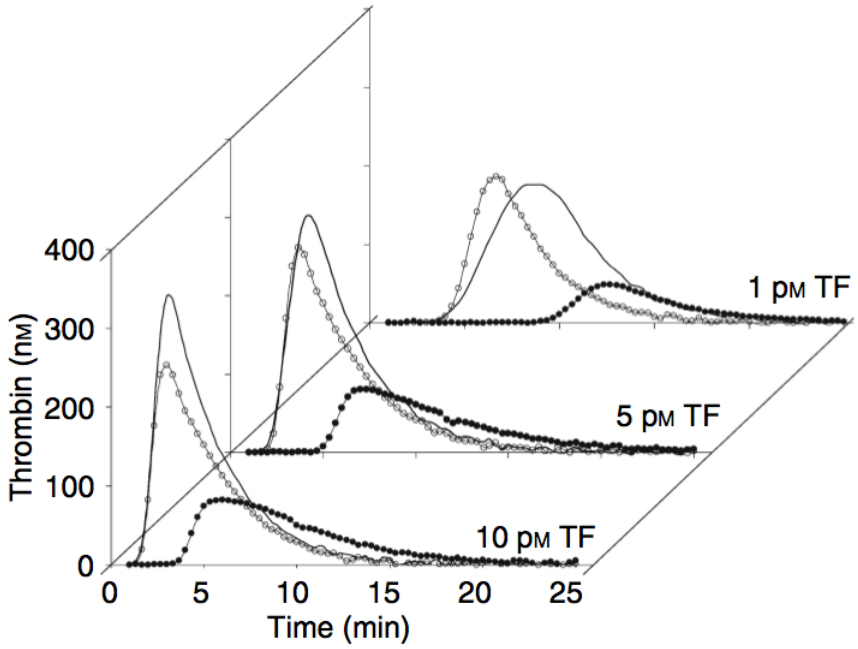

Figure 3.4: Thrombin generation triggered with the indicated TF concentrations in patient plasma, before (•) and 24 h after (○) vitamin K administration, and in PNP ().

still present in the patient's plasma at 72 h.

In order to enhance the contribution of the APC pathway, thrombin generation was also evaluated in the presence of the venom Protac (not shown). This protein C activator substantially inhibited thrombin generation in PNP (61% reduction in ETP), but was poorly effective in the patient's plasma, both before (21% reduction) and after (25% reduction) vitamin K administration. The ETP measured in plasma from a protein S deficient subject (PS:C and PS:Ag, 40%) was poorly modified (11% decrease) after the addition of Protac.

FXa generation was measured with a specific FXa fluorogenic substrate. The FXa generation after extrinsic activation (Table 3.1) substantially increased following vitamin K administration (from 31% to 77% of PNP), and was maintained at 72 h (64%of PNP). The time to maximal FXa generation was consistently shortened at 24 h (from 3.3 to 1.0 min) and similar to that of PNP (1.1 min). The FXa generation levels at baseline were comparable with FX antigen levels, and higher than FX activity in the coagulation assay, further confirming the presence of dysfunctional FX molecules (Fig. 3.3).

3.5 Discussion

The genotyping and characterization of the coagulation phenotype in the VKCFD2 patient provided us with information that significantly extends the knowledge of this rare condition, and has general implications for vitamin K-dependent regulation of plasma factor levels.

It is intriguing that among the several CpG sites (n = 30), potential hot spots for missense changes in VKORC1, only that contained in codon 98 and responsible for very low VKORC1 activity has been found to be mutated in VKCFD2 patients from different countries [5]. Differently, several VKORC1 mutations have been associated with warfarin resistance [5,28,29], a major clinical phenotype in the anticoagulant therapy. However, themutational pattern of VKCFD2 is based on very few cases, which prevents an informative comparison.

The homozygous VKORC1 Arg98Trp change has been found associated with heterogeneous clinical pictures, ranging fromfatal intracerebral hemorrhage after birth [6] to moderate/severe bleeding in adult life in the proposita. Noticeably, this Italian patient is also homozygous for FVII polymorphisms predicting further reduction in FVII levels, which could exacerbate bleeding tendency. From these observations we infer that VKCFD2, caused by a single VKORC1 mutation, interacts with other genetic/environmental factors with a major role in the clinical phenotype. The variations of coagulation param-

eters observed in the proposita before vitamin K supplementations further support the role of environmental components.

The homozygous condition for the VKORC1 Arg98Trp substitution provided us with a valuablemodel to investigate in vivo the effect of the limited availability of vitamin K hydroquinone on coagulation factor levels, and to compare biosynthetic efficiency of hepatocytes subjected to genetically induced depletion of vitamin K hydroquinone with those poisoned by coumarin derivatives. FIX, as strongly indicated by its normal antigen levels, showed the lowest sensitivity to the defective vitamin K cycle, either induced by the genetic deficiency of VKORC1 or warfarin therapy [8,11].

Interestingly, we found that the functional levels of procoagulant factors before vitamin K supplementation, which integrate several biosynthetic and secretion steps in vivo, inversely correlated with the affinity of the vitamin Kdependent carboxylase for coagulation factor propeptides. Our observation supports the hypothesis that binding affinities, found in vitro to vary over a considerable range [25] and to influence the extent of γ-carboxylation [30], contribute to predict activity level differences in vivo, particularly for FVII, FX and prothrombin levels and to a lesser extent for FIX.

The similarly reduced protein levels of FVII, FX and PC in the presence of a wide range of activity levels, produced a gradient in specific activity among factors (Fig. 3.3), with PC displaying the lowest value. The major effect of VKORC1 deficiency on this inhibitor does not appear to be mediated by a preferential impairment in biosynthesis/secretion [14]. The low affinity of PC formembranes [31], further decreased by reduced γ-carboxylation of the Gla domain, should substantially contribute to the extremely reduced PC functional levels in clotting assays, in the lower range of those observed in patients on stabilized warfarin treatment [32].

Vitamin K supplementation enabled us to investigate temporal variations in levels of the vitamin K-dependent factors, their effects on overall function tests and clinical coagulation phenotype. An intravenous single dose of vitamin K resulted in a fast, efficient and sustained normalization of coagulation times, as indicated by the integrated assays we used.

The fast amelioration of clotting times and of coagulation factor activity levels at 4 h after vitamin K supplementation could reflect, in addition to a *de novo* biosynthesis, a short time release of proteins accumulated at the intracellular level because of impaired γ-carboxylation. This complexity limits our ability to measure in plasma the steady state conditions of the vitamin K-dependent factors. The restoration of the carboxylase activity by vitamin K supplementation could be also favoured by DT-diaphorase, a NAD(P)H dehydrogenase, that might play a role in the generation of the hydroquinone form of vitamin K in the presence of a high concentration of vitamin K

quinone [33], as probably occurs after vitamin K intravenous administration.

The evaluation of the thrombin generation offered several quantitative parameters clearly demonstrating the beneficial effects of vitamin K supplementation, particularly the markedly shortened lag-time and time to peak. In the presence of normalized or borderline activity levels of all procoagulant factors and PC, the partial restoration of protein S levels would contribute to the short time parameters of thrombin generation, and to the low response to Protac. Comparison of thrombograms obtained with different TF concentrations provided clear evidence for the gain of information obtained at 1 pM TF concentration, potentially mimicking the physiological trigger and able to highlight the effect of reduced protein S levels [34].

The follow up of the coagulation factor levels after transient vitamin K-induced increase permits a parallel evaluation of their natural decay in plasma, of great interest for replacement regimens in inherited deficiencies. Strikingly, the differential decrease in functional levels of endogenous procoagulant vitamin K-dependent factors from the peak to 72 h supports the lowest half-life values previously estimated in plasma for infused single factors.

The functional rescue and the uneventful clinical course after invasive diagnostic procedures suggest that intravenous vitamin K administrations should be considered the first-line treatment in this condition. However, intravenous and oral long-term administrations should be tested in a formal study to evaluate the effectiveness of vitamin K administration in prophylactic terms.

Taken together our findings in VKCFD2 validate in vivo the cellular and molecular investigations focused on regulation of circulating factor level activity by vitamin K cycle components, and provide evidence for the interplay between coagulation factor specific biosynthesis and decay after vitamin K supplementation.

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Chapter 4

Reduced FVII and FVIII levels and shortened thrombin-generation times during a healthy diet in middle-aged women with mild to moderate CVD risk.

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4.1 Abstract

Background: No experimental study has investigated the effect of whole-diet therapies on a wide range of hemostatic parameters, and their relationship with metabolic and inflammatory markers. Such information was sought in middle-aged women with moderate cardiovascular disease (CVD) risk subjected to an integrated healthy diet. **Methods**: Forty-nine premenopausal womenwere screened for C-reactive protein levels $\geq$1 mg L^{-1} and at least one additional CVD risk factor. Sixteen women (age: 43-54 years) were selected and received a 12-week diet (four phases) integrating National Cholesterol Education Program-Adult Treatment Panel-III recommendations with components of a Mediterranean-style diet. **Results**: We observed a reduction in body mass index (BMI) (P = 0.001), waist circumference (P = 0.005), total (P = 0.011) and low-density lipoprotein (LDL) cholesterol levels (P = 0.035). Antigen levels of coagulation factor (F)VII (P = 0.003) and FVIII (P = 0.005) were clearly reduced by dietary intervention, which also appeared to decrease circulating tissue factor but not fibrinogen and von Willebrand factor (VWF) antigen levels. Levels of FVIII and tumor necrosis factor-a, among the inflammation markers, showed the highest correlation, particularly before the intervention (r = 0.55, P = 0.032). Only this cytokine influenced FVIII variation over time, thus highlighting new relations between coagulation and cellular components of inflammation. The functional effect of diet on coagulation was indicated by markedly prolonged thrombin generation initiation and propagation times (lag time, P = 0.002; time to peak, P = 0.005). **Conclusions**: The changes observed in coagulation initiation and amplification phases, body composition and lipid profile could translate into a remarkable decrease in the risk for cardiovascular disease. Our observations suggest novel relationships between coagulation and inflammatory components.

4.2 Introduction

As diet affects the hemostatic system [1,2], it is a suitable target for interventions aimed at reducing the risk for cardiovascular disease (CVD) [3,4]. Increased levels of hemostatic factors, including fibrinogen, factor (F) VIII, FVII, tissue factor (TF) and von Willebrand factor (VWF) have been related to increased cardiovascular disease (CVD) risk [5-9], in line with the key role of coagulation factors in thrombus formation. Furthermore, the causal contribution of coagulation factor levels to CVD is supported, although indirectly, by the clinical benefit of anticoagulant therapy in patients with acute

coronary syndrome [10,11].

During the past decades, the overall CVD mortality has declined but only a modest reduction has been observed among women [12], which might suggest that primary prevention programs are less effective in women than in men. Although changes in life-style in premenopausal women might represent an effective prevention strategy aimed at enduring risk factor modifications, little is known about hemostatic variations induced by diet in this population.

The aim of this work was to provide a detailed evaluation of the impact of a defined intervention strategy on the hemostatic system in pre-menopausal middle-aged women with a mild to moderate risk for CVD. In particular, we investigated the effects of an integrated healthy diet on a wide panel of hemostatic variables and on an overall coagulation functional test. The rapid turnover of most coagulation factors would enabled us to efficiently monitor the response to the intervention. Coagulation variations were also investigated in relation to specific metabolic and inflammatory parameters previously associated with thrombotic cardiovascular events [13,14].

4.3 Methods

4.3.1 Study population

A group of middle-aged overweight (BMI $\geq$25 kg m^{-2}) women was invited to participate in this study. Forty-nine volunteers were screened for eligibility in accordance with the following inclusion criteria: C-reactive protein (CRP) $\geq$1 mg L^{-1} in two different assessments; and one or more of the following CVD risk factors (waist circumference 88 cm; triglycerides $\geq$150 mg dL^{-2}; high-density lipoproteins (HDL)-cholesterol <50 mg dL^{-2}; arterial blood pressure $\geq$130/85 mmHg; plasma glucose $\geq$110 mg dL^{-2}). Exclusion criteria were: average daily ethanol intake >30 g over the past 5 years; clinical diagnosis of diabetes mellitus according to the American Diabetes Association Criteria [15]; diagnosis of menopause according to the American Association of Clinical Endocrinologists [16]; history of CVD; current therapy with hormonal drugs, anti-thrombotics and/or anticoagulants, angiotensin converting enzyme (ACE) inhibitors, angiotensin II receptor blockers and vitamin supplementation. Fifteen women had CRP levels <1 mg L^{-2}; eight were using ACE inhibitors or hormonal replacement therapy, one was menopausal, two had diabetes, one did not have any of the abovestated CVD risk factors, and six declined to participate. Sixteen women agreed to participate and completed an independently validated dietary questionnaire on alcoholic intake and eating habits.

4.3.2 Intervention

The protocol of this open-label, non-randomized intervention includes four phases (84 days). We integrated the National Cholesterol Education Program-Adult Treatment Panel-III (NCEP-ATPIII) dietary recommendations with the guidelines of the scientific advisory committee of the American Heart Association, based on the effects induced by a Mediterraneanstyle diet on the risk of CVD [17]. During the first 21 days (T_0- T_{21}), all subjects received an isocaloric diet (carbohydrates 57%, proteins 13% and lipids 30%) according to Therapeutic Lifestyle Changes - ATPIII [4]. The energy intake was calculated for every subject based on basal metabolic rate and physical activity levels. Participants were not allowed to consume any alcoholic beverage, extra virgin olive oil, fish and green or black tea, rich in omega-3, polyphenols and antioxidants. 180 mL day^{-1} of monovarietal dry white wine (11% alcoholic strength) was added at T_{21}. This alcoholic beverage provided 20 g of alcohol/day intake. Based on the postulated anti-inflammatory effects, during the third phase ($T_{42}T_{63}$), the diet was integrated with extra virgin olive oil, low index carbohydrates, salmon and nuts. In the last phase ($T_{63}T_{84}$), participants were treated as in T_0T_{21}.

A daily food intake diary monitored compliance with the protocol during the study, and after each phase a clinician assessed any adverse effects. The Human Research Ethics Committee of the Azienda Ospedaliera-Universitaria of Ferrara approved the study protocol.

4.3.3 Anthropometric parameters

Anthropometric measurements were taken according to standardized procedures. Fat mass was also determined by tetrapolar bioelectric impedance analysis (Dietosystem Ltd., Milan, Italy).

4.3.4 Lipids and inflammation markers

After overnight fasting, blood samples were collected at the beginning of each study phase. Serum and plasma samples aliquots were stored at -80°C. Total serum cholesterol and triglycerides levels were determined using standard enzymatic techniques (Roche Diagnostics, GmbH, Basel, Switzerland). To measure HDL-Cholesterol, apo B-containing lipoproteins were precipitated from plasma using phosphotungstic acid and Mg^{2+}. LDL cholesterol was calculated according to Friedewald's formula. High-sensitivity CRP (hsCRP) levels were measured (inter-assay coefficient of variation = 5%) by particle-enhanced immunonephelometry (Roche Diagnostics, GmbH). Serum

interleukin-6 (IL-6) and tumor necrosis factoralpha (TNF-α) were quantified using a commercial ELISA (BioSource International Inc.,Camarillo,CA,USA). Intra- and inter-assay variation coefficients for IL-6 and TNF-α ranged from 1.8%to 5.4% and from 0.9% to 9.9%, respectively.

4.3.5 Plasma coagulation factors

Antigen levels of TF, FVII and FVIII were determined using a commercial ELISA (TF American Diagnostica, Greenwich, CT, USA; FVII and FVIII Affinity Biologicals Inc., Ancaster, Canada). The intra-assay coefficients of variation for TF, FVII and FVIII assays were 12.8%, 7% and 8.7%, respectively, while the inter-assay coefficients were 14.7%, 7.3% and 7.4%, respectively. To evaluate the VWF antigen levels, we used the ELISA protocol which is previously described [18]. The intraassay and inter-assay coefficients of variation for VWF ELISA assay were 6.5% and 15%, respectively. Antigen levels of FVII, FVIII and VWF were expressed as a percentage of the healthy plasma in-house pool. Fibrinogen levels were measured using a prothrombin time-derived method with a turbidimeter kit (Diagen, Thame, UK).

4.3.6 Thrombin generation measurements

Calibrated automated thrombin activity measurement was performed as described elsewhere [19]. Before testing, platelet poor plasma (PPP) samples were centrifuged at 23 000 x g at 4°C for 1 h. Coagulation was triggered in recalcified PPP by addition of 1 pM recombinant human TF and 4 μM phospholipids (20% phosphatidyl serine and 80% phosphatidyl choline) at 37°C. All experiments were carried out in duplicate. The assay variability was lower than 3%. The slope of thrombin generation curves was calculated using the following formula: {Peak (nM)/[ttpeak (min)-lag time (min)]}.

4.3.7 DNA isolation and analysis of genetic polymorphisms

DNAwas extracted from peripheral blood leukocytes using the salting-out method. The ABO blood group of patients was determined by genotyping as previously described [20]. FVII gene polymorphisms were analyzed as described [21].

4.3.8 Statistical analysis

Data are presented as mean ± standard deviation for parameters with normal distribution and as median with interquartile range for parameters with skewed distribution (thrombin generation, hsCRP, IL-6, TNF-α). To approximate a normal distribution, these variables were analyzed after log-transformation. Pair-wise correlations were estimated using Pearson's correlation coefficient. Differences in mean values across study phases were analyzed using analysis of variance (ANOVA) for repeated measures and analysis of covariance (ANCOVA). After multiple comparisons, P-values were adjusted with Bonferroni's method. Statistical and graphical data analyses were performed using Stata 9 (StataCorp. 2005. Stata Statistical Software: Release 9. College Station, TX: StataCorp LP) and R language (R Foundation, version 2.6.1).

4.4 Results

4.4.1 Variation in body composition, lipid and inflammatory parameters

Variation in anthropometric, lipid and inflammatory parameters of the 16 women who completed the study protocol are summarized in Table 4.1. The subjects showed a clear and progressive reduction in BMI ($P = 0.001$) throughout the study period. The BMI decline was paralleled by a reduction in waist circumference ($P = 0.005$) and, although as a trend, in fat mass ($P = 0.096$). Total and LDL cholesterol levels were significantly reduced over time with the most important changes observed after the first study phase (T_{21} vs. T_0; $P < 0.05$). The degree of change in anthropometric and lipid parameters was in good agreement with previous reports [22]. HDL-cholesterol and triglyceride levels did not show appreciable variation over time, even after moderate alcohol intake, reported to affect their levels.

At baseline, median CRP was 2.2; nine women had values higher than 2 mg L^{-1} and five higher than 3 mg L^{-1}. The inflammatory profile did not significantly change throughout the study although all inflammatory markers, and particularly IL-6, tended to decrease over time.

4.4.2 Variation in coagulation factors levels

To evaluate the effects on hemostatic components, we determined the antigen levels of clotting factors mainly synthesized in the liver (FVII, FVIII and fibrinogen) or in other tissues (VWF and TF) (Fig. 4.1).

FVII levels showed an appreciable variation ($P = 0.003$) and the lowest value was observed at T_{63} (15.2% lower than T_0; $P < 0.05$), in the presence of both alcohol and anti-inflammatory components. However, the decrease was already present at T_{42} after alcohol introduction (9.5% lower comparing T_{42} vs. T_0; $P < 0.01$). The variation pattern did not differ upon exclusion of subjects heterozygous for the -402 A and -323 10 bp insertion FVII alleles, known to be associated with increased and decreased FVII levels, respectively [21,24].

A positive correlation ($r = 0.32$, $P = 0.004$) was observed between FVII and triglyceride values, a well-known determinant of circulating FVII levels [23]. However, the FVII level variation was influenced neither by parallel variations in plasma triglyceride nor by fat mass modification, as indicated by multiple linear regression analysis (ANCOVA adjusted for triglycerides and fat mass).

During dietary treatment, FVIII antigen levels significantly decreased ($P = 0.005$). The reduction was borderline significant at T_{21}, when all subjects had the same isoenergetic non anti-inflammatory diet, was maximal at T_{63} (15.1% compared with T_0, $P < 0.05$) and persisted even in the last phase when alcohol and anti-inflammatory food intake was interrupted.

We found that overall FVIII values were positively correlated with CRP ($r = 0.24$, $P = 0.034$), an important cytokine of the acute phase response, and TNF-α ($r = 0.24$, $P = 0.043$), a pro-inflammatory cytokine associated with obesity [25]. The positive relation between FVIII and TNF-α levels was also detected before the intervention ($r = 0.55$, $P = 0.033$). FVIII level variation was not significant after adjustment for TNF-α values (ANCOVA). Differential adjustment for other inflammation markers, CRP and IL-6, did not produce a similar effect on FVIII pattern. Over time, the FVIII variations were not influenced by body fat modifications. Adjustment for blood groups [26] did not affect FVIII level variation.

Circulating fibrinogen levels, which are an independent CV risk factor [27], did not show appreciable variations between the different study phases and were similar to those measured in healthy Italian women [28]. Among the different inflammatory markers measured, only CRP showed a strong positive relation ($r = 0.54$, $P < 0.001$) with fibrinogen levels at each diet phase.

Although the statistical analysis of TF levels in plasma is complicated by the pronounced inter-individual and intraindividual changes, TF levels appeared to decrease in a stepwise manner during the alcohol and anti-inflammatory food intake. During treatment, white blood cell counts and pro-inflammatory protein (IL-6, CRP, TNF-α) distributions could not explain the TF level variations.

VWF antigen, a potential marker of endothelial dysfunction or activation, did not show significant variations over time. A positive relation between FVIII and VWF levels was clearly detectable, with the highest values at the beginning of the study ($r = 0.58$, $P = 0.018$) and in the presence of both alcohol and anti-inflammatory components ($r = 0.68$, $P = 0.004$). The variation of the ratio between FVIII and VWF levels showed a pattern similar to that of FVIII levels (data not shown).

4.4.3 Functional variations of the hemostatic system

We measured thrombin generation in plasma samples using a low concentration of TF supplied with a non-limiting concentration of phospholipids, as a sensitive way to monitor the interplay of procoagulant and anticoagulant components.

The lag time and time to peak (ttPeak) were significantly modified during diet treatment (Fig. 4.2). We observedmarkedly prolonged times, and particularly the median lag time increased from 6.3 (T_0) to 8.5 min (T_{42}). Addition of anti-inflammatory components (T_{63}) did not prolong these times further, which overlapped at the beginning and end of the study. The endogenous thrombin potential (ETP) and peak parameters did not show appreciable variation during the study and were clearly correlated with FVIII antigen levels (ETP vs. FVIII $r = 0.48$, $P < 0.001$; Peak vs. FVIII $r = 0.49$, $P < 0.001$), as previously observed [29]. The reference plasma showed ETP and peak values (1285.2 ± 197.4 nmol $min^{-1}1$ and 159.1 ± 42.4 nmol, respectively) similar to those observed in the study population. The modification of body composition did not influence any thrombin generation parameters considered.

To further evaluate the relationship between the thrombin generation rate and the dietary treatments, the slope of thrombin generation curves was calculated. As indicated by multivariate regression analysis, this parameter was determined by FVII ($\beta = 0.39$, $P = 0.01$) and FVIII ($\beta = 0.35$, $P < 0.01$) levels but did not show appreciable variation over time (data not shown).

4.5 Discussion

Although several studies have been conducted on the effects of whole-diet therapies on CV risk factors [30,31], no experimental study has investigated the effect on a wide panel of haemostatic parameters, and their relationship with metabolic and inflammatory markers. The present study reports original observations about the beneficial effects of an integrated healthy diet in

	T_0	T_{21}	T_{42}	T_{63}	T_{84}	
	Isocaloric NCEP-ATPIII diet					
		+20 g day^{-1} alcohol				
Phases			+anti-inflammatory components			*P*
BMI (kg m^{-2})	29.2 ± 2.7	28.4 ± 2.7	28.0 ± 2.4	27.8 ± 2.4	27.4 ± 2.6	0.001
Fat Mass (kg)	28.8 ± 4.8	27.6 ± 4.9	26.3 ± 5.2	26.0 ± 4.8	26.6 ± 7.0	0.096
Waist circumference (cm)	86.7 ± 6.3	85.6 + 6.4	83.9 ± 6.4	83.1 ± 5.2	83.9 ± 5.6	0.005
Total cholesterol (mg dL^{-1})	238 ± 40	213 ± 35	209 ± 38	215 ± 45	222 ± 43	0.011
LDL-cholesterol (mg dL^{-1})	161 ± 43	141 ± 41	137 ± 39	137 ± 46	146 ± 39	0.035
HDL-cholesterol (mg dL^{-1})	58 ± 14	55 ± 15	53 ± 12	57 ± 13	57 ± 10	0.375
Triglycerides (mg dL^{-1})	98 (71–118)	77 (52–112)	96 (77–106)	90 (68–114)	90 (73–109)	0.127*
hsCRP (mg L^{-1})	2.2 (1.4–3.5)	1.9 (1.3–3.2)	1.9 (1.1–3.6)	1.8 (1.2–3.8)	1.5 (1.3–3.3)	0.517*
IL-6 (pg mL^{-1})	0.82 (0.6–1.2)	0.67 (0.6–0.8)	0.78 (0.6–1.1)	0.68 (0.4–0.9)	0.62 (0.5–0.8)	0.121*
TNF-α (pg mL^{-1})	4.6 (2.6–6.1)	4.3 (3.6–6.3)	4.1 (2.9–9.3)	4.6 (2.9–5.6)	4.1 (3.0–5.2)	0.626*

Table 4.1: Variation in body composition, lipid and inflammatory parameters during dietary phases. Data expressed as mean ± SD. P-values refer to analyses of variance for repeated measures. Triglycerides, hsCRP, IL-6 and TNF-α are expressed as median and interquartile range (25th and 75th quartile). *P-values refer to analyses of variance for repeated measures after log-transformation of the dependent variable.

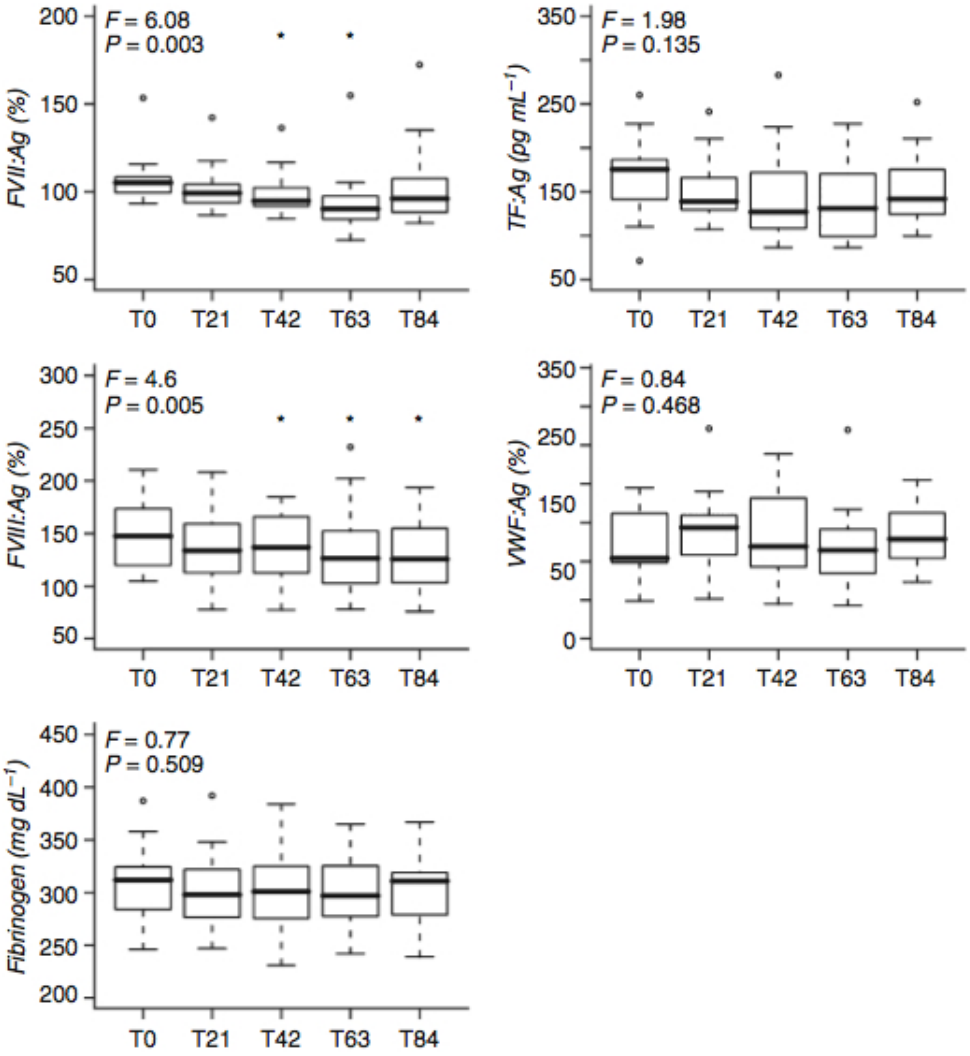

Figure 4.1: Over-study variation of hemostatic factor levels. The black line inside the boxes represents the median value and distribution outliers are indicated with blank circles (∘). The asterisks indicate significant variation ($P < 0.01$) with respect to T_0 after Bonferroni's adjustment for multiple comparisons.

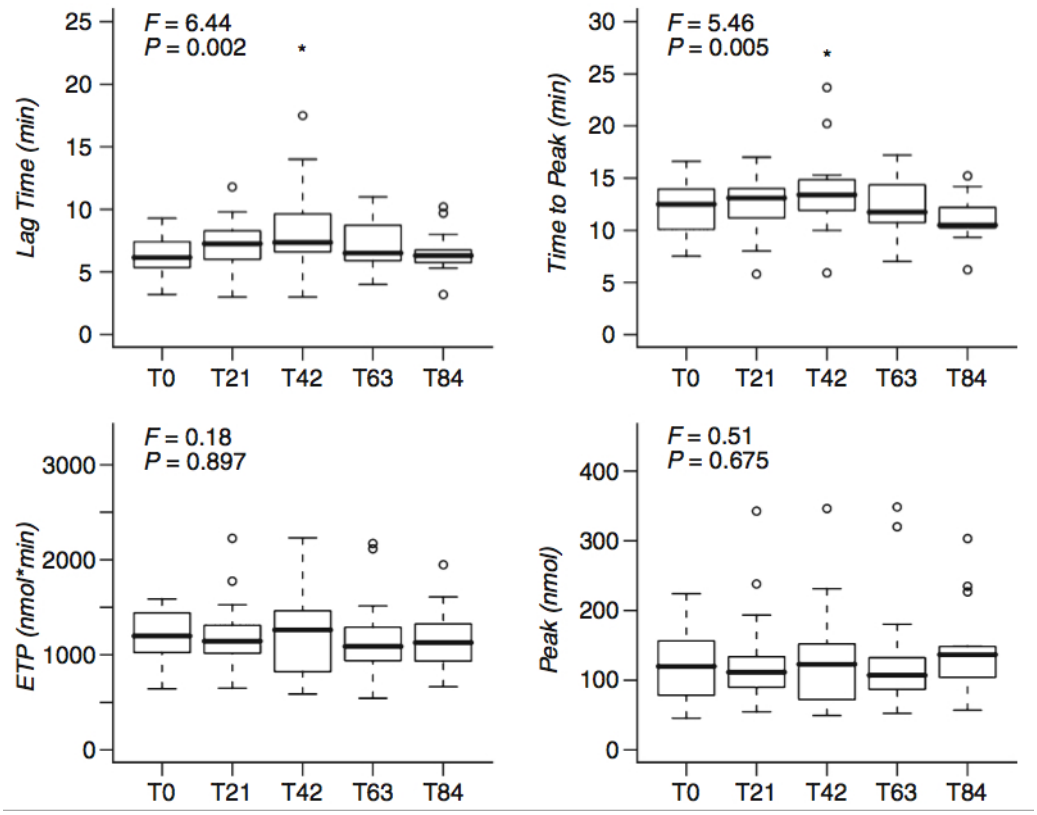

Figure 4.2: Over-study variation of thrombin generation parameters. The black line inside the boxes represents the median value and distribution outliers are indicated with blank circles (○). The asterisks indicate significant variation ($P < 0.01$) with respect to T_0 after Bonferroni's adjustment for multiple comparisons.

a selected middle-aged female population, characterized by high CRP levels and at least one additional risk factor for CVD.

Biologically and clinically relevant modifications of body composition, lipids, coagulation factor levels and thrombin generation parameters were observed. The reduction in anthropometric and lipid parameters, and particularly BMI, waist circumference, total and LDL cholesterol levels, would translate into a remarkable decrease in the risk of CVD.

The antigenic determination of the hemostatic factors, increased levels of which are recognized or candidate risk factors for CVD, favored the quantitative evaluation of dietinduced biosynthetic changes, and avoided potential drawbacks of clotting assays.

FVII and FVIII levels were remarkably reduced, whereas fibrinogen concentration did not show appreciable changes during the course of the study. These observations indicate differential effects of diet on those blood coagulation factors that are predominantly produced by the liver, a key organ involved in themetabolic changes observed. Further differences were observed between the stepwise decrease of FVII and FVIII levels, for which FVIII was not counteracted by removing alcohol and anti-inflammatory components, and persisted at the end of the study. VWF levels were strongly correlated with those of FVIII, but poorly modified by dietary treatments. This observation, and the similar pattern observed for FVIII levels and FVIII/VWF ratio, further support a role of diet on modification of FVIII expression.

Diet-responsive anthropometric and metabolic parameters were found to be associated with coagulation factor levels but did not appreciably influence any hemostatic factor variations. This pointed towards as yet undiscovered components in the regulation of coagulation factor expression that were influenced by intervention.

We observed a clear association between coagulation factor levels and the inflammatory profile. Levels of TNF-α, a cytokine over-produced by adipocytes and macrophages of adipose tissue in the obese state [32], were correlated with those of FVIII and affected their variation over time. This suggests a previously unknown relationship. As a comparison, CRP, which is produced by hepatocytes, was strongly related both to fibrinogen [17] and FVIII but did not affect the FVIII variation over time. These observations suggest the presence of inflammation- driven crosstalk between hepatic and extra-hepatic sites for specific regulation of coagulation factor genes.

The functional effects of the dietary treatments were clearly demonstrated by a comprehensive functional evaluation of the coagulation pathway in plasma. This meant that the influence of dietary changes upon hemostatic components could be examined and correlated with the degree of blood hypercoagulability. We observed significantly prolonged initiation and propaga-

tion phases of thrombin generation, which exploit the enzymatic and cofactor activity of FVII and FVIII, respectively. In spite of the reduction of FVII and FVIII levels over time and of the estimated influence of these factors on thrombin generation rate, we did not observe significant relations between the variations in thrombin generation times and of FVII and FVIII levels. However, the large number of components, potentially modified by the intervention, that might influence the thrombin generation assay, could mask these relations.

Some limitations have to be taken into account when interpreting our findings. The sample size, limited by restrictive inclusion criteria and particularly by alcohol assumption and the need for persistently increased CRP levels, precludes detection of small effects of the intervention (i.e. IL-6 and TF levels). On the other hand, the changes observed even in a small sample are likely to be biologically and clinically significant. Finally, the study design, without a formal control group, might be prone to outcome modifications as a result of secular changes even in the absence of a true intervention effect. However, the finding of specific and biologically plausible relationships does not suggest the presence of this type of bias.

In conclusion, the integrated healthy diet produced a differential effect on coagulation factors, and composite effect on metabolic and coagulation parameters. The observed variations suggest novel relationships between coagulation and inflammatory components. The intervention, which is a feasible approach, sensibly reduced risk factors for CVD in middle-aged premenopausal women, an ideal target for nonpharmacologic primary prevention.

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Chapter 5

Stimulation of P2 (P2X7) receptors in human dendritic cells induces the release of tissue factor-bearing microparticles

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5.1 Abstract

Receptors for extracellular nucleotides are the focus of increasing attention for their ability to cause release of plasma membrane vesicles (microparticles, MPs). Here, we show that monocyte-derived human dendritic cells (DCs) stimulated with a P2X7 receptor (P2X7R) agonist undergo a large release of MPs endowed with procoagulant activity. Functional and Western blot studies revealed that MPs contain the membrane-bound form of tissue factor (TF), a glycoprotein acting as essential cofactor of activated factor VII and triggering blood coagulation. Quiescent DCs express the membrane-bound (full length), as well as truncated alternatively spliced TF forms. DC reactivity to anti-TF Abs disappeared almost completely on stimulation with ATP or benzoyl ATP (BzATP), as shown by immunoblot and confocal microscopy analysis. Concurrently, TF reactivity and activity appeared in the vesicular fraction, indicating that MPs are important carriers for the dissemination of full-length TF form. Activity of MP-bound TF, comparable to that of relipidated recombinant TF, was dose dependently inhibited by the addition of a specific anti-human TF antibody. We infer that a large fraction of this protein, and its procoagulant potential, are "deliverable"after physiological or pathological stimuli. These findings might have implications for triggering and propagating coagulation in healthy and atherosclerotic vessels.

5.2 Introduction

Dendritic cells (DCs) were originally identified as antigen-presenting cells critical for antigen capture and processing and for the activation of naive T lymphocytes (1-3). DCs are the focus of increasing attention for their immunomodulatory activity, for their role in resistance to infections and tumors, and in the induction of tolerance toward normal cell constituents (4). DCs express several P2 receptor (P2R) subtypes, a feature that makes them eminently responsive to extracellular nucleotides, as shown by ATP or UTP-dependent modulation of cytokine production (5-7), upregulation of chemokine receptors (8), and stimulation of chemotaxis (9). Interestingly, sensitivity to extracellular nucleotides changes during DC maturation and in different DCs subpopulations (9, 10).

Nucleotides are present in millimolar concentrations in the cytoplasm and in the nanomolar range in the extracellular milieu under quiescent conditions. Very active and ubiquitous nucleotide-metabolizing enzymes (ectonucleotidases) have a key role in keeping the extracellular nucleotide concentration low (11, 12). Nucleotides may be released by virtually any cell via

lytic and nonlytic pathways and P2R present on the DC surface may act as sensors to monitor their extracellular levels. Although very few studies have investigated the actual ATP concentration at inflammatory sites, in vivo observations provide clear evidence for sustained ATP release at foci of tissue injury and inflammation (13). This may be also relevant for atherosclerosis, as a very recent report shows that ATP secretion by macrophages modulate macrophage adhesion to the endothelium, and thus macrophage recruitment to the atheroma (14).

On the basis of pharmacological, functional, and cloning data, two P2R subfamilies have been so far described: P2YR and P2XR (15, 16). P2YR are seven membrane-spanning, G-protein-coupled receptors. Their activation triggers generation of inositol 1,4,5- trisphosphate and release of Ca^{2+} from intracellular stores. P2YR are ubiquitous, being expressed by monocytes, macrophages, dendritic cells, neurons, smooth and striated muscle cells, as well as epithelial and endothelial cells (17, 18). P2XR are plasma membrane channels selective for monovalent and divalent cations that are directly activated by extracellular ATP. These channels were originally identified in mammalian sensory neurons, and subsequently found also in smooth muscle cells, fibroblasts, and immune cells (17, 19).

DCs express high levels of a peculiar purinergic receptor subtype, previously known as P2Z (20) and later named P2X7R (21). The P2X7R differs from the other P2XR for its extended carboxy-terminal domain that endows this receptor with the ability to form large plasma membrane pores permeable to small hydrophilic molecules. An interesting property of the P2X7R pore is its reversibility: removal of ATP triggers resealing of the plasma membrane. Stimulation of P2X7R by BzATP induces emission of IL-1β containing microvesicles from the human monocyte cell line THP-1 (22) and from mouse microglial cells (23).

Circulating microvesicles also referred to as microparticles (MPs) are released into the bloodstream by a variety of cells. Because of the exposure of negatively charged phospholipids, in particular, phosphatidylserine, MPs might provide surfaces that support coagulation. The finding that circulating leukocyte-derived MPs contain tissue factor (TF) further suggests an important procoagulant function of MPs (24-26).

TF is a transmembrane glycoprotein that acts as the cellular receptor and essential cofactor for activated factor VII (FVIIa). At the site of vascular injury, the TF/FVIIa complex triggers coagulation by activating factor IX and X, ultimately resulting in thrombin formation (27). Moreover, the FVIIa-TF complex may also play a role in the migration and proliferation of vascular smooth muscle cells (28), in vascular remodeling and in plaque neovascularization (29) and thereby in promoting plaque destabilization.

TF is constitutively expressed in skin, organ surfaces, vascular adventitia, and epithelialmesenchymal surfaces, where it acts as a sort of hemostatic envelope (30, 31). In whole blood (blood-borne TF), TF is present in two forms: a high MW MP-bound, and a low MWsoluble form (asTF) produced by alternative splicing.

Altered expression of TF has implications in several diseases, particularly atherothrombosis, where it is crucial in thrombus formation after plaque rupture. TF is thought to be one of the main determinants of the thrombogenicity of the atherosclerotic plaque, and it has been shown that the enhanced activity of extracellular TF in the lipid core is directly related to the presence of TF-bearing-MPs (32). Moreover, elevated levels of TF-bearing MPs were found to be associated with cardiovascular disease (33, 34). Although many observations indicate the involvement of TF-bearing MPs in atherothrombosis, the cellular and molecular mechanisms responsible for their release are poorly known.

In the present paper, we investigated the release of TF associated to MPs in monocyte-derived DCs stimulated with the P2X7R agonist benzoyl ATP (BzATP).

5.3 Material and Methods

5.3.1 Reagents

ATP was purchased from Roche (Roche Diagnostics SpA, Monza, Italy); BzATP, benzamidine and phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma (Sigma- Aldrich, Milan, Italy); EDTA was from Baker (J. T. Baker, Phillipsburg, NJ, USA); Percoll and Ficoll-Paque were obtained from Pharmacia (Pharmacia Biotech AB, Uppsala, Sweden).

5.3.2 DC purification

DCs were obtained from peripheral blood of healthy donors, as described previously (35). Briefly, monocytes were separated by a two-step Ficoll and Percoll gradient, and then cultured in 2 mM l-glutamine and 25 mM HEPES-containing RPMI 1640 medium (EuroClone, Milan, Italy) complemented with 10% heat-inactivated FBS (Invitrogen, San Giuliano Milanese, Italy), 1 mM sodium pyruvate, 1% nonessential amino acids, 200 U/ml penicillin, 20 μg/ml streptomycin (EuroClone), and 0.05 mM 2-mercaptoethanol (Invitrogen) at 37°C with 5% CO_2, in the presence of 200 U/ml IL-4 and 100 ng/ml GM-CSF (Peprotech, Rocky Hill, NJ, USA).

5.3.3 MP purification

To increase MP recovery, DCs were stimulated in a solution containing 300 mM sucrose, 1 mM K2HPO4, 1 mM MgSO4, 5.5 mM glucose, and 20 mM HEPES, 1 mM $CaCl_2$, pH 7.4 with KOH (sucrose solution). After stimulation of the cells with 200 μM BzATP or 3 mM ATP, supernatants were collected and 1 mM EDTA was added. The protease inhibitors benzamidine and PMSF were also added. Floating cells and cell debris were eliminated by centrifuging supernatants for 5 min at 160 g. MPs were purified by centrifugation of cell supernatants at 100,000 g for 90 min, at 4°C. Vesicle pellets were then resuspended in the same solution. In samples used for tissue factor activity measurements, proteases inhibitors were omitted.

5.3.4 Microscopy analysis

DCs were detached from Petri dishes by using 2 mM EDTAcontaining cold PBS, and then plated ($2x10^5$) onto 24-mm glass coverslips (Merck Eurolabs, Lutterworth, UK). Experiments were performed in the sucrose solution described above. Morphological changes and MP shedding were analyzed by mounting coverslips in a thermostatted Leyden chamber (model TC-202A; Medical Systems Corp., NY, USA), placed onto the stage of an inverted Nikon Eclipse TE300 microscope (Nikon Corp., Tokyo, Japan). The images were captured with a back-illuminated CCD camera (Princeton Instruments, Trenton, NJ, USA) using the Metamorph software (Universal Imaging Corporation, West Chester, PA). DCs for electron microscope analysis were detached from flasks with 2 mM EDTA-containing cold PBS, and cell pellets were fixed with 2.5% glutharaldeide. Samples for electron microscopy were processed by the Centro di Microscopia Elettronica of the University of Ferrara (Ferrara, Italy).

5.3.5 Determination of TF cofactor activity

The MPs suspension (10μl) was incubated with 2 nM FVIIa (Novo Nordisk, Bagsvrd, Denmark) at 37°C for 10 min in the presence of 5 mM $CaCl_2$ (final volume, 30 μl). 10 nM zymogen factor X (FX) (HTI, Essex Junction, VT, USA) and 200 μM of a specific fluorogenic substrate for activated FX (Spectrozyme FXa, American Diagnostic, Stamford, CT, USA) were then added in a final volume of 100 μl. Fluorescence (excitation 360 nm, emission 465 nm) was immediately measured over time through a SpectraFluor Plus microplate reader (Tecan, Austria). The assay was standardized by using serial dilutions (1/7.5-1/480) of Innovin (Dade Bearing, Marburg, Germany)

in 20 mM HEPES, 150 mM NaCl, 0.1% PEG 8000, 5 mM CaCl2, 100 μM phospholipid vesicles (PLves 20:80 phosphatidylserine/phosphatidylcoline), pH 7.4. Inhibition of TF activity by a sheep polyclonal anti-human TF antibody (HTI) was assessed in a FXa generation assay. To this purpose, the MPs suspension was preincubated for 20 min at room temperature with increasing concentrations of the antibody (0-6 nM). The residual cofactor activity was expressed as a percentage of activity in the absence of antibody.

5.3.6 Western blot analysis

DCs and MPs were lysed by three freeze-thawing cycles, mixed with a solution containing 60 mM Tris, 2% SDS, 2.5% β-mercaptoethanol, 10% glycerol, and bromphenol blue and boiled for 5 min. Samples were run on a SDS-PAGE (4-12%, Bio-Rad, Hercules, CA) and then transferred onto a nitrocellulose filter (Protran, Schleicher & Schuell, Dassel, Germany). The filter was incubated with a rabbit polyclonal anti-human TF antibody (1 mg/ml, American Diagnostics, Stamford, CT). Secondary antibody was a HRP-conjugated polyclonal goat anti-rabbit IgG (1 mg/ml, Dako Cytomation, Denmark). Chemiluminescence was detected with the Supersignal West Femto maximum sensitivity substrate (Pierce Biotechnology, Rockford, IL, USA).

5.3.7 Immunocytochemistry and confocal analysis

DCs, previously plated onto 13-mm glass coverslips, were incubated for 10 min at 37°C in a sucrose solution in the presence or absence of 200 μM BzATP. Cells were then rinsed twice with PBS. All of the subsequent procedures were carried out at 4°C, under gentle shaking. Cells were blocked in 5% goat serum containing PBS for 2 min and then incubated for 1 h with 40 μg/ml of rabbit anti-human TF IgG, (American Diagnostics). After 3 washes with PBS (10 min each), samples were incubated with 25 μg/ml goat TRITC-conjugated antirabbit IgG (Sigma-Aldrich) for 1 h in the dark, washed 3 times with PBS for 20 min, and fixed for 1 h in 2% paraformaldeyde. Coverslips were then washed 3 times in PBS and mounted onto glass slides in the presence of Pro Long Antifade solution (Molecular Probes, Leiden, The Netherlands). Confocal images were acquired with a Zeiss LSM 510 confocal microscope equipped with a plan-Apochromat 63x oil immersion objective (Carl Zeiss, Arese, Italy). The 543-nm excitation wavelength was provided by a HeNe laser source. All images were obtained at a 12% laser potency and with a pinhole diameter of 135 μm. Amplifier and detector optimizing parameters were maintained constant for all of the experiments. When required, a single 3D projection of confocal images on the z axis was ob-

tained with the LSM examiner software (Carl Zeiss). Fluorescence emission was quantitated starting from 3D projections with the cell imaging software MetaMorph (Universal Imaging), as described previously (36). Data were acquired from 10 to 15 cells per coverslip. An average of 10 coverslips was analyzed for each experimental condition. Data are expressed in fluorescence arbitrary units (FU) and shown as mean plus se. Tests of significance were performed by Students t test and ANOVA by means of GraphPad Instat 3.06 software (GraphPad Software, Inc., San Diego, CA, USA).

5.3.8 RT-PCR

RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA) and analyzed by RT-PCR (Access RT-PCR System, Promega, Madison WI, USA) using primers annealing in exon 4 (forward: 5'-GAACGGACTTT-AGTCAGAAGG-3') and 6 (reverse: 5'-TGACCACAAATACCACAGCTCC-3') of TF gene. RT-PCR for the house-keeping GAPDH gene (forward: 5'-CCACCCATGGCAAATTCCATGGCA-3' and reverse: 5'-TCTAGACGGC-AGGTCAGGTCCACC-3') was also performed. Amplified fragments were separated by electrophoresis on a 3% agarose gel, and visualized with ethidium bromide.

5.4 Results

5.4.1 Stimulation of DCs with BzATP induces MP release

Human DCs challenged with 200 μM BzATP released a large amount of MPs (Fig. 5.1). Particle release started within seconds of stimulation, usually at one pole of the cell, and rapidly spread to the whole cell body. Vesicle shedding was preceded by cell contraction and rounding and was paralleled by a striking increase in plasma membrane blebbing and emission of philopodia. Electron microscopy analysis revealed that BzATP stimulation induced dramatic changes in cell morphology and a striking plasma membrane reorganization (Fig. 5.2). Similar results were obtained by stimulation with 3 mM ATP (not shown).

5.4.2 MPs show FVIIa cofactor activity

We then asked whether MPs released by DCs showed procoagulant activity. MPs obtained from different donors were assessed by using a functional assay

for TF, based on its property to behave as a cofactor for FVIIa in FXa generation. MPs had a TF-like activity that was dependent on the amount of MPs used in the test (Fig. 5.3).

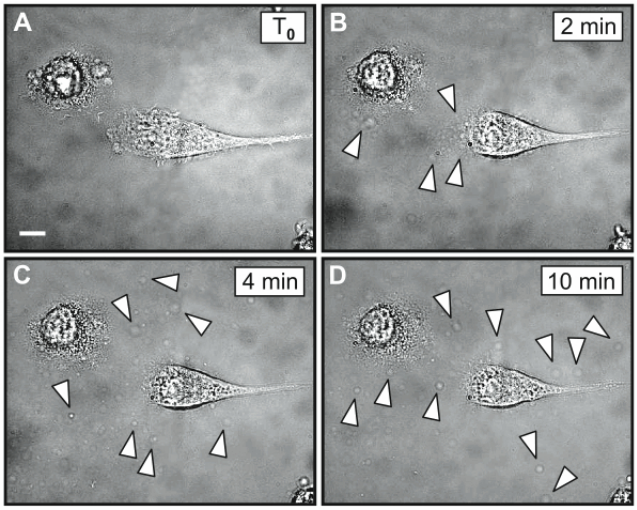

Figure 5.1: P2X7 receptor stimulation induces cell shrinkage and MP shedding from human dendritic cells (DCs). Cells were seeded onto glass coverslips and stimulated with 200 μM BzATP at 37°C, as indicated in Materials and Methods. Images were acquired at 5-s intervals with the Nikon Eclipse T-300 microscopy set up described in Materials and Methods. Arrowheads indicate shedded MPs. Scale bar=10 μm.

Activity of MPs was compared to that of Innovin, a well-known reagent for TF-dependent activation of blood coagulation. MP activity ranged between 0.6 and 0.1 nM TF, with a mean value of 38.9±13.8 pM TF/μg MP protein, i.e., 1.2 fmol/μg MP protein, as the assay was run in 30 μl. Because DCs release ∼18±7μg of MPs/106 cells, we calculate an activity of released TF of ∼700 pM TF/10^6 cells, i.e., 21.6 fmol/10^6 cells. To rule out the possibility that the FXa generation activity was due to MPs phospholipid content, phospholipid preparations devoid of TF were also tested. Under these conditions, no fluorescence increase was observed.

As a further proof of a specific role of MP-associated TF in FXa generation, assays were performed in the presence of increasing concentration of a specific antihuman TF antibody. As shown in Fig. 5.4, the antibody was able to reduce, in a dose-dependent manner, the cofactor activity present in the MP preparation.

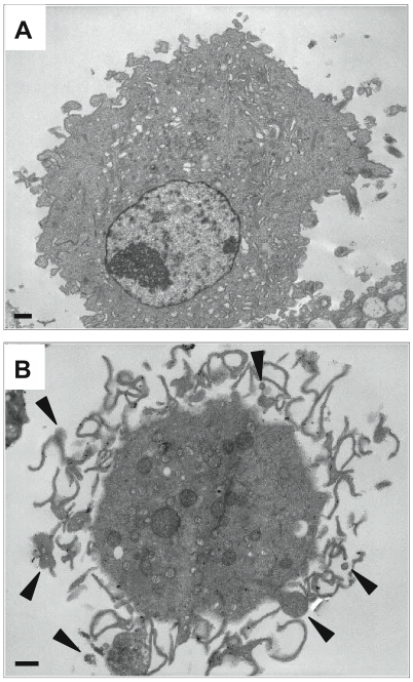

Figure 5.2: Electron microscopy of BzATP-stimulated DCs. Cells were stimulated as in Fig. 5.1, detached from flasks, fixed with glutharaldeide, and processed for electron microscopy, as described in Materials and Methods. Arrowheads indicate plasma membrane extrusions, blebs, and MPs in the process to be released. Scale bars =1μm.

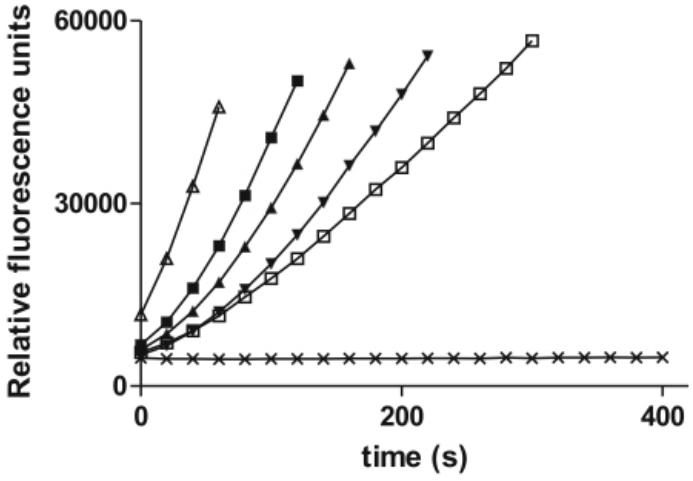

Figure 5.3: TF cofactor activity in MPs from DCs. FXa generation was evaluated as relative fluorescence over time and compared to recombinant TF (Innovin) and phospholipid vesicles, as controls. ■ MPs (13.5 μg), ▲ MPs (6.75 μg), ▼ MPs (4.5 μg), △TF (0.6 nM) from Innovin, □ TF (0.1 nM) from Innovin, x Phospholipid vesicles only.

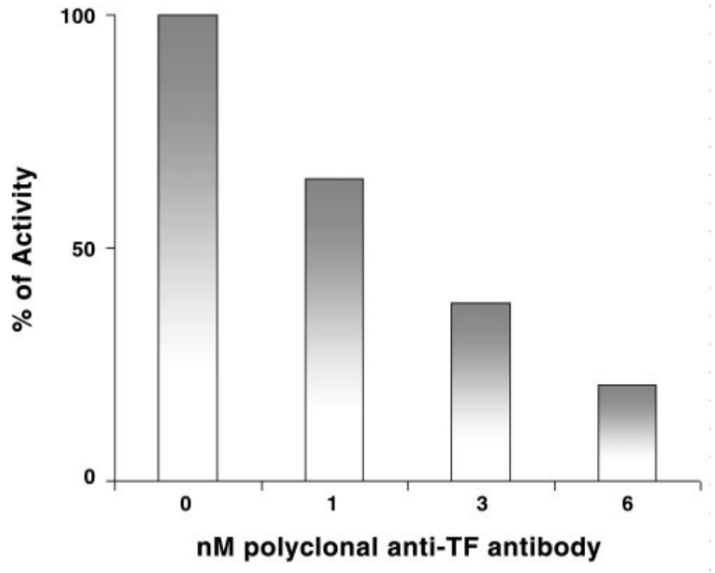

Figure 5.4: Inhibition of MP TF cofactor activity by an anti-TF antibody. Activity was expressed as a percentage of FXa generation in the absence of polyclonal anti-human TF antibody (HTI, VT, USA). Duplicate determinations of one of three similar experiments.

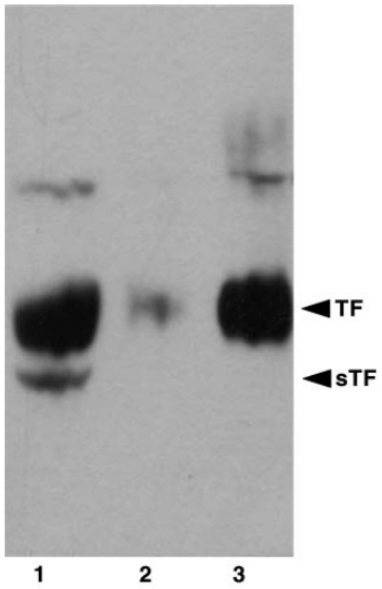

Figure 5.5: Expression of TF mRNA in DCs. Lane 1, BzATP-treated DCs; lane 2, unstimulated DCs. TF, transmembrane form; sTF, soluble form. M, molecular weight markers.

5.4.3 Human DCs express the mRNA both for transmembrane and asTF

TF mRNA expression in stimulated and unstimulated DCs was investigated by RT-PCR analysis. To allow the detection of both full-length (transmembrane) and soluble (lacking the exon 5- encoded transmembrane domain) forms of TF transcripts, primers flanking exon 5 were designed.

The amplified fragments (307 bp and 147 bp, Fig. 5.5) are compatible with the expression in stimulated or unstimulated human DCs of the full length and soluble (asTF) forms of TF, respectively.

5.4.4 TF protein is associated to MPs released by DCs

The presence of TF protein in DCs and MPs was evaluated through Western blot analysis with a specific polyclonal anti-human TF antibody. Immunoreactivity to TF was very high in untreated DCs (Fig. 5.6, lane 1), where two major bands compatible with the presence of both forms of TF protein were clearly distinguishable. A faint band with high molecular weight, likely corresponding to TF dimers, was also present. Comparison of the intensity of the bands clearly indicated that the transmembrane form was predominant. Interestingly, the TF immunoreactivity was almost completely lost on stimulation with BzATP (Fig. 5.6, lane 2), thus suggesting that TF was released

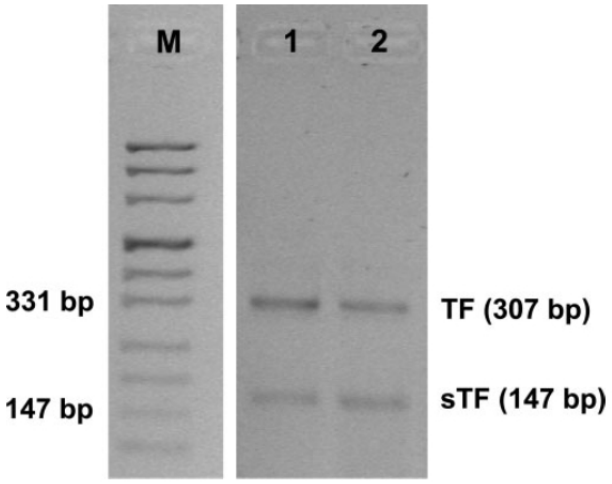

Figure 5.6: Western blot analysis of TF in DCs and MPs. Lane 1, untreated DCs; lane 2, DCs stimulated with 200 μM BzATP for 10 min; lane 3, MPs from BzATP-stimulated DCs. Arrows indicate the membrane-bound (TF) and the soluble (sTF) forms.

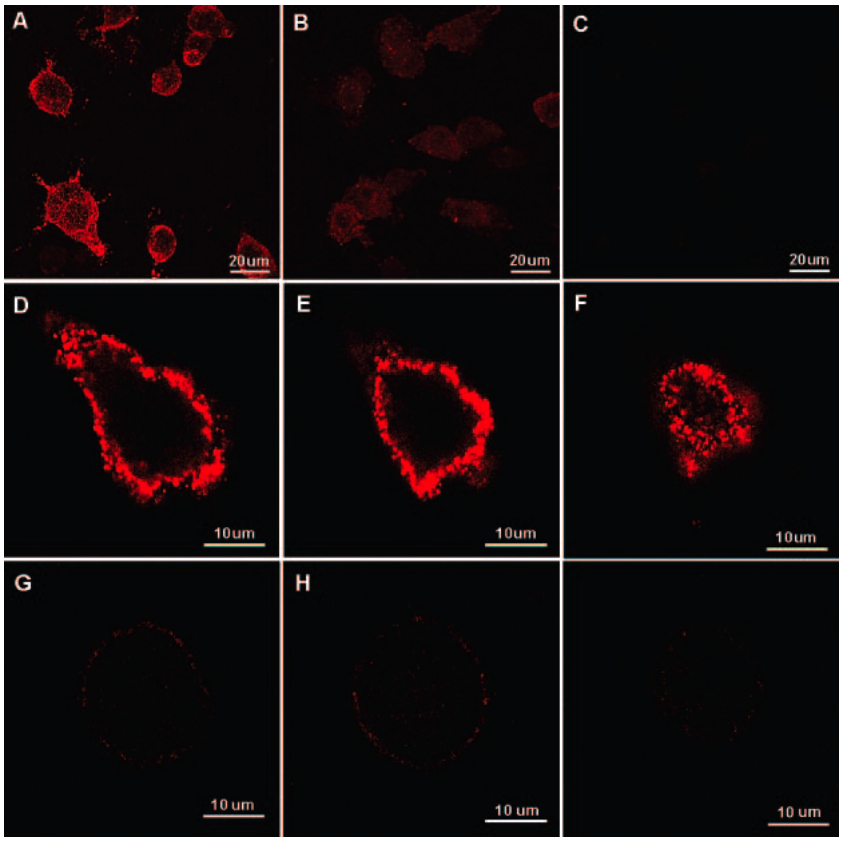

Figure 5.7: Loss of plasma membrane expression of TF on stimulation with BzATP. DCs were layered onto glass coverslips and either left untreated (A, C-F), or stimulated with 200 μM BzATP for 10 min (B, G-I). Both quiescent and BzATP-stimulated cells were then incubated in the presence of an anti-TF antibody and further processed for immunofluorescence analysis as described in Materials and Methods. C) DCs were incubated with the secondary antibody without prior treatment with the anti-TF antibody. A-C show a z axis projection of the different confocal sections, while D-I show a single confocal plane cut through the basal (D, G), equatorial (E, H), or apical (F, I) section of the cells. Images shown are representative of four separate experiments from which at least 15 fields were acquired for each experimental condition.

from the cells. In keeping with this hypothesis, the MPs shed from BzATP-stimulated DCs showed an intense immunostaining for the full-length form of TF (Fig. 5.6, lane 3). On the contrary, no immunoreactivity for the asTF form was detected on the MPs. These show that a large amount of TF is released from DCs by MP shedding.

5.4.5 Expression of TF on DC plasma membrane is drastically reduced on BzATP stimulation

Changes in TF expression on cell surface were monitored by confocal microscopy. To this purpose, DCs were incubated with a specific TF antibody. Confocal microscopy analysis revealed a punctate, bright fluorescence mainly localized on the plasma membrane and in the peripheral cytoplasm. Fluorescence intensity drastically declined on stimulation with BzATP (Fig. 5.7). DCs incubated in the absence of primary Ab were fully negative, confirming that fluorescence was not due to unspecific binding of the secondary antibody (Fig. 5.7C). Panels A-C show a projection on the z axis of the different confocal slices, while panels D-I show single confocal planes (basal, D, G; equatorial E, H; apical, F, I).

5.5 Discussion

The crucial role of TF in the initiation of coagulation (27) as obligatory cofactor of FVIIa, in the migration and proliferation of vascular smooth muscle cells (28), in vascular remodeling, and in plaque neovascularization (29) makes TF expression levels of extreme importance in the control of early steps of the coagulation cascade and in the thrombogenicity of the atherosclerotic plaque. Interestingly, increased levels of circulating TF have been found to be associated with cardiovascular disease (34, 37). In addition, the Cys186- Cys209 disulfide bond on the surface of TF might be modified by protein disulfide isomerase (PDI) that inhibits TF thrombogenicity in a nitric oxide-dependent fashion, thus further linking oxidative stress and coagulation (38).

The procoagulant activity of MPs found in human atherosclerotic plaques (32) and the presence at these sites of DCs, expressing high levels of the P2X7R, suggested to us that ATP released by damaged or infiltrating inflammatory cells might trigger release of TF-bearing MPs via stimulation of the P2X7R. To address this issue, we investigated 1) the ability of human DCs to release MPs on stimulation of the P2X7R and 2) the presence of MP-bound TF.

We found that DCs express the mRNA encoding the full-length form of TF and, to a lesser extent, the alternatively spliced TF mRNA, as found in other cell types of hematopoietic lineage (39). The presence of both TF forms in DCs was confirmed at the protein level by Western blot analyses. Stimulation of DCs with a potent P2X7 receptor agonist such as BzATP induced a massive release of MPs. The membrane-bound form was found to be mainly associated with the MPs, suggesting that most of asTF is directly released into extracellular space on DC stimulation. TF on MPs was found to be functional as shown by a fluorogenic assay that allowed to quantitatively evaluate TF cofactor activity for FVIIa in the stimulation of the physiological substrate FX.

In our experimental set-up, the addition of DCsderived MPs produced generation of activated FX, comparable to that triggered by relipidated recombinant TF. Although a much more extensive experimentation is clearly required, these data strongly suggest that MPs are an important source of procoagulant activity specifically due to TF and not to their phospholipid content as phospholipid vesicles lacking TF were fully inactive. In further support of the specific effect of TF-bearing MPs, the procoagulant activity was largely inhibited by an anti-TF antibody.

It is reported that MPs shedding can be triggered by exogenous stimulants or occur spontaneously even from quiescent cells (40). A higher rate of MP release is observed in apoptotic cells. Among exogenous stimuli, extracellular ATP has been identified as one of the most potent triggers for MP shedding, mainly through activation of the P2X7R, although involvement of other P2 receptors is also likely (22, 23). The physiological significance of shed MPs is not entirely clear, and even their biochemical features are still under investigation. However, it is well known that MPs spontaneously shed by activated or apoptotic monocytes or endothelial cells have a high TF content and a high procoagulant activity (40).

Our data show that activation of the P2X7R triggers at the same time a large MP shedding and a massive TF release. Strikingly, analyses of TF bands in DCs before and after treatment with BzATP revealed that the vast majority of TF was lost, mainly through MP shedding. This observation was strongly corroborated by results from immunostaining of TF and confocal microscopy, which showed a punctate immunoreactivity, mainly localized to the membrane/submembrane area. A similar distribution was recently described in rabbit smooth muscle cells and in HEK293 cells transfected with TF-GFP (41), although in DCs, membraneassociated TF immunoreactivity was higher than in muscle cells or TF transfectants. Our observations provide a clear evidence that activation of the P2X7R can trigger depletion of TF from DCs via shedding of rapidly diffusible MPs. The intrinsic structural

components of the MPs, such as phosphatidylserine, favor the assembly of coagulation macromolecular complexes and are likely to participate in the procoagulant activity together with TF, which is absolutely required to sustain the catalytic activity of FVIIa.

These results indicate that conditions characterized by large release of ATP into the extracellular space create a strong procoagulant microenvironment due not only to ADP generation from ATP via CD39L1 (NTPDase2) (42), but also to ATP-stimulated release of TF-bearing MPs. While it is as yet unknown whether extracellular nucleotides are able to trigger MP release from platelets (the main circulating source of readily releasable ADP and ATP), we show here that they maybe be potentially very important for DCs.

DCs and other mononuclear phagocytes are known to infiltrate the vessel wall and be a major constituent of the atheroma. Platelet aggregation on a dysfunctional endothelium, or at a disrupted plaque, may quickly release large amounts of ATP (this nucleotide is costored with ADP within platelet-dense granules) and generate a propagating wave of procoagulant activity of which TF-bearing MP may be a main constituent. It is well known that MPs are a major determinant of plaque thrombogenicity (32). TF release from DCs might also be precipitated by factors other than platelet aggregation, as it is increasingly appreciated that ATP is released at sites of inflammation by immune cells via nonlytic pathways (43). Extracellular ATP might also have an important role in atheroma formation or in its evolution by stimulating cytokine secretion, release of proteases or, as recently shown by Wong and colleagues (14), by modulating monocyte/macrophage recruitment into the plaque.

Very recently, the mechanism of release of the proinflammatory cytokines IL-1β and IL-18 has stirred hot interest (44, 45). Two nonalternative vesicle-mediated pathways seem to be likely involved: 1) a first one based on atypical secretory lysosomes (46), and 2) a second one based on the release of MPs (22). To our knowledge, the possible participation of secretory lysosomes in TF release has not been investigated but we cannot exclude that this pathway might also be involved. Our current protocol for DC stimulation and MP harvest included incubation of DCs in low salt buffer, an experimental condition that on one hand improves MP recovery, but on the other that might also enhance TF targeting to MPs and obscure the contribution of secretory lysosomes or other possible export pathways. In any case, whichever the relative contribution of these two pathways to TF externalization, DC-derived MPs also contain IL-1β (47), thus it might well be that activated DCs release membrane-bound boluses of costored proinflammatory and procoagulant factors.

An often asked question is whether ATP is the true physiologically rele-

vant stimulus for the P2X7R. Scattered evidence suggests that the P2X7R might also be activated by different stimuli such as antimicrobial peptides released by polymorphonuclear leukocytes (48). This might suggest that additional proinflammatory factors that are generated in the atheromatous plaque or at other sites of vessel wall inflammation may cause release of TF-bearing MPs by acting at the P2X7R.

In conclusion, our observations identify a novel pathway for TF release from DCs and provide new insights into the mechanisms underlying the generation and spreading of procoagulant activity in immunity and inflammation.

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Chapter 6

General discussion

6.1 General discussion

During the last decades, there has been increasing attention to plasma levels of specific coagulation factors, or inhibitors, or to functional coagulation parameters able to predict a risk for thrombotic or haemorrhagic events. The Thrombin generation assay, aimed at monitoring the generation of the last effector of the clotting cascade, is certainly a valuable candidate. The features of the Thrombin generation curve provide information on different steps of the process, the lag phase on the initiation phase triggered by the extrinsic pathway and the peak on the propagation phase characterized by a thrombin burst, which is eventually completely inhibited by plasma protease inhibitors (termination). The area under the curve, known as the endogenous thrombin potential (ETP), represents the total amount of active thrombin formed (Fig. 6.1).

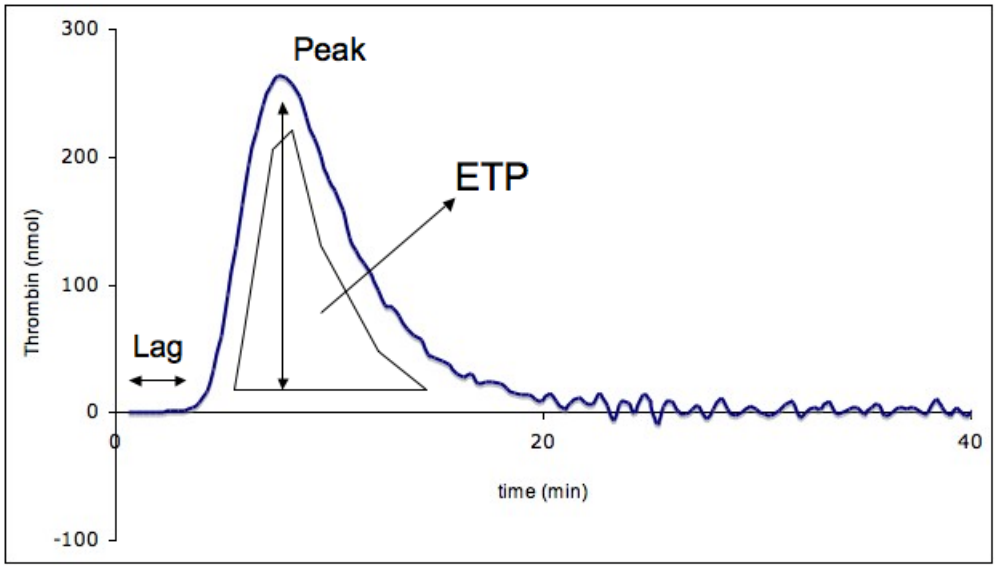

Figure 6.1: Thrombogram and parameters

Several studies have shown that the ETP is a good overall indicator of prothrombotic [1–6] and haemorrhagic tendency [1, 7–10]. However, only sporadic attempts have been made to elucidate the effects of single coagulation factors on thrombin generation [11–13], thus leaving the relationship between levels of coagulation factors/inhibitors and the various parameters of the thrombin generation curve (lag time, ETP and peak height) still an open question.
A recent study addressed this issue in a healthy population [14]. In particular, the thrombin generation parameters were evaluated by varying the

concentration of the procoagulant trigger, Tissue Factor, and by adding anticoagulant factors such as Thrombomodulin and Activated protein C to plasma samples. Under each condition, the lag time reflects the initiation phase of thrombin generation, while the peak height and the ETP (which are highly correlated) probe the propagation and termination phases of coagulation. Thrombin generation test could be a useful overall assay to identify the impact on the clotting process by fluctuations of levels of key protein of coagulation.
A number of molecular mechanisms, acting at different levels of the gene information flow, could produce variations in the expression of these proteins. The genetic component, producing a lifelong effect, is certainly the major determinant of levels in most factors.
Beside the gene mutations responsible for disease states i.e. hemophilia or thrombosis, there is a number of more subtle gene changes, the so-called polymorphisms, that are able to tune coagulation protein or activity levels. Gene changes might influence the expression of clotting proteins by occurring either in their specific genes or in genes encoding for proteins involved in the correct biosynthesis of coagulation factors (modifiers).
The evidence that, not infrequently, the same mutation is associated to different clinical phenotypes, support the hypothesis that individual polymorphisms confer a marginal to moderate risk factor in pathology that becomes evident only across many thousands of individuals.

Intragenic components

All these observations are consistent with the common idea that several pathologies are caused by the interplay among multiple genetic and environmental factors which jointly concur to the development of the disease.
This is the case of the Coronary Artery Disease (CAD), a multifactorial disease in which are involved many biochemical pathways and intermediate phenotypes (e.g. hyperlipidemia, diabetes, hypertension), each in turn under the control of many genes. Evidence that a hypercoagulable state is associated with increased mortality has been provided [15, 16], and so it is biologically plausible that the simultaneous presence of several genetic variations with modest effects on the hemostatic process could be relevant in rising the risk of the major thrombotic complication, i.e. Myocardial Infarction (MI). We investigated [Martinelli et al. 2008] the combined effect of several common prothrombotic polymorphisms (fibrinogen β-chain -455 G/A, Prothrombin 20210 G/A, Factor V Leiden, Factor V R2, Factor VII A1/A2, Factor VII -402 G/A) on the risk for MI, in a selected population of high risk patients with angiographically documented, advanced CAD. The prevalence of MI

increased linearly with an increasing number of unfavorable alleles.
The association between genotypes with variations in the thrombin generation parameters was subsequently investigated in a subset of these patients, to reveal potential acquired risk-phenotype due to the stratication of variables. In thrombin generation measurement, the presence of FV Leiden, the FII G20210A mutations have been already found to be associated with increased thrombin generation [17, 18]. It was experimentally proved that the lag time of thrombin generation was influenced by fibrinogen and FVII, in line with the notion that the coagulation cascade is initiated by binding of TF to FVIIa; moreover determinants of the amount of thrombin formed were again fibrinogen and also prothrombin level but with high procoagulant stimuli, possibly because only a fraction of the prothrombin present in plasma is activated at low TF [14].
In our study, the thrombin generation activity was assessed as a function of the number of procoagulant alleles (fibrinogen β-chain -455 A, Prothrombin 20210 A, Factor V Leiden, Factor V R2, Factor VII A1, Factor VII -402 A). The selected patients were divided in order to form three groups matched for age and sex representing increasing risk-factor groups.

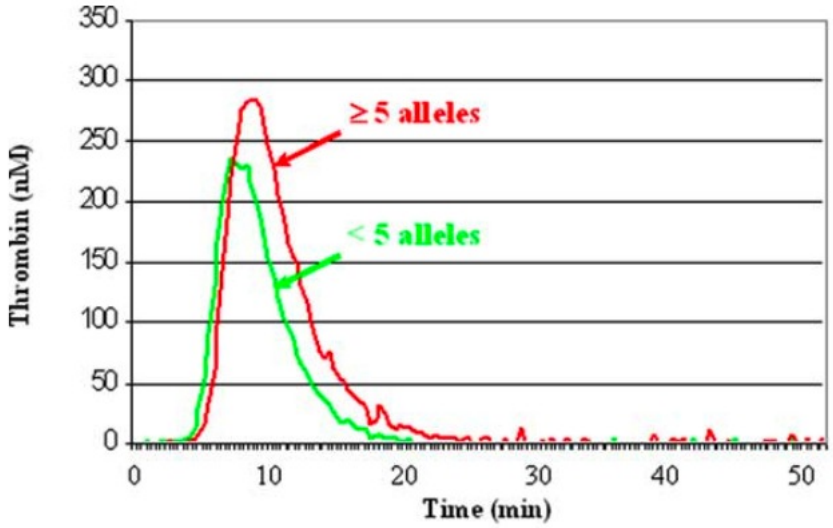

Figure 6.2: Comparison between the median thrombin generation activity curves in groups stratified on the basis of number of procoagulant alleles, with a threshold level at 5 alleles.

We found a clear association between the number of procoagulant alleles and thrombin generation parameter i.e. Endogenous Thrombin Potential (ETP) Fig. 6.2. While individual genetic susceptibility variants are of limited clinical use, the combined information from a number of these variants can

permit the identification of groups of people at high and low risk of developing a complex trait such as MI [19, 20].
This approach, applied on a larger scale, could be a predictive model for risk evaluation, and the thrombin generation assay, measuring the cumulative effect of pro-thrombotic tendencies, in the frame of an integrated study, may be suitable for this purpose.

Extragenic components

Significant variations in clotting proteins levels can originate from direct intragenic variation but also by variations in genes encoding proteins involved in the biosynthesis and processing of clotting factors. A valuable model to assess the influence of modifiers on the levels of different circulating vitamin-k-dependent clotting factors is represented by deficiency of components of the vitamin-k-cycle, essential for the γ-carboxylation. γ-carboxylation is a key post-translational modification of many coagulation factor, which is essential for proper folding of their Gla domain and for the interaction with negatively charged phospholipid surfaces. Previous studies have identified a mutation in the vitamin K 2,3-epoxide reductase complex subunit 1 (VKORC1) gene that is responsible for impaired γ-carboxylation of vitamin-k-dependent clotting factors. We took advantage from the identification of this mutation in a italian patient to perform an extended analysis of the coagulation phenotype associated to VKORC1 deficiency [Marchetti et al. 2008].
Antigen protein's levels at baseline were markedly reduced except for FIX, whose levels appeared to be poorly influenced by defective Vitamin K cycle. Functional levels of procoagulant proteins before intervention, inversely correlated with the affinity of the vitamin-K dependent carboxylase for coagulation factor propeptides, according to the hypothesis that weaker affinity for the carboxylase, results in more efficiently carboxylated product due to an increase in off-rate and hence altered catalytic turnover [21]. Time course analysis evidenced that a single dose of vitamin K normalized coagulation times and plasma factor levels and activity. This partial restored normal phenotype persist over 24 hours.
The thrombin generation assay was also exploited to evaluate the effects on the overall coagulation phenotype. In order to highlight different contribution of vitamin k-dependent clotting factor to thrombin generation, increasing TF concentrations were used to trigger the reaction Fig. 6.3. Testing the thrombotic potential under different procoagulant stimuli emphasize the beneficial effect of vitamin-K supplementation. All thrombin generation assay's parameters were significantly restored, after vitamin k injection, compared with a normal plasma control.

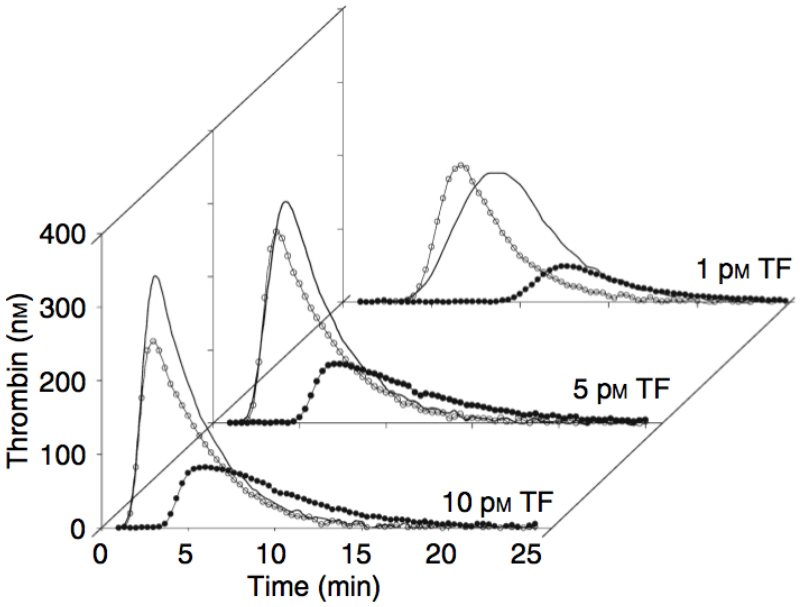

Figure 6.3: Thrombin generation triggered with the indicated TF concentrations in patient plasma, before (•) and 24 h after (○) vitamin K administration, and in PNP (-).

In addition, differential TF concentrations led others quantitative considerations. Since observed partial restored PS levels could influence the initiation phase of thrombin generation [14, 22, 23], shortened time parameters of thrombin generation, lag time and time to peak, clearly contribute to this hypothesis. All these observations provide evidence for the interplay between coagulation factor specific biosynthesis and decay, after vitamin K supplementation.

Acquired components

It is well-established that acquired components, also related to human behaviour and habits, have a strong influence on the expression of many coagulation factor, and might represent independent risk factor for disease.
Conditions such as obesity [24, 25], oral contraception [26, 27], hormone replacement therapy [27], age [28], alcohol intake [29] and potentially smoking [30], could enhance risk for thrombotic events.
Epidemiological studies have demonstrated that in a given population, diet, with other several environmental factors, is an important determinant of risk of vascular disease and mortality [31].
Hence the effect of whole-diet therapies on hemostatic parameters and their

relationship with metabolic and inflammatory markers, could represent an interesting approach to address this issue.
Our study [Passaro et al. 2008] was aimed at evaluating the effects of a Mediterranean style diet on coagulation parameters that represent risk factor for cardiovascular disease (CVD) [32].
Measurements were conducted in a middle aged population of overweighted woman with high levels of C-Reactive Protein (CRP), an important cytokine of acute phase response, and at least another CVD risk factor. Observed reduction of anthropometric and lipid parameters, as already reported [33], would translate into a remarkable decrease in the risk of CVD.
After progressive introduction of proved anti-inflammatory dietary elements, such as wine [34, 35] or fish [36], all inflammatory markers, and particularly IL-6, tended to decrease over time, even though not significantly.
We found a clear association between coagulation factor levels and the inflammatory profile. Levels of Tumor Necrosis Factor-α, a cytokine over-produced by adipocytes and macrophages of adipose tissue in the obese state [37], were correlated with those of FVIII and affected their variation over time. The observed variations suggest novel relationships between coagulation and inflammatory components. It has been reported that increasing levels of hemostatic factors, including fibrinogen, FVIII, FVII, TF and vWF are related to increased CVD risk [38–42], in line with the key role of coagulation factors in thrombus formation; moreover, plasma from females is more procoagulant than in males, as indicated by the tendency towards shorter lag times and higher ETPs and thrombin peaks [14]. This suggest that the eventual influence of dietary changes upon hemostatic components could be examined and correlated with the degree of blood hypercoagulability.
We found that FVII and FVIII levels were remarkably reduced during the course of the study, and FVIII decrease persisted over the dietary intake. Also temporal parameters of thrombin generation, lag time and tt peak, were significantly prolonged Fig. 6.4, perhaps reflecting changes related with initial stages of coagulation cascade. In spite of this evidences, we did not observed significant relations between variations in thrombin generation times and FVII and FVIII levels.
Moreover, since the "rate"of thrombin generation is considered the key variable to stable fibrin clot formation [43, 44], we have calculated the slope of thrombin generation curves, but no significant correlation was observed over time. However a large number of components potentially modified by the intervention and not directly quantified, might influence the thrombin generation assay, masking possible relations.
Changes observed overtime reinforce the relationships between coagulation, environment and inflammatory components; and suggest monitoring of co-

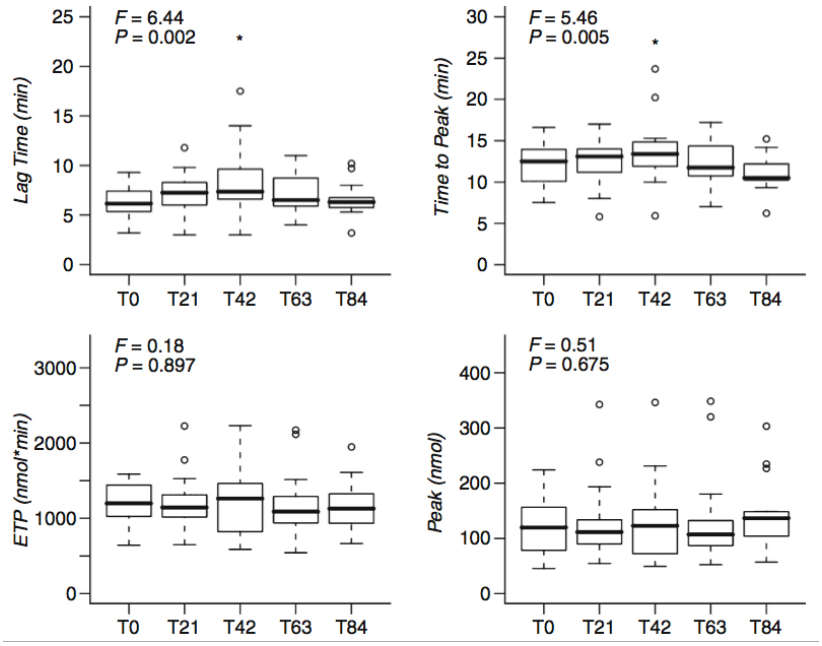

Figure 6.4: Over-study variation of thrombin generation parameters. The black line inside the boxes represents the median value and distribution outliers are indicated with blank circles (∘). The asterisks indicate significant variation ($P < 0.01$) with respect to T_0 after Bonferroni's adjustment for multiple comparisons.

agulation parameters as an informative tool for evaluating short-term effects of diet therapy.

Cellular components

Mechanisms involved in the intracellular and extracellular trafficking of proteins could represent another important level of control of coagulation factor levels. This is certainly the case for Tissue factor (TF). In fact, it has been demonstrated that, beside the transmembrane and the soluble forms, TF circulates into the blood stream bound to cell-derived microparticles (MPs). TF-bearing MPs, providing a suitable phospholipid surfaces for the assembly of coagulation complexes [47, 48], have been receiving increasing attention in recent years as potential vehicles of biological informations through blood vessels. The crucial role of TF in the initiation of coagulation [45] as obligatory cofactor of FVIIa, in migration and proliferation of vascular smooth muscle cells [46], in vascular remodeling, and in plaque neo-vascularization, makes TF expression levels of extreme importance in the control of early steps of the coagulation cascade and in the thrombogenicity of the atherosclerotic plaque.

In our study [Baroni et al. 2007] we tested the hypothesis that dendritic cells, an important components of the atherosclerotic plaque, can express and release TF-bearing MPs upon stimulation.
Our data clearly showed that blood monocyte-derived human dendritic cells are able to express, endogenous TF mRNA and protein also in a "soluble"variant engendered from an alternative splicing process. It has been previously demonstrated that DCs upon stimulation with benzoyl ATP, a potent agonist of the more physiologic ATP, through Its signalling via P2X7 receptors, are able to release MPs [49] Fig. 6.5. In our study we demonstrated that a fraction of these MPs contains TF.
We found TF to be functional as shown by a fluorogenic assay allowing to

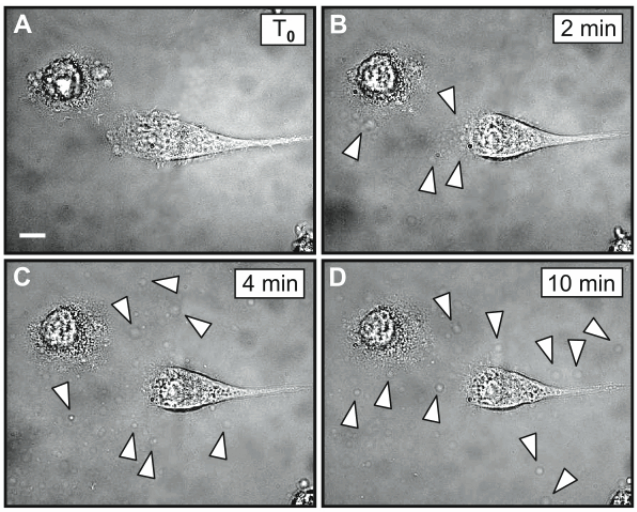

Figure 6.5: P_2X_7 receptor stimulation induces cell shrinkage and MP shedding from human dendritic cells (DCs).

quantitatively evaluate TF cofactor activity for FVIIa in the stimulation of the physiological substrate FX.
Blood DC migrating into the sites of vascular injury or inflammation, are activated under high thrombin concentrations and secrete proinflammatory cytokines, which subsequently modulate coagulation or other physical activation [50]. Under certain stimuli, such as plaque erosion or rupture, the concomitant presence of platelets, that may quickly release large amounts of ATP (costored with ADP within platelet-dense granules), and Dendritic cells, expressing high levels of the P_2X_7 receptors, may predispose to sustained thrombin generation leading to the acute thrombotic event. This complex regulatory network evidences the potential physiological significance of shed MPs, as confirmed by our observations, even if their biochemical features are

still under investigation.

6.2 Conclusions

Our studies provide evidence for genetic and environmental determinants of coagulation factor levels in plasma, and for their integrated effects on generation of thrombin. The interplay between these components is responsible for ample individual variations in the coagulation phenotype. These findings have implications on the diagnosis, prophylaxis and therapy of coagulation diseases, and encourage further investigations to better understand the clinical significance of the molecular heterogeneity of the human hemostatic proteome.

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Chapter 7

Summary Publications

Summary

Background

The maintenance of blood fluidity within the vascular system is ensured by the balance between procoagulant and anticoagulant components. Hemostasis and blood coagulation are processes regulated by a complex network of interactions, involving plasma/tissue proteins and cells. Numerous epidemiologic studies indicate that deciencies in blood coagulation proteins cause serious bleeding problems, whereas the deciencies in coagulation inhibitors and certain mutations in coagulation factors may lead to thrombotic disorders. The coagulation process has been conceptualized as being dependent primarily on adequate levels of the coagulation proteins. From clinical studies it is assumed that the normal concentration range of proteins involved in blood coagulation and regulation of this process may vary in the average blood sample from 50% to 150% of their mean plasma values. This suggests that a wide range of factor levels is compatible with normal hemostatic function; however, even within the normal range, variations can affect the rate and extent of thrombin generation. As a consequence, the stimulus/response which follows tissue factor presentation to blood and the subsequent expression of thrombin activity is highly variable even in the normal population. In addition, the qualitative performance of these proteins is governed by many molecular events which are influenced both by genetic background, and by environmental processes that alter coagulation proteins during circulation and may produce very different phenotypic responses. All these considerations emphasize the importance in measuring and monitoring variations of coagulation factor levels, providing potential correlations with non physiologic conditions. Also, the evidence of how the coagulation proteins are involved in the acute phase of inflammation and several other human diseases, confirm that analysis of their level changes and modulation, represent the way required for appropriate diagnosis and therapeutic intervention.

Aims

A number of molecular mechanisms intragenic, extragenic and environmental, acting at different levels of the gene information ow, could produce variations in the expression of coagulation protein or activity levels. To get inside this complex regulative network we report four different studies aimed at elucidate aspects of clotting factor modulation. Study of oscillations in coagulation factor levels, due to inherited or environmental factor, could establish novel relationships between coagulation and inflammatory components, or with determinant of other human pathologies.

Methods

Methods are included in articles published in 2007/2008; they are focused on thrombin generation assay, an overall assay measuring the amount of thrombin produced in plasma samples in standard condition. This method evidences subjects coagulation-phenotype, allowing the correlation with other clotting factors levels.

Main Results

The combined effect of ten common prothrombotic polymorphisms as a determinant of MI was tested in a population of 804 subject, half of whom affected by severe Coronary Artery Disease (CAD). The number of procoagulant alleles was significantly associated with the Endogenous Thrombin Potential (ETP), similarly, subjects with a high number of procoagulant alleles had significantly higher ETP values as compared to subjects with fewer alleles.

Similar approach was used to investigate a rare bleeding disorder, vitamin K-dependent clotting factor deficiency type 2 (VKCFD2), which offered several quantitative parameters providing us information on vitamin K-dependent regulation of plasma factor levels; a markedly variation in Thrombin Generation parameters and others clotting factors measurements improved the identification of a clear coagulation phenotype.

We also probed the effects of an integrated healthy diet on a wide panel of hemostatic and inflammatory variables. The changes observed in coagulation initiation and amplification phases, body composition and lipid profile could translate into a remarkable decrease in the risk for cardiovascular disease, suggesting novel relationship between coagulation and inflammation components.

Finally we investigate how Tissue Factor-bearing Microparticles was generated by monocyte-derived Dendritic Cells stimulated with the P2X7R agonist benzoyl ATP (BzATP); our observations identify a novel pathway for tissue factor release, providing new insights into the mechanisms underlying the generation and spreading of procoagulant activity. The activity of this potential circulating trigger of coagulation and inflammation stimuli could be involved in progression of several pathology and inflammation process.

Conclusions

Our studies provide quantitative evidence for genetic and environmental determinants of coagulation factor levels in plasma, and for their integrated effects on generation of thrombin. The consequent variation of coagulation phenotype produces ample temporal and individual variations in coagulation function. These findings have implications on the diagnosis, preven-

tion/prophylaxis and therapy of coagulation diseases, and encourage further investigations to better understand the clinical significance of the molecular heterogeneity of the human hemostatic proteome.

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