Materials and Methods

Reagents

Human coagulation factors (aPC, IIa, VIIa, IXa, Xa, XIa, XIIa) and plasmin were purchased from Haematologic Technologies Inc. (Essex Junction, VT). Active plasma kallikrein and high molecular weight kininogen were from Enzyme Research Laboratories (South Bend, IN). Coagulation factor-deficient plasmas were obtained from George King Bio-Medicals Inc. (Overland Park, KS). Angiotensin converting enzyme (ACE), caspase-1, cathepsin G, cathepsin S, complement C1s, complement C3b, chymase, elastase, MMP-8, MMP-9, protease 3, tryptase, and H-D-Phe-Pro-Arg-chloromethyl ketone (PPACK) were from Calbiochem (La Jolla, CA). Human endothelin-converting enzyme-1 (ECE-1) and human chemerin DuoSet ELISA kit were products of R&D Systems (Minneapolis, MN). Microparticle (MP) reagent was purchased from thrombinoscope BV (Netherlands). Gly-Pro-Arg-Pro (GPRP) and kaolin were purchased from Sigma (St. Louis, MO). All the peptides (> 95% purity) and oligonucleotide primers in this study were from Elim Biopharm (Hayward, CA). FXI levels in plasma from FXI deficient patients and matched controls were measured on an ACL Futura Plus coagulometer (Instrumentation Laboratories, Milan, Italy) using HemosIL® reagents (Instrumentation Laboratory, Milan, Italy).

Generation of CHO-S clones expressing human chemerin forms

A cDNA encoding human chemerin (NM_002889) from Open Biosystems (Huntsville, AL) was used as the template for chemerin form cloning. Template cDNAs were amplified by PCR using Platinum Pfx DNA polymerase (Invitrogen, Carlsbad, CA) to create various chemerin isoforms of chem163S, chem158K and chem163S$_{R162A}$. His-tag, mutations and stop codons were inserted into the primers (Supplementary Table 2) to create different

chemerin expression constructs using the ubiquitous chromatin opening element (UCOE) vector, pCET-1019AS-puro (Millipore, MA). Plasmids encoding chemerin forms and the 6his-chemerin forms were verified by sequencing before transfection into CHO-S cells by DMRIE-C reagent (Invitrogen) according to the manufacturer's protocol. After 48 h, the cells were seeded at a density of 5×10^4 cells/mL in 100 μL medium (50% CHO Medium 5/ 50% CD-CHO medium, 8 mM L-glutamine, and 1% HT supplement) in 96-well plates and clones selected with 10 μg/mL puromycin. After 12 days, expression of human chemerin forms was assessed by ELISA.

Protein production and purification

Chemerin forms were produced and purified as described previously [16]. CHO-S cells stably expressing the 6his-chem163S were seeded into 500 mL of CD-CHO medium at 1×10^5 cells/mL. After 4 days of culture at 37 °C, the conditioned medium was collected by centrifugation, filtered through a 0.22 μm filter, and applied to a HisTrap™ FF column (GE Healthcare). The column was pre-equilibrated with binding buffer containing 20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4. The 6his-chem163S protein was eluted with 20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4 using an AKTA fast protein liquid chromatography system (GE Healthcare, Little Chalfont, UK) and collected in 5 mL fractions. Fractions containing chemerin were identified by SDS-PAGE analysis and pooled. The purified proteins were analyzed by SDS-PAGE. The molecular mass and purity of the protein was confirmed by MALDI-TOF mass spectrometry with internal standards of 2,466, 3,660, and 5,734 Da included in the analysis (PAN Facility, Stanford University). For storage, 0.1% protease-free BSA was added, and aliquots were kept at −80°C.

In vitro enzyme cleavage assays

Purified chem163S (10 μm) was incubated with 100 nM enzymes at 37 °C for 30 min in PBS containing 2 mM $CaCl_2$ and 0.1 mM $ZnCl_2$ and the reaction terminated by dithiothreitol (DTT; 10 mM) before analysis by SDS-PAGE and mass spectrometry.

FXIa cleavage of 6his-chem163S in assay buffer

FXIa （100 nM） and 6his-chem163S (10 μM) were incubated in FXIa buffer (50 mM Hepes, 125 mM NaCl, 5 mM $CaCl_2$ and 0.1 mg/mL BSA) at 37 °C for 30 min. Reactions were terminated by addition of 100 nM of PPACK (Calbiochem) and products analyzed by MALDI-TOF mass spectrometry. The amounts of 6his-chem163S consumption and 6his-chem158K generation were determined by chem163S and chem158K ELISAs.

Specific ELISAs for chemerin forms

Specific ELISAs for human chemerin forms were performed as previously described [32,40]. In brief, either a commercially available mouse monoclonal anti-human chemerin antibody (R&D Systems, Minneapolis, MN) or anti-his-tag antibody (Thermofisher, Waltham, MA) were used as the capture antibody. The antibody (4 μg/mL) was coated in PBS buffer onto ELISA plates, and nonspecific binding sites blocked with 1% BSA in PBS. Purified recombinant proteins were used as standards to construct the calibration curves. Samples and standards were diluted with 1% BSA in PBS and incubated in the wells for 2 h. After washing with 0.05% Tween 20 in PBS, the samples were incubated with the specific antibodies (500 ng/ml). For anti-chem163S antibody, 10 μg/mL chem158K peptide was added to remove residual cross-reactivity. For anti-chem157S antibody, 10 μg/mL chem163S peptide was added to remove residual cross-reactivity. For anti-chem155A antibody, 10 μg/mL chem163S and 10 μg/ml chem157S peptides were added. The plates were then processed as described [40] and the concentrations of chemerin forms calculated from the

calibration curves of the purified chemerin standards.

Kinetic analysis of FXIa cleavage of chemerin peptides

To determine the kinetic constants for cleavage of the C-terminal sequences of chemerin and FIX by FXIa, peptides (1 μM ~ 3 mM, Supplementary Table 3) representing the C-terminus and the FXIa cleavage site in FIX were treated with FXIa (30 nM) in FXIa assay buffer for 30 min at 37°C before terminating the reaction with 100 nM PPACK. 100 μl of each reaction mixture was loaded onto a Zorbax Eclipse Plus C18 (4.6 ×□150 mm) column (Agilent, Santa Clara, CA) and separated with a 0 ~ 40% acetonitrile gradient in 0.1% trifluoroacetic acid (v/v) by HPLC. The concentration of peptides present in the reaction mixture was determined from the area under the curve for that peptide compared to a standard curve of that peptide run separately. The standard curves were constructed with peptides (1 μM ~ 3 mM). The values for K_m and k_{cat} were determined by fitting to the Michaelis-Menten equation by nonlinear regression analysis using Prism v6 (Graphpad, San Diego, CA). The experiments were performed three times independently, and the data were pooled for analysis.

Preparation of platelet-rich plasma and platelet-poor plasma

Blood was drawn into tubes containing 3.8% sodium citrate (BD Biosciences) and platelet-rich plasma (PRP) prepared by centrifugation at 250 × *g* for 10 min at room temperature. Platelet-poor plasma (PPP) was prepared by centrifugation of the PRP at 1200 × *g* for 10 min at room temperature.

Cleavage of chemerin in PPP, PRP and FXI-depleted plasma

MP reagent (5 μM), GPRP (5 mM), kaolin (5 μg/mL), 6his-chem16S (10 μM) and Ca^{++} (5 mM) were incubated in human pooled normal plasma (George King Bio-medical,

Inc.) or FXI-depleted plasma (Heamatologic Technologies Inc.) at 37°C. In some experiments hirudin (1.5 U/mL) was added to block thrombin activity in PolyP triggered PRP. The reactions were terminated by addition of PPACK (100 μM) at various time points between 0 min and 30 min.

Preparation of plasma for chemerin form ELISAs

Human blood samples were obtained under protocols approved by Stanford University Medical Center, Partners Healthcare Institutional Review Boards or the Ethical Committee of the University of Perugia and informed consent was obtained from donors. Details Plasma (up to 2 mL) before and after contact phase activation was mixed with 100 μl of heparin-agarose (Sigma) and complete protease inhibitor (Roche Applied Science). After incubation at 4°C for 2 h, the heparin-agarose beads were pelleted and washed extensively with PBS, and chemerin was eluted with 0.8 M NaCl in PBS, all in the presence of complete protease inhibitor. The eluted proteins were diluted with 1% BSA in PBS for analysis by ELISAs for the specific chemerin forms.

Factor XIa chromogenic assay.

MP reagent (5 μM), GPRP (5 mM), kaolin (5 μg/mL), and FXIa chromogenic substrate D-LPR-ANSNH-C3H7•2HCl (Haematologic Technologies Inc.) at 50 μM were diluted in plasma at 37°C. The reactions were initiated by recalcifying either PRP or PPP with Ca^{++} at 5 mM, followed by monitoring the fluorescence intensity at excitation/emission 352/470 nm using a FLUOROSKAN ASCENT FL fluorescent plate reader (Thermo Electron Corp.). The assay was repeated at least four times independently. Calibration curves were constructed with FXIa (0.1 nM ~ 100 nM).

Calcium mobilization assay

Cells expressing human CMKLR1 (hCMKLR1/L1.2 cells) were labeled with Quest Fluo-8 AM dye (AAT Bioquest, Sunnyvale, CA) in HHBS buffer (Hanks buffered saline plus 20 mM Hepes pH 7.0) containing 2.5 mM probenecid and 0.04% Pluronic F-127 at 37 °C for 30 min in the wells of a 96-well plate (12.5×10^4 cells/well). The dye-loaded cells were stimulated with peptides representing different chemerin forms at room temperature, and the fluorescence intensity was followed at excitation/emission 495/535 nm using a FLUOROSKAN ASCENT FL fluorescent plate reader. The assay was repeated at least four times independently for each protein or peptide using three different preparations of peptides.

Statistics

Comparison of two groups was by Student's t test; multigroup comparisons were by two-way ANOVA analysis. The correlation analysis was calculated with two-tailed Pearson correlation coefficients. The analysis was carried out using Prism v6 (GraphPad, La Jolla, CA). Values of $P < 0.05$ were considered significant.

Supplementary Table 1: Characteristics of FXI-deficient patients and healthy controls

FXI Deficient Patients	FXI (IU/ml)	Age	Sex	BMI	ISTH bleeding score*	Bleeding phenotype
P001	0.02	43	F	22	2	Mild epistaxis Easy bruising
P002	0.33	63	M	25	0	absent
P003	0.35	9	F	19	0	absent
P004	0.35	70	F	48	0	absent
P005	0.37	66	M	19	0	absent
P006	0.40	52	M	22	0	absent
P007	0.40	26	M	23	1	Epistaxis during infancy
P008	0.18	28	M	22	0	absent
P009	0.41	20	M	23	0	absent
P010*	0.03	46	M	21	17	bleeding following mucocutaneous procedures (wisdom teeth, skin biopsy, large skin excision for melanoma), and post traumatic intramuscular bleeds
P011*	0.09	70	M	27	18	Mucosal bleeding after trauma or procedures (epistaxis with boxing, surgery for deviated nasal septum) excessive post op bleeding (appendectomy, knee meniscus surgery)
Healthy Controls						
C001	1.13	38	F	22	0	absent
C002	1.07	59	M	24	0	absent
C003	0.96	13	F	19	0	absent
C004	0.84	67	F	23	0	absent
C005	0.91	61	M	20	0	absent
C006	0.77	47	M	22	0	absent
C007	0.93	31	M	23	0	absent
C008	0.97	33	M	23	0	absent
C009	1.01	20	M	22	0	absent

Subjects indicated by the same number are matched (e.g. P001 is the patient and C001 the matched control)

* Plasma samples from patients P010 and P011 were only used for the experiments in Table 4

FXI levels in patients were assessed on at least two different occasions

BMI= body mass index.

The ISTH bleeding score is a quantitative measure of the bleeding phenotype, calculated after administration of a standardized questionnaire (*Rodeghiero F et al. J Thromb Haemost. 2010;8:2063-5)*

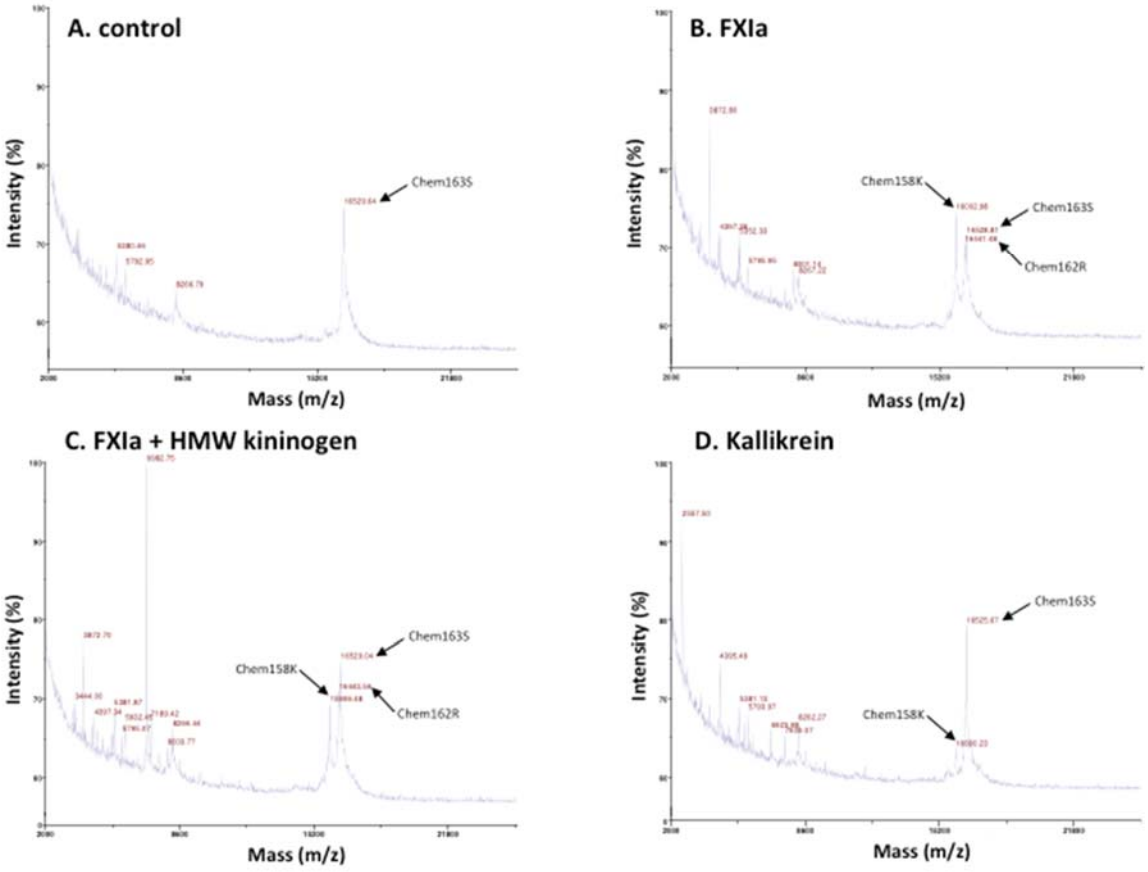
Supplementary Table 2. Sequence of Specific Primers

Construct	Primer	Sequence
6his-chem163S	Forward	5'-A**GCCGGC**CACCATGCGACGGCTGCTGATCCCTCTGGCCCTGTGGCTGGGTGCGGTGGGCGTGGGCGTCGCCCATCATCACCATCACCATGAGCTCACGG-3'
	Reverse	5'-A**GCTAGC**TTAGCTGCGGGGCAGGGCCTT-3'
6his-chem163S$_{R162A}$	Forward	5'-A**GCCGGC**CACCATGCGACGGCTGCTGAT-3'
	Reverse	5'-A**GCTAGC**TTAGCTGCGGGGCAGGGCCTT-3'
6his-chem158K	Forward	5'-A**GCCGGC**CACCATGCGACGGCTGCTGAT-3'
	Reverse	5'-A**GCTAGC**TTACTTGGAGAAGGCGAAC-3'

Supplementary Table 3. FIX and chemerin peptides.

	Sequence	Molecular mass (Dalton)	pI
FIX-10mer	FNDFTRVVGG	1111.2	5.8
FIX-6mer	FNDFTR	798.8	5.8
chem-15mer	YFPGQFAFSKALPRS	1715.9	10.0
chem-15mer$_{R162A}$	YFPGQFAFSKALPAS	1630.8	8.6
chem-14mer	YFPGQFAFSKALPR	1628.8	10.0
chem-14mer$_{R162A}$	YFPGQFAFSKALPA	1543.7	8.6
chem-10mer	YFPGQFAFSK	1191.3	8.6

Supplementary Figure 1



Chem163S (10 μ M) was treated for 30 min at 37° C with A) buffer, B) 100nM FXIa, C) 100 nM + 100 nM single-chain HMW kininogen and D) 100nM kallikrein before stopping the reaction with 10 mM DTT and analyzing the products by MALDI-TOF.