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Detection of bone and cartilage-related proteins in plasma of patients with a bone fracture using liquid chromatography-mass spectrometry

Lovorka Grgurevic[1], Boris Macek[2], Dragan Durdevic[3], and Slobodan Vukicevic[1]*

1 Laboratory of Mineralized Tissues, School of Medicine, Zagreb, Croatia

2 Max-Planck-Institute for Biochemistry, Department of Proteomics and Signal Transduction, Am Klopferspitz 18, 82152 Martinsried, Germany

3 Clinic of Traumatology, Draskoviceva 19, Zagreb, Croatia

Correspondence to:

Slobodan Vukicevic, MD, Ph.D., Laboratory of Mineralized Tissues, School of Medicine, University of Zagreb, Salata 11, 10000 Zagreb, Croatia, E-mail: vukicev@mef.hr; tel: +385 1 4566 812

Running title: Bone and cartilage-related proteins in human plasma

Abstract

Following bone fracture, a large number of growth factors, cytokines, and their cognate receptors involved in the repair process are active at the fracture site. To determine whether they appear in the patients' blood as candidate biomarkers for following the outcome of healing we analyzed in the plasma of 25 patients with an acute bone fracture following affinity plasma purification, SDS gel electrophoresis and liquid chromatography - tandem mass spectrometry (LC-MS/MS). Two hundred and thirteen nonredundant proteins were identified in the *in-gel* analysis of pooled plasma proteins. Gene ontology (GO) analysis indicated that a majority of detected proteins were of extracellular origin, whereas only a small number were of intracellular (cytosole and nucleus) origin. A significant proportion of detected proteins was involved in the cell growth and proliferation, transport and coagulation. Twelve proteins were potentially related to bone and cartilage metabolism, and several have not been previously identified in the plasma, including: TGF-β induced protein IG-H_3, cartilage acidic protein 1, procollagen C proteinase enhancer protein and TGF-β receptor III.

Introduction

The blood is rich with a large amount of previously unstudied molecules that could reflect the ongoing physiologic state of various tissues. As blood flows through most of the tissues of the human body the origins of plasma proteins are various. In the complex mixture of a plasma proteome, albumin and other carrier proteins are present in a high abundance, as well as proteins that originate from circulating blood cells. Almost all cells in the body communicate directly or indirectly with blood and upon damage or cell death tissue-specific proteins are released into the bloodstream. Therefore, most of potential undiscovered biomarkers will be eventually found in the plasma fraction, where much less abundant proteins enter the blood from the surrounding tissue.

Bone undergoes continuous turnover and remodeling consisting of bone formation and bone resorption, two opposite and well-balanced processes. The various bone serum and urinary markers are usually classified according to the metabolic process indicating low and high, decreased or increased bone turnover [1].

Following fracture, a large number of growth factors, cytokines, and their cognate receptors involved in bone repair are highly expressed at the fracture site in the first hours following injury. It is presumed that some or all of these factors initiate active repair process acting on the cells of the bone marrow, periosteum, and external soft tissues adjacent to the fracture site. Skeletal tissues are the main source of such proteins, while some are released from associated inflammatory cells at the site of injury [2, 10].

In this study we analyzed proteins as candidate biomarkers expressed in the plasma of patients with an acute bone fracture. The plasma proteins of patients were characterized by SDS gel electrophoresis and affinity purification followed by tandem mass spectrometry LC-MS/MS.

Following identification of proteins those associated with bone and cartilage metabolism were singled out and analyzed. Some of characterized proteins have not yet been identified in the circulation and their presence or quantity could reflect the extent of injury and the success of the fracture repair.

Materials and methods

Plasma collection

Human blood plasma samples were supplied by the Clinic of Traumatology in Zagreb. The approval for the collecting samples was obtained from the institutional Ethics Committee. Blood samples from 25 adult humans (21-60 years of age) of both genders with a single long bone fracture were drawn into syringes containing 3.8% sodium citrate to form an anticoagulant-to-blood ratio (v/v) 1:9. Plasma was obtained by centrifugation (15 min at 3000xg), and aliquots of each adult blood sample were pooled for the further analysis. Aliquot samples were stored at -80°C until analysis.

Affinity column purification

Pooled plasma of patients with a single-bone fracture (80ml) was diluted 2-fold with 10 mM sodium phosphate buffer (pH 7), and applied to a heparin Sepharose column (Amersham Pharmacia Biotech), previously equilibrated with 10 mM sodium phosphate buffer (pH 7). Bound proteins were eluted from the column with 10 mM sodium phosphate buffer (pH 7) containing 1 M and 2 M NaCl. Eluted fractions were precipitated with saturated ammonium sulfate (SAS) to a final concentration of 35%.

SDS gel electrophoresis and *in-gel* digestion

SDS-PAGE was run on a NuPAGE 10% Bis-Tris gel (Invitrogen, Carlsbad, USA) using MOPS SDS buffer system, and subsequently stained with Coomassie staining kit (NuPAGE, Invitrogen), as instructed by the manufacturer. After staining, each of the seven gel lanes was sliced in 12 pieces and the corresponding pieces were combined as indicated in Figure 1. The pieces were

then subjected to in-gel reduction, alkylation and trypsin digestion as described previously [4]. Gel pieces were washed two times with acetonitrile/25 mM NH_4HCO_3, reduced by incubation with 10 mM dithiothreitol (DTT) for 45 minutes at 56ºC and carboxyamidomethylated by incubation in 55 mM iodoacetamide for 45 minutes at room temperature. Trypsin (Promega) was added to dried gel pieces (150 ng per piece, diluted in 25 mM NH_4HCO_3) and incubated overnight at 37°C. Tryptic peptides were extracted with formic acid/acetonitrile/H_2O (10:20:70); and 100% acetonitrile, dried and resuspended in trifluoroacetic acid/acetonitrile/H_20 (1:2:97) for MS analysis.

Mass spectrometry

Tryptic peptides were analyzed by a liquid chromatography-mass spectrometry (LC-MS). Agilent 1100 nanoflow HPLC system (Agilent Technologies) was coupled to a LTQ-Orbitrap mass spectrometer (Thermo Scientific) using a nano-electrospray LC-MS interface (Proxeon Biosystems). Peptides were loaded on a home-made 75 μm C_{18} HPLC column in solvent "A" (0.5% acetic acid in Milli-Q water) and eluted with a 70-minute segmented linear gradient of 10-60% solvent "B" (80% acetonitrile, 0.5% acetic acid in Milli-Q water) at a flow rate of ca. 250 nL/min.

Mass spectrometer was operated in the positive ion mode. Each measurement cycle consisted of a full MS scan acquired in the orbitrap analyzer at a resolution of 60000, and MS/MS fragmentation of the five most-intense ions in the linear ion trap analyzer. To further improve mass accuracy, the lock-mass option was used as described previously [9]. This has resulted in a typical peptide average absolute mass accuracy of less than 1 ppm.

Peak lists were generated using in-house developed software (Raw2msm) [9], and searched against concatenated forward and reverse ("decoy") IPI human database (version 3.13) using

Mascot search engine (Matrix Science). Searches were done with trypsin specificity (2 missed cleavages allowed), carboxyamidomethylation as fixed modification, and oxidized methionine as variable modification. Precursor ion and fragment ion mass tolerances were 10 ppm and 0.5 Da, respectively.

Results of the database search were validated in the MSQuant software (http://msquant.sourceforge.net). Only peptides with a mass deviation lower than 5 ppm were accepted; two peptides were required for protein identification.

Gene ontology (GO) analysis was performed using ProteinCenter software package (Proxeon Biosystems).

Results

Gene ontology analysis of characterized plasma proteins

Pooled plasma samples were subjected to heparin affinity chromatography to enrich for proteins specific for bone and cartilage, majority of which are known to have heparin binding domains. This has also partially removed highly abundant plasma proteins, such as albumin, immunoglobulins, transferin and haptoglobulin. Fractions of interest were collected, precipitated with ammonium sulphate and separated on 1D SDS-PAGE gel (Figure 1). Gel bands were excised, digested with trypsin and analyzed by LC-MS/MS. Peptide fragmentation spectra were searched against the human IPI protein database, and the results of the database search were validated using MSQuant software. Only peptides with a mass deviation lower than 5 ppm were accepted; two peptides were required for protein identification, which led to an overall false-positive rate of less than 1% at both the peptide and the protein level.

In total, two hundred and thirteen nonredundant proteins were identified in the in-gel analysis of pooled plasma proteins from patients with a single bone fracture and listed in Table 1.

Gene ontology (GO) analysis of plasma proteins showed that a majority (63.8%) of detected proteins were of extracellular origin, whereas only a small number (7.5%) were of intracellular (cytosole and nucleus) origin. Interestingly, we also detected a relatively high number (35.2%) of membrane related proteins (Figure 2 A).

According to the molecular function analysis, 37.6% of detected proteins had catalytic properties, 18.3% were classified as signal transducers, and 13.1% as transporters (Figure 2 B).

In terms of biological activity, a significant proportion of detected proteins were involved in the cell growth and proliferation (21.1%), transport (23.9%) and coagulation (13.1%) (Figure 2 C).

Identification of bone- and cartilage-related proteins

From the list of detected proteins we singled out 12 proteins which could be related to bone and cartilage metabolism (Table 2). Among them there were proteins not previously identified in the plasma, like cartilage acidic protein 1 (CRTAC-1) which was identified with 28 peptides and an average peptide Mascot score of 53. A molecule also related to the cartilage metabolism was the Splice isoform A of the proteoglycan-4 or lubricin, identified with 2 peptides and an average peptide Mascot score of 60.

Transforming growth factor beta receptor III was identified in the plasma for the first time with 4 specific peptides and an average Mascot score of 44, as well as the transforming growth factor beta induced protein IG-H3, with 20 peptides and an average peptide Mascot score of 57.

Among extracellular matrix proteins not previously detected in the plasma was the alpha 3 type VI collagen isoform 1 identified with 2 peptides and an average peptide Mascot score of 60.

Previously identified extracellular matrix proteins of interest for bone repair included: isoform long of collagen alpha-1 (XVIII) chain precursor or endostatin with 5 peptides and an average peptide Mascot score of 36, splice isoform 2 of collagen alpha 3 (VI) chain precursor with 10 identified peptides and an average peptide Mascot score of 62, extracellular matrix protein 1 precursor with 57 identified peptides and an average peptide Mascot score of 54, and type IV collagenease precursor or matrix metalloproteinase-2 (MMP2) with 3 identified peptides and an average peptide Mascot score of 74 (Table 2). MMP-2 degrades extra-cellular proteins and disrupts the subendothelial basement membrane, thus enabling the transmigration of inflammatory cells. Another metalloproteinase inhibitor 1 (TIMP-1) was identified with 5 peptides and an average peptide Mascot score of 49 (Table 2).

Discussion

In this study we used state-of-the art proteomics techniques to characterize proteins in the plasma of patients with an acute bone fracture. Gene ontology showed a variety of different proteins, among which several have not been previously detected in the blood and could reflect the bone and cartilage stages of bone regeneration. Among them CRTAC-1, a glycosylated extracellular matrix molecule secreted by chondrocytes from the human articular cartilage. In the cell culture it was described as a candidate marker to distinguish the chondrocyte-like phenotype and activity from osteoblast-like and mesenchymal stem cells [15]. Thus its presence in the plasma of patients with an acute fracture could indicate the normal development and function of cartilaginous callus formation within the first week after the fracture and then its replacement by bone in the following weeks. In parallel CRTAC-1 could also indicate a concomitant joint cartilage injury immediately following an accident. In this way it may help distinguish between fractures with and without damaged joint cartilage, which would make CRTAC-1 an ideal marker for the various stages of the fracture repair. In the following study we need a precise time-related follow up of the plasma profile of CRTAC-1 in patients with a bone fracture with and without injured joint cartilage.

Splice isoform A of the proteoglycan-4 is a secreted, cytoprotective glycoprotein, a product of the gene proteoglycan 4 and a major component of the synovial fluid participating in the boundary lubrication of synovial fluids [11, 12, 13]. It prevents protein deposition onto cartilage from synovial fluid, controls the adhesion-dependent synovial growth, and inhibits the adhesion of synovial cells to the cartilage surface. It is highly expressed by synoviocytes and could serve as a marker of their activity following injury. It has been previously identified in the plasma [5].

The fracture healing process might be associated with a distinctive enzyme activity pattern at the fracture site, which may be reflected in their respective plasma/serum concentrations of various enzymes in their activity pattern. Thus, variations in the concentration of TIMP-1 and MMP-1 in the period following the fracture could have an important influence on the bone healing, as well as on other mechanisms leading to the development of a nonunion [6].

Discovery of circulating TßRIII was surprising since it is known that it has an essential role in the murine and chick development and that TßRIII knockout mice have an embryonic lethal phenotype. TßRIII acts as a TGF-ß co-receptor, concentrating ligand on the cell surface and enhancing ligand binding to the signaling TGF-ß receptor TßRII.[8] It is well known that transforming growth factor ß1 (TGF-ß1) and its receptor TßRII together with extracellular matrix proteins osteocalcin and collagen type I have an important role in the process of fracture healing. This result might add TßRIII to a list of novel biomarkers for following fracture repair. Recently, it was shown that TßRIII has also an important function as a suppressor of breast and prostate cancer progression [3, 16]. The possibility of following the cancer progression by detection of TßRIII in plasma should be further examined, especially knowing the role of TGFβ-1 and related family members in the progression of tumor growth and metastasis [14].

TGF-β IG-H_3 adhesion protein in plasma may play an important role in the cell-collagen interactions and binding to type I, II and IV collagens and may have an important role in the endochondral bone formation. It may also serve as a potential biomarker for the progression of successful bone healing. Additional studies will be needed to demonstrate the potential of these newly discovered plasma proteins as potential biomarkers for following the fracture healing and related disorders in humans.

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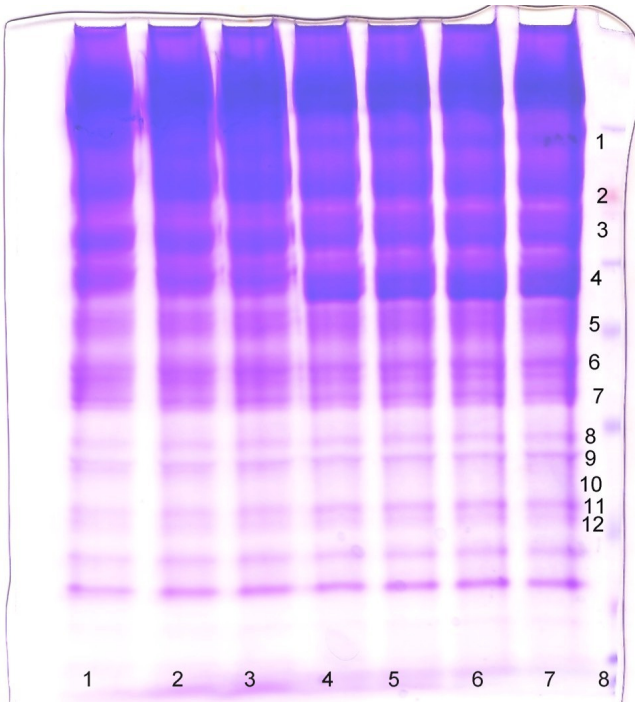
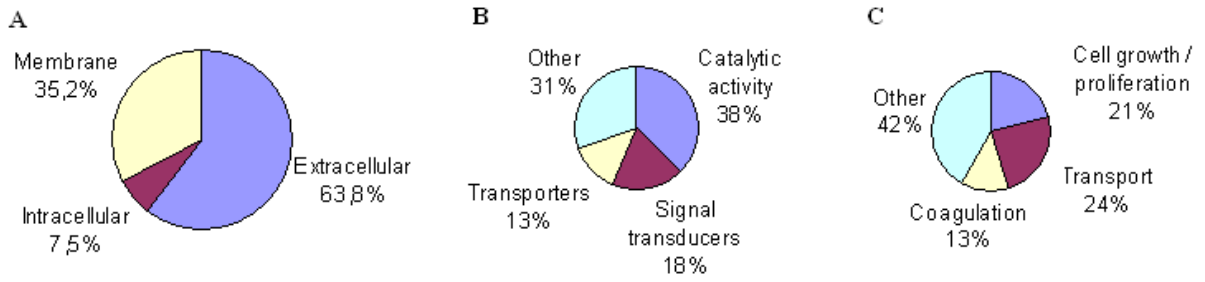
Description of figures:

Figure 1. Pooled plasma protein separation by one-dimensional SDS gel



Pooled plasma of patients with a single-bone fracture was applied to a heparin Sepharose column (Amersham Pharmacia Biotech). Bound proteins were eluted from the column with 10 mM sodium phosphate buffer (pH 7) containing 1 M NaCl (lane 4-7) and 2 M NaCl (lane 1-3), lane 8 molecular mass marker . The numbers in the column indicate gel lanes sliced and prepared for MS analysis. Gel was stained with a Comassie Brilliant Blue.

Figure 2. Protein categorization using gene ontology (GO) component terms



Total nonredundant proteins identified from pooled plasma of patients with a single bone fracture were compared according to the following categories: (A) subcellular localization, (B) molecular function and (C) biological process.

Table 1. Proteins identified by a tandem mass spectrometry LC-MS/MS in pooled purified plasma from patients with fracture

IPI Accession number	Protein name
IPI00000137.1	N-acetylglucosamine-1-phosphotransferase subunit gamma precursor
IPI00000138.1	Alpha-1,3-mannosyl-glycoprotein 2-beta-N-acetylglucosaminyltransferase
IPI00000816.1	14-3-3 protein epsilon
IPI00001753.1	Myosin-4
IPI00002147.4	Chitinase-3-like protein 1 precursor
IPI00003176.1	Serine protease HTRA1 precursor
IPI00003351.2	Extracellular matrix protein 1 precursor
IPI00003590.2	Isoform 1 of Sulfhydryl oxidase 1 precursor
IPI00004957.1	Angiopoietin-related protein 3 precursor
IPI00006114.4	Pigment epithelium-derived factor precursor
IPI00006154.1	Isoform Long of Complement factor H-related protein 2 precursor
IPI00006510.1	Tubulin beta-1 chain
IPI00006543.2	Complement factor H-related 5
IPI00006662.1	Apolipoprotein D precursor
IPI00007118.1	Plasminogen activator inhibitor 1 precursor
IPI00007199.4	Protein Z-dependent protease inhibitor precursor
IPI00007221.1	Plasma serine protease inhibitor precursor
IPI00007858.1	Myosin-13
IPI00008556.1	Isoform 1 of Coagulation factor XI precursor
IPI00009865.1	Keratin, type I cytoskeletal 10
IPI00009920.2	Complement component C6 precursor
IPI00010295.1	Carboxypeptidase N catalytic chain precursor
IPI00010779.4	Tropomyosin 4
IPI00010896.2	
IPI00011252.1	Complement component C8 alpha chain precursor
IPI00011261.2	Complement component C8 gamma chain precursor
IPI00011264.1	Complement factor H-related protein 1 precursor
IPI00013004.1	Isoform 1 of Pyridoxal kinase
IPI00016915.1	Insulin-like growth factor-binding protein 7 precursor
IPI00017256.5	Ras suppressor protein 1
IPI00017530.1	Ficolin-2 precursor
IPI00011264.1	Complement factor H-related protein 1 precursor
IPI00018219.1	Transforming growth factor-beta-induced protein ig-h3 precursor
IPI00018305.3	Insulin-like growth factor-binding protein 3 precursor

Table 1. Continued

IPI Accession number	Protein name
IPI00019359.3	Keratin, type I cytoskeletal 9
IPI00019579.1	Complement factor D precursor
IPI00019580.1	Plasminogen precursor
IPI00019581.1	Coagulation factor XII precursor
IPI00019591.1	Isoform 1
IPI00020091.1	Alpha-1-acid glycoprotein 2 precursor
IPI00020996.3	Insulin-like growth factor-binding protein complex acid labile chain precursor
IPI00021263.3	
IPI00021304.1	Keratin, type II cytoskeletal 2 epidermal
IPI00021364.1	Properdin precursor
IPI00021439.1	Actin, cytoplasmic 1
IPI00021440.1	
IPI00021727.1	C4b-binding protein alpha chain precursor
IPI00021841.1	
IPI00021842.1	Apolipoprotein E precursor
IPI00021854.1	Apolipoprotein A-II precursor
IPI00021885.1	Isoform 1 of Fibrinogen alpha chain precursor
IPI00021891.5	Isoform Gamma-B of Fibrinogen gamma chain precursor
IPI00022200.2	alpha 3 type VI collagen isoform 1 precursor
IPI00022229.1	Apolipoprotein B-100 precursor
IPI00022371.1	Histidine-rich glycoprotein precursor
IPI00022391.1	Serum amyloid P-component precursor
IPI00022392.1	Complement C1q subcomponent subunit A precursor
IPI00022394.2	Com
IPI00022395.1	Complement component C9 precursor
IPI00022418.1	Isoform 1 of Fibronectin precursor
IPI00022426.1	AMBP protein precursor
IPI00022434.2	ALB protein
IPI00022488.1	Hemopexin precursor
IPI00022822.4	Isoform Long of Collagen alpha-1(XVIII) chain precursor
IPI00022895.7	Alpha-1B-glycoprotein precursor
IPI00022937.3	Coagulation factor V
IPI00023006.1	Actin, alpha cardiac muscle 1
IPI00023728.1	Gamma-glutamyl hydrolase precursor

Table 1. Continued

IPI Accession number	Protein name
IPI00024825.2	Isoform A of Proteoglycan-4 precursor
IPI00025204.1	CD5 antigen-like precursor
IPI00025276.1	Isoform XB of Tenascin-X precursor
IPI00025862.1	C4b-binding
IPI00026314.1	Isoform 1 of Gelsolin precursor
IPI00026689.4	Hypothetical protein DKFZp686L20222
IPI00027507.1	Complement factor H-related protein 3 precursor
IPI00027780.1	72 kDa type IV collagenase precursor
IPI00027827.2	Extracellular superoxide dismutase [Cu-Zn] precursor
IPI00028091.2	Actin-like protein 3
IPI00028413.7	Inter-alpha-trypsin inhibitor heavy chain H3 precursor
IPI00029061.2	Selenoprotein P precursor
IPI00029193.1	Hepatocyte growth factor activator precursor
IPI00029236.1	Insulin-like growth factor-binding protein 5 precursor
IPI00029739.4	Isoform 1 of Complement factor H precursor
IPI00029863.4	Alpha-2-antiplasmin precursor
IPI00032179.2	Antithrombin III variant
IPI00032220.3	Angiotensinogen precursor
IPI00032258.4	
IPI00032291.1	Complement C5 precursor
IPI00032292.1	Metalloproteinase inhibitor 1 precursor
IPI00032311.4	Lipopolysaccharide-binding protein precursor
IPI00032328.1	Isoform HMW of Kininogen-1 precursor
IPI00041065.3	Hyaluronan-binding protein 2 precursor
IPI00043083.1	Beta-parvin
IPI00060715.1	BTB/POZ domain-containing protein KCTD12
IPI00154742.5	25 kDa protein
IPI00163207.1	Isoform 1 of N-acetylmuramoyl-L-alanine amidase precursor
IPI00164623.4	187 kDa protein
IPI00165438.2	Muscle type neuropilin 1
IPI00168728.1	FLJ00385 protein (Fragment)
IPI00178083.2	29 kDa protein
IPI00183968.4	tropomyosin 3 isoform 1
IPI00186903.3	Isoform 2 of Apolipoprotein-L1 precursor

Table 1. Continued

IPI Accession number	Protein name
IPI00216134.3	tropomyosin 1 alpha chain isoform 7
IPI00216699.1	Isoform 2 of Unc-112-related protein 2
IPI00216773.4	ALB protein
IPI00218192.2	Isoform 2 of Inter-alpha-trypsin inhibitor heavy chain H4 precursor
IPI00218732.2	Serum paraoxonase/arylesterase 1
IPI00219018.6	Glycer
IPI00219465.4	Transcobalamin-2 precursor
IPI00219682.5	Erythrocyte band 7 integral membrane protein
IPI00219713.1	Isoform Gamma-A of Fibrinogen gamma chain precursor
IPI00220327.2	Keratin, type II cytoskeletal 1
IPI00220350.1	Isoform Beta-3B of Integrin beta-3 precursor
IPI00220642.6	14-3-3 protein gamma
IPI00220644.8	pyruvate kinase 3 isoform 2
IPI00220701.3	Isoform 2 of Collagen alpha-3(VI) chain precursor
IPI00289831.4	Isoform PTPS of Receptor-type tyrosine-protein phosphatase S precursor
IPI00291262.3	Clusterin precursor
IPI00291866.5	Plasma protease C1 inhibitor precursor
IPI00291867.3	Complement factor I precursor
IPI00292218.3	Hepatocyte growth factor-like protein precursor
IPI00292530.1	Inter-alpha-trypsin inhibitor heavy chain H1 precursor
IPI00292950.4	Heparin cofactor 2 precursor
IPI00293925.2	Isoform 1 of Ficolin-3 precursor
IPI00294004.1	Vitamin K-dependent protein S precursor
IPI00294193.4	Isoform 1 of Inter-alpha-trypsin inhibitor heavy chain H4 precursor
IPI00294395.1	Complement component C8 beta chain precursor
IPI00295976.5	Isoform 1 of Integrin alpha-IIb precursor
IPI00296099.6	Thrombospondin-1 precursor
IPI00296165.5	Complement C1r subcomponent precursor
IPI00296176.2	
IPI00296537.3	Isoform C of Fibulin-1 precursor
IPI00296608.6	Complement component C7 precursor
IPI00297284.1	Insulin-like growth factor-binding protein 2 precursor
IPI00297550.7	Coagulation factor XIII A chain precursor

Table 1. Continued

IPI Accession number	Protein name
IPI00297779.6	T-complex protein 1 subunit beta
IPI00298497.3	Fibrinogen beta chain precursor
IPI00298828.3	Beta-2-glycoprotein 1 precursor
IPI00298860.5	Growth-inhibiting protein 12
IPI00298971.1	Vitronectin precursor
IPI00298994.5	271 kDa protein
IPI00299145.8	Keratin, type II cytoskeletal 6E
IPI00299547.4	Neutrophil gelatinase-associate
IPI00299738.1	Procollagen C-endopeptidase enhancer 1 precursor
IPI00302592.2	filamin 1
IPI00303476.1	ATP synthase subunit beta, mitochondrial precursor
IPI00303963.1	C
IPI00304273.2	Apolipoprotein A-IV precursor
IPI00304865.3	transforming growth factor, beta receptor III
IPI00305461.2	Inter-alpha-trypsin inhibitor heavy chain H2 precursor
IPI00306311.8	Pleckstrin
IPI00328609.3	Kallistatin precursor
IPI00328703.1	OAF homolog
IPI00329775.7	Isoform 1 of Carboxypeptidase B2 precursor
IPI00333828.4	Serpin A11 precursor
IPI00339228.1	Isoform 8 of Fibronectin precursor
IPI00382436.1	Ig lambda chain V-III region SH
IPI00382606.1	Factor VII active site mutant immunoconjugate
IPI00383111.2	57 kDa protein
IPI00384280.5	Prenylcysteine oxidase precursor
IPI00384444.4	Keratin, type I cytoskeletal 14
IPI00384938.1	Hypothetical protein DKFZp686N02209
IPI00385429.1	collectin sub-family member 11 isoform b
IPI00387025.1	Ig kappa chain V-I region DEE
IPI00387099.1	Ig kappa chain V-I region Rei
IPI00387113.1	Ig kappa chain V-III region B6
IPI00387120.1	Ig kappa chain V-IV region Len
IPI00399007.5	Hypothetical protein DKFZp686I04196 (Fragment)
IPI00418153.1	Hypothetical protein DKFZp686I15212

Table 1. Continued

IPI Accession number	Protein name
IPI00418163.3	complement com
IPI00418495.4	Platelet
IPI00419424.3	IGKV1-5 protein
IPI00426051.3	Hypothetical protein DKFZp686C15213
IPI00430808.1	Hypothetical protein
IPI00430842.3	IGHA1 protein
IPI00431645.1	HP protein
IPI00448925.3	IGHG1 protein
IPI00451624.1	Isoform 1 of Cartilage acidic protein 1 precursor
IPI00465248.5	enolase 1
IPI00465378.1	Apolipoprotein A-V precursor
IPI00465439.4	
IPI00472073.1	HLA class I histocompatibility antigen, B-59 alpha chain precursor
IPI00472610.2	IGHM protein
IPI00477090.5	IGHM protein
IPI00477597.1	Isoform 1 of Haptoglobin-related protein precursor
IPI00477644.2	26 kDa protein
IPI00477992.1	complement component 1, q subcomponent, B chain precursor
IPI00478003.1	Alpha-2-macroglobulin precursor
IPI00478493.3	HP protein
IPI00479116.1	Carboxypeptidase N subunit 2 precursor
IPI00479708.5	IGHM protein
IPI00549291.4	IGHM protein
IPI00550991.3	Isoform 1 of Alpha-1-antichymotrypsin precursor
IPI00553177.1	Alpha-1-antitrypsi
IPI00641368.4	Tsukushi precursor
IPI00641737.1	Haptoglobin precursor
IPI00643034.2	Isoform 1 of Phospholipid transfer protein precursor
IPI00643041.2	GTP-binding nuclear protein Ran
IPI00643525.1	Complement component 4A
IPI00646909.2	Tubulin alpha-8 chain
IPI00654888.3	Kallikrein B, plasma (Fletcher factor) 1
IPI00719373.1	IGLC1 protein
IPI00745872.2	Isoform 1 of Serum albumin precursor

Table 1. Continued

IPI Accession number	Protein name
IPI00783024.1	Myosin-reactive immunoglobulin heavy chain variable region (Fragment)
IPI00783987.1	Complement C3 precursor (Fragment)
IPI00784822.1	Hypothetical protein
IPI00785050.1	Hypothetical protein
IPI00785200.1	Hypothetical protein
IPI00787629.1	similar to Apolipoprotein
IPI00790993.1	104 kDa protein
IPI00794487.1	Immunoblobulin light chain (Fragment)
IPI00807531.1	IGHG1 protein

Table 2.

Protein name	IPI accession number	Number of identified peptides	Average peptides Mascot score	Previously dentified in plasma	GO console: Molecular function	GO console: Cellular component	GO console: Biological Process
Extracellular matrix protein 1 precursor	IPI00003351.2	57	54	+	-Signal transducer activity -Structural molecule activity -transporter activity	Extracellular	-cell communication -metabolism -regulation of biological process -transport
Transforming growth factor beta induced protein IG-H3 precursor	IPI 00018219.1	20	57	-	-protein binding	Extracellular	-cell proliferation - regulation of biological process -sensory perception
Splice isoform 1 of cartilage acidic protein 1 precursor	IPI00451624.1	28	53	-	-metal ion binding	-proteasom -Golgi complex	
Splice isoform 2 of collagen alpha 3 (VI) chain precursor	IPI00220701.3	10	62	+	-enzyme regulator activity -protein binding - Structural molecule activity	Extracellular	-development -transport
Type IV collagenease precursor	IPI00027780.1	3	74	+	- catalytic activity - enzyme regulator activity - Metal ion binding -	Extracellular	-development -metabolism
Alpha 3 type VI collagen isoform 1 precursor	IPI00022200.2	2	60	-	-enzyme regulator activity -protein binding -Structural molecule activity	Extracellular	-development -transport
Procollagen C proteinase enhancer protein precursor	IPI00299738.1	10	58	-	- nucleic acid binding -protein binding	Extracellular	-development -metabolism
Transforming growth factor beta receptor III	IPI00304865.3	4	44	-	-receptor activity -signal transducer activity	-Golgi	-cell communication -development

Table 2. continued

Isoform Long of Collagen alpha-1(XVIII) chain precursor	IPI00022822.4	5	36	+	-Metal ion binding -protein binding -structural molecular activity	Extracellular	-cell death -cell motility -cell organization and biogenesis -cell proliferation -development -regulation of biological process -sensory per ception -transport
Hyaluronan binding protein 2 precursor	IPI00041065.3	7	51	+	- catalytic activity	Extracellular	-metabolism
Metalloproteinase inhibitor 1 precursor	IPI00032292.1	5	49	+	-catalytic activity -enzyme regulator activity -Metal ion binding	Extracellular	-cell proliferation -development -regulation of biological process -metabolism
Splice isoform A of proteoglycan-4 precursor	IPI00024825.2	2	60	+		Extracellular	Cell proliferation

Individual peptide Mascot scores > 27 indicate identity or extensive homology ($p<0.05$).