Effects of Storage Time and Exogenous Protease Inhibitors on Plasma Protein Levels

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Abstract

Plasma biomarker analysis requires intact unbiased starting material. We analyzed the effects on plasma protein profiles of protease inhibitor cocktails and preprocessing storage. Plasma from 12 healthy subjects collected with and without protease inhibitors was prepared immediately and after 2 hours of room temperature storage. The samples were analyzed by a multiplexed enzyme-linked immunosorbent assay that captured 99 chemokines and cytokines. Unsupervised hierarchical analysis clustered the samples into 4 groups: one composed predominantly of samples processed immediately, another of samples processed after 2 hours, and the remaining two a mix of immediate and 2-hour samples. The mixed and immediate group protein profiles were similar. However, among the immediate and 2-hour samples, the levels of 37 factors differed significantly: all were greater after 2 hours. The dramatic changes in protein levels during storage were independent of protease inhibitors and are likely due to cytokine production and/or release by leukocytes and platelets.

Proteomic analysis of clinical samples is being used extensively to mine the proteome for specific biomarkers and patterns of disease. The accurate analysis of biomarkers requires highest quality starting material. Many biomarker studies use serum or plasma samples.[1-6] When analyzing plasma or serum protein levels, it is critical to collect, preserve, and store the samples using procedures that minimize protein changes. During prolonged room temperature storage of whole blood, proteins are subject to degradation due to endogenous proteolytic activity.

There are many naturally occurring proteases that are characterized by the nature of their active center. These proteases include serine, cysteine, aspartic, and matrix metalloproteases. Serine proteases contain a serine and histidine in their active center, whereas cysteine proteases contain a cysteine; metalloproteases, a cation such as Zn^{2+}, Ca^{2+}, or Mn^{2+}; and aspartic, an aspartic acid group **Table 1**.

Plasma contains many proteases. Many of the proteases in plasma are released from activated, dying, or lysed neutrophils[7-9] or mononuclear phagocytes.[10] Plasma also contains an abundance of protease inhibitors whose function is to arrest the activity of proteolytic enzymes. These protease inhibitors include α_1-protease inhibitor,[11] tissue inhibitor of metalloprotease (TIMP),[12] α_2-macroglobulin,[12] and plasminogen activator inhibitor-1. However, because of the large quantities and variety of proteases that can be released into the blood by neutrophils and mononuclear phagocytes such as neutrophil elastase, matrix metalloprotease (MMP)-2, and MMP-9,[13,14] protease inhibitors normally found in plasma are not completely effective.

One method that is used to preserve the integrity of plasma or serum proteins is to separate and freeze plasma immediately after the blood sample is obtained.[13] However, this is not always

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Table 1
Protease Inhibitors and the Types of Proteases They Affect

	Protease Type				
Inhibitor	Serine	Cysteine	Metallo	Aspartic	Other
α-1 Protease inhibitor	X				
α_2-Macroglobulin					Broad spectrum
Tissue inhibitor of metalloproteases			X		
Aprotinin	X				
Leupeptin	X	X			
Pepstatin				X	
Chymostatin					α-, β-, γ-, δ-Chymotrypsin
EDTA			X		
Phenyl-methyl-sulfonyl fluoride	X	X			
Di-isopropyl fluorophosphate	X				
E-64		X			
Pefabloc SC	X				

possible when collecting samples from patients enrolled in clinical studies because the facilities to separate and freeze plasma often are separate from the clinics or patient care facilities where the samples are collected. As a result, several hours may elapse before the samples arrive in the laboratory and can be processed. During this time, cell metabolism or activation of proteolytic activity may critically change plasma soluble factor content. This in turn may impact on the overall quantitative and qualitative analysis of the proteins present at the time of collection and result in an inaccurate protein profile.

An alternative method to prevent the degradation of soluble factors is to add protease inhibitors to blood samples. The collection of blood into calcium chelators such as EDTA or citrate not only prevents the activation of the coagulation cascade, but the chelators also inhibit calcium-dependent proteases. Specific cocktails of protease inhibitors are sometimes added to blood samples. During the last 2 decades, protease inhibitors have been developed that act on various proteases (Table 1). At least one blood collection tube manufacturer has produced special collection tubes that contain a cocktail of protease inhibitors intended to prevent protein degradation during sample storage.

The purposes of this study were to determine whether the levels of soluble factors differ between plasma separated from whole blood and frozen immediately after collection and plasma prepared and frozen 2 hours after collection and to determine whether any changes in factor levels that occur can be prevented by the collection of blood into tubes containing a cocktail of protease inhibitors.

Materials and Methods

Study Design

Blood from 12 healthy subjects was collected into 2 standard EDTA vacuum tubes (K_2 EDTA 10.8 mg, 6 mL Vacutainer Plus, Sterile Interior, Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) and 2 EDTA vacuum tubes with protease inhibitors (BD P100v1.0 Blood Collection System for Use in Protein Analysis, 8.5 mL, Becton Dickinson Vacutainer Systems). Plasma was prepared immediately from blood from 1 standard EDTA tube (E1) and 1 EDTA plus protease inhibitors tube (P1). Blood in the other EDTA (E2) and EDTA plus protease inhibitor (P2) tubes was stored at room temperature for 2 hours before preparing plasma. For both sets of tubes, after plasma preparation, aliquots were made, immediately placed on dry ice, and subsequently stored at –80°C. Samples underwent only 1 thaw before the testing of 99 soluble factors by a multiplexed enzyme-linked immunosorbent assay (ELISA).

For the second part of the study, blood from 3 healthy subjects was collected into 3 different types of tubes: standard EDTA, EDTA plus protease inhibitors-BD P100v1.0, and EDTA plus protease inhibitors-BD P100v1.1 (Becton Dickinson Vacutainer Systems). The P100v1.1 tubes have the same protease inhibitors as the P100v1.0 tubes, but they have a modified inner capsule designed to improve the separation of plasma from the cellular element of whole blood. Two collections were performed for each type of tube; one was processed immediately and the other was processed after 2 hours of storage as described in the preceding paragraph.

Plasma Separation and Storage

Two plasma separation protocols were used. For blood collected from the first 6 subjects, the collection tubes were centrifuged at 3,500 rpm (2,500*g*) for 8 minutes (Sorval Legend T, Kendro Laboratory Products, Newtown, CT). Plasma was removed and placed in a 5-mL plastic tube on ice. One-milliliter aliquots of plasma were placed in cryovials (2-mL Nunc, catalog No. 363401, Nagle Nunc, Rochester, NY) and snap-frozen on dry ice and ethanol. After the plasma was frozen, the vials were transferred to –80°C for storage. Blood from the last 6 subjects was prepared in the same manner except that it was centrifuged at 2,500 rpm (1,600*g*) for 15 minutes (Sorval Legend T).

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Analysis of Plasma Proteins

The levels of 99 soluble factors were assessed on an ELISA-based platform (Pierce Search Light Proteome Arrays, Boston, MA) consisting of multiplexed assays that measured up to 16 proteins per well in standard 96-well plates.[1] The arrays were produced by spotting 2 × 2, 3 × 3, or 4 × 4 patterns of different monoclonal antibodies into each well of a 96-well plate. Following a typical sandwich ELISA procedure, signals were generated by using a chemiluminescent substrate. The light produced at each spot in the array was captured by imaging the entire plate with a commercially available cooled CCD camera. The data were reduced using image analysis software that calculated exact values (pg/mL) based on standard curves.

Each sample was tested for the following 99 human proteins: granulocyte colony-stimulating factor (G-CSF); granulocyte-macrophage colony-stimulating factor (GM-CSF); interferon (IFN)-α; IFN-γ; interleukin (IL)-1α; IL-Rα; IL-1β; IL-2; IL-3; IL-4; IL-5; IL-6; IL-7; IL-8; IL-9; IL-10; IL-11; IL-12p40; IL-12p70; IL-13; IL-15; IL-16; IL-17; IL-18; tumor necrosis factor (TNF)-α; epithelial cell–derived neutrophil activating protein 78 (ENA-78); Eotaxin/CCL11; Eotaxin 2; Exodus II; growth-related oncogene-α (CXCL1); inducible 309; IFN-inducible protein 10 (CXCL10); IFN-inducible T-cell α chemoattractant (CXCL11); lymphotactin; monocyte chemoattractant protein (MCP)-1 (CCL2); MCP-2 (CCL8); MCP-3 (CCL7); MCP-4 (CCL13); macrophage-derived chemokine; monokines induced by interferon (CXCL9); macrophage inflammatory protein (MIP)-1α (CCL3); MIP-1β; MIP-3α (CCL20); MIP-3β (CCL19); neutrophil chemoattractant peptide (NAP)-2 (CXCL7); stromal-derived factor-1β (CXCL12); regulated on activation, normal T cell–expressed and secreted (RANTES/CCL5); thymus and activation regulated chemokine (CCL17); angiopoietin-2; fibroblast growth factor basic (FGF-β); keratinocyte growth factor (KGF); hepatocyte growth factor (HGF); heparin-binding epidermal growth factor; human growth hormone; platelet-derived growth factor BB (PDGF-BB); TIMP-1; TIMP-2; vascular endothelial growth factor (VEGF); MMP-1; MMP-2; MMP-3; MMP-8; MMP-9; MMP-10; MMP-13; brain-derived neurotrophic factor; ciliary neurotrophic factor; leukemia inhibitory factor; serum amyloid A; pregnancy associated plasma protein-A; receptor for activation of NF-κ β (RANK); RANK-L; osteoprotegrin; plasminogen activator inhibitor-1 (PAI-1) total; PAI-1 active; vascular cell adhesion molecule; intracellular adhesion molecule (ICAM-1); L-selectin; E-selectin; TNF receptor (TNFR)1; TNFR2; IL-2 receptor (IL-2R); IL-2Rγ; IL-6R; CD40 ligand (CD40L); leptin; adiponectin (ACRP-30); amphiregulin; ApoB-100; Apo-A1; fibrinogen; IL-1Rα; myeloperoxidase; aminoterminal proBNP (NT-proBNP); nerve growth factor β; CD14; C-reactive protein; myeloid progenitor inhibitory factor-1 (MPIF-1); and neutrophin-3. The levels of 2 of the 99 factors, IL-3 and MPIF-1, were consistently below the level of detection of the assay, and they were excluded from analysis.

Statistical Analysis

A paired 2-tailed *t* test of natural log transformed data was used to compare the plasma factor levels between groups. Differences were considered significant at a cutoff *P* value of .01 or less. Relatedness in cytokine expression patterns in samples collected in EDTA and EDTA-protease inhibitor tubes was tested by applying unsupervised and supervised Eisen hierarchical clustering methods[15] to the data set encompassing the 97 cytokines across all samples.

Results

Comparison of Factor Levels in Plasma and Plasma Plus Protease Inhibitors Prepared Immediately and 2 Hours After Collection

Unsupervised hierarchical clustering was applied to the 97 soluble factors across the 48 plasma samples prepared immediately and after 2 hours of storage. The 48 plasma samples clustered into 4 groups. The first group comprised predominantly samples processed immediately (immediate group, samples E1 and P1), the second included samples processed after 2 hours (2-hour storage group, samples E2 and P2), and the remaining 2 groups were a mix of samples (mixed groups, samples E1, E2, P1, and P2) processed immediately and after 2 hours of storage at room temperature. All 4 groups contained an approximately equal mix of samples of plasma and plasma plus protease inhibitors.

The group composed predominantly of samples processed immediately after collection, the immediate group **Figure 1** (red bar), was made up of 10 samples, 9 of which were processed immediately. Among the 9 samples processed immediately, 5 were plasma plus protease inhibitors (P1-01, P1-03, P1-11, P1-08, and P1-05) and 4 were plasma without protease inhibitors (E1-01, E1-03, E1-08, and E1-05). The other sample was plasma plus protease inhibitors prepared 2 hours after collection (P2-03).

The group made up predominantly of samples processed 2 hours after collection, the 2-hour group, contained 16 samples, including 13 that were processed 2 hours after collection (Figure 1, yellow bar). These 13 samples included 7 plasma (E2-01, E2-10, E2-09, E2-05, E2-06, E2-04, and E2-12) and 6 plasma plus protease inhibitors (P2-01, P2-10, P2-09, P2-02, P2-11, and P2-12). The 3 samples from blood processed immediately after collection included 2 plasma plus protease inhibitor (P1-09 and P1-12) and 1 plasma without protease inhibitors (E1-12).

The remaining 2 groups were a mix of plasma samples prepared immediately and after 2 hours of storage: mixed

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group 1 (Figure 1, blue bar) and mixed group 2 (Figure 1, black bar). Both mixed groups were made up of 11 samples, and both included 6 samples prepared immediately after collection and 5 prepared after 2 hours of storage. Mixed group 1 included 3 plasma samples prepared immediately (E1-10, E1-07, and E1-02), 3 samples of plasma plus protease inhibitors prepared immediately (P1-07, P1-10, and P1-02), 3 plasma samples prepared after 2 hours (E2-07, E2-03, and E2-02), and 2 samples

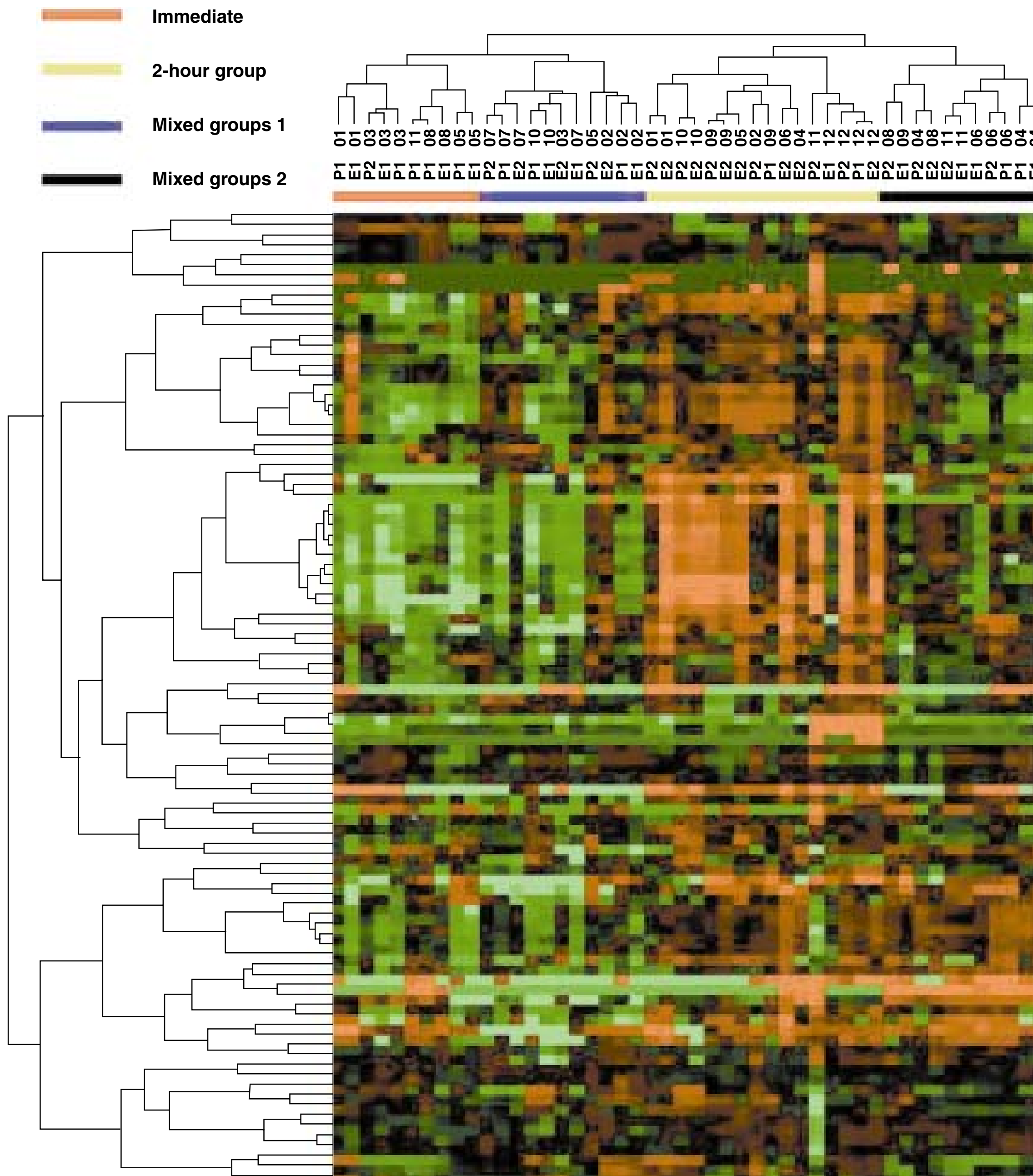

Figure 1 Unsupervised hierarchical clustering of soluble factors in plasma prepared immediately and after storage of whole blood collected with and without protease inhibitors. Blood from 12 healthy subjects (01-12) collected into EDTA and plasma tubes was prepared immediately (E1) or after 2 hours of room temperature storage (E2). Blood from the same 12 donors also was collected into tubes with EDTA plus protease inhibitors and was processed immediately (P1) or 2 hours after collection (P2). The levels of 97 soluble factors were measured using a multiplex enzyme-linked immunosorbent assay. Hierarchical clustering applied to the data set encompassing the 97 soluble factors and 48 samples clustered the samples into 4 groups: immediate, 2-hour, mixed 1, and mixed 2. Higher factor levels are shown in red and lower factor levels in green.

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of plasma plus protease inhibitors prepared after 2 hours (P2-07 and P2-05). Mixed group 2 included 4 plasma samples prepared immediately (E1-09, E1-11, E1-06, and E1-04), 2 plasma plus protease samples prepared immediately (P1-06 and P1-04), 3 plasma plus protease inhibitor samples prepared after 2 hours (P2-08, P2-04, and P2-06), and 2 plasma samples processed after 2 hours (E2-08 and E2-11).

Overall, the proteomic profiles of samples in the 2 mixed groups and the immediate group were similar, but the profile of the 2-hour group differed from the others. A cluster of 22 factors with levels greater in the 2-hour group distinguished the 2-hour group from the others. This cluster included 7 growth factors (FGF, VEGF, HGF, KGF, G-CSF, GM-CSF, and PDGF-BB), 6 interleukins (IL-2, IL-4, IL-5, IL-6, IL-7, and IL-10), 3 chemokines (NAP-2/CXCL7, ENA-78/CXCL5, and RANTES/CCL5), and 2 IFNs (IFN-α and IFN-γ) **Figure 2**.

Plasma samples from donors 1 through 6 were processed by centrifugation at 2,500*g* for 8 minutes and plasma samples

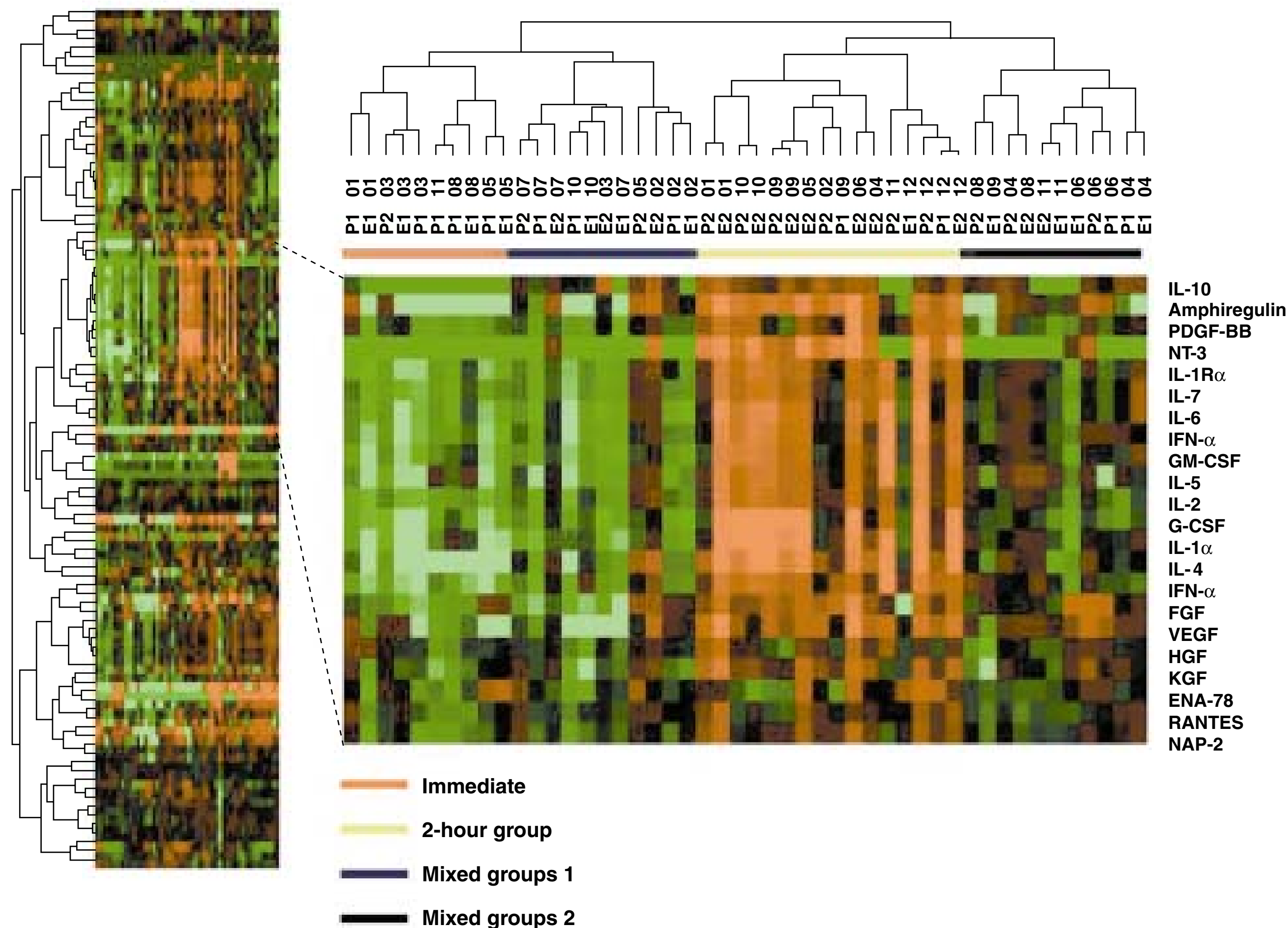

Figure 2 Factors that distinguish plasma samples prepared from blood immediately and 2 hours after collection. Blood from 12 healthy subjects collected into EDTA and plasma was prepared immediately (E1) or after 2 hours of room temperature storage (E2). Blood from the same 12 donors also was collected into tubes with EDTA plus protease inhibitors and was processed immediately (P1) or 2 hours after collection (P2). The levels of 97 soluble factors were measured using a multiplex enzyme-linked immunosorbent assay. Hierarchical clustering was applied to the data set encompassing the 97 soluble factors and 48 samples. The 48 samples were clustered into 4 groups. The proteomic profile of 3 groups, the immediate group, mixed groups 1, and mixed groups 2 were similar, but the 2-hour group differed from the others. A cluster of 22 factors were higher in the 2-hour group. Higher factor levels are shown in red and lower factor levels in green. ENA, epithelial cell–derived neutrophil activating protein; FGF, fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; HGF, hepatocyte growth factor; IFN, interferon; IL, interleukin; KGF, keratinocyte growth factor; NAP, neutrophil chemoattractant peptide; NT-3, neutrophin-3; PDGF-BB, platelet-derived growth factor BB; RANTES, regulated on activation, normal T cell–expressed; VEGF, vascular endothelial growth factor.

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from donors 7 through 12 by centrifugation at 1,600*g* for 15 minutes. When unsupervised hierarchical clustering was used to analyze the 12 plasma samples prepared immediately across the 97 factors, the samples were divided into 2 groups. Both groups were a mix of samples prepared using the 2 centrifugation protocols **Figure 3A**. Similarly, when unsupervised hierarchical clustering was used to analyze plasma samples prepared after 2 hours of storage, samples of plasma plus protease inhibitors prepared immediately and of plasma plus protease inhibitors processed after 2 hours, the samples clustered into 2 or 3 groups, but for none of the 3 types of samples did samples prepared using the 2 centrifugation protocols segregate into separate groups **Figure 3B**, **Figure 3C**, and **Figure 3D**, suggesting that centrifugal speed was not a critical factor affecting protein profiling.

Comparison of Factor Levels in Plasma Prepared Immediately and 2 Hours After Collection

The levels of all 97 factors were compared using paired *t* tests among plasma samples prepared immediately and those prepared 2 hours after collection (all E1 vs all E2). This analysis revealed that 37 factors differed significantly between the 2 groups. The mean levels of all 37 factors were greater in samples prepared 2 hours after collection than those prepared immediately after collection ($P < .01$) **Table 2**. The average increase in factor levels after 2 hours of storage ranged from 1.24- to 17.5-fold (Table 2). Most of the 37 proteins with increased levels following storage for 2 hours were interleukins, metalloproteases, and growth factors.

Comparison of Factor Levels in EDTA Plus Protease Inhibitors Plasma Prepared Immediately and 2 Hours After Collection

The levels of 32 factors differed significantly between samples of plasma plus protease inhibitors prepared immediately (all P1 samples) and 2 hours after collection (all P2 samples) **Table 3**. The levels of 31 of 32 factors were greater in P2 than in P1 samples. The level of NT-proBNP decreased after 2 hours of storage at room temperature. The average change in factor levels ranged from 1.18- to 9.33-fold.

Comparison of Factor Levels in Samples of Plasma and Plasma Plus Protease Inhibitors

A comparison of protein levels in blood collected in plasma (all E1 samples) and plasma with protease inhibitors tubes prepared immediately after collection (all P1 samples) revealed significant differences in the levels of only 4 factors: PAI-1, Eotaxin, Exodus II, and NT-proBNP **Table 4**. PAI-1 levels were greater in plasma, and Eotaxin 2, Exodus II, and NT-proBNP were greater in plasma plus protease inhibitor plasma (Table 4).

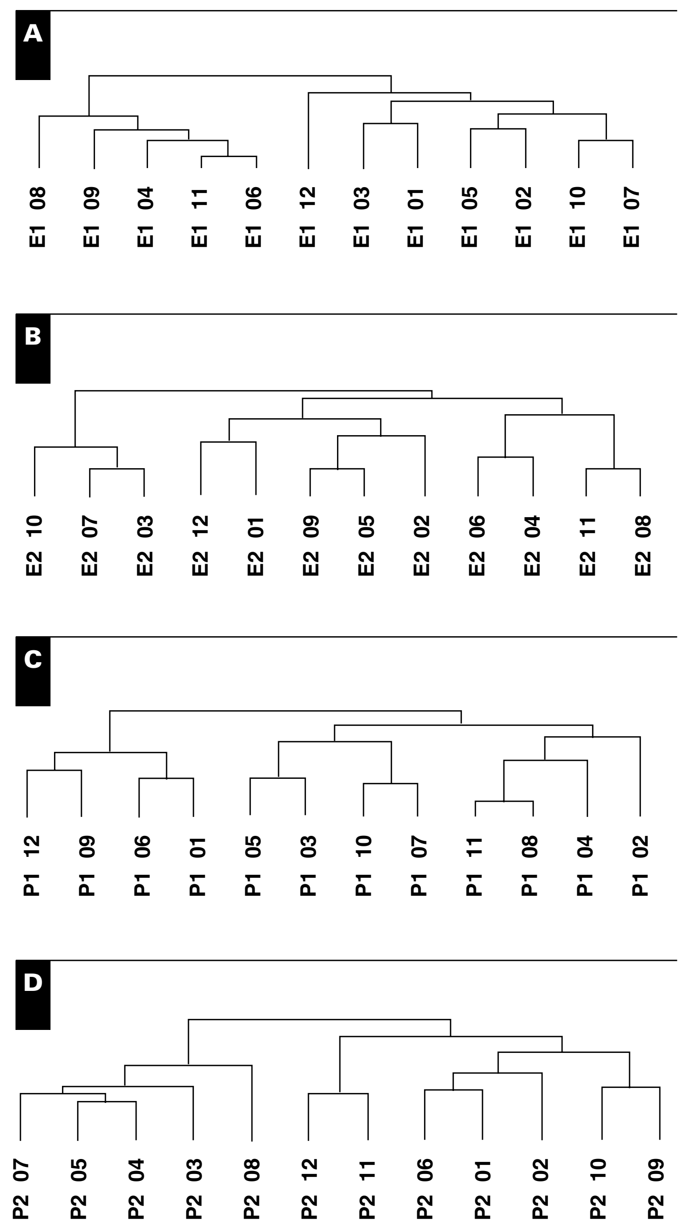

Figure 3 Effects of plasma preparation centrifugation protocol on factor levels. Plasma samples for donors 1 through 6 (01 to 06) were prepared by centrifugation for 8 minutes at 2,500*g*, and samples from donors 7 through 12 (07 to 12) were prepared by centrifugation for 15 minutes at 1,600*g*. Unsupervised hierarchical clustering was applied to 97 plasma factors across 12 plasma samples prepared immediately (**A**, E1), 12 plasma samples prepared after 2 hours of room temperature storage (**B**, E2), 12 plasma plus protease inhibitor samples prepared immediately (**C**, P1), and 12 plasma plus protease inhibitor samples prepared after 2 hours of room temperature storage (**D**, P2). Because there was no segregation of samples from donors 1 through 6 or 7 through 12, centrifuge force and duration had no effect on the samples.

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Table 2
Factors Whose Levels Differed in Plasma Processed Immediately Compared With After 2 Hours of Storage

	Plasma Factor Levels (pg/mL)*			
Factor	**Processed Immediately**	**Processed After 2 h**	**Fold Difference**	***P*** †
Amphiregulin	3.0 ± 5.0	53.4 ± 37	7.14 ± 5.89	.000006
IL-4	6.4 ± 5.5	101 ± 89	9.56 ± 6.32	.000047
IL-15	9.9 ± 2.9	15.2 ± 2.5	1.63 ± 0.41	.000060
IL-16	444 ± 102	556 ± 106	1.27 ± 0.16	.000063
MMP-13	867 ± 755	2,437 ± 1,223	3.86 ± 2.68	.000091
IFN-α	14.5 ± 10.8	66.7 ± 46.3	7.64 ± 11.20	.000097
IL-10	1.1 ± 1.7	5.7 ± 2.6	4.15 ± 3.59	.00013
IL-2	75 ± 31	409 ± 313	7.27 ± 10.1	.00018
IL-2Rγ	819 ± 1,102	1,979 ± 1,088	3.77 ± 2.51	.00019
β-NGF	132 ± 198	339 ± 178	4.42 ± 3.17	.00020
IL-6	7.6 ± 7.5	52.0 ± 41.5	11.4 ± 12.2	.00022
IL-12p70	11.5 ± 8.1	21.0 ± 7.3	2.38 ± 1.12	.00023
MIP-3β	109 ± 53	161 ± 79	1.46 ± 0.37	.00024
IFN-β	7.3 ± 4.0	52.1 ± 42.0	11.3 ± 15.2	.00024
IL-1Rα	358 ± 233	1,758 ± 1,241	6.98 ± 7.81	.00026
HGF	197 ± 90	376 ± 265	1.91 ± 0.76	.00027
IL-9	453 ± 254	940 ± 235	2.70 ± 1.84	.00028
Eotaxin 2	58.8 ± 16.3	103.3 ± 49.5	1.74 ± 0.56	.00032
G-CSF	119 ± 60	2,186 ± 2,088	20.8 ± 24.2	.00034
IL-12p40	111 ± 79	177 ± 70	2.07 ± 0.86	.00034
IL-5	4.9 ± 3.7	27.6 ± 19.5	5.65 ± 4.11	.00034
NT-3	0.4 ± 1.4	39.0 ± 45.9	—	.00039
TNF-α	54.7 ± 26.1	96.6 ± 32.8	2.04 ± 0.83	.00045
IL-1α	9.4 ± 7.7	115 ± 100	17.5 ± 24.3	.00050
PDGF-BB	127 ± 79	645 ± 767	6.02 ± 7.35	.00054
IL-7	21 ± 16	85.7 ± 62.5	5.67 ± 6.91	.00073
GM-CSF	214 ± 163	1,201 ± 948	11.0 ± 18.7	.00076
TGF-α	100 ± 70	265 ± 140	2.99 ± 1.54	.00099
KGF	20.9 ± 12.8	85.1 ± 65.5	4.07 ± 4.56	.00182
MMP-10	1,280 ± 1,666	2,089 ± 817	2.44 ± 1.24	.00204
IL-1β	49.1 ± 12.5	110.8 ± 68.0	2.41 ± 1.64	.0023
MMP-2	457,211 ± 186,517	580,716 ± 164,158	1.35 ± 0.33	.0024
PAI-1 total	10,711 ± 9,700	11,713 ± 11,511	1.31 ± 0.49	.0039
CD40L	361 ± 231	616 ± 538	1.67 ± 0.74	.0047
BDNF	118,351 ± 25,038	143,347 ± 25,756	1.24 ± 0.27	.0065
MMP-1	8,392 ± 9,497	13,370 ± 10,880	1.95 ± 1.10	.0067
FGF	104 ±123	297 ± 254	3.63 ± 3.76	.0088

BDNF, brain-derived neurotrophic factor; β-NGF, nerve growth factor β; FGF, fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; HGF, hepatocyte growth factor; IFN, interferon; IL, interleukin; KGF, keratinocyte growth factor; MIP, macrophage inflammatory protein; MMP, matrix metalloprotease; NT-3, neutrophin-3; PAI, plasminogen activator inhibitor; PDGF-BB, platelet-derived growth factor BB; TGF, transforming growth factor; TNF, tumor necrosis factor.

* Data are given as mean ± SD. Fold difference represents the mean SD of the factor level after 2 hours divided by the level immediately after processing for each of the 12 samples.

† *P* values were calculated using natural log transformed data and paired *t* tests.

Similarly, a comparison of the levels of factors in plasma (all E2 samples) and plasma plus protease inhibitors prepared after 2 hours of storage (all P2 samples) revealed significant differences in the levels of 4 factors: PAI-1, MMP-2, TIMP-2, and ICAM-1 **Table 5**. The levels of PAI-1 and MMP-2 were greater in plasma than in plasma plus protease inhibitor, and the levels of TIMP-2 and ICAM-1 were greater in plasma plus protease inhibitor samples.

Analysis of the Level of Factors in Protease Inhibitor PI 1.1 Tubes

We found little difference between plasma prepared from blood collected in EDTA and EDTA plus protease inhibitors; however, an improved version of the PV1.0 blood collection tube, the PV1.1, was made available during the study. For comprehensiveness of our study, we extended the analysis to 3 additional healthy donors and included blood collections in PV1.1 tubes. Blood was collected into 3 types of tubes, EDTA, EDTA plus protease inhibitor PV1.0, and EDTA plus protease inhibitor PV1.1. Plasma was prepared immediately and 2 hours after collection. Hierarchical clustering was applied to 97 factors across the 18 samples, and the samples were clustered into 2 groups **Figure 4**. One group contained 10 samples, 6 processed after 2 hours and 4 processed immediately. The other group contained 8 samples, 5 processed immediately and 3 after 2 hours. The samples collected in EDTA alone, EDTA plus protease inhibitor PV1.0, and EDTA plus protease inhibitor PV1.1 were found

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Table 3
Factors Whose Levels Differed Between Plasma Containing Protease Inhibitors Processed Immediately and After 2 Hours of Storage

	Plasma Plus Protease Inhibitor Factor Levels (pg/mL)*			
Factor	Processed Immediately	Processed After 2 h	Fold Difference	P†
IL-2	73.5 ± 55.3	315 ± 319	4.71 ± 4.42	.000049
IL-5	5.3 ± 4.7	41.3 ± 70.3	5.79 ± 7.24	.000096
IL-1Rα	358 ± 306	1,619 ± 1,574	5.67 ± 5.67	.00014
MMP-10	861 ± 745	1,639 ± 696	2.51 ± 0.97	.00014
NAP-2	1,374,271 ± 653,243	2,213,232 ± 939,458	1.73 ± 0.49	.00018
MMP-9	41,105 ± 14,021	68,235 ± 22,245	1.78 ± 0.64	.00018
IL-2Rγ	606 ± 854	1,376 ± 759	5.22 ± 4.00	.00022
IL-4	8.9 ± 10.7	81.1 ± 93.1	4.82 ± 3.41	.00022
IL-7	17.2 ± 9.5	79.8 ± 80.1	5.19 ± 5.24	.00034
Eotaxin 2	76.4 ± 25.3	107.0 ± 44.1	1.39 ± 0.28	.00037
IFN-α	17.3 ± 19.5	84.9 ± 125.4	7.00 ± 9.80	.00041
β-NGF	117 ± 139	237 ± 139	4.16 ± 3.01	.00043
PDGF-BB	174 ± 146	321 ± 220	2.17 ± 1.30	.00059
IL-1β	57.6 ± 44.0	109.6 ± 38.0	2.62 ± 1.35	.00076
IL-6	6.2 ± 4.3	49.6 ± 59.7	6.55 ± 7.93	.00097
TGF-α	119 ± 145	206 ± 101	2.90 ± 1.99	.0011
KGF	30.1 ± 15.0	55.6 ± 28.1	2.20 ± 1.26	.0014
IL-1α	13.1 ± 9.7	99.0 ± 125.0	7.72 ± 9.81	.0014
G-CSF	138 ± 96	1,535 ± 2,290	9.33 ± 13.83	.0016
ENA-78	808 ± 671	1,104 ± 872	1.50 ± 0.47	.0016
MIP-3β	127 ± 75	148 ± 82	1.18 ± 0.16	.0017
VEGF	26.7 ± 25.0	76.1 ± 46.1	3.19 ± 2.97	.0018
PAI-1 total	6,216 ± 7,706	6,770 ± 6,990	2.32 ± 1.42	.0020
FGF	134 ± 133	205 ± 132	2.32 ± 1.50	.0025
GM-CSF	209 ± 127	1,453 ± 2,120	8.97 ± 14.27	.0032
Amphiregulin	14.8 ± 20.9	35.2 ± 22.8	2.17 ± 0.86	.0036
IFN-γ	9.7 ± 7.7	54.2 ± 66.8	5.75 ± 9.00	.0047
MMP-13	797 ± 1,149	1,747 ± 1,134	7.58 ± 11.20	.0058
MMP-3	1,461 ± 1,084	2,142 ± 966	1.86 ± 1.11	.0075
NT-proBNP	69.0 ± 70.2	41.5 ± 41.0	0.74 ± 0.61	.0094
TARC	230 ± 169	361 ± 247	2.02 ± 1.22	.0095
IL-10	2.3 ± 2.0	4.1 ± 2.2	1.74 ± 0.80	.0098

β-NGF, nerve growth factor β; ENA, epithelial cell–derived neutrophil activating protein; FGF, fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; KGF, keratinocyte growth factor; MIP, macrophage inflammatory protein; MMP, matrix metalloprotease; NAP, neutrophil chemoattractant peptide; NT-proBNP, aminoterminal proBNP; PAI, plasminogen activator inhibitor; PDGF-BB, platelet-derived growth factor BB; TARC, thymus and activation regulated chemokine; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.
* Data are given as mean ± SD. For factors that increased after 2 hours the fold difference represents the mean ± SD of the factor level after 2 hours divided by the level immediately after processing of all 12 samples. For factors that decreased after 2 hours, the fold difference represents the mean ± SD of the factor level immediately after processing divided by the level after 2 hours of all 12 samples.
† *P* values were calculated using natural log transformed data and paired *t* tests.

in similar proportions in both groups. Although not enough donors were studied to make definite conclusions, there did not seem to be differences among the 3 types of samples.

Discussion

We compared the levels of 97 factors in plasma prepared from blood collected in EDTA and EDTA plus protease inhibitors that was processed immediately and after 2 hours of room temperature storage. We expected to find that the levels of many factors would fall due to proteolytic degradation during storage. However, in plasma without added protease inhibitors none of the factor levels fell in 2 hours, and the levels of more than one third of the factors increased. The collection of blood into tubes with protease inhibitors had little effect on the factor levels during the 2 hours of storage.

The levels of a wide variety of factors increased during 2 hours of room temperature storage; interleukins, growth factors, and MMPs were most likely to be affected. Because these types of factors are produced by leukocytes and platelets, the increase in the levels of these factors may be due to the release of stored proteins by leukocytes and platelets or by the de novo production of these factors. It is not likely the cell death and lysis is responsible for the increased factor levels. Even leukocytes with the shortest life span, granulocytes, remain viable for several hours. Neutrophils, lymphocytes, monocytes, or platelets might release factors as part of normal metabolism or following stimulation during the collection and storage process.

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Table 4
Factors Whose Levels Differed Between Plasma and Plasma Containing Protease Inhibitors Processed Immediately After Collection

Factor	Factor Plasma Levels (pg/mL)*			P†
	Plasma	Plasma Plus Protease Inhibitors	Fold Difference	
PAI-1 active	10,711 ± 9,700	6,215 ± 7,706	3.92 ± 2.82	.00027
Eotaxin 2	59 ± 16	76 ± 25	1.31 ± 0.26	.0010
Exodus II	27 ± 14	46 ± 20	2.13 ± 1.19	.0042
NT-proBNP	26 ± 35	69 ± 70	1.89 ± 1.38	.0073

NT-proBNP, aminoterminal proBNP; PAI, plasminogen activator inhibitor.
* Data are given as mean ± SD.
† *P* values were calculated using natural log transformed data and paired *t* tests.

Table 5
Factors Whose Levels Differed Between Plasma and Plasma Containing Protease Inhibitors Processed 2 Hours After Collection

Factor	Factor Levels (pg/mL)*			P†
	Plasma	Plasma Plus Protease Inhibitors	Fold Difference	
PAI-1 active	11,713 ± 11,511	6,770 ± 6,990	2.18 ± 0.88	.00027
MMP-2	580,716 ± 164,158	495,659 ± 132,686	1.18 ± 0.17	.0030
TIMP-2	255,071 ± 44,148	310,004 ± 58,452	1.23 ± 0.23	.0040
ICAM-1	510,740 ± 146,212	567,770 ± 157,816	1.12 ± 0.13	.0081

ICAM, intracellular adhesion molecule; MMP, matrix metalloprotease; PAI, plasminogen activator inhibitor; TIMP, tissue inhibitor of metalloprotease.
* Data are given as mean ± SD.
† *P* values were calculated using natural log transformed data and paired *t* tests.

Several studies have found that serum and plasma factors are stable for many hours following separation from cells. When plasma is separated from cells and is stored with protease inhibitor cocktails, plasma proteomic profiles remain stable for at least 48 hours.[16] However, blood cell metabolism continues for many hours after blood is collected. When unseparated whole blood is stored at room temperature, several changes in plasma analytes occur.[13] Within 4 hours, oxygen levels and pH fall, and carbon dioxide levels rise. Hemoconcentration due to the movement of water into cells also can cause slight changes in soluble factor levels, but these changes do not occur unless plasma remains in contact with cells for 24 hours or more.[13] Our data suggest that within 2 hours of collection, leukocytes and platelets produce and/or release significant quantities of interleukins, growth factors, and MMPs.

Cell stimulation could cause the production and release of factors, although the samples were collected in tubes containing EDTA, a calcium chelator that not only prevents the activation of the coagulation cascade, but also prevents complement cascade activation and cation-dependent platelet and leukocyte activation. In addition, blood collection tubes are coated with silicon to prevent hemolysis and damage to other cells. Nevertheless, it still is possible that cells contain in their cytoplasm soluble proteins that are ready to be released and are released nonspecifically or that the EDTA is blocking only signaling and de novo protein synthesis but not the release of already synthesized proteins.

Based on the results of this study, we recommend that plasma be separated from whole blood and frozen immediately after the blood has been collected. When blood is processed in this manner, there is no advantage to collecting blood in tubes containing protease inhibitors.

The levels of many soluble factors increase when plasma samples are kept at room temperature for 2 hours before processing as we demonstrated by high-throughput protein array analysis. The collection of blood in a protease inhibitor cocktail blood collection tube had little effect on protein preservation. Our findings contribute to the establishment of standard methods for plasma collection and handling and shed light on the importance of minimizing handling time before freezing. A 2-hour delay in processing and freezing plasma may, in fact, trigger a storm of soluble factors that can lead to confounding results in any type of subsequent proteomic analysis.

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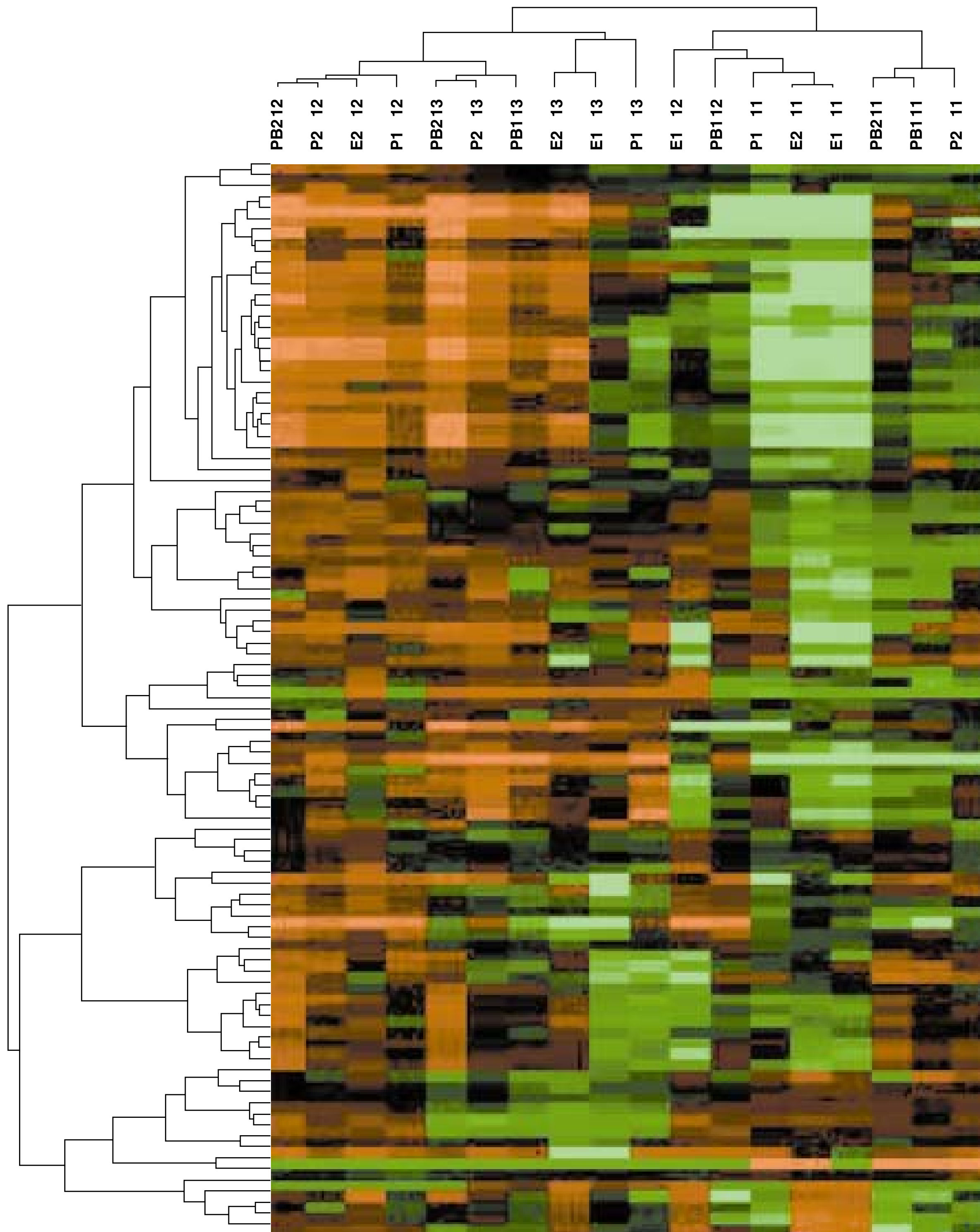

Figure 4 Comparison of the effects of 2 different protease inhibitor cocktails collection systems on plasma factor levels. Blood from 3 donors was collected in tubes with EDTA (E), EDTA plus protease inhibitor cocktail PV1.0 (P), or protease inhibitor cocktail PV1.1 (PB), and plasma was prepared immediately (E1, P1, and PB1) or after 2 hours of room temperature storage (E2, P2, and PB2). Unsupervised hierarchical clustering applied to 97 plasma factors across 18 plasma samples prepared from the 3 donors separated the samples into 2 groups with no difference in the distribution of samples prepared from blood collected into the 2 different protease inhibitor tubes. Higher factor levels are shown in red and lower factor levels in green.

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