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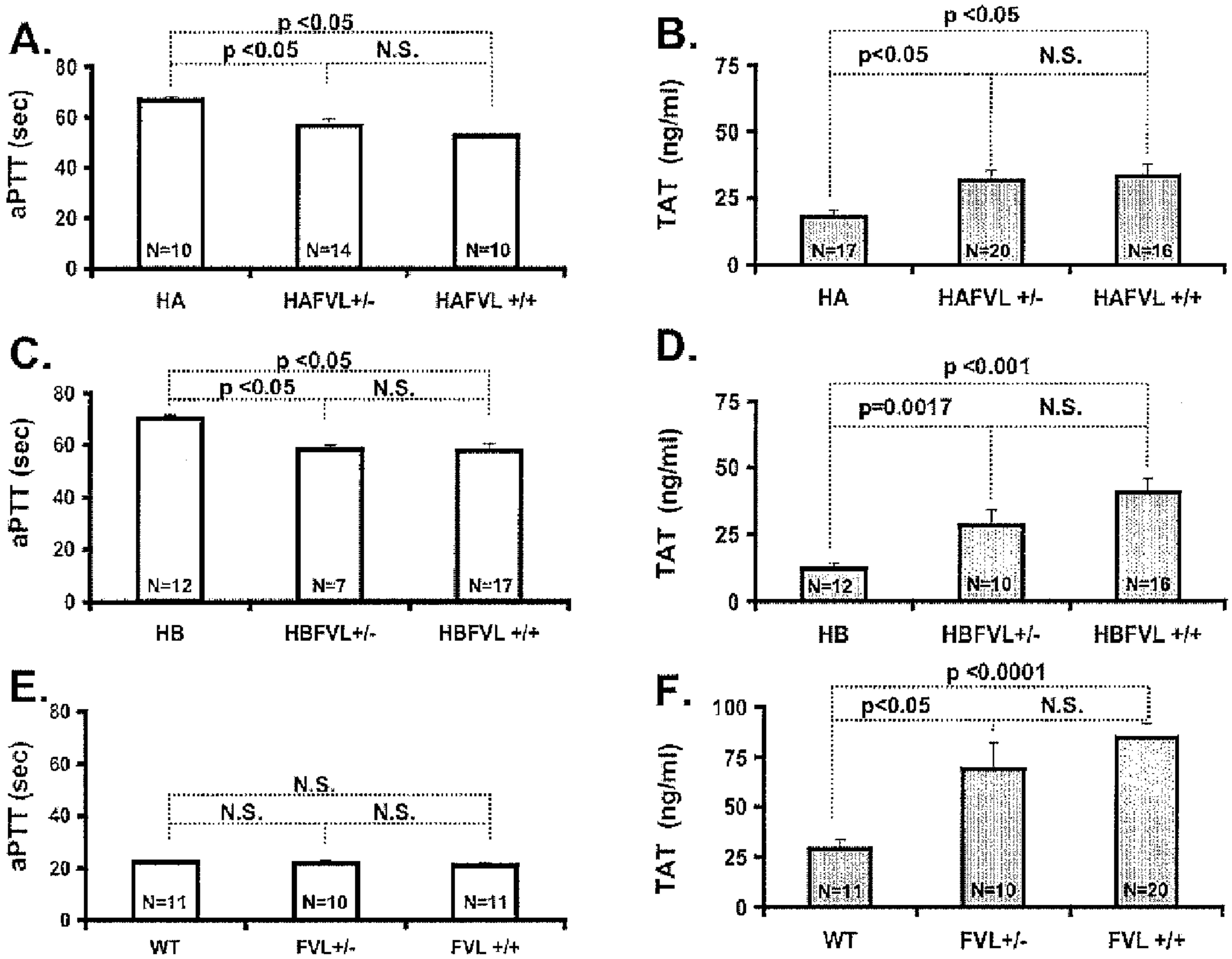
(54) Title: COMPOSITIONS AND METHODS FOR MODULATING HEMOSTASIS USING VARIANT FORMS OF ACTIVATED FACTOR V



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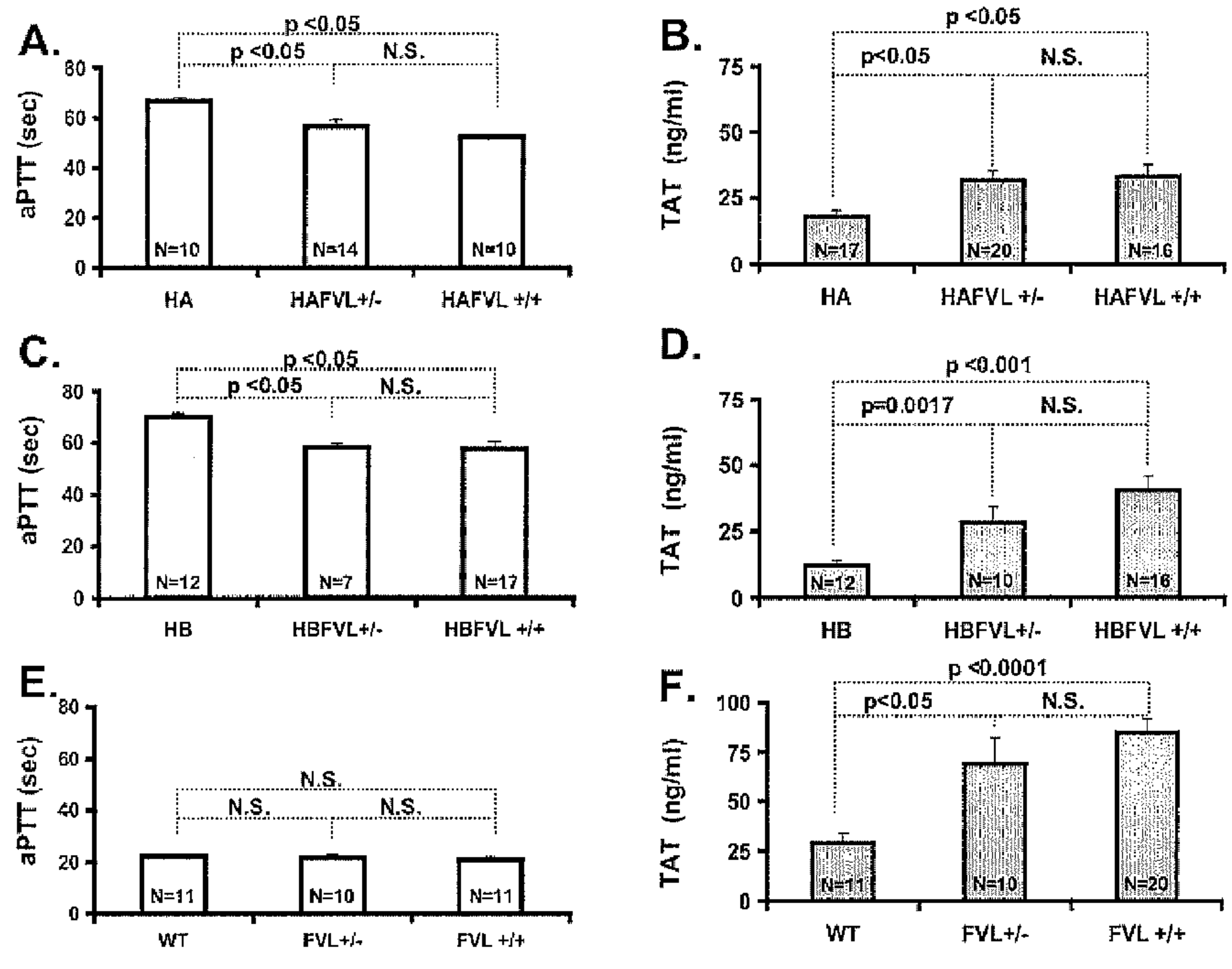

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(57) Abstract: Methods for the treatment of coagulation disorders using Factor V/Va variants are provided.

COMPOSITIONS AND METHODS FOR MODULATING HEMOSTASIS USING VARIANT FORMS OF ACTIVATED FACTOR V

By Rodney M. Camire
Valder R. Arruda

This application claims priority under 35 U.S.C. §119(e) to US Provisional Application No. 60/776,124 filed February 23, 2006, the entire contents being incorporated by reference herein as though set forth in full.

Pursuant to 35 U.S.C. §202(c), it is acknowledged that the U.S. Government has certain rights in the invention described herein, which was made in part with funds from the National Institutes of Health, Grant Number K01 DK60580-01 and P01-HL74124-01 (Project 2).

FIELD OF THE INVENTION

The present invention relates the fields of medicine and hematology. More specifically, the invention describes therapeutic strategies using activated forms of FV and derivatives thereof for modulating the coagulation cascade in patients in need thereof.

BACKGROUND OF THE INVENTION

Several publications and patent documents are cited throughout the specification in order to describe the state of the art to which this invention pertains. Each of these citations is incorporated herein by reference as though set forth in full.

Hemophilia is an X-linked hemorrhagic disorder resulting from mutations in either the FVIII (hemophilia A) or FIX (hemophilia B) genes, with an incidence of approximately 1:5,000 male individuals worldwide. Affected individuals commonly present spontaneous hemorrhages and prolonged bleeding after trauma or surgery. Severely affected subjects present FVIII or FIX levels lower than 1% of normal and comprise the majority of clinically symptomatic cases. The remaining patients have mild to moderate disease with factor levels of 1-30%[1].

The clinical presentation of hemophilia is essentially indistinguishable for FVIII or FIX deficiency. However, there is clear evidence that the clinical phenotype of hemophilia varies among patients with similar residual factor levels or even with the same underlying mutation [2-4]. Therefore, it is possible that other genetic or acquired factors influence the hemophilia phenotype. The current understanding of the genetic basis of venous thrombosis provides an opportunity to determine whether these risk factors could improve the hemophilia phenotype.

Thrombin generation is in part controlled by activated protein C (APC), which is formed by limited proteolysis of the zymogen protein C by the thrombin-thrombomodulin complex. The anticoagulant effect of APC results from the inactivation of both factors Va and VIIIa on membrane surfaces[5]. The most common inherited thrombophilia results from a mutation in the FV gene (Arg 506 to Gln) known as FV Leiden (FVL). Because Arg 506 is the initial cleavage site for APC, FVL is inactivated at approximately one tenth the rate of normal FVa [6], which result in high thrombin levels that create a procoagulant state.

FVL is the most commonly investigated modifier of the hemophilia phenotype because it is present in 2-5% of the Caucasian population [7, 8]. Initial reports suggested the amelioration of the severe hemophilia A phenotype among subjects with FVL [9, 10]. Further studies, however, failed to demonstrate the clinical impact of such association. In screening over 700 hemophilia subjects, 35 cases of FVL have been identified. In only half of these cases (14 hemophilia A and 1 hemophilia B) the association was considered beneficial in terms of frequency of bleeds and/or factor consumption over time [9-14]. The reasons for these discrepancies are not clear but could relate to the small number of subjects, differences in age groups, the presence of underlying infectious diseases, and the retrospective nature of the study. The results of a pediatric study have been informative in this matter since many of the complications common among adults are not confounding factors in children. This case-control study demonstrated that among hemophilia A children with FVL or with other thrombophilia risk factors, the onset of the first bleeding episode was delayed [14].

There is also *in vitro* evidence that the FVL mutation can modify thrombin generation in FVIII [15] or FIX deficient plasma [16]. Moreover, the assessment of the fibrinolytic system in hemophilia revealed that thrombin-induced clots in FVIII or FIX deficient plasma were lysed prematurely [17]. Therefore, the enhanced generation of thrombin by FVL may also increase the resistance of the fibrin clot to premature lysis [18, 19].

SUMMARY OF THE INVENTION

To gain insight into the discrepancies between clinical and laboratorial assessments of the impact of FVL on hemophilia, we took advantage of the availability of genetically engineered mice for severe hemophilia and FVL. These murine models provide the opportunity to address the role of this thrombotic risk factor in modifying the hemophiliac phenotype *in vivo* with minimal influence from environmental factors. Using these unique model systems in conjunction with biochemical assays and real-time imaging of clot formation in living animals, we have found that activated forms of FV can beneficially modify the hemophilia phenotype.

A pharmaceutical composition comprising the activated forms of FV as well as cleavage resistant forms of activated FV of the invention in a biologically compatible carrier which may be directly infused into a patient is also provided. Another preferred aspect of the invention includes methods for the treatment of a hemostasis related disorder in a patient in need thereof comprising administration of a therapeutically effective amount of the activated forms of FV containing pharmaceutical compositions described herein. Such methods should have efficacy in the treatment of disorders where a pro-coagulant is needed and include, without limitation, hemophilia A and B, hemophilia A and B associated with inhibitory antibodies, coagulation factor deficiency, vitamin K epoxide reductase C1 deficiency, gamma-carboxylase deficiency, bleeding associated with trauma, injury, thrombosis, thrombocytopenia, stroke, coagulopathy, disseminated intravascular coagulation (DIC); over-anticoagulation treatment disorders, Bernard Soulier syndrome, Glanzman thromblastemia, and storage pool deficiency.

Another aspect of the invention, includes host cells expressing the variant activated forms of FV of the invention in order to produce large quantities thereof. Methods for isolating and purifying the activated forms of FV are also disclosed.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. Clotting assays in murine models of hemophilia or Factor V Leiden (FVL) and in normal controls. Panels A, C, and E: Modified one stage activated partial thromboplastin assay (aPTT) in mouse plasma. Panels B, D and F: Thrombin-antithrombin complex (TAT) levels in mice plasma. The numbers of animals per group are indicated. Adult hemophilia A (HA) or hemophilia B (HB) mice without

FVL were compared with animals heterozygous (+/-) or homozygous (+/+) for FVL. *P* values were calculated by ANOVA. NS: not significant.

Fig. 2. Hemostatic assessment following tail clip assay in hemophilic mice crossed with Factor V Leiden (FVL). Blood loss was measured by hemoglobin content of the saline solution by optical density at A_{575} post tail clipping of hemophilia mice. Panel A: Hemophilia A (HA) mice, HA heterozygous (+/-) and homozygous for FVL (+/+) were compared. Panel B: Littermates hemophilia B (HB), HB heterozygous (+/-) and homozygous for FVL (+/+) were compared. The numbers of animals per group are indicated. *P* value was calculated by t-test.

Fig. 3. Platelet deposition in arterial thrombi in mice upon laser-induced endothelial damage using fluorescence signals. Upper panels represent data from hemophilia A (HA) mice and bottom panels are data from hemophilia B (HB) mice. Platelet deposition was monitored using an anti-CD41Alexa-555 labeled antibody and composite images at ~120 seconds post laser injury are shown. Thrombus formation was assessed at the baseline (panel A) and after protein replacement (panel B). Hemophilic mice homozygous or heterozygous for FVL are shown in panels D to F. Hemophilic mice injected with 30μg of FVa (Panel C).

Fig. 4. Time course of platelet accumulation in arterial thrombi. Platelet deposition in developing thrombin over time was monitored with anti-CD41 Alexa-55 labeled antibody. The arbitrary relative fluorescent unit (RFU) represents the median platelet-derived fluorescence for several thrombi. Panel A represents hemophilia B mice (HB), HB mice infused with human F.IX concentrated and hemostatically normal C57Bl/6 mice as control (CT). Panel B represents hemophilia A (HA) or HB mice homozygous for Factor V Leiden (FVL) and naïve hemophilic mice. Panels C and D represents hemophilia A (HA) or (HB) naïve mice or injected with purified FV or FVa proteins. These experiments are representative of multiple thrombi (> 5 thrombi/mouse) in (2-4) mice per group, as shown in table 2.

Fig. 5 Schematic representation of FV, FV-810 and FVa. The sequence above FV-810 indicates which B-domain elements have been deleted. IIa cleavage sites are indicated as well as the molecular weight of the various fragments.

DETAILED DESCRIPTION OF THE INVENTION

The role of factor V Leiden (FVL) as a modifier of the severe hemophilia phenotype is still unclear. We used mice with hemophilia A or B crossed with FVL to elucidate *in vivo* parameters of hemostasis. Real-time thrombus formation in the microcirculation was monitored by deposition of labeled platelets upon laser-induced endothelial injury using widefield microscopy in living animals. No thrombi formed in hemophilic A or B mice following vascular injuries. However, hemophilic mice, either heterozygous or homozygous for FVL, formed clots at all injured sites. Injection of purified activated FV into hemophilic A or B mice could mimic the *in vivo* effect of FVL. In contrast to these responses to a laser injury in a microvascular bed, FVL did not provide sustained hemostasis following damage of large vessels in a ferric chloride carotid artery injury model, despite of the improvement of clotting times and high circulating thrombin levels. Together these data provide evidence that FVL has the ability to improve the hemophilia A or B phenotype, but this effect is principally evident at the microcirculation level following a particular vascular injury. Our observations may partly explain the heterogeneous clinical evidence of the beneficial role of FVL in hemophilia.

Thus, in accordance with one aspect of the invention, methods for the treatment of hemophilia are provided. An exemplary method entails administration of an effective amount of an activated form of FV or a derivative thereof to patient to enhance clot formation, thereby ameliorating the symptoms of hemophilia. This treatment initiates coagulation in individuals who lack intrinsic pathway proteins, have inhibitors to these proteins, or who have some other hemostatic abnormality which would benefit by administration of an activated form of FV. Administration of active FV or engineered derivatives of an activated form of FV which have the activated form of FV-like properties to by-pass deficiencies in the intrinsic or extrinsic pathway is disclosed.

Protein replacement therapy using recombinant or plasma-derived forms of factor VIII (or B-domain deleted FVIII), factor IX, or factor VIIa (NovoSeven) is currently the mainstay of hemophilia care. While this treatment regime has limitations, it is very effective and has helped thousands of patients. Over the past 20 years significant progress has been made by several groups in understanding the biochemistry of FV. Factor V circulates in plasma as a single chain procofactor at a concentration of 7 μg/mL (20 nM) and has a half-life of ~12 hours. It is a large (M_r = 330,000, 2196 amino acids) heavily glycosylated, single chain, multi-domain (A1-A2-B-A3-C1-C2) protein which is synthesized in the liver and is homologous to factor VIII. Factor V is secreted as an inactive procofactor and cannot function in the prothrombinase complex (FXa, FVa, anionic membranes, and calcium). This is consistent with the observation that FV binds very weakly, if at all, to FXa and prothrombin, and indicates that proteolytic conversion of FV to FVa leads to appropriate structural changes which impart cofactor function.

Thrombin is established as the most robust activator of FV. Proteolysis occurs at Arg^{709}, Arg^{1018}, and Arg^{1545} generating FVa_{IIa}, a heterodimer composed of an N-terminal 105 kDa heavy chain associated via Ca^{2+} ions to the C-terminal 74/71 kDa light chain. See Figure 5. The large, heavily glycosylated B domain, spanning amino acids 710-1545, is not necessary for cofactor activity and is released during activation.

In addition to using activated factor V (FVa), we have engineered single chain FV derivatives in which large segments of the B-domain have been deleted (For example, $FVdes^{811-1491}$; FV-810, Journal of Biological Chemistry, (2004) **279:** 21643-21650; Figure 5). We have also made additional derivatives which have cofactor-like properties as well. For example, FV-902 (factor V des903-1491) also has activity profiles that are similar to FV-810 and the activated form of FV. These B-domain truncated derivatives exhibit functional proprieties equivalent to FVa, even in the absence of intentional proteolysis. The usefulness of these derivatives in the context of this application are: 1) they are secreted from the mammalian cell line in a single-chain form, and do not require intentional proteolytic activation with thrombin or factor Xa; 2) they have activities that are comparable to two-chain active factor Va; 3) they do not need to be further processed with thrombin; and 4) they may be more stable in plasma compared to two-chain factor Va (i.e have better half-lives).

Additional useful derivatives include:

FV-810; factor V lacking amino acids 811-1491; (published JBC, 279, 2004, 21643-21650)

FV-859; factor V lacking amino acids 860-1491;

FV-866; factor V lacking amino acids 867-1491;

FV-902; factor V lacking amino acids 903-1491;
FV-924; factor V lacking amino acids 923-1491;
FV-937; factor V lacking amino acids 938-1491;
FV-956; factor V lacking amino acids 957-1491;

Others include:

FV-1033-B58-s131; factor V lacking amino acids 1034-1491 with amino acids 900-1030 exchanged with amino acids 907-1037 of factor VIII;
FV-1033-B58-s104; factor V lacking amino acids 1034-1491 with amino acids 904-1007 exchanged with amino acids 972-1075 of factor VIII;
FV-1033-B58-s46; factor V lacking amino acids 1034-1491 with amino acids 963-1008 exchanged with amino acids 1032-1077 of factor VIII;
Each of the above derivatives exhibit functional properties which are comparable to two chain the activated form of FV.

In order to enhance stability of the molecules *in vivo* and provide resistance or protection the protein C pathway each of the above derivatives could be modified at:

Arg506
Arg306
Arg679

While in theory any amino acid could be exchanged at this site, Gln is preferable.

Thus, the methods and compositions of the invention could be used to treat patients with hemophilia A and hemophilia B; patients with hemophilia A and B who have inhibitory antibodies to either factor VIII or factor IX, respectively; and other hemostatic disorders.

I. Definitions:

Various terms relating to the biological molecules of the present invention are used hereinabove and also throughout the specification and claims.

The phrase "activated form of FV" refers to a modified form of FV which has been genetically altered such that it exhibits cofactor activity comparable to two-chain FVa in the absence of intentional proteolysis and in the absence of specific cofactors.

Preferred sites for amino acid alterations in the parent FV molecule are described above.

The phrase “hemostasis related disorder” refers to bleeding disorders such as hemophilia A and B, hemophilia A and B patients with inhibitory antibodies, deficiencies in coagulation Factors, VII, IX and X, XI, V, XII, II, von Willebrand factor, combined FV/FVIII deficiency, vitamin K epoxide reductase C1 deficiency, gamma-carboxylase deficiency; bleeding associated with trauma, injury, thrombosis, thrombocytopenia, stroke, coagulopathy, disseminated intravascular coagulation (DIC); over-anticoagulation associated with heparin, low molecular weight heparin, pentasaccharide, warfarin, small molecule antithrombotics (i.e. FXa inhibitors); and platelet disorders such as, Bernard Soulier syndrome, Glanzman thromblastemia, and storage pool deficiency.

With reference to nucleic acids of the invention, the term "isolated nucleic acid" is sometimes used. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous (in the 5' and 3' directions) in the naturally occurring genome of the organism from which it originates. For example, the "isolated nucleic acid" may comprise a DNA or cDNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the DNA of a prokaryote or eukaryote.

With respect to RNA molecules of the invention, the term "isolated nucleic acid" primarily refers to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from RNA molecules with which it would be associated in its natural state (i.e., in cells or tissues), such that it exists in a "substantially pure" form (the term "substantially pure" is defined below).

With respect to protein, the term "isolated protein" or "isolated and purified protein" is sometimes used herein. This term refers primarily to a protein produced by expression of an isolated nucleic acid molecule of the invention. Alternatively, this term may refer to a protein which has been sufficiently separated from other proteins with which it would naturally be associated, so as to exist in "substantially pure" form.

The term "promoter region" refers to the transcriptional regulatory regions of a gene, which may be found at the 5' or 3' side of the coding region, or within the coding region, or within introns.

The term “vector” refers to a small carrier DNA molecule into which a DNA sequence can be inserted for introduction into a host cell where it will be replicated. An “expression vector” is a specialized vector that contains a gene or nucleic acid sequence with the necessary regulatory regions needed for expression in a host cell.

The term "operably linked" means that the regulatory sequences necessary for expression of a coding sequence are placed in the DNA molecule in the appropriate positions relative to the coding sequence so as to effect expression of the coding sequence. This same definition is sometimes applied to the arrangement of coding sequences and transcription control elements (e.g. promoters, enhancers, and termination elements) in an expression vector. This definition is also sometimes applied to the arrangement of nucleic acid sequences of a first and a second nucleic acid molecule wherein a hybrid nucleic acid molecule is generated.

The term "substantially pure" refers to a preparation comprising at least 50-60% by weight the compound of interest (e.g., nucleic acid, oligonucleotide, protein, etc.). More preferably, the preparation comprises at least 75% by weight, and most preferably 90-99% by weight, of the compound of interest. Purity is measured by methods appropriate for the compound of interest (e.g. chromatographic methods, agarose or polyacrylamide gel electrophoresis, HPLC analysis, and the like).

The phrase "consisting essentially of" when referring to a particular nucleotide sequence or amino acid sequence means a sequence having the properties of a given SEQ ID NO:. For example, when used in reference to an amino acid sequence, the phrase includes the sequence per se and molecular modifications that would not affect the basic and novel characteristics of the sequence.

The term "oligonucleotide," as used herein refers to primers and probes of the present invention, and is defined as a nucleic acid molecule comprised of two or more ribo- or deoxyribonucleotides, preferably more than three. The exact size of the oligonucleotide will depend on various factors and on the particular application for which the oligonucleotide is used.

The term "probe" as used herein refers to an oligonucleotide, polynucleotide or nucleic acid, either RNA or DNA, whether occurring naturally as in a purified restriction enzyme digest or produced synthetically, which is capable of annealing with or specifically hybridizing to a nucleic acid with sequences complementary to the probe. A probe may be either single-stranded or double-stranded. The exact length of the probe will depend upon many factors, including temperature, source of probe and method of use. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide probe typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides.

The probes herein are selected to be "substantially" complementary to different strands of a particular target nucleic acid sequence. This means that the probes must be sufficiently complementary so as to be able to "specifically hybridize" or anneal with their respective target strands under a set of pre-determined conditions. Therefore, the probe sequence need not reflect the exact complementary sequence of the target. For example, a non-complementary nucleotide fragment may be attached to the 5' or 3' end of the probe, with the remainder of the probe sequence being complementary to the target strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the probe, provided that the probe sequence has sufficient complementarity with the sequence of the target nucleic acid to anneal therewith specifically.

The term "specifically hybridize" refers to the association between two single-stranded nucleic acid molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally used in the art (sometimes termed "substantially complementary"). In particular, the term refers to hybridization of an oligonucleotide with a substantially complementary sequence contained within a single-stranded DNA or RNA molecule of the invention, to the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence.

The term "primer" as used herein refers to an oligonucleotide, either RNA or DNA, either single-stranded or double-stranded, either derived from a biological system, generated by restriction enzyme digestion, or produced synthetically which, when placed in the proper environment, is able to act functionally as an initiator of template-dependent nucleic acid synthesis. When presented with an appropriate nucleic acid template, suitable nucleoside triphosphate precursors of nucleic acids, a polymerase enzyme, suitable cofactors and conditions such as a suitable temperature and pH, the primer may be extended at its 3' terminus by the addition of nucleotides by the action of a polymerase or similar activity to yield a primer extension product.

The primer may vary in length depending on the particular conditions and requirements of the application. For example, in diagnostic applications, the oligonucleotide primer is typically 15-25 or more nucleotides in length. The primer must be of sufficient complementarity to the desired template to prime the synthesis of the desired extension product, that is, to be able to anneal with the desired template strand in a manner sufficient to provide the 3' hydroxyl moiety of the primer in appropriate juxtaposition for use in the initiation of synthesis by a polymerase or similar enzyme. It is not required that the primer sequence represent an exact complement of the desired template. For example, a non-complementary nucleotide sequence may be attached to the 5' end of an otherwise complementary primer. Alternatively, non-complementary bases may be interspersed within the oligonucleotide primer sequence, provided that the primer sequence has sufficient complementarity with the sequence of the desired template strand to functionally provide a template-primer complex for the synthesis of the extension product.

The term "percent identical" is used herein with reference to comparisons among nucleic acid or amino acid sequences. Nucleic acid and amino acid sequences are often compared using computer programs that align sequences of nucleic or amino acids thus defining the differences between the two. For purposes of this invention comparisons of nucleic acid sequences are performed using the GCG Wisconsin Package version 9.1, available from the Genetics Computer Group in Madison, Wisconsin. For convenience, the default parameters (gap creation penalty = 12, gap extension penalty = 4) specified by that program are intended for use herein to compare sequence identity. Alternately, the Blastn 2.0 program provided by the National Center for Biotechnology Information(at http://www.ncbi.nlm.nih.gov/blast/; Altschul et al., 1990, J Mol Biol 215:403-410) using a gapped alignment with default parameters, may be used to determine the level of identity and similarity between nucleic acid sequences and amino acid sequences.

II. Preparation of activated forms of FV Encoding Nucleic Acid Molecules, Polypeptides and derivatives thereof

A. Nucleic Acid Molecules

Nucleic acid molecules encoding the activated forms of FV or derivative thereof of the invention may be prepared by using recombinant DNA technology methods. The availability of nucleotide sequence information enables preparation of isolated nucleic acid molecules of the invention by a variety of means. For example, nucleic acid sequences encoding the activated forms of FV or derivative thereof polypeptide may be isolated from appropriate biological sources using standard protocols well known in the art.

Nucleic acids of the present invention may be maintained as DNA in any convenient cloning vector. In a preferred embodiment, clones are maintained in a plasmid cloning/expression vector, such as pBluescript (Stratagene, La Jolla, CA), which is propagated in a suitable E. coli host cell. Alternatively, the nucleic acids may be maintained in vector suitable for expression in mammalian cells. In cases where post-translational modification affects the activated forms of FV or derivative thereof function, it is preferable to express the molecule in mammalian cells.

The activated forms of FV or derivative thereof-encoding nucleic acid molecules of the invention include cDNA, genomic DNA, RNA, and fragments thereof which may be single- or double-stranded. Thus, this invention provides oligonucleotides (sense or antisense strands of DNA or RNA) having sequences capable of hybridizing with at least one sequence of a nucleic acid molecule of the present invention. Such oligonucleotides are useful as probes for detecting the activated form of FV expression.

B. Proteins

A full-length activated form of FV or derivative thereof polypeptide of the present invention may be prepared in a variety of ways, according to known methods. The protein may be purified from appropriate sources, e.g., transformed bacterial or animal cultured cells or tissues which express the activated form of FV, by immunoaffinity purification. However, this is not a preferred method due to the low amount of protein likely to be present in a given cell type at any time.

The availability of nucleic acid molecules encoding an activated form of FV or derivative thereof polypeptide enables production of the activated form of FV or derivative thereof using in vitro expression methods known in the art. For example, a cDNA or gene may be cloned into an appropriate in vitro transcription vector, such as pSP64 or pSP65 for in vitro transcription, followed by cell-free translation in a suitable cell-free translation system, such as wheat germ or rabbit reticulocyte lysates. In vitro transcription and translation systems are commercially available, e.g., from Promega Biotech, Madison, Wisconsin or BRL, Rockville, Maryland.

Alternatively, according to a preferred embodiment, larger quantities of the activated form of FV or derivative thereof may be produced by expression in a suitable prokaryotic or eukaryotic expression system. For example, part or all of a DNA molecule encoding the activated form of FV for example, may be inserted into a plasmid vector adapted for expression in a bacterial cell, such as E. coli or a mammalian cell such as CHO or Hela cells. Alternatively, in a preferred embodiment, tagged fusion proteins comprising the activated form of FV or derivative thereof can be generated. Such activated form of FV or derivative thereof-tagged fusion proteins are encoded by part or all of a DNA molecule, ligated in the correct codon reading frame to a nucleotide sequence encoding a portion or all of a desired polypeptide tag which is inserted into a plasmid vector adapted for expression in a bacterial cell, such as E. coli or a eukaryotic cell, such as, but not limited to, yeast and mammalian cells. Vectors such as those described above comprise the regulatory elements necessary for expression of the DNA in the host cell positioned in such a manner as to permit expression of the DNA in the host cell. Such regulatory elements required for expression include, but are not limited to, promoter sequences, transcription initiation sequences, and enhancer sequences.

The activated form of FV or derivative thereof proteins, produced by gene expression in a recombinant prokaryotic or eukaryotic system may be purified according to methods known in the art. In a preferred embodiment, a commercially available expression/secretion system can be used, whereby the recombinant protein is expressed and thereafter secreted from the host cell, to be easily purified from the surrounding medium. If expression/secretion vectors are not used, an alternative approach involves purifying the recombinant protein by affinity separation, such as by immunological interaction with antibodies that bind specifically to the recombinant protein or nickel columns for isolation of recombinant proteins tagged with 6-8 histidine residues at their N-terminus or C-terminus. Alternative tags may comprise the FLAG epitope, GST or the hemagglutinin epitope. Such methods are commonly used by skilled practitioners.

The activated form of FV or derivative thereof proteins, prepared by the aforementioned methods, may be analyzed according to standard procedures. For example, such proteins may be subjected to amino acid sequence analysis, according to known methods.

As discussed above, a convenient way of producing a polypeptide according to the present invention is to express nucleic acid encoding it, by use of the nucleic acid in an expression system. A variety of expression systems of utility for the methods of the present invention are well known to those of skill in the art.

Accordingly, the present invention also encompasses a method of making a polypeptide (as disclosed), the method including expression from nucleic acid encoding the polypeptide (generally nucleic acid). This may conveniently be achieved by culturing a host cell, containing such a vector, under appropriate conditions which cause or allow production of the polypeptide. Polypeptides may also be produced in in vitro systems, such as reticulocyte lysate.

III. Uses of the activated form of FV or derivative thereof- Encoding Nucleic Acids and Proteins

The activated form of FV polypeptide or derivative thereof or nucleic acids encoding the same having altered coagulation activities may be used according to this invention, for example, as therapeutic and/or prophylactic agents which modulate the blood coagulation cascade. The present inventors have discovered that these molecules can be altered to increase coagulation.

A. In a preferred embodiment of the present invention, the activated form of FV or derivative thereof may be administered to a patient via infusion in a biologically compatible carrier, preferably via intravenous injection. The activated form of FV or derivative thereof of the invention may optionally be encapsulated into liposomes or mixed with other phospholipids or micelles to increase stability of the molecule. The activated form of FV or derivative thereof may be administered alone or in combination with other agents known to modulate hemostasis (e.g., Factor VIIa, FIX, FVIII or FX/Xa and derivatives thereof). An appropriate composition in which to deliver the activated form of FV or derivative thereof may be determined by a medical practitioner upon consideration of a variety of physiological variables, including, but not limited to, the patient's condition and hemodynamic state. A variety of compositions well suited for different applications and routes of administration are well known in the art and are described hereinbelow.

The preparation containing the purified activated forms of FV or derivative thereof contains a physiologically acceptable matrix and is preferably formulated as a pharmaceutical preparation. The preparation can be formulated using substantially known prior art methods, it can be mixed with a buffer containing salts, such as NaCl, $CaCl_2$, and amino acids, such as glycine and/or lysine, and in a pH range from 6 to 8.

Until needed, the purified preparation containing the factor V/Va analog can be stored in the form of a finished solution or in lyophilized or deep-frozen form. Preferably the preparation is stored in lyophilized form and is dissolved into a visually clear solution using an appropriate reconstitution solution.

Alternatively, the preparation according to the present invention can also be made available as a liquid preparation or as a liquid that is deep-frozen.

The preparation according to the present invention is especially stable, i.e., it can be allowed to stand in dissolved form for a prolonged time prior to application.

The preparation according to the present invention can be made available as a pharmaceutical preparation with the activated form of FV or derivative thereof in the form of a one-component preparation or in combination with other factors in the form of a multi-component preparation.

Prior to processing the purified protein into a pharmaceutical preparation, the purified protein is subjected to the conventional quality controls and fashioned into a therapeutic form of presentation. In particular, during the recombinant manufacture, the purified preparation is tested for the absence of cellular nucleic acids as well as nucleic acids that are derived from the expression vector, preferably using a method, such as is described in EP 0 714 987.

Another feature of this invention relates to making available a preparation which contains an activated form of FV or derivative thereof with a high stability and structural integrity and which, in particular, is free from inactive factor V/Va analog intermediates and autoproteolytic degradation products and which can be produced by activating a factor V analog of the type described above and by formulating it into an appropriate preparation.

The pharmaceutical preparation may contain dosages of between 10-1000 μg/kg, more preferably between about 10-250 μg/kg and most preferably between 10 and 75 μg/kg, with 40 μg/kg of the variant factor V polypeptide being particularly preferred. Patients may be treated immediately upon presentation at the clinic with a bleed. Alternatively, patients may receive a bolus infusion every one to three hours, or if sufficient improvement is observed, a once daily infusion of the activated form of FV or derivative thereof described herein.

B. The activated form of FV or derivative thereof or derivative thereof-Encoding Nucleic Acids

The activated form of FV or derivative thereof-encoding nucleic acids may be used for a variety of purposes in accordance with the present invention. In a preferred embodiment of the invention, a nucleic acid delivery vehicle (i.e., an expression vector) for modulating blood coagulation is provided wherein the expression vector comprises a nucleic acid sequence coding the activated form of FV or derivative thereof polypeptide, or a functional fragment thereof as described herein. Administration of the activated form of FV or derivative thereof-encoding expression vectors to a patient, results in the expression of the activated form of FV or derivative thereof polypeptide which serves to enhance coagulation. In accordance with the present invention, the activated form of FV or derivative thereof encoding nucleic acid sequence may encode the activated form of FV or derivative thereof polypeptide as described herein whose expression modulates hemostasis.

Expression vectors comprising the activated form of FV or derivative thereof nucleic acid sequences may be administered alone, or in combination with other molecules useful for modulating hemostasis. According to the present invention, the expression vectors or combination of therapeutic agents may be administered to the patient alone or in a pharmaceutically acceptable or biologically compatible composition.

In a preferred embodiment of the invention, the expression vector comprising nucleic acid sequences encoding the activated form of FV or derivative thereof is a viral vector. Viral vectors which may be used in the present invention include, but are not limited to, adenoviral vectors (with or without tissue specific promoters/enhancers), adeno-associated virus (AAV) vectors of multiple serotypes (e.g., AAV-2, AAV-5, AAV-7, and AAV-8) and hybrid AAV vectors, lentivirus vectors and pseudo-typed lentivirus vectors [e.g., Ebola virus, vesicular stomatitis virus (VSV), and feline immunodeficiency virus (FIV)], herpes simplex virus vectors, vaccinia virus vectors, and retroviral vectors.

In a preferred embodiment of the present invention, methods are provided for the administration of a viral vector comprising nucleic acid sequences encoding an activated form of FV or derivative thereof, or a functional fragment thereof. Adenoviral vectors of utility in the methods of the present invention preferably include at least the essential parts of adenoviral vector DNA. As described herein, expression of the activated form of FV or derivative thereof polypeptide following administration of such an adenoviral vector serves to modulate hemostasis.

Recombinant adenoviral vectors have found broad utility for a variety of gene therapy applications. Their utility for such applications is due largely to the high efficiency of in vivo gene transfer achieved in a variety of organ contexts.

Adenoviral particles may be used to advantage as vehicles for adequate gene delivery. Such virions possess a number of desirable features for such applications, including: structural features related to being a double stranded DNA nonenveloped virus and biological features such as a tropism for the human respiratory system and gastrointestinal tract. Moreover, adenoviruses are known to infect a wide variety of cell types in vivo and in vitro by receptor-mediated endocytosis. Attesting to the overall safety of adenoviral vectors, infection with adenovirus leads to a minimal disease state in humans comprising mild flu-like symptoms.

Due to their large size (~36 kilobases), adenoviral genomes are well suited for use as gene therapy vehicles because they can accommodate the insertion of foreign DNA following the removal of adenoviral genes essential for replication and nonessential regions. Such substitutions render the viral vector impaired with regard to replicative functions and infectivity. Of note, adenoviruses have been used as vectors for gene therapy and for expression of heterologous genes.

For a more detailed discussion of the use of adenovirus vectors utilized for gene therapy, see Berkner, 1988, Biotechniques 6:616-629 and Trapnell, 1993, Advanced Drug Delivery Reviews 12:185-199.

It is desirable to introduce a vector that can provide, for example, multiple copies of a desired gene and hence greater amounts of the product of that gene. Improved adenoviral vectors and methods for producing these vectors have been described in detail in a number of references, patents, and patent applications, including: Mitani and Kubo (2002, Curr Gene Ther. 2(2):135-44); Olmsted-Davis et al. (2002, Hum Gene Ther. 13(11):1337-47); Reynolds et al. (2001, Nat Biotechnol. 19(9):838-42); U.S. Patent Nos. 5,998,205 (wherein tumor-specific replicating vectors comprising multiple DNA copies are provided); 6,228,646 (wherein helper-free, totally defective adenovirus vectors are described); 6,093,699 (wherein vectors and methods for gene therapy are provided); 6,100,242 (wherein a transgene-inserted replication defective adenovirus vector was used effectively in in vivo gene therapy of peripheral vascular disease and heart disease); and International Patent Application Nos. WO 94/17810 and WO 94/23744.

For some applications, an expression construct may further comprise regulatory elements which serve to drive expression in a particular cell or tissue type. Such regulatory elements are known to those of skill in the art and discussed in depth in Sambrook et al. (1989) and Ausubel et al. (1992). The incorporation of tissue specific regulatory elements in the expression constructs of the present invention provides for at least partial tissue tropism for the expression of the activated form of FV or derivative thereof or functional fragments thereof. For example, an E1 deleted type 5 adenoviral vector comprising nucleic acid sequences encoding the activated form of FV or derivative thereof under the control of a cytomegalovirus (CMV) promoter may be used to advantage in the methods of the present invention.

Exemplary Methods for Producing Adenoviral Vectors

Adenoviral vectors for recombinant gene expression have been produced in the human embryonic kidney cell line 293 (Graham et al., 1977, J. Gen. Virol. 36:59-72). This cell line is permissive for growth of adenovirus 2 (Ad2) and adenovirus 5 mutants defective in E1 functions because it comprises the left end of the adenovirus 5 genome and, therefore, expresses E1 proteins. E1 genes integrated into the cellular genome of 293 cells are expressed at levels which facilitate the use of these cells as an expression system in which to amplify viral vectors from which these genes have been deleted. 293 cells have been used extensively for the isolation and propagation of E1 mutants, for helper-independent cloning, and for expression of adenovirus vectors. Expression systems such as the 293 cell line, therefore, provide essential viral functions in trans and thereby enable propagation of viral vectors in which exogenous nucleic acid sequences have been substituted for E1 genes. See Young et al. in The Adenoviruses, Ginsberg, ed., Plenum Press, New York and London (1984), pp. 125-172.

Other expression systems well suited to the propagation of adenoviral vectors are known to those of skill in the art (e.g., HeLa cells) and have been reviewed elsewhere.

Also included in the present invention is a method for modulating hemostasis comprising providing cells of an individual with a nucleic acid delivery vehicle encoding the activated form of FV or derivative thereof polypeptide and allowing the cells to grow under conditions wherein the activated form of FV or derivative thereof polypeptide is expressed.

From the foregoing discussion, it can be seen that the activated form of FV or derivative thereof polypeptide or nucleic acids encoding the same may be used in the treatment of disorders associated with aberrant blood coagulation.

C. Pharmaceutical Compositions

The expression vectors of the present invention may be incorporated into pharmaceutical compositions that may be delivered to a subject, so as to allow production of a biologically active protein (e.g., the activated form of FV or derivative thereof polypeptide or functional fragment or derivative thereof). In a particular embodiment of the present invention, pharmaceutical compositions comprising sufficient genetic material to enable a recipient to produce a therapeutically effective amount of the activated form of FV or derivative thereof polypeptide can influence hemostasis in the subject. Alternatively, as discussed above, an effective amount of the variant Factor V polypeptide may be directly infused into a patient in need thereof. The compositions may be administered alone or in combination with at least one other agent, such as a stabilizing compound, which may be administered in any sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose, and water. The compositions may be administered to a patient alone, or in combination with other agents which influence hemostasis.

In preferred embodiments, the pharmaceutical compositions also contain a pharmaceutically acceptable excipient. Such excipients include any pharmaceutical agent that does not itself induce an immune response harmful to the individual receiving the composition, and which may be administered without undue toxicity. Pharmaceutically acceptable excipients include, but are not limited to, liquids such as water, saline, glycerol, sugars and ethanol. Pharmaceutically acceptable salts can also be included therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles. A thorough discussion of pharmaceutically acceptable excipients is available in Remington's Pharmaceutical Sciences (Mack Pub. Co., 18th Edition, Easton, Pa. [1990]).

Pharmaceutical formulations suitable for parenteral administration may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiologically buffered saline. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

For topical or nasal administration, penetrants appropriate to the particular barrier to be permeated are used in the formulation. Such penetrants are generally known in the art. The pharmaceutical compositions of the present invention may be manufactured in any manner known in the art (e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping, or lyophilizing processes).

The pharmaceutical composition may be provided as a salt and can be formed with many acids, including but not limited to, hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents than are the corresponding, free base forms. In other cases, the preferred preparation may be a lyophilized powder which may contain any or all of the following: 1-50 mM histidine, 0.1%-2% sucrose, and 2-7% mannitol, at a pH range of 4.5 to 5.5, which is combined with buffer prior to use.

After pharmaceutical compositions have been prepared, they may be placed in an appropriate container and labeled for treatment. For administration of the activated form of FV or derivative thereof-containing vectors, such labeling would include amount, frequency, and method of administration.

Pharmaceutical compositions suitable for use in the invention include compositions wherein the active ingredients are contained in an effective amount to achieve the intended therapeutic purpose. Determining a therapeutically effective dose is well within the capability of a skilled medical practitioner using the techniques provided in the present invention. Therapeutic doses will depend on, among other factors, the age and general condition of the subject, the severity of the aberrant blood coagulation phenotype, and the strength of the control sequences regulating the expression levels of the activated form of FV or derivative thereof polypeptide. Thus, a therapeutically effective amount in humans will fall in a relatively broad range that may be determined by a medical practitioner based on the response of an individual patient to vector-based activated form of FV or derivative thereof treatment.

D. Administration

The variant activated form of FV polypeptides, alone or in combination with other agents may be directly infused into a patient in an appropriate biological carrier as described hereinabove. Expression vectors of the present invention comprising nucleic acid sequences encoding the activated form of FV or derivative thereof, or functional fragments thereof, may be administered to a patient by a variety of means (see below) to achieve and maintain a prophylactically and/or therapeutically effective level of the activated form of FV or derivative thereof polypeptide. One of skill in the art could readily determine specific protocols for using the activated form of FV or derivative thereof encoding expression vectors of the present invention for the therapeutic treatment of a particular patient. Protocols for the generation of adenoviral vectors and administration to patients have been described in U.S. Patent Nos. 5,998,205; 6,228,646; 6,093,699; 6,100,242; and International Patent Application Nos. WO 94/17810 and WO 94/23744., which are incorporated herein by reference in their entirety.

The activated form of FV or derivative thereof encoding adenoviral vectors of the present invention may be administered to a patient by any means known. Direct delivery of the pharmaceutical compositions in vivo may generally be accomplished via injection using a conventional syringe, although other delivery methods such as convection-enhanced delivery are envisioned (See e.g., U.S. Pat. No. 5,720,720). In this regard, the compositions may be delivered subcutaneously, epidermally, intradermally, intrathecally, intraorbitally, intramucosally, intraperitoneally, intravenously, intraarterially, orally, intrahepatically or intramuscularly. Other modes of administration include oral and pulmonary administration, suppositories, and transdermal applications. A clinician specializing in the treatment of patients with blood coagulation disorders may determine the optimal route for administration of the adenoviral vectors comprising the activated form of FV or derivative thereof nucleic acid sequences based on a number of criteria, including, but not limited to: the condition of the patient and the purpose of the treatment (e.g., enhanced or reduced blood coagulation).

The present invention also encompasses AAV vectors comprising a nucleic acid sequence encoding the activated form of FV or derivative thereof polypeptide.

Also provided are lentivirus or pseudo-typed lentivirus vectors comprising a nucleic acid sequence encoding an activated form of FV or derivative thereof polypeptide

Also encompassed are naked plasmid or expression vectors comprising a nucleic acid sequence encoding an activated form of FV or derivative thereof polypeptide.

The Example set forth below is provided to illustrate certain embodiments of the invention. It is not intended to limit the invention in any way.

EXAMPLE I

The following materials and methods are provided to facilitate the practice of the present invention.

Animals

The local Animal Care and Use Committee approved all procedures. Murine models for severe FVIII [20] or FIX deficiency [21] were crossed with mice carrying FVL (on C57Bl6 background) generated by knock-in technology as previously described [22, 23]. The hemophilia B model was generated on a C57Bl/6-129 mixed background [21, 24] and further crossed into C57Bl/6 for additional five generations. The hemophilia A mice were on C57Bl/6-129 mixed background. Through a series of breedings we obtained hemophilia A or B mice of all three expected FVL genotypes, which allow comparison littermate mice.

Coagulation assays

Blood samples obtained by tail clipping were collected into 3.8% sodium citrate (9 parts of blood: 1 part anticoagulant). Clotting factor activity was determined by a modified one stage assay incubating 50 μl of human FIX- or FVIII-deficient plasma with 50 μl of automated activated partial thromboplastin time (aPTT) reagent (Organon Teknika, Durham, NC), and a total of 50 μl of undiluted test sample. Fifty microliters of 25 mM $CaCl_2$ were added, and time to clot formation was measured using Stat4 Coagulation Instrument (Diagnostic Stago, Parsipanny, NJ). Thrombin-antithrombin (TAT) complexes were measured by Enzygnost TAT enzyme-linked immunosorbent assay (ELISA) purchased from Dade Behring (Marburg, Germany), as previously described, which present high cross reactivity with murine TAT [25, 26].

Tail clipping assay

Mice were anesthetized and the distal portion of the tail (2.5-3 mm of diameter) was cut and immersed in 37°C saline solution. Bleeding time measurements exceeding 10 min were stopped by suture of the tail. The blood loss was determined by measuring the absorbance of hemoglobin (A_{575} nm) in the saline solution in which the tail was placed, as reported [26].

Ferric chloride ($FeCl_3$) carotid artery model

The carotid artery of adult mice was exposed, a Doppler flow probe (Model 0.5VB; Transonic Machinery Systems, Ithaca, NY) was placed on the surface of the exposed artery and a baseline blood flow measurement recorded. Subsequently, a 2 mm^2 piece of Whatman #1 paper soaked in ferric chloride (15% solution) was applied to the adventitial surface of the exposed artery for 2 minutes, after which it was removed, and carotid artery blood flow recorded. Time to carotid artery occlusion was defined as the time after initiation of arterial injury and the onset of stable occlusion[27].

Real-time widefield intravital microscope

The cremaster muscle of adult mice was exposed, stretched and pinned across the intravital microscopy tray. The rat anti-CD41 (murine platelet glycoprotein complex IIb/IIIa) Alexa-555 labeled antibody (Molecular Probes, Eugene, OR) was infused at a dose of 10 μg per mouse. Immediately after infusion of the antibody, a laser-induced injury was performed on the vessel wall of the cremasteric arterioles [28]. The injuries were performed using a pulse-nitrogen dye laser applied through the micropoint laser system (Phototonic Instruments St. Charles, Illinois). We used an Olympus BX6IWI fixed-stage motorized upright fluorescence microscope with a long-distance condenser and 40X water-immersion objective. Data analysis was carried out utilizing the Slidebook 4.0 software (Intelligent Imaging Innovations, Denver, CO). Fluorescence data were captured digitally up to 10 milliseconds/event for 300 frames. The amount of platelet accumulation in the developing thrombi was determined by the sum of all pixel values of the platelet-specific signal and expressed as relative fluorescence unit (RFU), an arbitrary unit in which the integrated platelet fluorescence intensity is determined.

Assessing effects of FV or FVa proteins in the hemostasis of hemophilia mice.

Human FV was isolated from plasma and recombinant FVa was prepared as described before [29]. Purification of both proteins was carried out using an immunoaffinity column containing anti-human F.V antibody [29]. For *in vitro* activation of FV, 20 nM FV was incubated with murine or human thrombin (Haematological Technologies, Inc. Essex-Junction, VT) at concentration of 0.25 nM at 37°C. Samples were withdrawn from the reaction mixtures at several time points and the specific cofactor activity was determined by a PT-based assay using FV-deficient plasma. To determine the cleavage of FV by murine or human thrombin, 300 nM of single chain FV was incubated for several time intervals with thrombin (1 nM). Samples were removed and analyzed by SDS-PAGE. Next, FVa was infused *via* the tail vein into hemophilia B mice and blood samples were collected by tail clipping prior to protein infusion, and after 15 and 120 minutes for determination of FVa levels, aPTTs, and TAT levels. Next, we injected human FVa (30-60 μg/mouse) or FV (60-120 μg/mouse) through the jugular vein of hemophilia A or B mice and monitored clot formation in the intravital microscopy over a period of two hours.

Statistical analysis

Comparison of data obtained from distinct experimental groups was analyzed using JMP version 4.0.2 (SAS Institute Inc. Cary, NC).

RESULTS

Hemophilia A and B mice with FVL present improved clotting times.

The determination of clotting activity for hemophilia A mice homozygous or heterozygous for FVL mutation revealed shortening of the aPTT values when compared to hemophilia A mice without FVL (Fig. 1A). Similar improvement on the aPTT values was also determined for hemophilia B mice with FVL (Fig. 1B). We next determined TAT levels, to verify whether the improvement of the aPTT values was associated with increased thrombin generation. This immunoassay was developed to detect human TAT but also presents high cross-reactivity to murine TAT [25, 26]. There was a good correlation between shortening of the aPTT and increased levels of TAT (Fig. 1, panels C and D). However, TAT levels of hemophilia mice with FVL did not reach those of FVL without hemophilia (Fig. 1, panel F).

Blood loss following tail- clipping is reduced among hemophilia B mice carrying FVL.

We next tested whether the mild improvement of the clotting times *in vitro* was associated with *in vivo* hemostatic performance. Blood loss was measured during a ten minute period after sectioning the distal part of the tail. No difference was seen among hemophilia A with or without FVL (Fig. 2A), whereas the blood loss among hemophilia B mice homozygous for FVL was reduced when compared with mice without the mutation (Fig. 2B).

No sustained thrombus formation following carotid artery injuryin hemophilia mice with FVL.

All normal mice (n=5) or FVL homozygous mice (n=5) tested without hemophilia presented full vessel occlusion (Table 1), which was characterized by the interruption of blood flow within 6 to 8 min post vessel injury. In contrast, no vessel occlusion was detected in hemophilia A mice without FVL (Table 1). In two out of ten hemophilia A mice with FVL, only a transient reduction of the blood flow was detected, but not at the levels to suggest full lumen occlusion (Table 1). Similar findings were determined among hemophilia B homozygous for FVL, with only one of the eight mice developing a complete and one a transient vessel occlusion.

Table 1: Carotid artery occlusion following $FeCl_3$-induced injury model.

Genotype	N. of mice	N. of mice with occlusion		Occlusion time ± SD
		Transient	Complete	
WT	5	0	5	6.3 ± 2 min
FVL (+/+)	5	0	5	6.5 ± 1 min
HA	5	0	0	-
HA/FVL (+/-)	5	1	0	-
HA/FVL (+/+)	5	1	0	-
HB	5	0	0	-
HB/FVL (+/-)	4	0	0	-
HB/FVL (+/+)	8	1	1	6 min

WT: wild-type controls; FVL: Factor V Leiden; HA: hemophilia A; HB: hemophilia B.

FVL restores the ability to form thrombi in hemophilic mice at the microcirculation level

We monitored platelet accumulation during real-time imaging of the laser-induced endothelial damage in the microcirculation. The composite image consisted of a brightfield image of the thrombus and fluorescence image of platelets (Fig.3). In normal mice (n= 3), we determined that all injured arteriole sites (n=30) resulted in clot formation. In addition, we characterized the thrombus formation of FVL homozygous mice (n= 4), and as expected, clots formed in all (n= 40) injured sites (Table 2).

Table 2: Summary of thrombus formation following laser-induced endothelial damage.

Genotype	N. of mice	N. of sites injured	N. of clots (%)
WT	3	30	30 (100)
FVL	4	40	40 (100)
Hemophilia A			
FVL (+/+)	5	45	45 (100)
FVL (+/-)	6	43	43 (100)
FVL (-/-)	8	39	0*
F.VIII infusion	3	8	8 (100)
Hemophilia B			
FVL (+/+)	4	40	40 (100)
FVL (+/-)	3	18	18 (100)
FVL (-/-)	4	16	0*
F.IX infusion	3	21	21 (100)
Infusion of FVa			
Hemophilia A	2	8	8 (100)
Hemophilia B	2	13	13 (100)
Infusion of FV			
Hemophilia A	2	10	0*
Hemophilia B	2	11	0

WT: wild-type controls; FVL: Factor V Leiden; (+) Denotes presence of FVL mutation. For infusion experiments, purified F.IX (Mononine, Aventis Behring, Kankakee, IL) and recombinant F.VIII (Kogenate FS, Bayer, West Haven, CT) were used.
* Fischer's exact test was used for statistical analysis comparison between hemophilic mice with and without FVL ($P < 0.001$) or mice infused with FV and FVa ($P < 0.001$).

In contrast, no thrombus formation was detected in mice with hemophilia A (n= 8) or B (n= 4) following successive vascular injuries to a total of 68 injury sites, averaging 3-10 sites per mouse (Fig. 3, panel A; Table 2). However, when these animals received intravenous injection of human purified FVIII or FIX concentrates at doses to achieve ~100% of normal levels, clots formed in all injury sites (Fig. 3, panel B).

We next tested hemophilia A (n= 5) or B mice (n=4) homozygous for FVL. Clot formation was observed in all 85 injured sites (ranging from 5-10 sites/mouse) and remained stable for the duration of the experiment (Fig. 3 panels E and F). Interestingly, similar results were obtained in hemophilia A (n= 6) or hemophilia B (n= 3) mice heterozygous for FVL. In this group, a total of 61 injury sites were analyzed and thrombus formation was consistently detected (Fig. 3, panel D).

Time course of platelet deposition is shown in Fig. 4 for groups of hemophilic mice. The data is represented by the median values of RFU derived from the platelet deposition, which represent a relative comparison among different groups tested. There was a increase in platelet deposition over time for both hemophilia models with FVL as compared to hemophilia mice without the mutation (Fig. 4, panel B).

Infusion of purified FVa induces thrombus formation in murine models of hemophilia A or B

Initially we have determined that murine thrombin activates human FV in a similar fashion to human thrombin. To test if transient increment in the FV or FVa levels could mimic the *in vivo* effect of FVL, we injected mice with purified human FV or FVa. Because FVa is secreted in the active form, there is no requirement for thrombin activation of the purified protein [29]. Therefore, direct interpretation of these data is not confounded by traces of thrombin in the protein solution infused. Infusion of 30 μg of FVa/mouse into hemophilia B mice resulted in discrete shortening of the aPTT from 77±3 seconds at baseline to 72±4 seconds at both time points (15 or 120 minutes) post infusion. These data were in good agreement with increased levels of TAT from 15±5 ng/ml (at baseline) to 69±10 and 67±22 ng/ml at 15 and 120 minutes, respectively. We next monitored clot formation in the intravital microscopy. Prior to the protein infusion, no clot formation was observed in both hemophilia A and B mice, as expected. Following infusion of 30 μg or 100 μg of FVa, platelet accumulation was readily observed at all injury sites (Fig. 4, panel C and D). In contrast, when mice were injected with the procofactor FV at comparable doses to achieve similar plasma concentration (0.180 or 0.360 pmoles), no clot formation was detected. Collectively these results were similar for both hemophilia models (Table 2 and Fig. 4, panels C-D).

Discussion

The assessment of the clinical impact of FVL on the hemophilia phenotype has been controversial and hampered by the complex interaction of other acquired and genetic modifying factors. Therefore, the use of murine models minimizes the influence of several acquired factors. The occurrence of spontaneous bleeding episodes in murine models for severe hemophilia A or B is rare. Thus, to properly address the effect of FVL on the severe hemophilia phenotype, a series of *in vivo* hemostatic tests were imposed upon these animal models.

Hemophilia mice with FVL mutation presented improvement in hemostasis, as determined by shortening of the aPTT-based assays and increased TAT levels, which reflects enhanced thrombin generation. To address whether improvement on *in vitro* parameters of hemostasis had any relevant impact on the hemophilia phenotype *in vivo*, mice underwent a series of hemostatic challenges.

In one model a mechanical injury was induced by transection of the tail vessels; in a second, the injury was induced by a ferric chloride chemical to the carotid artery. The latter model is characterized by oxidative injury that disrupts the endothelium and exposes the subendothelium [30]. Hemophilia B mice homozygous for FVL presented modest improvement of hemostasis in both methods when compared with littermates without FVL whereas among hemophilia A models no improvement was found. It is possible that differences in mouse strains may affect some of the hemostatic parameters, as already shown for FVL [22]. These data suggest that, upon injury of large vessels, FVL does not provide a major beneficial hemostatic effect.

The real-time imaging of thrombus formation developed by Furie and colleagues [28], in conjunction with the laser-induced endothelial injury model [31], provides a sensitive method for evaluation of hemostasis at the microcirculation of a living animal. It has been observed that the outcome of this method is not full occlusion of the vessel lumen; rather, endothelial cells appear to be activated instead of disrupted with less exposure of the subendothelium [28, 31]. In hemophilia mice with no FVL mutation, no clot was observed even upon successive laser expositions. However, following replacement of the missing clotting factor by intravenous infusion of human FVIII or FIX concentrates, clot formation was observed in all arteriole sites injured. Hemophilia A or B mice homozygous for the FVL mutation have restored ability to form a thrombus. Interestingly, among hemophilia mice heterozygous for FVL, thrombus formation is comparable to homozygous FVL. This is particularly interesting since most human subjects with hemophilia and FVL carried only one FVL allele.

When extrapolating data from murine models to humans it is important to consider the differences between human and murine FV such the origin of FV synthesis [32, 33] and differences in the APC pathway such as the ability of human aPC to properly inactivate murine FVa [34], and the absence of the plasma protein C inhibitor [35]. To better assess the role of the APC pathway as a modifier of the hemophilia phenotype, other components such as thrombomodulin or endothelial protein C receptor (EPCR) functions [36] need to be investigated. The vascular distribution of thrombomodulin and EPCR differs as a function of vessel size. The results of murine models are informative because improvements in hemostasis by FVL seem to be vessel-dependent (i.e. micro *vs.* macro circulation). Thrombomodulin concentration in the microcirculation is >1000-fold higher than in vessels of ~0.3 cm diameter, which facilitates binding to thrombin and consequently clotting activity is depressed. We speculate that in the presence of FVL, the continuous thrombin generation at the microcirculation may lead to high free thrombin; therefore, the coagulation is locally enhanced. In large vessels, such as the murine carotid artery (diameter of ~0.55mm), thrombin is likely free since thrombomodulin levels are relatively low [37, 38]. Therefore, the impact of the FVL is not sufficient to significantly alter hemostasis at the macrocirculation level.

Using human hemophilia A plasma, Mann and colleagues demonstrated that slow formation of FVa is an additional factor that impacts the impaired thrombin generation. Because free FXa is rapidly inactivated, the presence of fully active FVa complexed with FXa in the prothrombinase complex is critical to prevent FXa inactivation by antithrombin and tissue factor pathway inhibitors [39]. Further experiments showed that increasing FV levels up to 150% in hemophilia A plasma did not result in significant enhancement of thrombin, whereas adding FVL to levels of 100% or 150% enhanced thrombin generation by 3 and 5-fold, respectively [15]. Recently, a similar effect was determined in hemophilia B plasma [16]. Therefore, we hypothesized that an increase in FVa levels could mimic the effect of FVL in hemophilia mice. The results demonstrated that FVa, but not FV, has the ability of restoring the hemophilia phenotype at the microcirculation level, in a similar manner as findings indicate that FVL does.

There is evidence that in humans FV, but not FVL, presents cofactor activity in the APC-mediated inactivation of FVIIIa. Recently, Simioni et al demonstrated increased thrombin generation and risk for venous thrombosis among homozygous FVL and heterozygous for FVL with partial FV deficiency (pseudo-homozygous APC resistance) when compared to subjects genotyped as heterozygous for the FVL mutation (wild-type FV activity preserved) [40]. Here we found that littermate hemophilic mice heterozygous and homozygous for FVL presented improvement of both *in vitro* and *in vivo* hemostatic parameters at similar fashion. These data suggest that it is slow inactivation of FVa with consequently procoagulant activity that underlying the potential benefits of FVL in hemophilia. Therefore, it is possible that murine FV does not present cofactor activity on FVIIIa-inactivation by APC. Although these experimental conditions may present distinct underlying mechanisms, data obtained in both genetic models and protein injection demonstrate the beneficial effects of the enhancement in thrombin levels, as the final outcome, and suggest that other alternative therapeutic approaches for hemophilia could be investigated.

In summary, this work demonstrated that the FVL mutation enhances hemostasis in hemophilia A or B mice as judged by the improvement of the clotting times and by the *in vivo* ability to form clots. Because FVL has markedly beneficial effects at the microcirculation level in an injury that predominantly does not expose the subendothelium, the protection against hemorrhagic challenges are likely to impact minor bleedings, but not those trauma-induced hemorrhages. These data explain in part the heterogeneity of the clinical diversity observed for the severe hemophilia phenotype with FVL especially among adults.

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While certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications may be made thereto without departing from the scope and spirit of the present invention, as set forth in the following claims.

What is claimed is:

1. A method for the treatment of hemophilia in a patient in need thereof, comprising administering an effective amount of an activated form of FV variant or a derivative thereof, thereby enhancing clot formation in said patient and ameliorating the symptoms of acquired and inherited bleeding disorder..

2. The method of claim 1, wherein said derivative is selected from the group consisting of FV-810; factor V lacking amino acids 811-1491, FV-859; factor V lacking amino acids 860-1491; FV-866; factor V lacking amino acids 867-1491; FV-902; factor V lacking amino acids 903-1491; FV-924; factor V lacking amino acids 923-1491; FV-937; factor V lacking amino acids 938-1491; FV-956; factor V lacking amino acids 957-1491; FV-1033-B58-s131; factor V lacking amino acids 1034-1491 with amino acids 900-1030 exchanged with amino acids 907-1037 of factor VIII; FV-1033-B58-s104; factor V lacking amino acids 1034-1491 with amino acids 904-1007 exchanged with amino acids 972-1075 of factor VIII; and FV-1033-B58-s46; factor V lacking amino acids 1034-1491 with amino acids 963-1008 exchanged with amino acids 1032-1077 of factor VIII.

3. The method of claim 1, for the treatment of hemophilia A.

4. The method of claim 1, for the treatment of hemophilia B.

5. The method of claim 1, wherein said activated form of FV or derivative thereof is delivered intravenously.

6. The method of claim 1, wherein a vector encoding the activated form of FV or derivative thereof is administered to said patient.

7. The method of claim 1, wherein said variant is a pro-coagulant and said disorder is selected from the group consisting of hemophilia A and B, hemophilia A and B associated with inhibitory antibodies, coagulation factor deficiency, vitamin K epoxide reductase C1 deficiency, gamma-carboxylase deficiency, bleeding associated with trauma, injury, thrombosis, thrombocytopenia, stroke, coagulopathy,

disseminated intravascular coagulation (DIC); over-anticoagulation treatment disorders, Bernard Soulier syndrome, Glanzman thromblastemia, and storage pool deficiency.

8. The method of claim 7, wherein said over-anticoagulation treatment disorder results from administration of heparin, low molecular weight heparin, pentasaccharide, warfarin, small molecule antithrombotics and FXa inhibitors.

9. The method of claim 1, wherein said variant is administered intravenously at least once a day at a dosage between about 10 and 500 μg/kg.

10. An isolated nucleic acid molecule encoding an activated form of FV, wherein said activated form of FV is selected from the group consisting of FV-859, FV-866, FV-924, and FV-937.

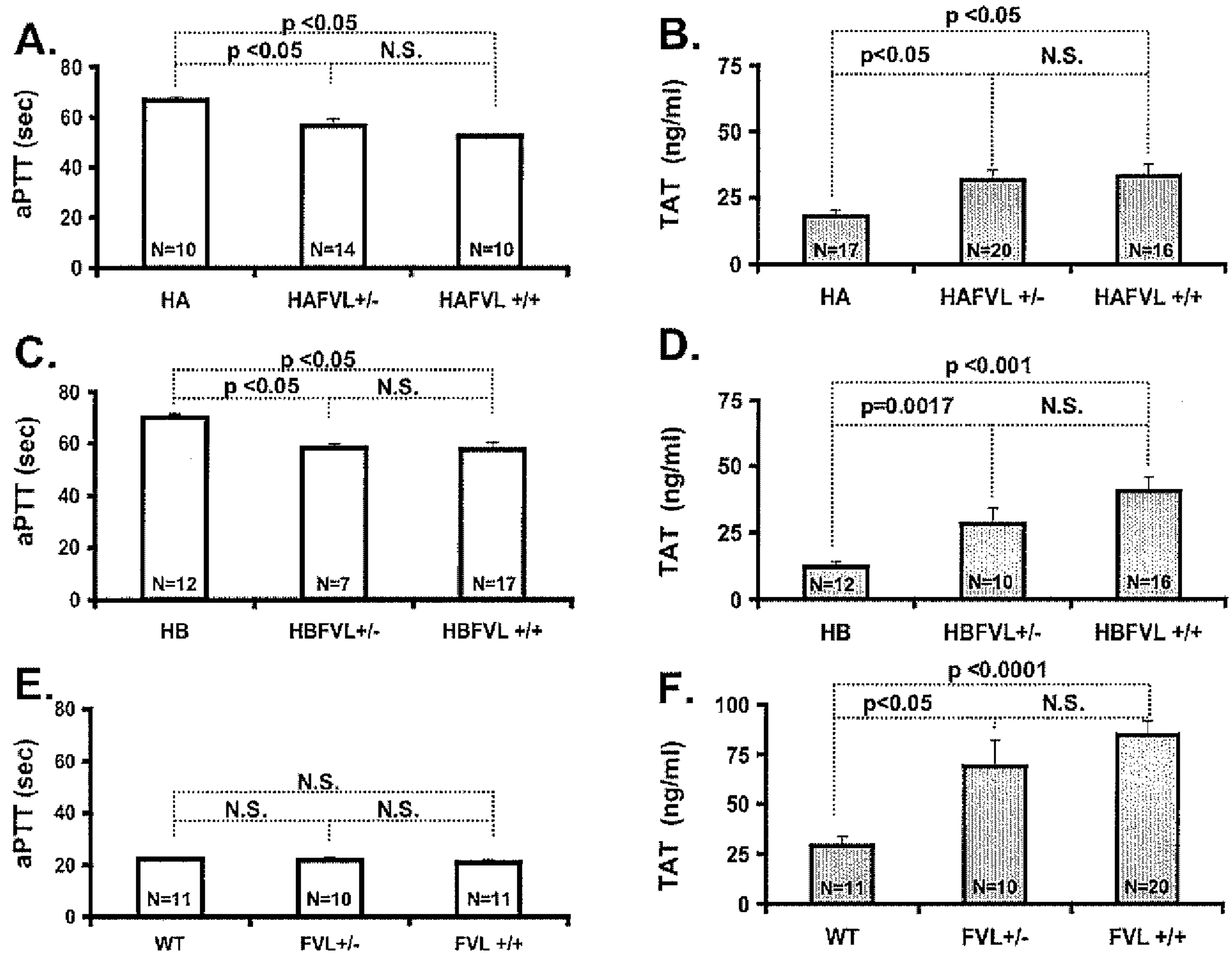
11. An isolated activated form of FV, wherein said activated form of FV is encoded by the nucleic acid molecule of claim 10.

12. An isolated nucleic acid molecule encoding an activated form of FV, wherein at least one of the arginine residues at positions 506, 306, and 679 of said activated form of FV is replaced with an amino acid other than arginine.

13. The nucleic acid molecule of claim 12, wherein the arginine residues are replaced with a glutamine.

14. The nucleic acid molecule of claim 12, wherein said activated form of FV is selected from the group consisting of FV-810; FV-859; FV-866; FV-902; FV-924; FV-937; FV-956; FV-1033-B58-s131; FV-1033-B58-s104; and FV-1033-B58-s46.

15. An isolated activated form of FV, wherein said activated form of FV is encoded by the nucleic acid molecule of claim 12.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N=14
N=10
HA
HAFVL+/-
HAFVL +/+
B.
p <0.05
p<0.05
N.S.
TAT (ng/ml)
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N=20
N=16
HA
HAFVL +/-
HAFVL +/+
C.
p <0.05
p <0.05
N.S.
aPTT (sec)
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N=7
N=17
HB
HBFVL+/-
HBFVL +/+
D.
p <0.001
p=0.0017
N.S.
TAT (ng/ml)
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N=10
N=16
HB
HBFVL+/-
HBFVL +/+
E.
N.S.
N.S.
N.S.
aPTT (sec)
N=11
N=10
N=11
WT
FVL+/-
FVL +/+
F.
p <0.0001
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N.S.
TAT (ng/ml)
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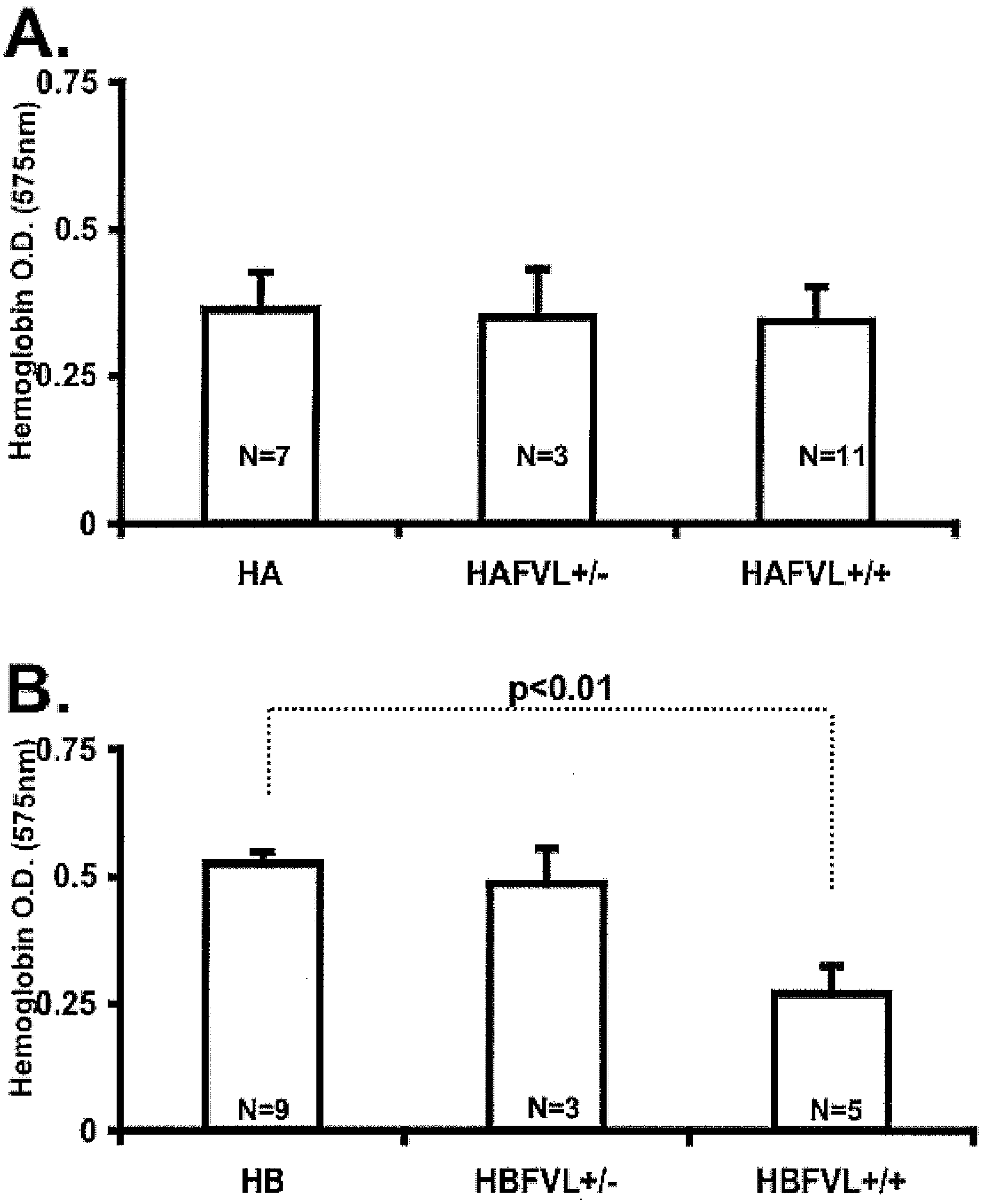
N=20
WT
FVL+/-
FVL +/+

Figure 1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0.5
0.25
0
N=7
N=3
N=11
HA
HAFVL+/-
HAFVL+/+
B.
p<0.01
Hemoglobin O.D. (575nm)
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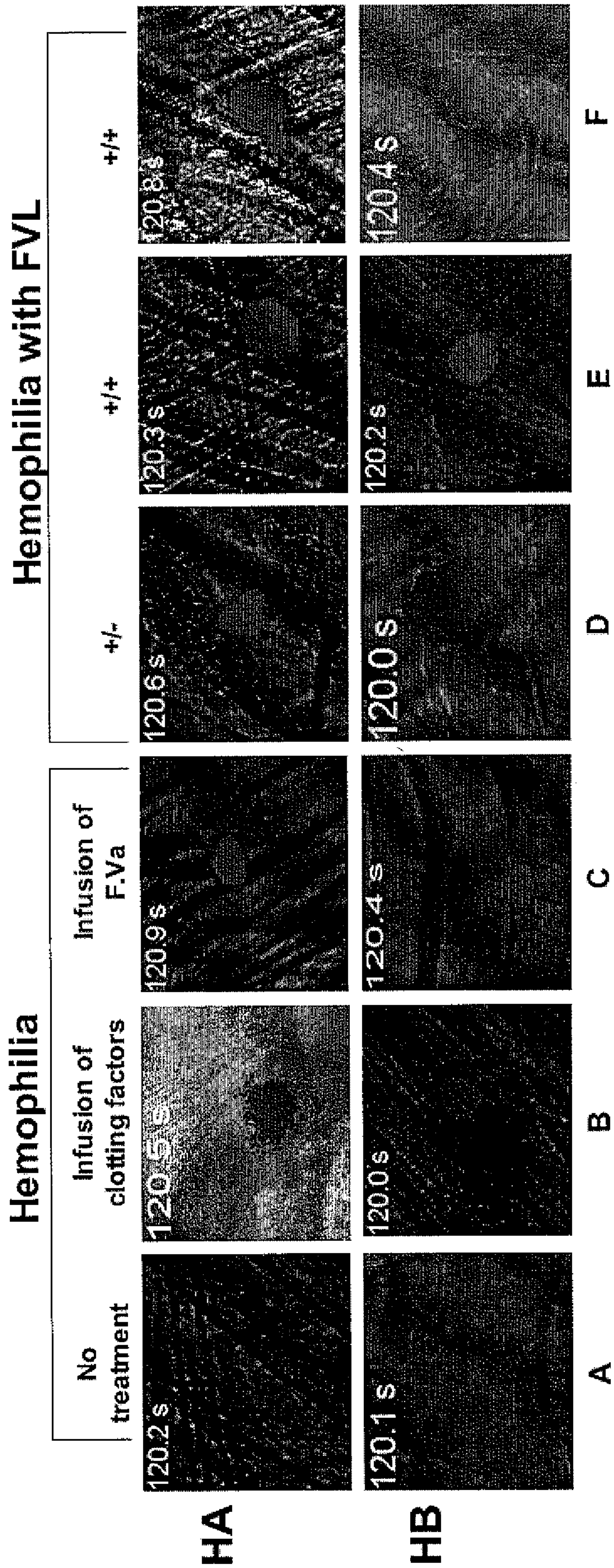
0.5
0.25
0
N=9
N=3
N=5
HB
HBFVL+/-
HBFVL+/+

Figure 2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Hemophilia with FVL
No treatment
Infusion of clotting factors
Infusion of F.Va
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+/+
+/+
HA
HB
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120.5 s
120.9 s
120.6 s
120.3 s
120.8 s
120.1 s
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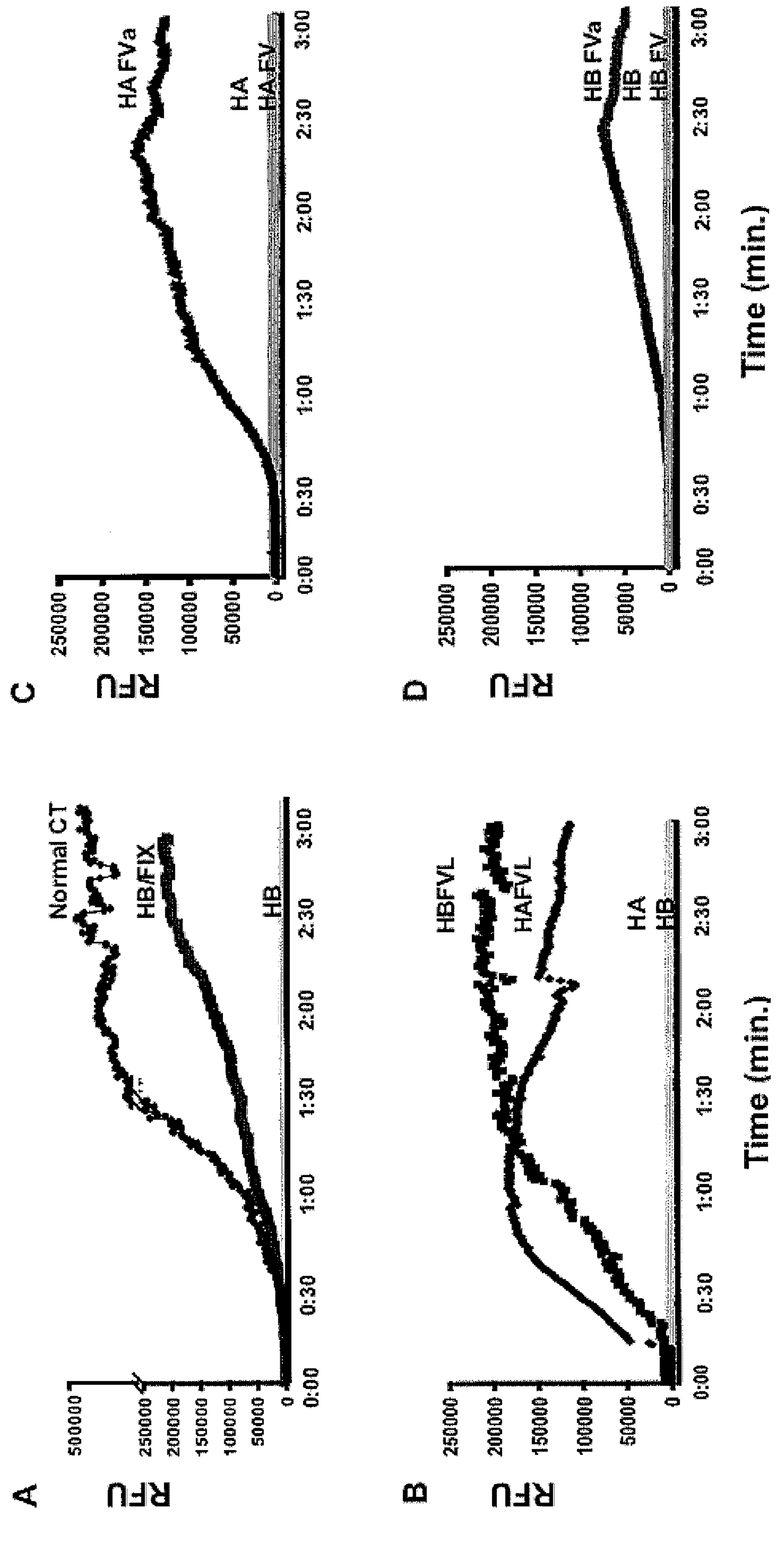
120.0 s
120.2 s
120.4 s
A
B
C
D
E
F

Figure 3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250000
200000
150000
100000
50000
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0:30
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2:00
2:30
3:00
Normal CT
HB/FIX
HB
Time (min.)
B
RFU
250000
200000
150000
100000
50000
0
HBFVL
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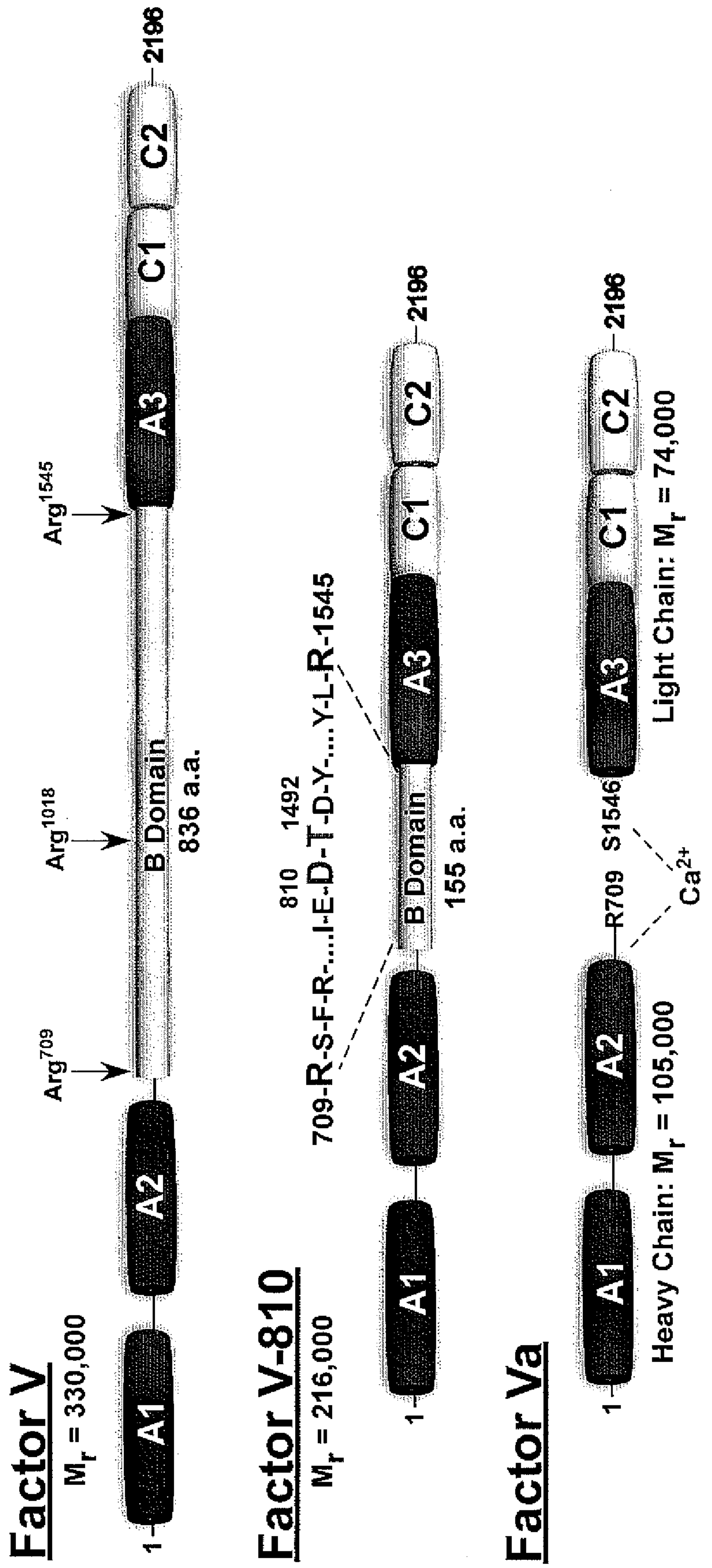
HB
C
RFU
HA FVa
HA
HA FV
D
RFU
HB FVa
HB
HB FV

Figure 4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A3
C1
C2
2196
Factor V-810
Mr = 216,000
1
A1
A2
810 1492
709-R-S-F-R-....I-E-D-T-D-Y-....Y-L-R-1545
B Domain
155 a.a.
A3
C1
C2
2196
Factor Va
1
A1
A2
R709
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Ca2+
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A3
C1
C2
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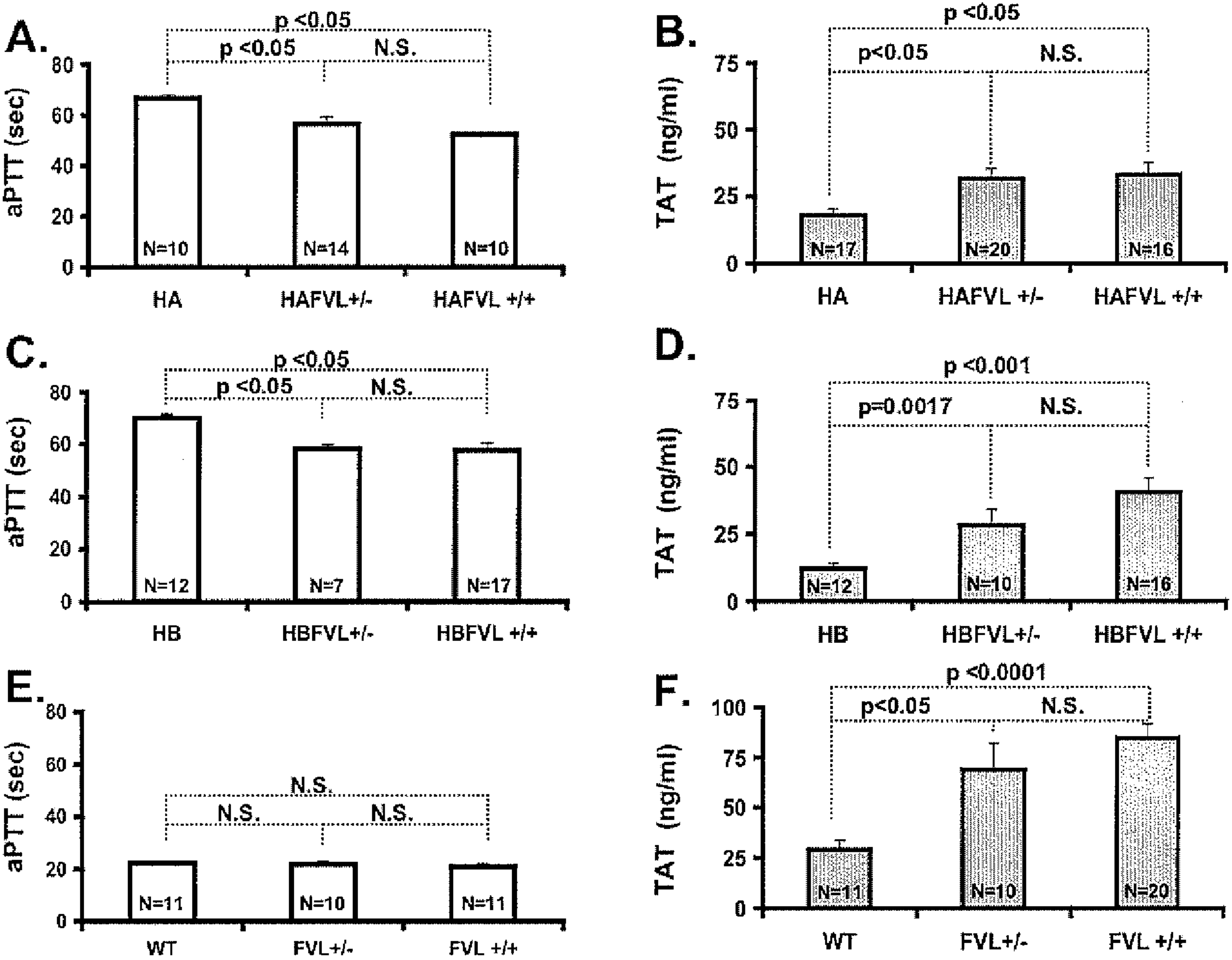
Light Chain: Mr = 74,000

Figure 5



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p <0.05
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20
0
N=10
N=14
N=10
HA
HAFVL+/-
HAFVL +/+
B.
p <0.05
p<0.05
N.S.
TAT (ng/ml)
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25
0
N=17
N=20
N=16
HA
HAFVL +/-
HAFVL +/+
C.
p <0.05
p <0.05
N.S.
aPTT (sec)
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N=17
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TAT (ng/ml)
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40
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TAT (ng/ml)
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75
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