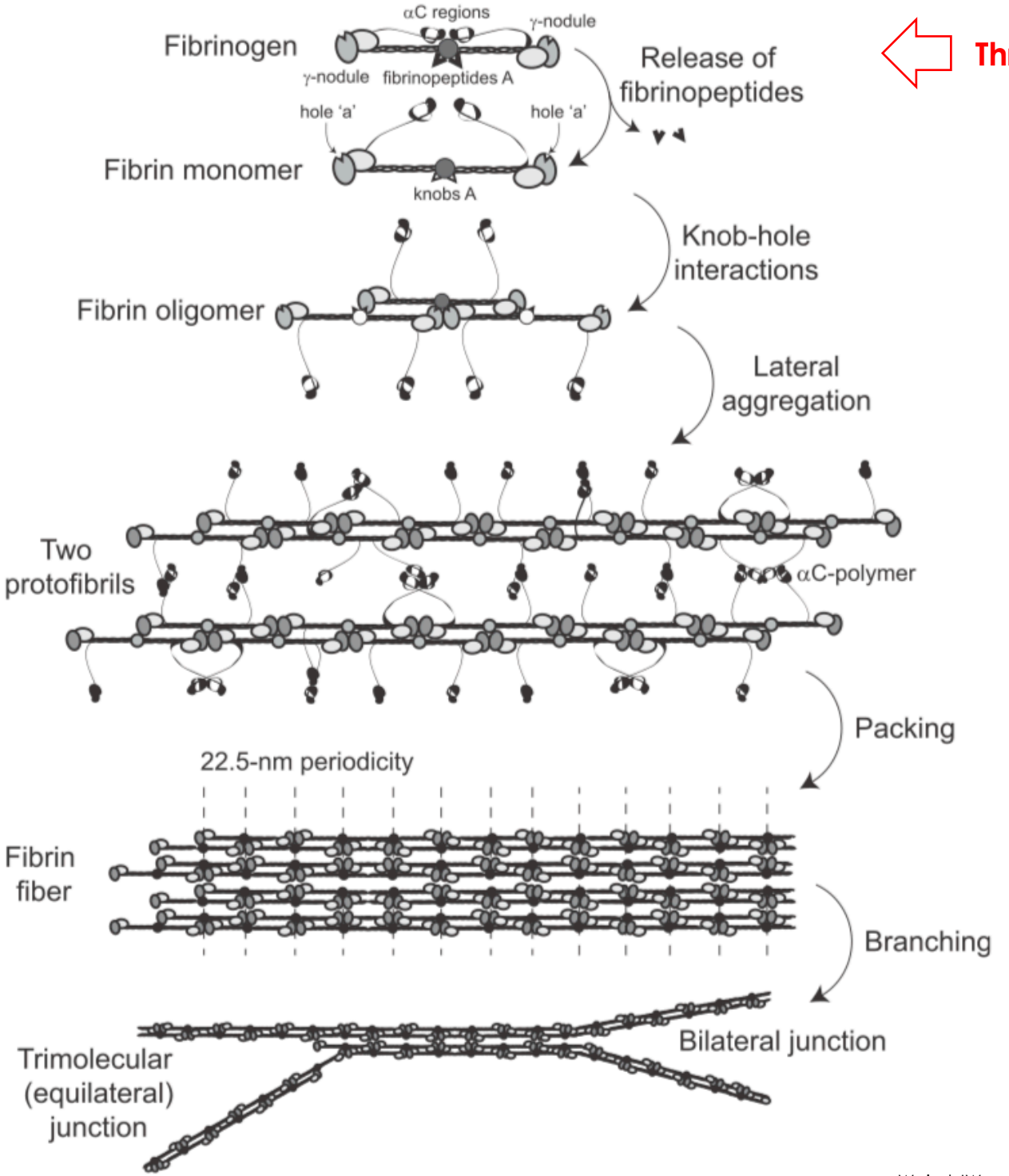
Thr Fibrinogen to fibrin

SOLUBLE

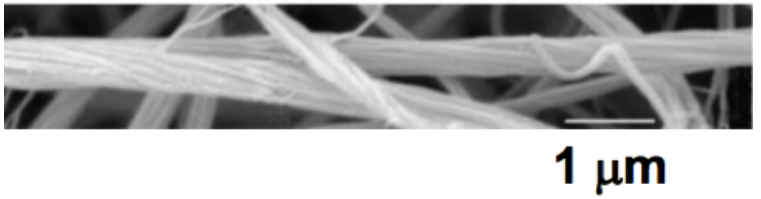
↓

INSOLUBLE



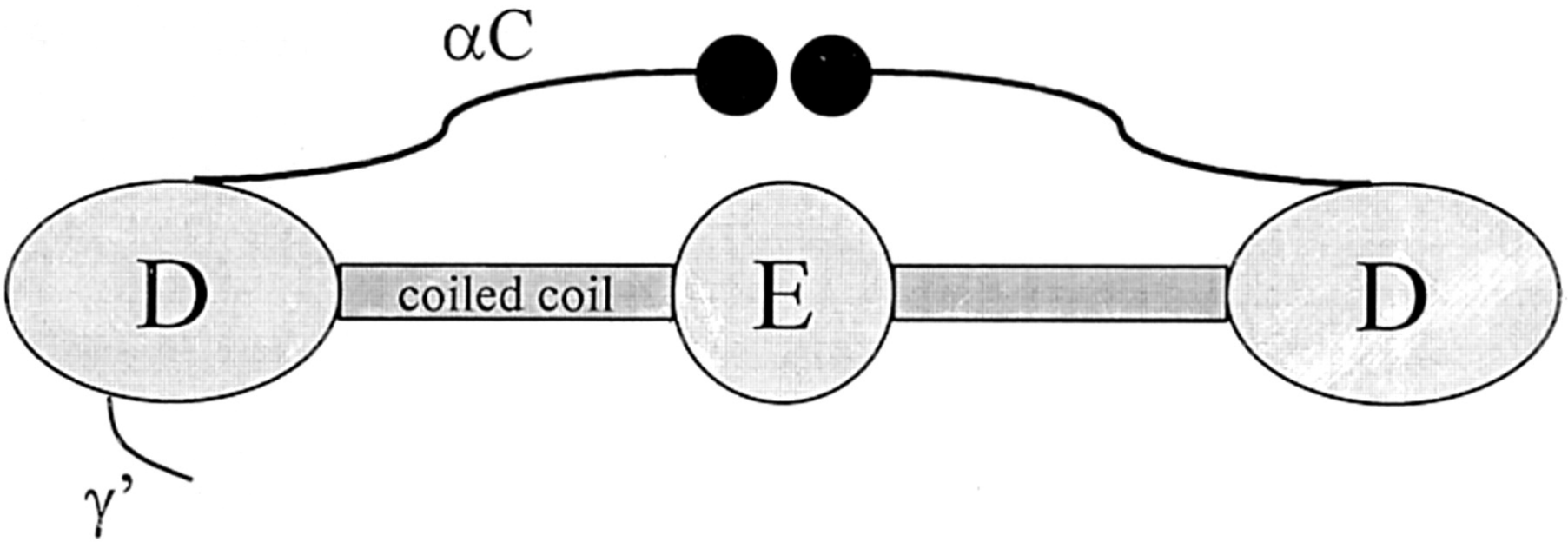
Critically important intermediate product, capable to undergo toward lateral aggregation (branching) -> 3D network

Fibrin fiber

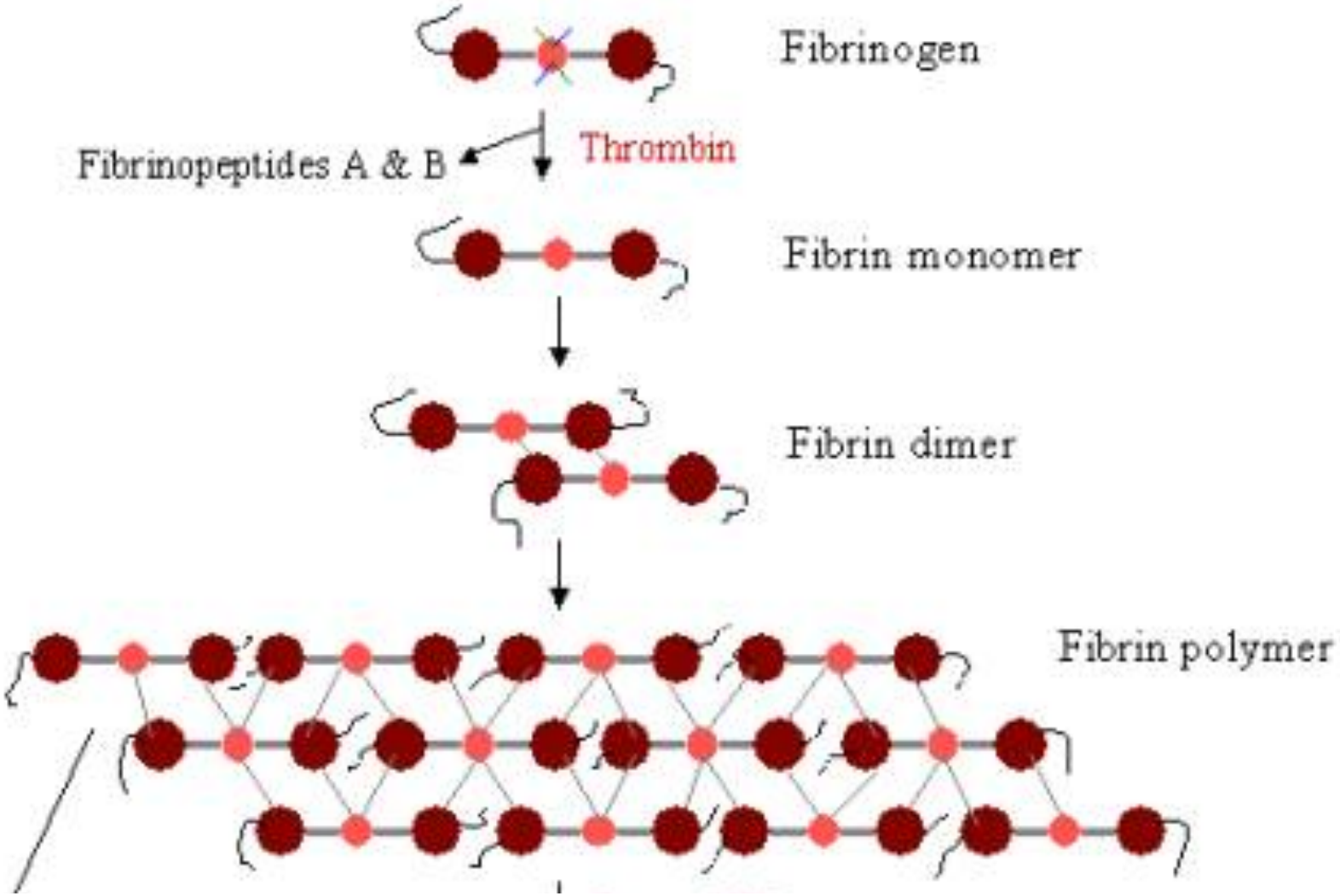


Weisel JW and Litinov RI Blood 20013

Schematic representation of the fibrinogen molecule.Fibrinogen consists of 6 polypeptide chains held together by disulfide bonds in a molecule with bilateral symmetry.

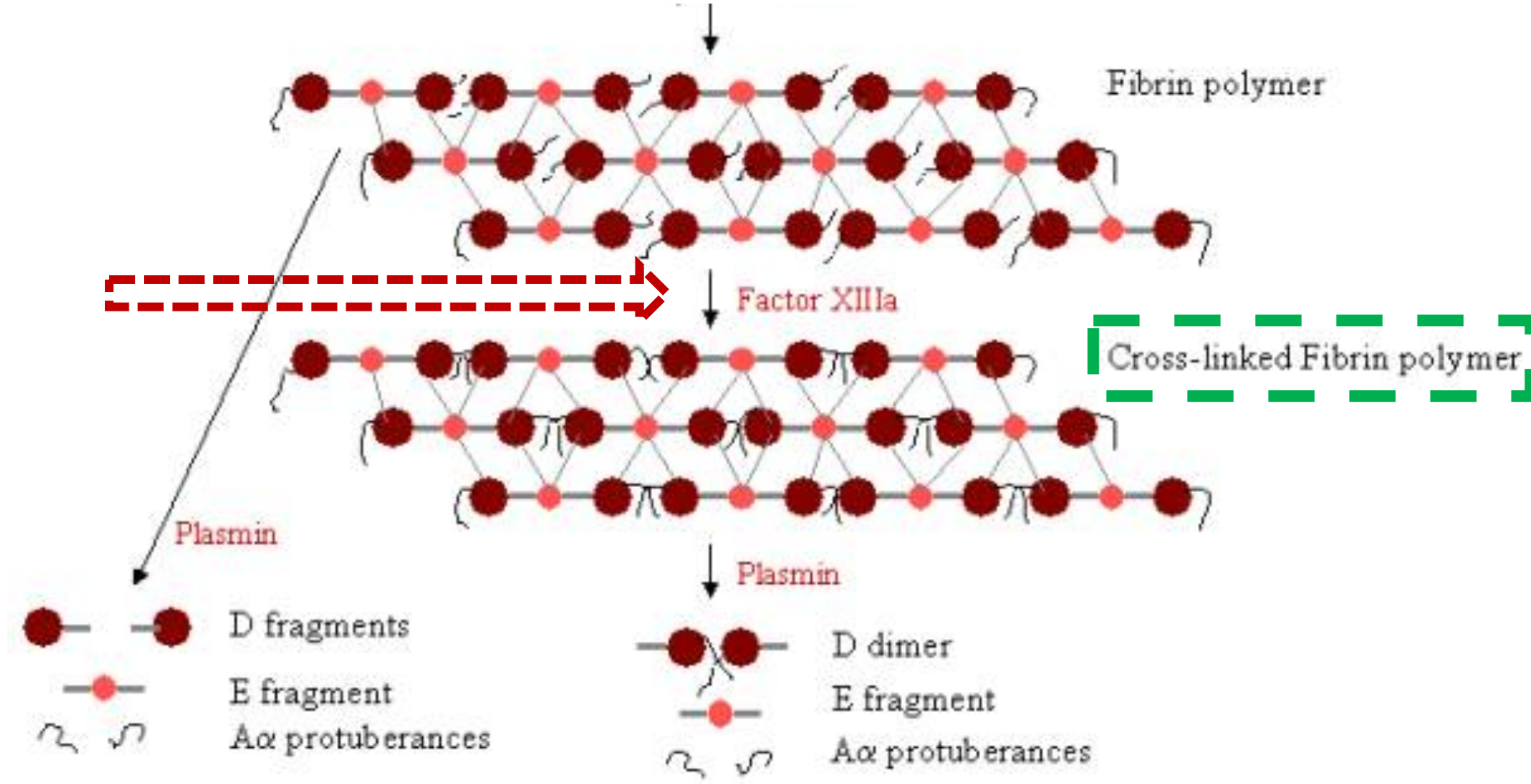


Robert A. S. Ariëns et al. Blood 2002;100:743-754

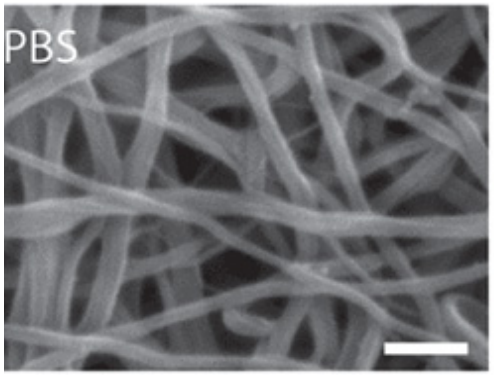


Parte finale coagulazione

Parte finale coagulazione «Cross-Linking» della Fibrina

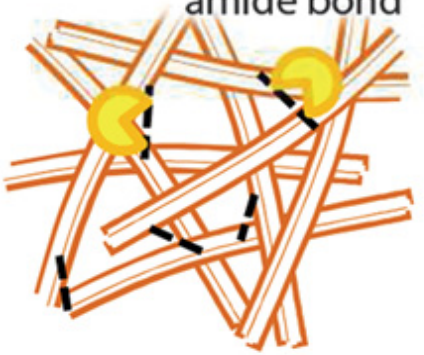


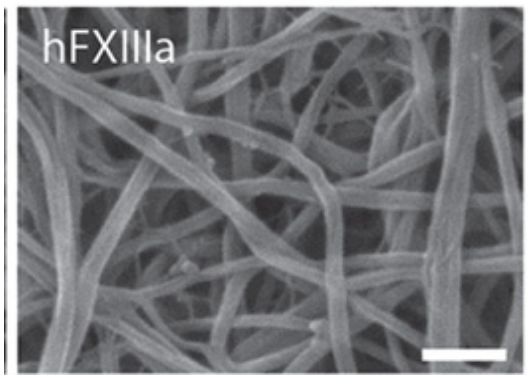
PBS



Factor XIIIa cross-linking

γ-Glutamyl-ε-lysyl amide bond





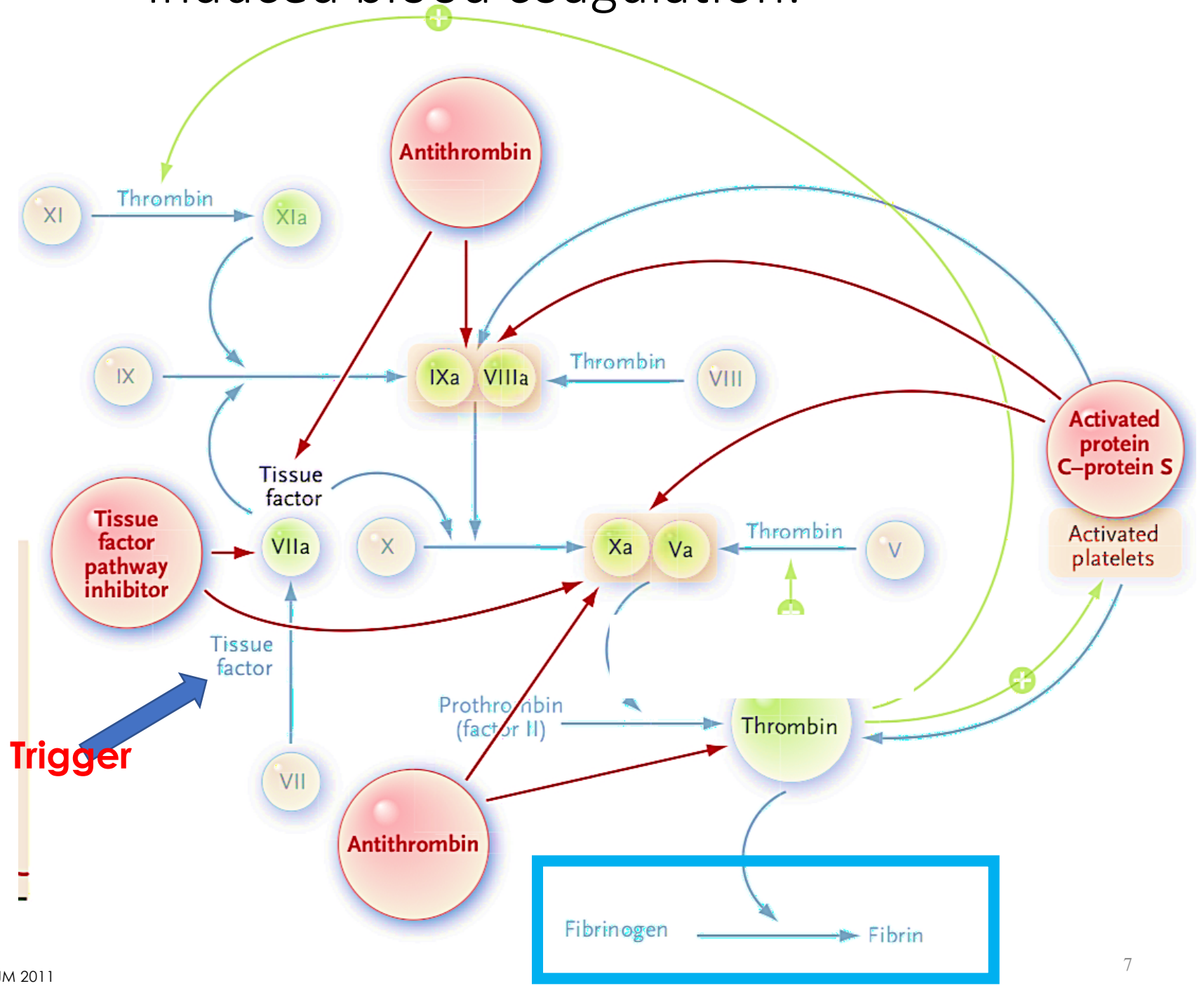
Studio del Cross-linking della fibrina nel plasma

Recalcified (10 mM, final) plasma was clotted with TF (1 pM) in the presence of increasing concentrations of the FXIIIa inhibitor T101.

Clots were dissolved and analyzed by western blotting with polyclonal anti-human fibrinogen antibody.

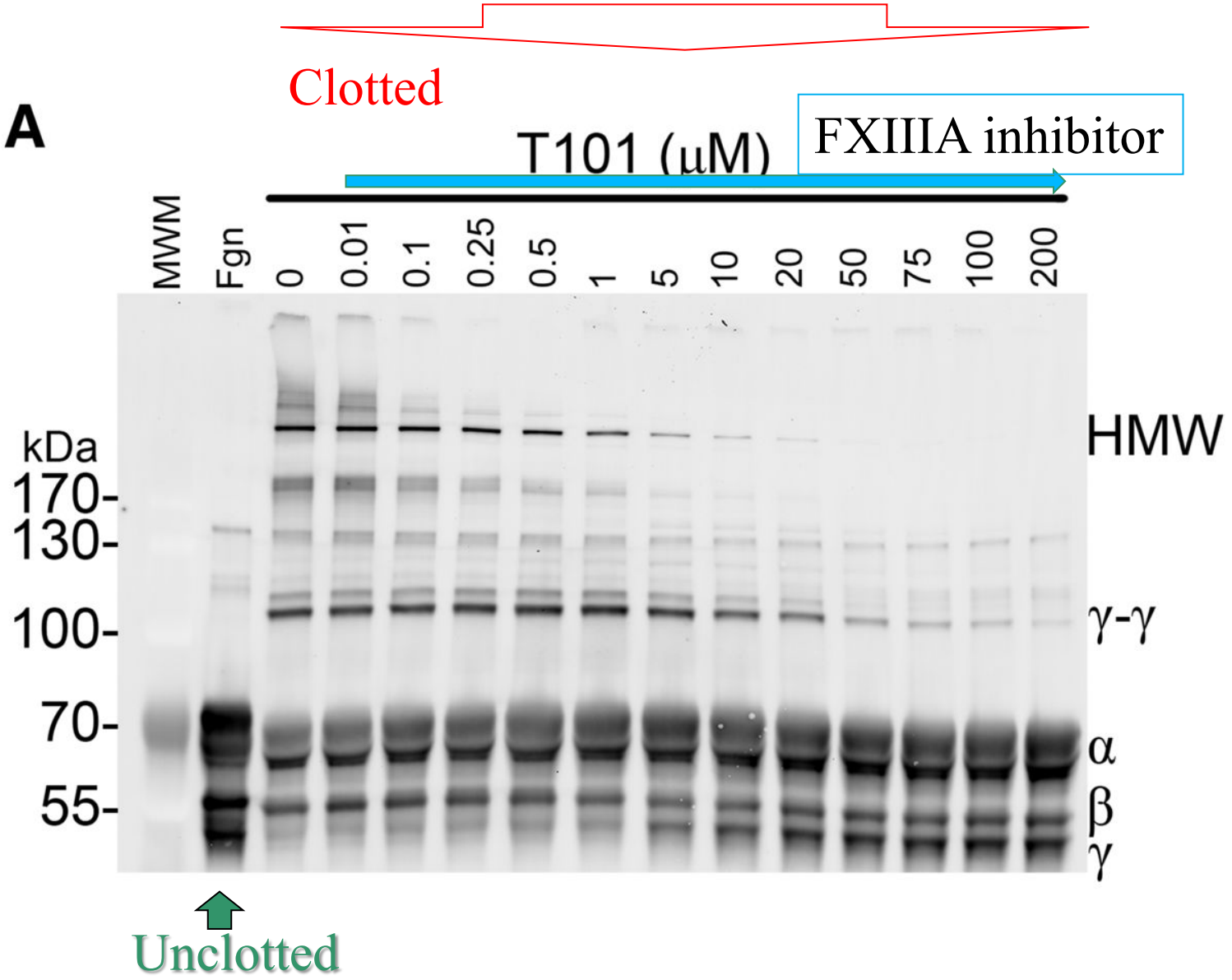
The second lane (Fgn) is unclotted plasma.

Induced blood coagulation:



Modified from Tripodi A NEJM 2011

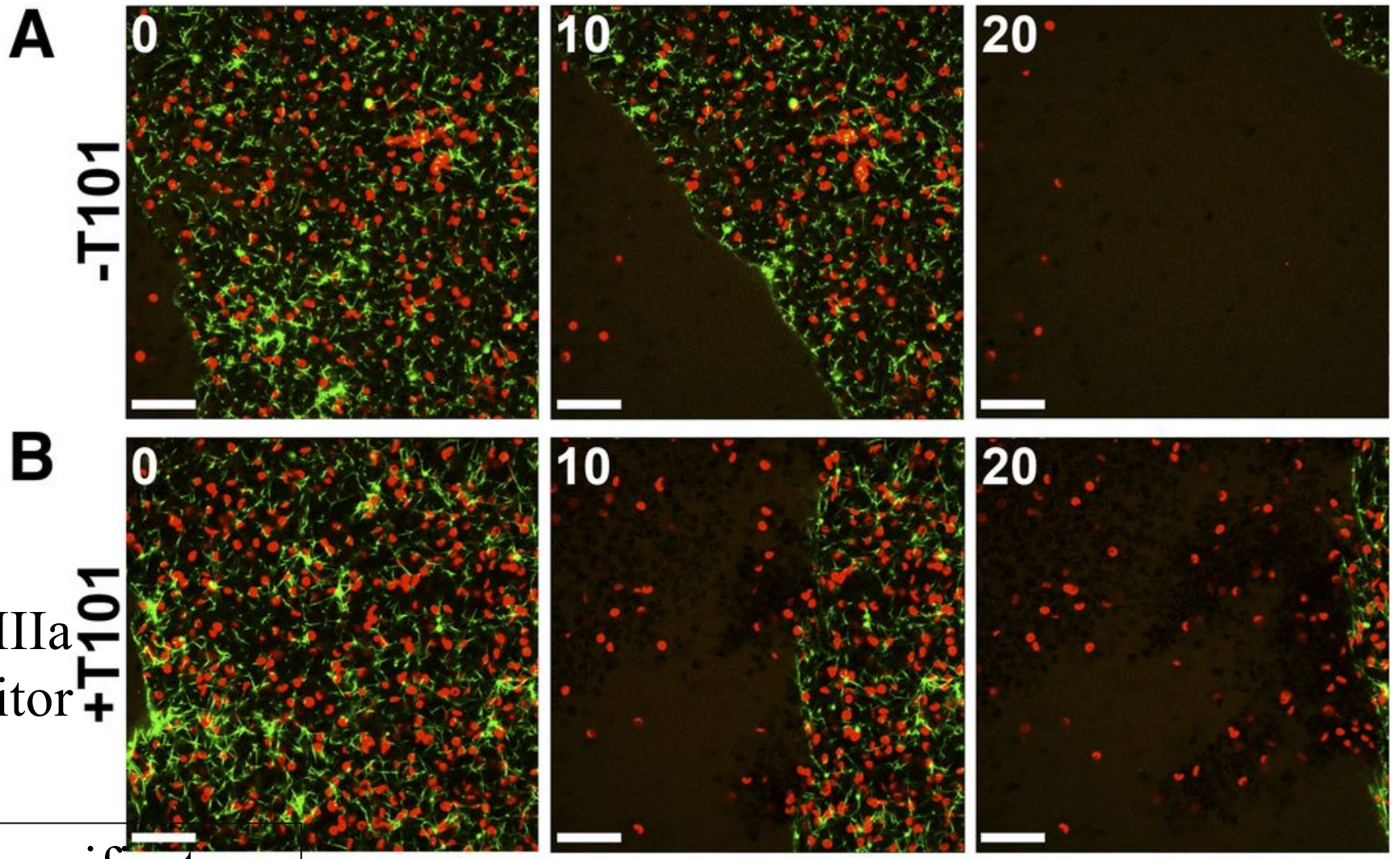
RBC retention is reduced at concentrations of T101 that inhibit α-chain crosslinking.



James R. Byrnes et al. Blood 2015;126:1940-1948

Clot formation and contraction in whole blood

RED octadecyl- rhodamine -labeled RBCs. GREEN -labeled fibrinogen,. Clot contraction



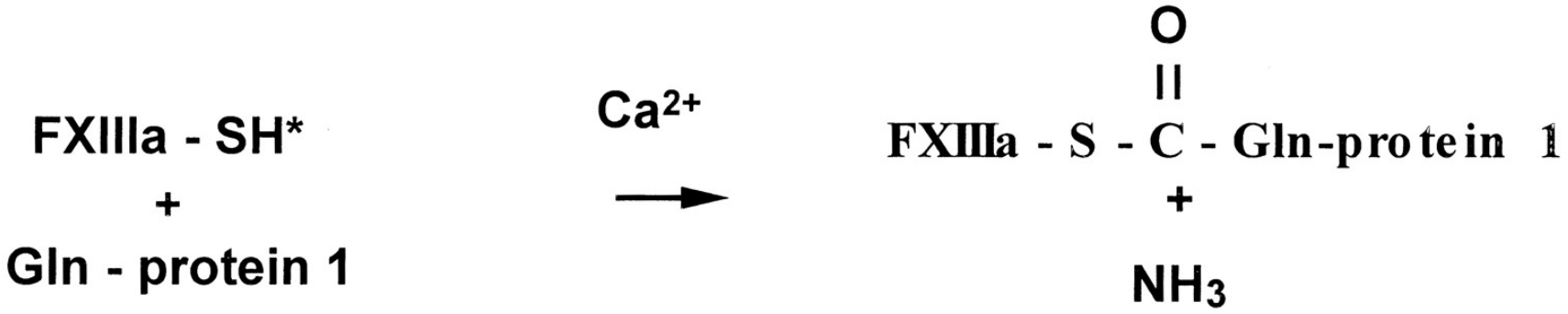
James R. Byrnes et al. Blood 2015;126:1940-1948

FXIIIa activity mantains RBCs within the clot during clot contraction.

blood

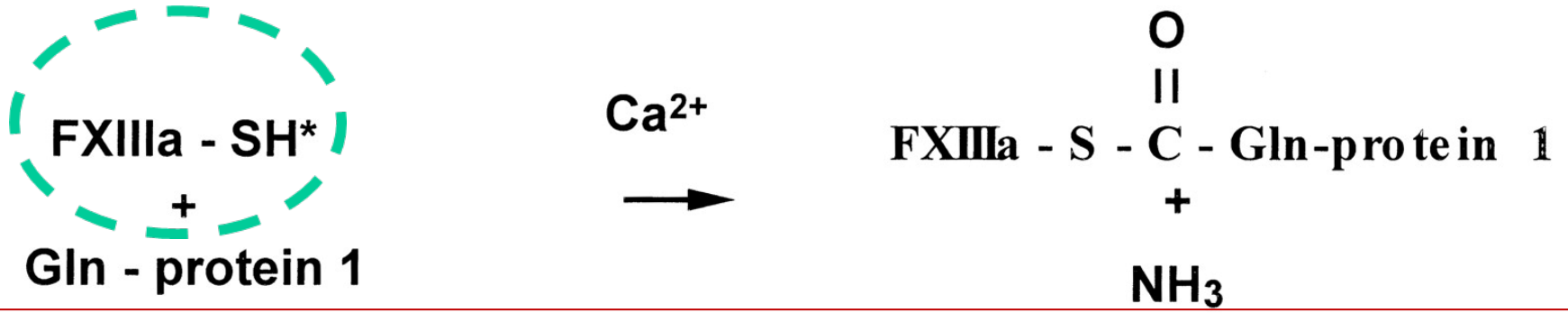
Cross-linking reaction catalyzed by activated factor XIII.Activated factor XIII

first forms a thioester bond with a selected protein-bound glutamine residue, releasing ammonia.

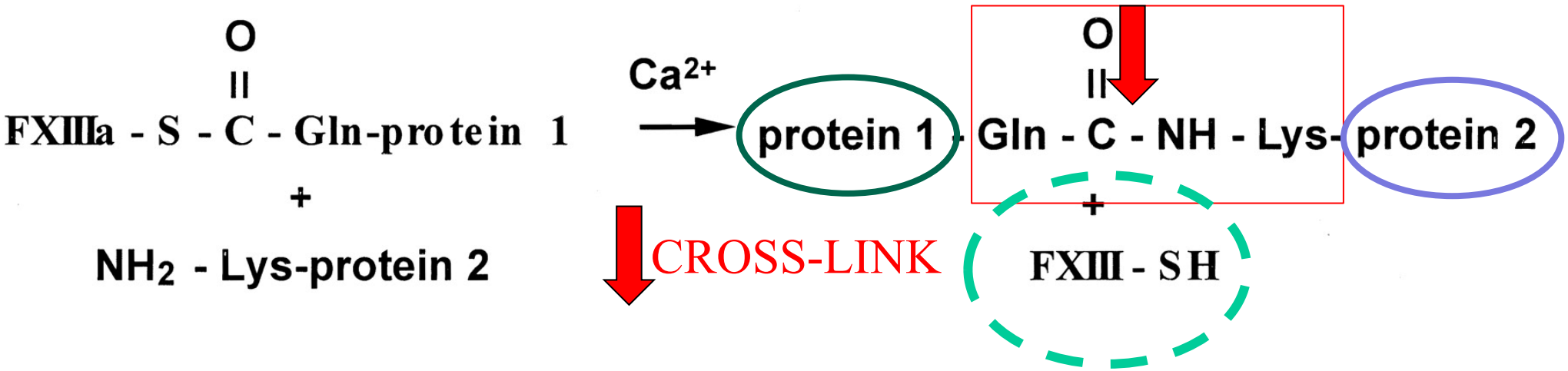


Robert A. S. Ariëns et al. Blood 2002;100:743-754

Cross-linking reaction catalyzed by activated factor XIII. Activated factor XIII first forms a thioester bond with a selected protein-bound glutamine residue, releasing ammonia, and …

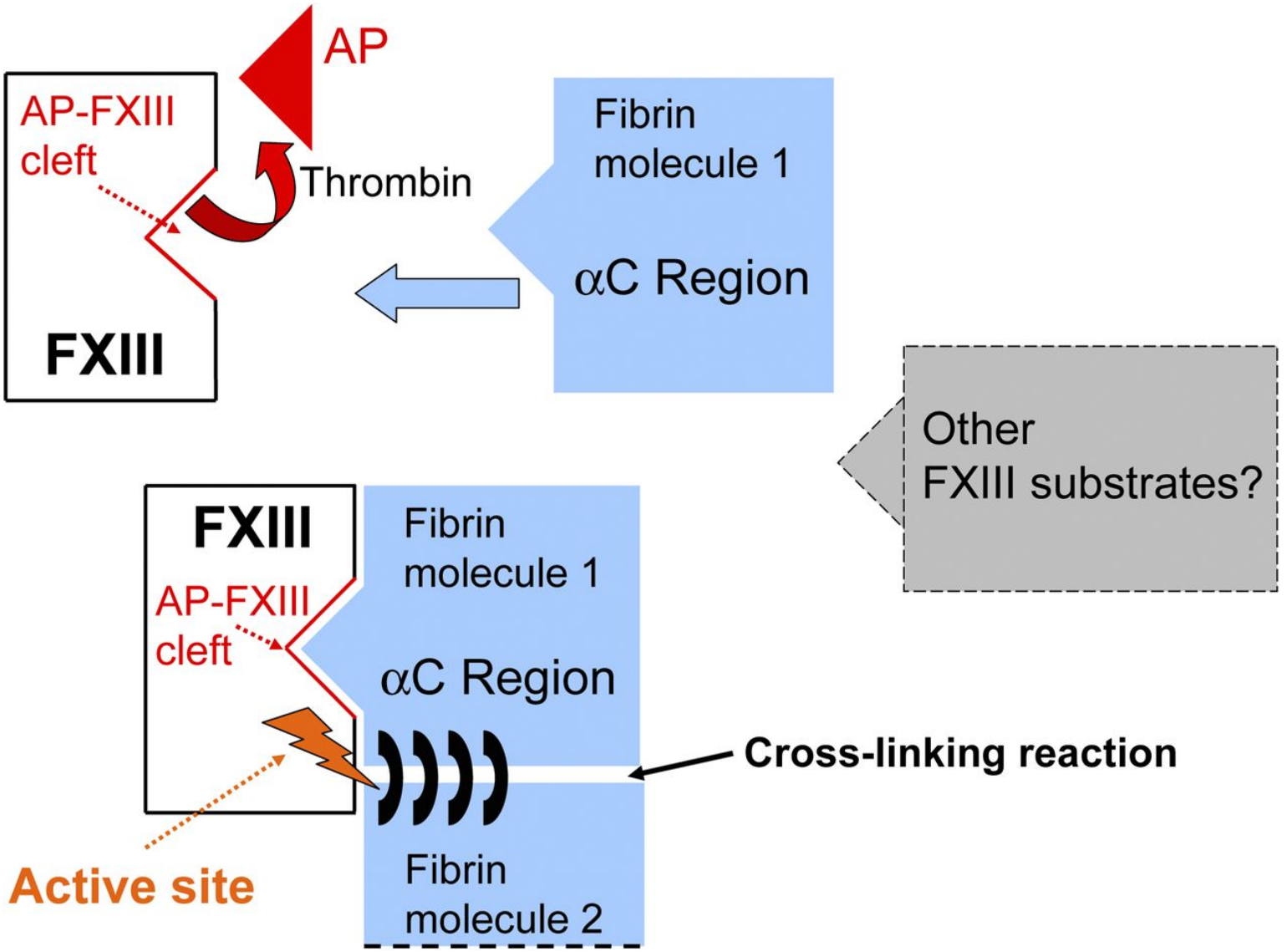


the thioester intermediate then reacts with a primary amine from a protein-bound lysine residue resulting in an amide or isopeptide bond



Robert A. S. Ariëns et al. Blood 2002;100:743-754

Simplified description of the interaction between activated FXIII and fibrin(ogen).



Hans P. Kohler Blood 2013;121:1931-1932

Studio del Cross-linking nel plasma

Table 1.

Factor XIII substrates

Substrate	Cross-linking site
Fibrin(ogen) γ-chain52–54	Gln398, Gln399, and Lys406
Fibrin(ogen) α-chain58–62	Gln221, Gln237, Gln328, Gln366, and 15 potential lysines from Lys208 to Lys606
α2-Antiplasmin67–69	Gln2
TAFI[150]	Gln2, Gln5, Gln292
PAI-2151 152	—
Fibronectin72 73	Gln3
Collagen72 80	—
Von Willebrand factor153 154	—
Vitronectin155 156	Gln93
Thrombospondin[157]	—
Factor V158 159	—
Actin160 161	—

Table 1.

Factor XIII substrates

Substrate	Substances with which it is cross-linked	Known or potential function
Fibrin(ogen) γ-chain52–54	Itself and α-chain	Clot stabilization
Fibrin(ogen) α-chain58–62	Itself and γ-chain	Clot stabilization
α2-Antiplasmin67–69	Lys303 fibrin α-chain	Resistance to fibrinolysis
TAFI[150]	Fibrin, itself	Resistance to fibrinolysis
PAI-2151 152	Lys148, Lys230, Lys413 fibrin α-chain	Resistance to fibrinolysis
Fibronectin72 73	Itself, fibrin, collagen	Migration of cells into the clot; wound healing
Collagen72 80	Fibronectin, fibrin	Stabilization of extracellular matrix
Von Willebrand factor153 154	Fibrin, collagen	Platelet adhesion to the clot
Vitronectin155 156	—	—
Thrombospondin[157]	Fibrin	—
Factor V158 159	Fibrin, platelets	Increased thrombin generation at the clot surface
Actin160 161	Fibrin	Clot retraction, stabilization of the platelet cytoskeleton

Coagulation factorXIIIa (FXIIIa) catalyzes cross-linking of Gln and Lys residues from many substrates during coagulation

? Identificare «tutti» i substrati del FXIIIa

A proteomic strategy based on a combination of

chromatographic separation

FXIIIa-specific labeling

High performance mass spectrometry.

Preparation of Plasma Samples

The plasma fraction was isolated after centrifugation at 950 rpm for 15 min.

EDTA was added to the plasma sample to a final concentration of 5 mM. Anticoagulant

The plasma was centrifuged at 13200 rpm and filtered through a 0.45-µm filter

Preparation of Plasma Samples

The plasma was centrifuged at 13200 rpm and filtered through a 0.45-µm filter

Filtered applied to a column containing the albumin-binding domain of protein G.

Cromatografia Affinita

Streptococcal protein G is a cell surface receptor protein with a multiple domain structure containing tandem repeats of **serum albumin-binding domains**

The albumin depleted flow through was collected and dialyzed against 40 mM Tris-HCl, 5 mM EDTA, pH 7.4 (buffer A),

Preparation of PROTEIN Plasma Samples 2

Applied to a 5-ml HiTrapQ column (GE Healthcare)

A strong anion exchange chromatography column for high-resolution, small-scale protein purification

The column was eluted using a linear gradient of NaCl flow rate of 2.5 ml/min.

Eluting fractions were monitored at 280 nm and pooled (five pools).

.

Preparation of PROTEIN Plasma Samples 3

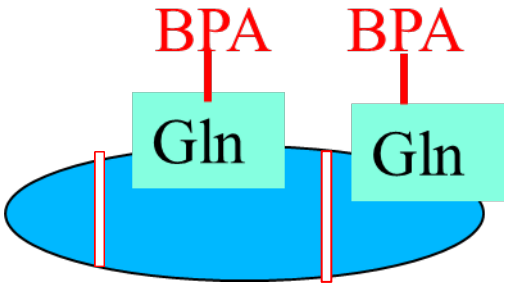
Eluting fractions were monitored at 280 nm and pooled (five pools).

All pools were dialyzed into 20 mM Tris-HCl, 137 mM NaCl, pH 7.4, and concentrated using either Centriprep **centrifugal filters** (Millipore) or Amicon Ultra centrifugal filters (Millipore) (molecular weight **cutoff**, **10 kDa**).

By **SDS-PAGE**, the filtrate did not contain any proteins.

MARCATURA ENZIMATICA (FXIII) PROTEINE PLASMATICHE

0. 3 mg of protein from each of the five HiTrapQ pools was incubated with FXIIIa in the presences of BPA (5-Biotinamido)PentylAmine) for 30, 60, and 180 min



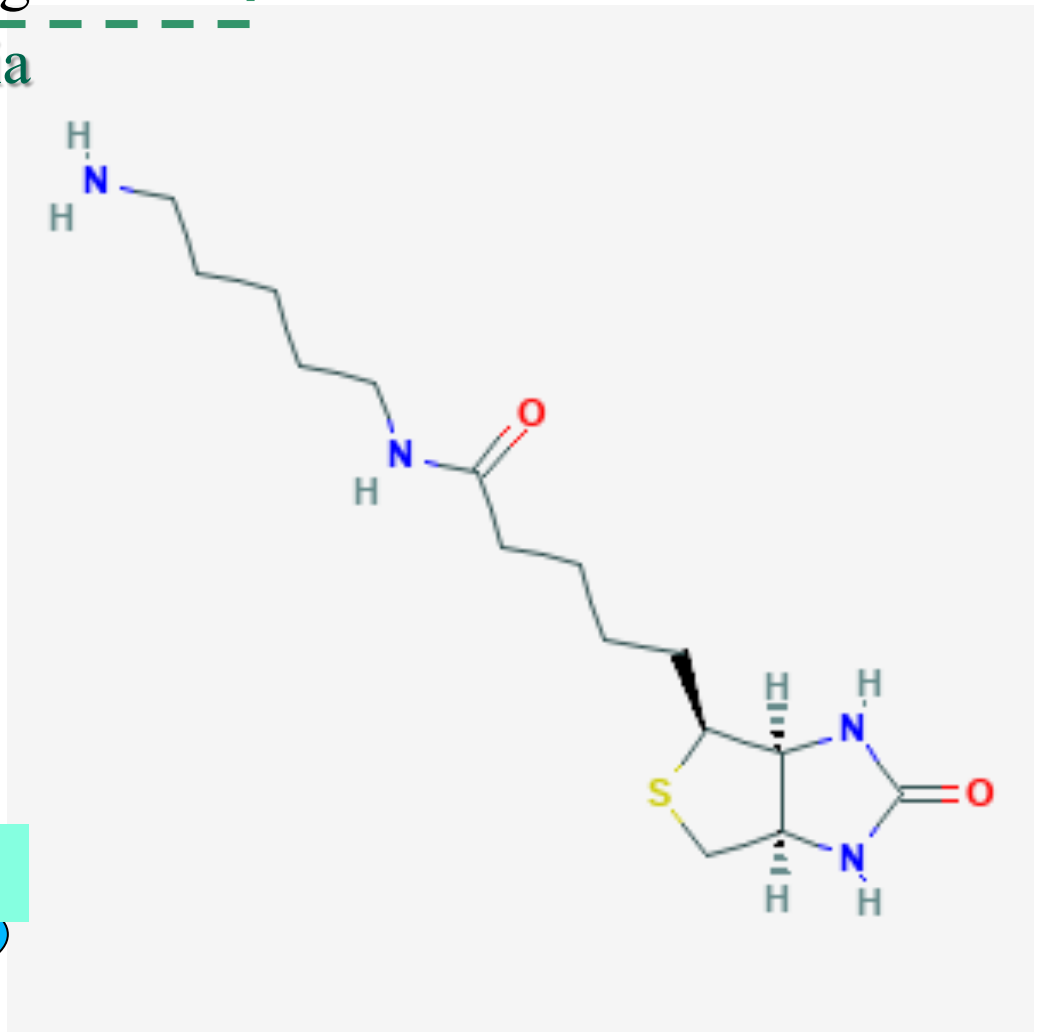
BPA-labeled peptides

BPA viene legato a «tutti i substrati del FXIIIa

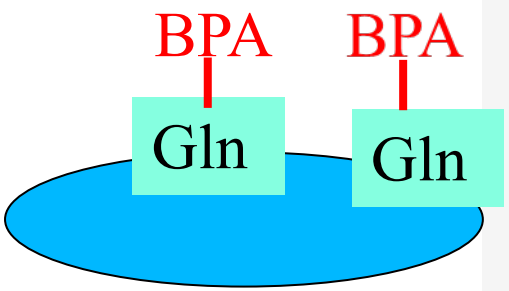
BPA (5-Biotinamido)PentylAmine

FXIIIa crosslinking to Gln

Ammina primaria

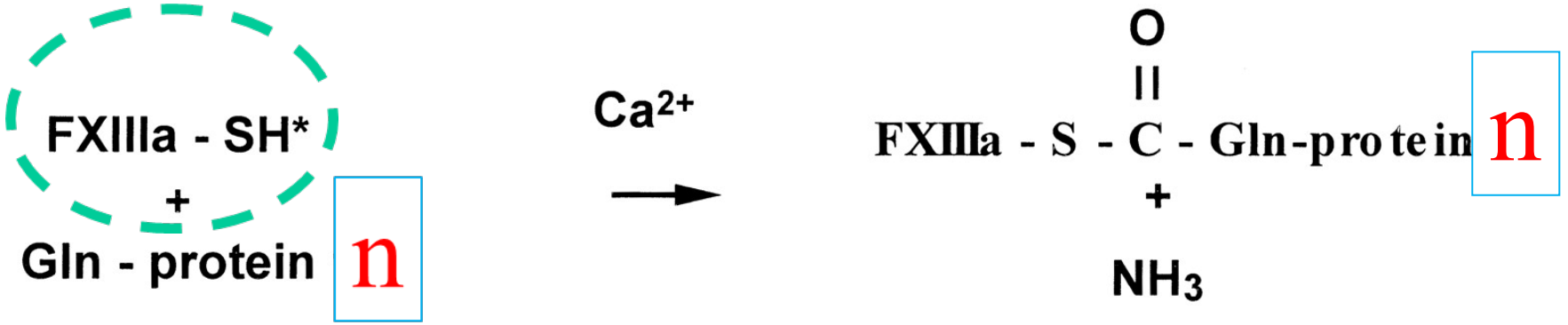


Biotina



MARCATURA ENZIMATICA (FXIII) PROTEINE PLASMATICHE

Activated factor XIII first forms a thioester bond with a selected protein-bound glutamine residue



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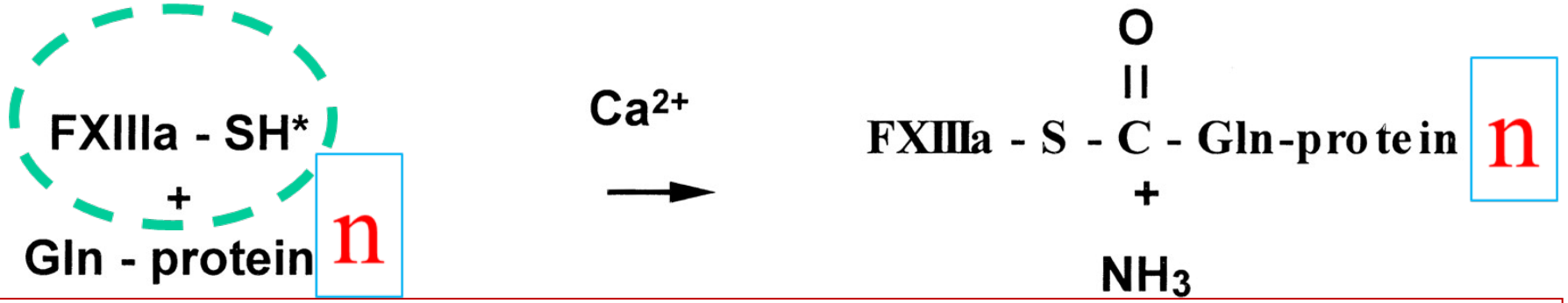
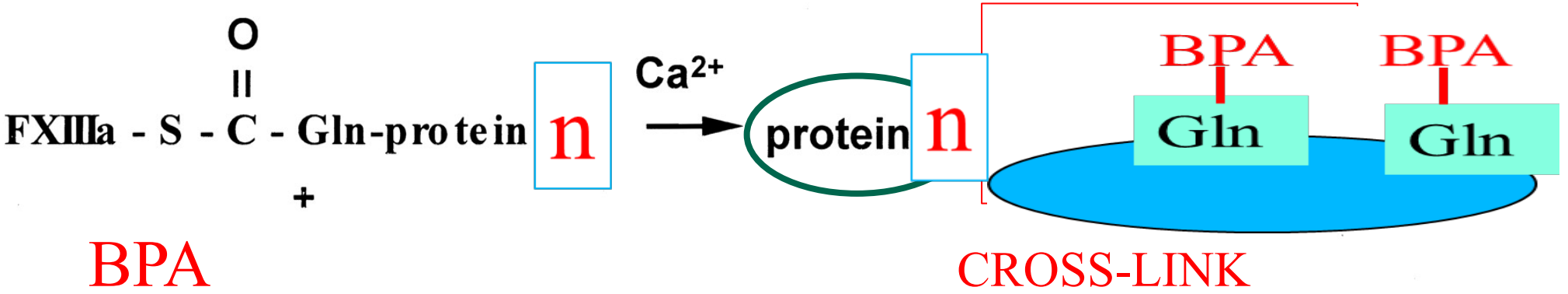


the thioester intermediate then reacts with a primary amine



BPA

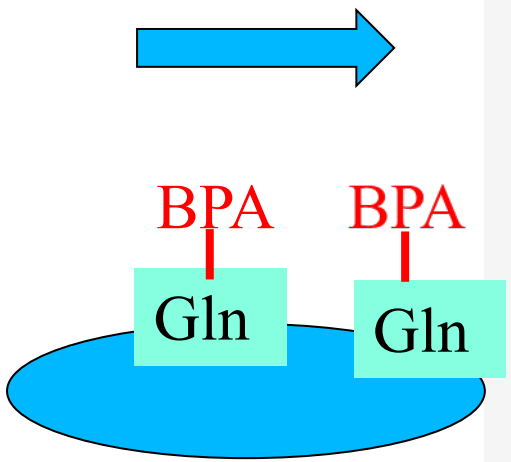
CROSS-LINK

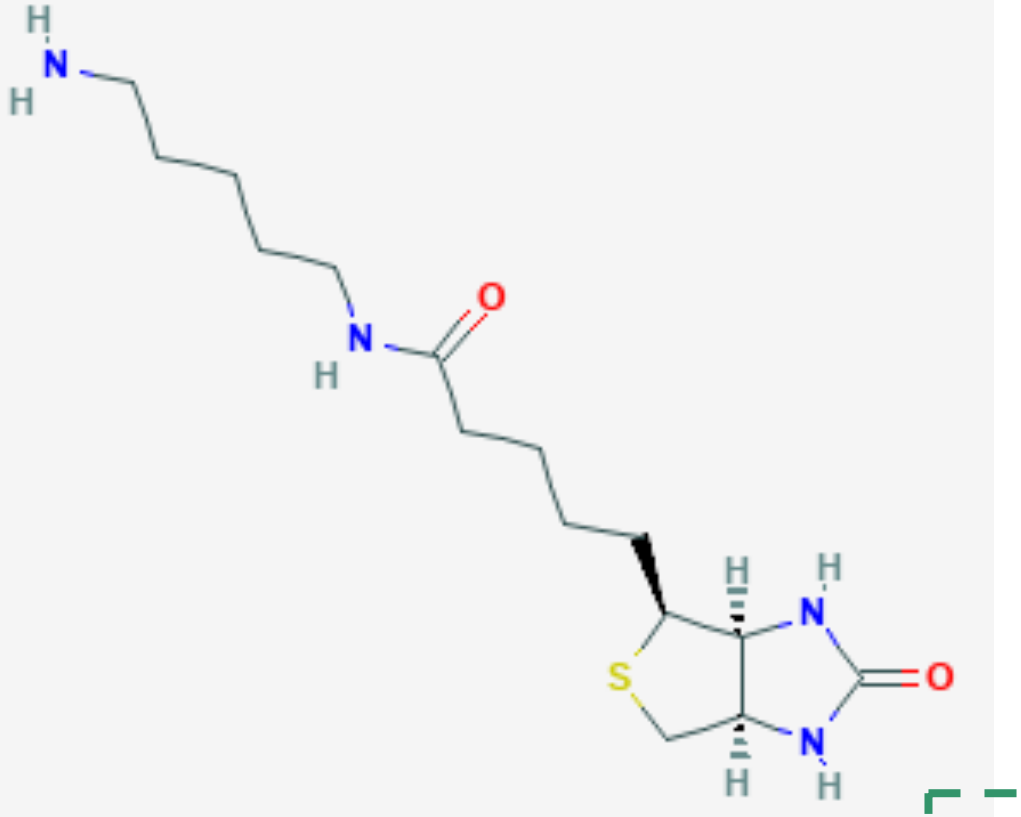
Robert A. S. Ariëns et al. Blood 2002;100:743-754

BPA (5-Biotinamido)PentylAmine

FXIIIa crosslinking to Gln

Ammina primaria





Biotina

Affinità Avidina

MARCATURA ENZIMATICA (FXIII) PROTEINE PLASMATICHE

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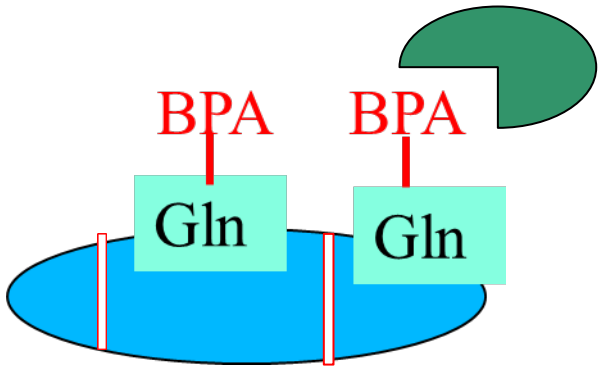
Avidin affinity column - BPA-labeled peptides



Avidin affinity column

Elution using 100 mM glycine, pH 2.8

tryptic digestion

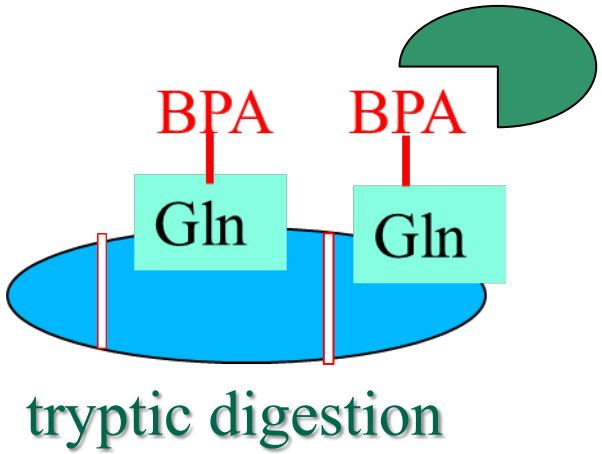
BPA-labeled peptides

MARCATURA ENZIMATICA (FXIII) PROTEINE PLASMATICHE

0. 3 mg of protein from each of the five HiTrapQ pools was incubated with FXIIIa in the presences of BPA (5-Biotinamido)PentylAmine) for 30, 60, and 180 min

Following tryptic digestion BPA-labeled peptides were obtained

Avidin affinity column - BPA-labeled peptides were eluted using 100 mM glycine, pH 2.8



BPA-labeled peptides

..*da diverse proteine ….*

Transglutaminase-catalyzed Incorporation of 5-(Biotinamido)pentylamine
FXIII was activated by incubation with thrombin (1 milliunit of thrombin/1 μg of FXIII) in 20 mm Tris-HCl, 137 mm NaCl, pH 7.4 for 1 h at 37 °C. A total of 300 μg of protein from each of the five HiTrapQ pools was incubated with FXIIIa at a 1:25 (w/w) ratio in 20 mm Tris-HCl, 137 mm NaCl, pH 7.4, containing 10 mm CaCl2, 0.5 mm DTT, and 10 mm BPA.

After an incubation period of 30, 60, or 180 min at 37 °C, the reaction was stopped by addition of EDTA to 15 mm. To identify endogenous FXIIIa activity, an identical set of control samples were incubated for 180 min without the addition of FXIIIa.

All labeled samples were lyophilized using a SpeedVac (Savant) and dissolved in 100 mm Tris-HCl, 6 m guanidine HCl, pH 8, containing 10 mm DTT followed by the addition of iodoacetamide to a final concentration of 30 mm. The reduced and alkylated samples were dialyzed into 20 mm NH4HCO3. The samples were concentrated using a SpeedVac (Savant).

A double digestion was performed with 2 × 1:40 (w/w) trypsin at 37 °C before addition of PMSF to a final concentration of 1 mm. The samples were lyophilized using a SpeedVac (Savant), dissolved in 100 mm NaH2PO4, 150 mm NaCl, pH 7, and applied to a monomeric avidin affinity column (Pierce) equilibrated in 100 mm NaH2PO4, 150 mm NaCl, pH 7.

After extensive washing, the BPA-labeled peptides were eluted using 100 mm glycine, pH 2.8, and desalted using self-packed micro columns containing POROS R2 (Applied Biosystems) prior to LC-MS/MS analysis (20). All samples were analyzed in three separate LC-MS/MS runs.

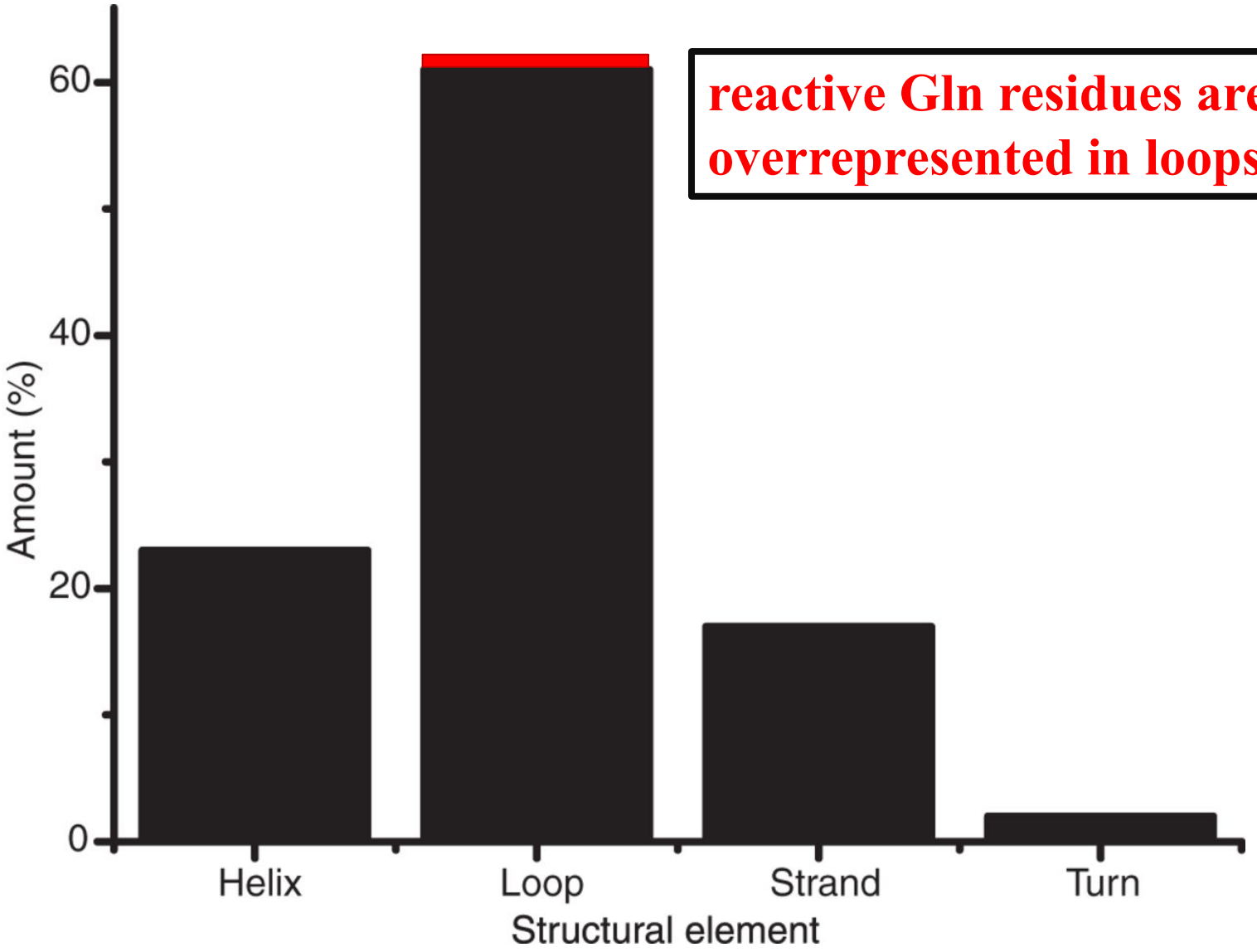
IDENTIFICAZIONE PEPTIDI

Identified by **LC-MS/MS** Spettrometria massa.

549 reactive Gln residues!

PDB e UniProt database = identificazione peptidi e e proteine

Secondary structure localization of reactive Gln residues (n=389)



Camilla Lund Nikolajsen et al. J. Biol. Chem. 2014;289:6526-6534

JBC

IDENTIFICAZIONE PEPTIDI

Identified by **LC-MS/MS** Spettrometria massa.

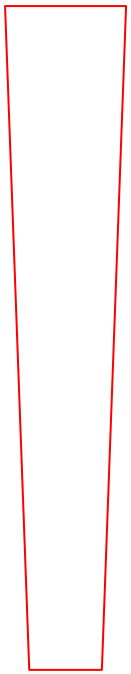
NUMERO DI CONTE

The identified substrates are listed according to the total number of spectral counts.

VARIAZIONE NEL TEMPO

The number of spectral counts was used to evaluate the **level** of BPA incorporation **over time**

High number of spectral counts



lower number of spectral counts

	Accession number	Name	Sites	Spectral counts			Clot ID
				30 min	60 min	180 min	
1	P02671	Fibrinogen α-chain	8	U	N	N	×
2	P01023	α_2-Macroglobulin	15	U	U	N	×
3	P00488	Coagulation factor XIII A chain	8	U	U	N	×
4	P00747	Plasminogen	20	U	U	N	×
5	P00734	Prothrombin	11	U	U	N	×
6	P19823	Inter-α-trypsin inhibitor heavy chain H2	16	U	U	N	×
7	P06727	Apolipoprotein A-IV	18	U	U	U	×
8	P01024	Complement C3	27	U	U	N	×
9	P02787	Serotransferrin	10	U	U	U	×
10	P0C0L5	Complement C4-B	19	U	U	N	
11	P19827	Inter-α-trypsin inhibitor heavy chain H1	11	U	N	N	×
12	P10909	Clusterin	5	U	U	U	×
13	P02679	Fibrinogen γ-chain	5	U	N	N	×
14	P08697	α_2-Antiplasmin	11	U	U	U	×
15	P07360	Complement component	7	U	U	N	

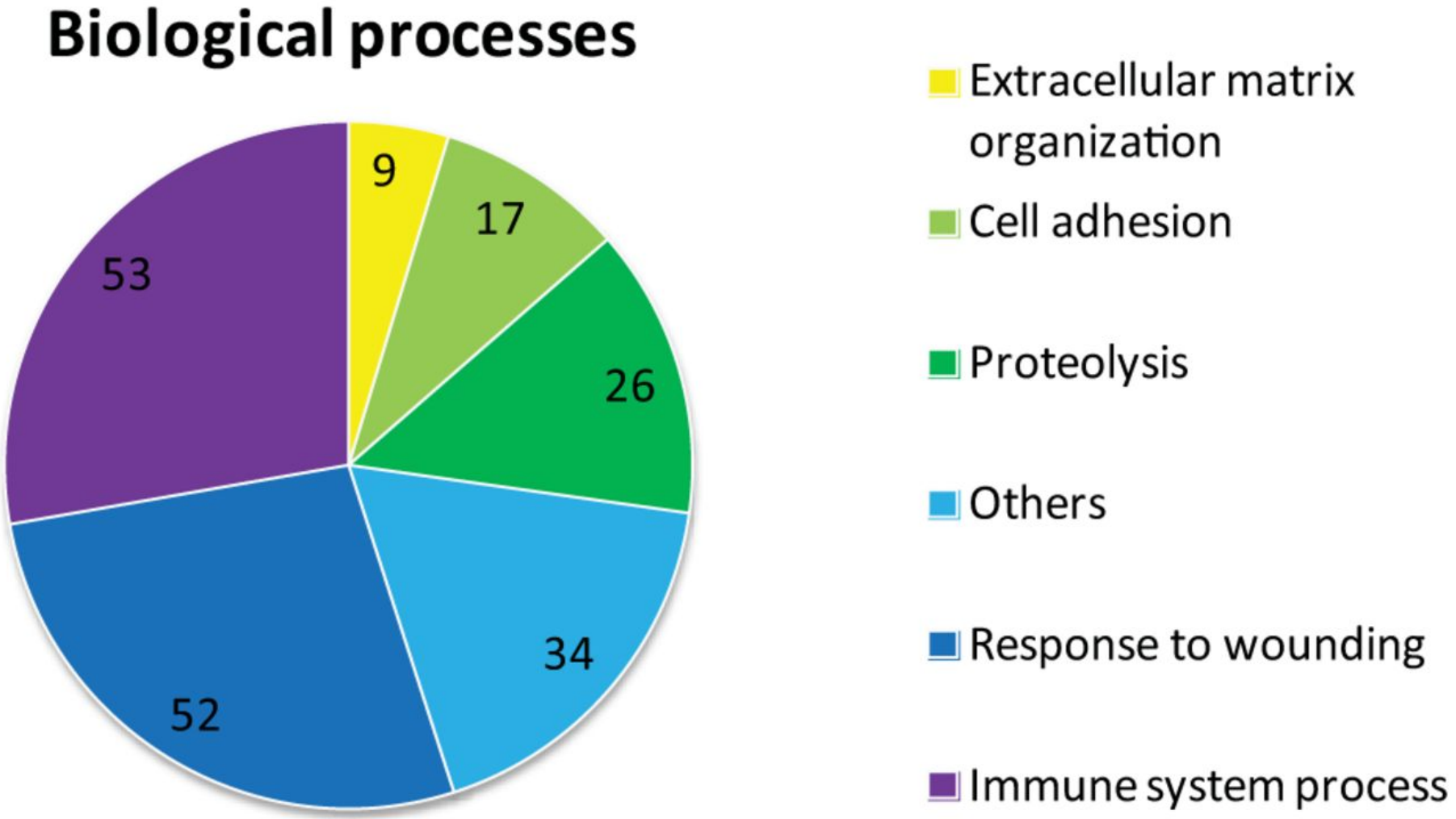
Sites = number of reactive Gln residue

Clot ID indicates that the substrate was cross-linked to the plasma clot.

U for up-regulated/D for down-regulated.
No regulation is indicated by N if the change is less than 30%.

		protein						
133	P11142	Heat shock cognate 71-kDa protein	1			U		
134	P04278	Sex hormone-binding globulin	1			U		
135	P62328	Thymosin β-4	1			U		
136	Q9UBG0	C-type mannose receptor 2	1			U		
137	P34931	Heat shock 70-kDa protein 1-like	1			U		
138	P01620	Ig κ-chain V-III region SIE	1			U		
139	Q92954	Proteoglycan 4	1			U		
140	Q76LX8	A disintegrin and metalloproteinase with thrombospondin motifs 13	1			U		
141	P05090	Apolipoprotein D	1			U		
142	Q99490	Arf-GAP with GTPase, ANK repeat, and PH domain-containing protein 2	1			U		
143	Q9H4A9	Dipeptidase 2	1			U		
144	P14314	Glucosidase 2 subunit β	1			U		
145	Q14697	Neutral α-glucosidase AB	1			U		
146	P55058	Phospholipid transfer protein	1			U		

Gene Ontology summary of the identified FXIIIa substrates.



Camilla Lund Nikolajsen et al. J. Biol. Chem. 2014;289:6526-6534

JBC

CoagulationfactorXIIIa(FXIIIa) catalyzes cross-linking of Gln and Lys residues during coagulation.

Results: 147 FXIIIa substrates were identified in human plasma

time-resolved analysis of the FXIIIa substrate proteome in plasma

ALTERNATIVA
(Verifica presenza peptidi nel coagulo)

The plasma fraction was recalcified and allowed to clot for 2h at37°C

To remove non covalently bound proteins, the clot was washed three times
3)20mM Tris-HCl, 2 M NaCl, 6 M guanidine HCl; and water.

The sample was boiled in 0.1% SDS and separated by SDS-PAGE.

Covalently cross-linked proteins were retained in the stacking gel and could be collected after

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The sample was digested with trypsin for16hat37°C.

The tryptic peptides were collected and micropurified, and were analyzed by mass spectrometry/ion chromatography

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Clot Formation and Purification

Blood was collected from healthy volunteers by fingerprick. The collected blood sample was immediately centrifuged for 1 min at 7000 × *g* to obtain the plasma fraction. The plasma fraction was allowed to clot for 2 h at 37 °C. To remove noncovalently bound proteins, the clot was washed three times for 20 min in each of the following buffers: 1) 20 mM Tris-HCl, 150 mM NaCl, pH 7.4; 2) 20 mM Tris-HCl, 2 M NaCl, pH 7.4; 3) 20 mM Tris-HCl, 2 M NaCl, 6 M guanidine HCl, pH 7.4; and 4) water. Finally the sample was boiled in sample buffer containing 0.1% SDS and separated by SDS-PAGE. Covalently cross-linked proteins were retained in the stacking gel and could be collected after electrophoresis. The SDS was removed by washing the gel piece in a microspin filter (molecular weight cutoff, 3 kDa) using: 1) water; 2) 50% acetonitrile containing 50 mM NH_4HCO_3; and finally, 3) 50 mM NH_4HCO_3. The sample was reduced, alkylated, and digested with trypsin for 16 h at 37 °C. The tryptic peptides were collected and micropurified using self-pack micro columns containing POROS R2 (20). The purified peptides were either analyzed by mass spectrometry directly or prefractionated by strong cation exchange. For strong cation exchange, the purified peptides were dissolved in 10 mM KH_2PO_4, 20% acetonitrile, pH 2.8 (Buffer A) and separated on a PolySULFOETHYL A column (PolyLC) equilibrated in buffer A. The peptides were eluted using a linear gradient of buffer B (500 mM KCL in buffer A) at 1% B/min using a flow rate of 150 µl/min. A total of 16 pools were collected and desalted using C18

Quantification of plasma clot proteins

A plasma clot was extensively washed and subjected to SDS-PAGE. The clot material that did not migrate into the gel was digested with trypsin and analyzed by LC-MS/MS.

The relative abundance of identified proteins was calculated using ion chromatography.

The calculation was based on the average intensity for the three most intense peptides from each protein.

The **14 quantified proteins** are all FXIIIa substrates.

CoagulationfactorXIIIa(FXIIIa) catalyzes cross-linking of Gln and Lys residues during coagulation.

Results: 147 FXIIIa substrates were identified in human plasma

48 of these were incorporated into the clot

Accession number	Name	Extracted ion chromatography quantitation
		%
P02671	Fibrinogen α-chain	40.0 ± 2.3
P02675	Fibrinogen β-chain	30.7 ± 4.0
P02679	Fibrinogen γ-chain	19.8 ± 2.9
P02751	Fibronectin	4.9 ± 1.0
P08697	α_2-Antiplasmin	1.6 ± 0.2
P02768	Serum albumin	0.6 ± 0.1
P04196	Histidine-rich glycoprotein	0.5 ± 0.1
P01857	Igγ-1 chain C region	0.5 ± 0.1
Q16610	Extracellular matrix protein 1	0.4 ± 0.0
P01023	α_2-Macroglobulin	0.2 ± 0.1
P00747	Plasminogen	0.2 ± 0.0
P01860	Igγ-3 chain C region	0.2 ± 0.0
P00488	Coagulation factor XIII A chain	0.1 ± 0.0
P01024	Complement C3	0.1 ± 0.0

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FINE

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time-resolved analysis of the FXIIIa substrate proteome in plasma

Combination of
chromatographic separation
FXIIIa-specific labeling and
High performance mass spectrometry.

Transglutaminase-catalyzed Incorporation of 5-(Biotinamido)pentylamine
FXIII was activated by incubation with thrombin (1 milliunit of thrombin/1 μg of FXIII) in 20 mm Tris-HCl, 137 mm NaCl, pH 7.4 for 1 h at 37 °C. A total of 300 μg of protein from each of the five HiTrapQ pools was incubated with FXIIIa at a 1:25 (w/w) ratio in 20 mm Tris-HCl, 137 mm NaCl, pH 7.4, containing 10 mm CaCl2, 0.5 mm DTT, and 10 mm BPA.

After an incubation period of 30, 60, or 180 min at 37 °C, the reaction was stopped by addition of EDTA to 15 mm. To identify endogenous FXIIIa activity, an identical set of control samples were incubated for 180 min without the addition of FXIIIa.

All labeled samples were lyophilized using a SpeedVac (Savant) and dissolved in 100 mm Tris-HCl, 6 m guanidine HCl, pH 8, containing 10 mm DTT followed by the addition of iodoacetamide to a final concentration of 30 mm. The reduced and alkylated samples were dialyzed into 20 mm NH4HCO3. The samples were concentrated using a SpeedVac (Savant).

A double digestion was performed with 2 × 1:40 (w/w) trypsin at 37 °C before addition of PMSF to a final concentration of 1 mm. The samples were lyophilized using a SpeedVac (Savant), dissolved in 100 mm NaH2PO4, 150 mm NaCl, pH 7, and applied to a monomeric avidin affinity column (Pierce) equilibrated in 100 mm NaH2PO4, 150 mm NaCl, pH 7.

After extensive washing, the BPA-labeled peptides were eluted using 100 mm glycine, pH 2.8, and desalted using self-packed micro columns containing POROS R2 (Applied Biosystems) prior to LC-MS/MS analysis (20). All samples were analyzed in three separate LC-MS/MS runs.

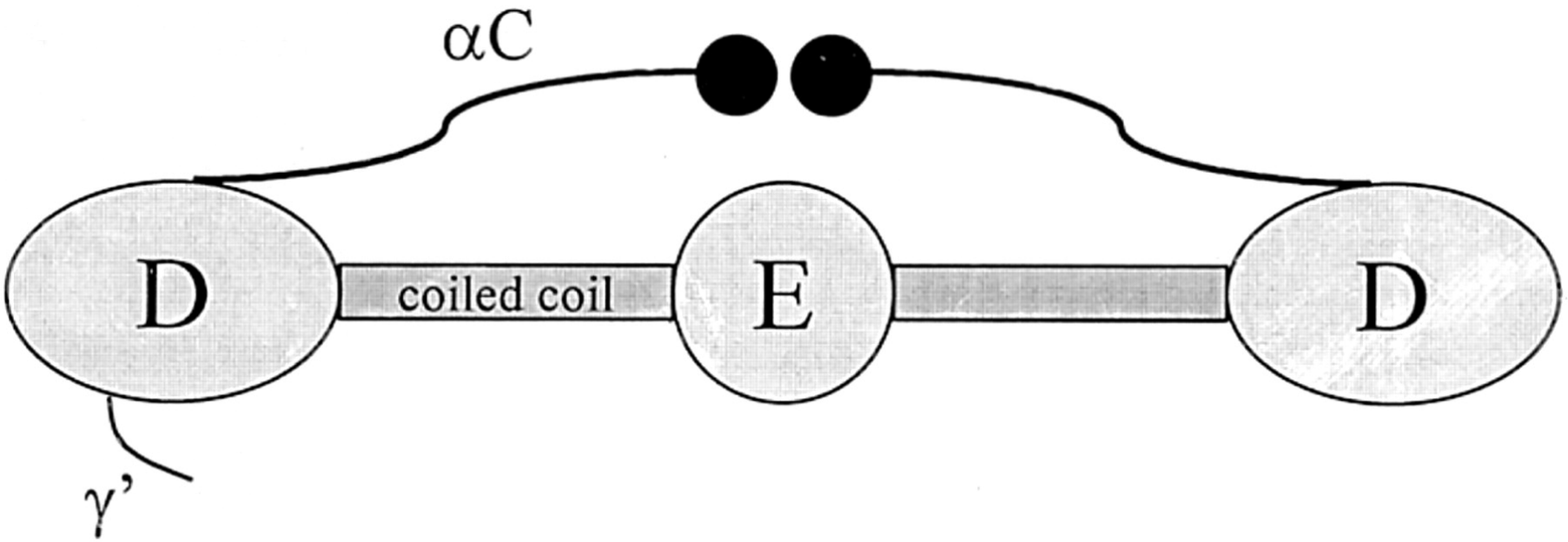
Clot Formation and Purification

Blood was collected from healthy volunteers by fingerprick. The collected blood sample was immediately centrifuged for 1 min at 7000 × *g* to obtain the plasma fraction. The plasma fraction was allowed to clot for 2 h at 37 °C. To remove noncovalently bound proteins, the clot was washed three times for 20 min in each of the following buffers: 1) 20 mM Tris-HCl, 150 mM NaCl, pH 7.4; 2) 20 mM Tris-HCl, 2 M NaCl, pH 7.4; 3) 20 mM Tris-HCl, 2 M NaCl, 6 M guanidine HCl, pH 7.4; and 4) water. Finally the sample was boiled in sample buffer containing 0.1% SDS and separated by SDS-PAGE. Covalently cross-linked proteins were retained in the stacking gel and could be collected after electrophoresis. The SDS was removed by washing the gel piece in a microspin filter (molecular weight cutoff, 3 kDa) using: 1) water; 2) 50% acetonitrile containing 50 mM NH_4HCO_3; and finally, 3) 50 mM NH_4HCO_3. The sample was reduced, alkylated, and digested with trypsin for 16 h at 37 °C. The tryptic peptides were collected and micropurified using self-pack micro columns containing POROS R2 (20). The purified peptides were either analyzed by mass spectrometry directly or prefractionated by strong cation exchange. For strong cation exchange, the purified peptides were dissolved in 10 mM KH_2PO_4, 20% acetonitrile, pH 2.8 (Buffer A) and separated on a PolySULFOETHYL A column (PolyLC) equilibrated in buffer A. The peptides were eluted using a linear gradient of buffer B (500 mM KCL in buffer A) at 1% B/min using a flow rate of 150 µl/min. A total of 16 pools were collected and desalted using C18

rate of 250 nl/min, using a 30 min gradient from 5 to 35% phase B (0.1% formic acid and 90% acetonitrile). The collected MS files were converted to Mascot generic format using the AB Sciex MS data converter beta 1.1 (AB Sciex) and the protein pilot Mascot generic format parameters. The generated peak lists were searched against the Swiss-Prot database (SwissProt_2013_07 containing 20264 human protein sequences) using in-house Mascot search engine (release version 2.3.02, Matrix Science).

Search parameters used for protein identification were: *Homo sapiens*, trypsin, two missed cleavages, carbamidomethyl (Cys) as fixed modification, and oxidation (Met) as variable modification. For the BPA-labeled samples, the corresponding modification was selected as a variable modification (Biotin:Thermo-21345). Peptide tolerance was 15 ppm, and MS/MS tolerance was 0.3 Da.

Schematic representation of the fibrinogen molecule.Fibrinogen consists of 6 polypeptide chains held together by disulfide bonds in a molecule with bilateral symmetry.



Robert A. S. Ariëns et al. Blood 2002;100:743-754