# Proteomic profile of circulating immune complexes in chronic Chagas disease

Short title: Circulating immune complexes in chronic Chagas disease

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SUMMARY

*Immune complexes (ICs) are the direct and real-time products of humoral immune responses. The identification of constituent foreign or autoantigens within ICs might bring new insights into the pathology of infectious diseases. We applied immune complexome analysis of plasma to the study of Chagas disease caused by Trypanosoma cruzi. Twenty sero-positive plasma samples including cardiac and/or megacolon determinate patients (n=11) and indeterminate (n=9) were analyzed along with 10 seronegative individuals to characterize the antigens bound to circulating ICs. We identified 39 T. cruzi antigens and 114 human auto-antigens specific to Chagas patients. Among those antigens, two T. cruzi antigens (surface protease GP63, glucose-6-isomerase) and six human auto-antigens (CD180 antigen, ceruloplasmin, fibrinogen beta chain, fibrinogen beta chain isoform 2 preprotein, isoform gamma-A of fibrinogen γ chain, serum paraoxonase) were detected in more than 50% of the patients tested. Human isoform short of complement factor H-related protein 2 and trans-sialidase of T.cruzi were more frequently found in the indeterminate (5/9 for both) compared to in the determinate Chagas (0/11, P = 0.046 for human, 1/11, P = 0.0498 for T.cruzi). The immune complexome could illustrate the difference of immune status between clinical forms of chronic Chagas disease.*

**Keywords** Cardiopathy; Chagas disease; immune complex; megacolon; tandem mass spectrometry

Nonstandard abbreviations: ICs, immune complexes; nano-LC-MS/MS, nano-liquid chromatography-tandem mass spectrometry; *T. cruzi, Trypanosoma cruzi*

## INTRODUCTION

Chagas disease, caused by the protozoan parasite *Trypanosoma cruzi* (*T. cruzi*), is endemic in Latin American countries and in the southern part of the United States, where the blood sucking triatomine insect vectors are widely distributed (1, 2). Almost all acute infections proceed to chronic disease, and after 10-20 years, 20-30% of the patients develop cardiac and/or gastrointestinal complications (3). Before the time when the parasites were detected in the affected heart or gut tissues by PCR, autoimmunity was suspected as a mechanism of pathology (4).

Immune complex (IC) is consisted of antibody and its antigen. Majority of the ICs is quickly eliminated from the blood flow by the innate immune system. Circulating ICs have been reported to increase followed by viral or parasitic infection (5-7); however, substantial amounts of ICs that bind to auto-antigens have been constantly observed in the healthy individuals (8). The biological role of circulating ICs remains poorly defined. Comprehensively identifying antigens in circulating ICs might bring new insights into the

pathology of complications. However, such profiling studies for circulating ICs had been very limited due to the lack of technology.

In the present study, immune complexome analysis was performed on plasma from Chagas disease patients with or without typical complications, along with specimens from seronegative individuals. These samples were profiled for foreign antigens and auto-antigens that had been incorporated into circulating ICs.

## MATERIALS AND METHODS

### Study sample

Plasma samples were collected from 20 chronic Chagas patients who were diagnosed as sero-positives at the Centro Nacional de Enfermedades Tropicales (Santa Cruz, Bolivia). These patients were clinically diagnosed as indeterminate Chagas (n=9; 38-50 years; 6 female) or determinate with cardiopathy and/or megacolon complications (n=11; 27-52 years; 8 female) including four patients of cardiopathy (38-48 years; 3 female), three megacolon complications (34-46 years; 2 female), and four patients with both cardiopathy and megacolon symptoms (27-52 years; 3 female). The inclusion criteria, classification, and clinical manifestations were described elsewhere (9, 10). Plasma samples from healthy Japanese donors (n=10; 30-65 years; 2 female) were collected at the Center for Health and Community Medicine, Nagasaki University. Each sample was stored at -80 °C pending analysis; each specimen was subjected to a single

pretreatment process and replicate nano-LC-MS/MS analyses (11, 12). All of the experimental protocols were approved by the Ethical Review Committee at the Institute of Tropical Medicine, Nagasaki University (No. 0210170018) and at the Centro Nacional de Enfermedades Tropicales. Written informed consent was obtained from each subject.

**Immune complexome analysis**

ICs in plasma were purified by magnetic beads with immobilized Protein A or Protein G (PureProteome®, Millipore, Darmstadt, Germany). Beads (40 $\mu$l) were washed with 500 $\mu$l of phosphate-buffered saline pH 7.4 (PBS, Wako Pure Chemicals, Osaka, Japan) and incubated with 10 $\mu$l of plasma samples diluted with PBS (1:9, v/v) for 30 min with gentle mixing. The beads with bound ICs were recovered with a magnet and washed three times with 500 $\mu$l of PBS. The beads were resuspended in 100 $\mu$l of 10 mM dithiothreitol (Wako) and incubated at 56 ºC for 45 min; then, 100 $\mu$l of 55 mM iodoacetamide (Tokyo Chemical Industry, Tokyo, Japan) were added and the mixture was incubated at room temperature for 30 min in the dark. Subsequently, trypsin (Promega, Madison, WI, USA) was added to a final concentration of 0.5 mg/ml, and the mixture was incubated overnight at 37 ºC. Trifluoroacetic acid (10%, Nacalai Tesque, Kyoto, Japan) was added to stop the digestion, and the supernatant containing the peptide digests of antigens and antibodies was recovered. Finally, the volume of this mixture was reduced to approximately 80 $\mu$l using reduced pressure. The peptide mixture (3 μl) was subjected to a nano-LC-electrospray ionization-tandem MS (LTQ-XL, Thermo Fisher Scientific, Waltham, MA, USA) equipped with the custom nanoLC system consisting of a LC-20AD LC pump (Shimadzu, Kyoto, Japan)

with LC flow splitter (Accurate, Dionex, Sunnyvale, CA, USA) and an HCT PAL autosampler (CTC Analytics, Zwingen, Switzerland). The sample was loaded onto a nano-precolumn (300 μm i.d. x 5.0 mm, L-C-18, Chemicals and Evaluation and Research Institute, Tokyo, Japan) in the injection loop. Peptides were separated by a nano HPLC column (75 $\mu$m i.d. x 15 cm, Acclaim PepMap100C18, 3 $\mu$m, Dionex) with gradient elution and ion-sprayed into MS with a spray voltage from 1.2 to 2.0 kV. The mass spectrometer was configured to optimize the duty cycle length with the quality of data acquired by progressing from a full scan of the sample to three tandem MS scans of the three most intense precursor masses (as determined by Xcaliber® software [Thermo Fisher Scientific] in real time). MS/MS data were extracted using Proteome Discoverer v.3.3 (Thermo Fisher Scientific). Spectra were searched against the public non-redundant protein database consisted of the forward and reverse sequences created in-house, including *T. cruzi* (downloaded from NCBI, 2013/06/24) and human (International Protein Index version 3.84 presented by The European Bioinformatics Institute). The filter criteria (single, double, and triple charge peptides with a correlation factor [XCorr] and protein probability [P]) were adjusted maintaining the empirically determined protein false discovery rate at 5%. Human proteins were identified with more than two unique peptides. On the other hand, *T. cruzi* proteins were identified with one unique peptide, and the one unique peptide was identical in all the patients positive for the corresponding *T. cruzi* protein. In the present study, each *T. cruzi* antigen were identified with an identical unique peptide. At the beginning of each day's measurement, the performance of nano-LC-MS/MS system was checked by confirming the sequence coverage of bovine serum albumin peptides (more than 70%).

**Data analysis**

Statistical analysis was performed using MedCalc version 11.0 statistical software (MedCalc Software, Ostend, Belgium) to compare the frequency of detected antigens between two groups (determinate and indeterminate). Two-tailed Fisher's exact test was used with the significance level set at $P < 0.05$.

RESULTS

The *T. cruzi* antigens and auto-antigens detected when using Protein A or Protein G beads are listed in Table 1 and 2. We identified 39 *T. cruzi* antigens (Table 1) and 113 human auto-antigens (Table 2); each of these antigens was found in at least two independent patients samples and was not found in the healthy donors. Among 39 *T. cruzi* antigens, surface protease GP63 (15/20) and glucose-6-isomerase (11/20) were found in more than 50% of the patients. Totally 113 human plasma proteins were detected as IC derived antigens specific for Chagas patients and six of them, CD180 (10/20), ceruloplasmin (14/20), fibrinogen beta chain (18/20), fibrinogen beta chain isoform 2 preprotein (17/20), isoform gamma-A of fibrinogen γ chain (17/20), serum paraoxonase (20/20) were found in more than 50% of the patients.

In an additional step, the patients were divided into two groups: determinate with cardiopathy or/and megacolon and indeterminate, and were statistically analyzed by comparing between groups based on the detection frequency. The human antigen, Isoform Short of Complement factor H-related protein 2 was detected significantly ($P = 0.046$) less frequently in the determinate group (0/11) than in the indeterminate

group (5/9). Furthermore, trans-sialidase (Accession: 71417633) associated ICs were more frequently in the indeterminate (5/9) compared to determinate group (1/11) (P = 0.0498).

When we compared the detection frequency between groups of cardiopathy, megacolon, and indeterminate, both *T. cruzi* hypothetical protein MOQ_002231 and hypothetical protein TCSYLVIO_003482 were detected significantly (P=0.045) more frequently in the megacolon group (5/7) than in the indeterminate group (0/9).

## DISCUSSION

Gene expression in *T. cruzi* is polycistronic. The levels of transcript usually do not correlate with the amounts of protein being produced; regulation of gene expression is achieved primarily at the post-transcriptional level (13-15). Thus, use of approaches such as microarrays and cDNA libraries to map expressed proteins do not yield consistent results. Proteomics-based approaches thus are extremely useful for reliably determining gene expression in *T. cruzi*. In recent years, many efforts have been concentrated on proteomic studies of whole-cell lysates from *T. cruzi* at different developmental stages (13, 16, 17) or of subcellular components/organelles (18, 19). However, these studies did not provide proteomic information on *in vivo* human immune responses against *T. cruzi* infection.

Blood serves as a useful tissue capable of detecting changes induced in the body during the course of *T. cruzi* infection and disease development (20). Antigenic proteins produced by *T. cruzi* trigger the adaptive immune response; however, those protective effects typically are not sufficient to eliminate the parasites from the body. This deficiency might reflect immune evasion by the parasite, as is well described for the

African trypanosome, which uses variant surface antigens (21). Recently, using an isolated beating rat heart model, Rodríguez-Angulo *et al* (2015) observed bradycardia and complete atrioventricular block after perfusing *T. cruzi* secreted proteins (22). In addition to a conventional immune response, an autoimmune response also has been proposed as an underlying mechanism in the pathogenesis of *T. cruzi* infection (21). Therefore, the comprehensive profiling of circulating IC-associated *T. cruzi* antigens and autoantigens reported here suggests some interesting insights into the pathology of Chagas disease.

As seen Table 1 and Table 2, only 7% of *T. cruzi* antigens or 12% of auto-antigens were recovered with both Protein A and Protein G beads. This indicates that the parallel use of the two beads recovered a wider range of antigens than that the use of either bead type alone. It is interesting that much more both *T. cruzi* antigens and auto-antigens identified with Protein A than Protein G and there is a little overlap between Protein G and Protein A immune complexome. The association constant between IgG and Protein G was reported to be 4-times higher than that between IgG and Protein A (23). It is known that IgG3 is captured on Protein G (but not Protein A) and IgM, IgA, IgD and IgE are captured on Protein A (but not Protein G). High association constant and binding to IgG3 of Protein G and distinct affinity of Protein A to IgM, IgA, IgD and IgE may contribute to more antigens identified with Protein G than Protein A and a little overlap between Protein A and Protein G.

Although the chronic Chagas patients keep high levels of *T.cruzi* specific antibodies, IC had never been analyzed before. In the present study, we found 39 trypanosomal antigens were bound to antibody in the circulation and the majority of those were revealed to be hypothetical instead of nominal antigens. Approximately 50% of the predicted protein-coding genes of the *T. cruzi* are annotated as hypothetical or conserved hypothetical proteins (24). And some of these proteins have been reported to localize various

organelles (25) or to show high probability of being secreted or membrane anchored, likely involved in host-cell invasion (26). Takiel *et al* (2009) screened an epimastigote-subtraced trypomatigote cDNA expression library by genetic immunization, in order to find new vaccine candidates for Chagas disease (27). As a result of this screening, 28 gene fragments were identified to improve *in vivo* protection, 19 of which were hypothetical proteins or unannotated *T. cruzi* open reading frames (27). Considering these previous findings, it is reasonable that many of *T. cruzi* antigens identified in this study were hypothetical proteins.

Among the parasite antigens incorporated into the circulating ICs, GP63 is relatively well analyzed with regards to its function. *T. cruzi* genes encoding a series of GP63 cell-surface GPI-anchored proteases are differentially expressed in a stage-specific manner, such that these proteins are more abundant in amastigotes than in epimastigotes or trypomastigotes (28). Anti-peptide antibodies against a C-terminal epitope present in a subset of GP63 proteins recognized the proteins at all life stages, and were shown to inhibit trypomastigote infection of host cells (29). Also, *in vitro* neutralization assays have indicated that anti-GP63 serum has a significant inhibitory effect on *T. cruzi* infection (30). In the present study, circulating IC-associated GP63 was detected not only in indeterminate Chagas disease patients, but also in Chagas disease patients with cardiopathy and/or megacolon complications. The high levels of ICs associated with GP63 in the peripheral blood of chronic Chagas disease patients suggest two possible explanations. First, the accumulation of these ICs may reflect constant production of the corresponding antigens, consistent with the over-production of GP63 as part of the parasites' immune evasion strategy. Second, the accumulation of these ICs may indicate a disturbance in the turnover of the ICs. This

possibility might reflect the formation of aggregated IC particles of a size inappropriate for phagocytosis by scavenger cells, or the failure to activate ICs sufficiently to bind to Fc receptors.

Another predominant trypanosomal antigen forming ICs in the patients' plasma was glucose-6-isomerase, as shown in Table 1. During infection of the human body, parasites are limited to using glycolysis of host sugars for ATP production. Glucose-6-isomerase is an enzyme that catalyzes the conversion of glucose-6-phosphate to fructose-6-phosphate in the second step of glycolysis. Glucose-6-isomerase has been reported to show relatively high enzymatic activity in *T. cruzi* epimastigotes, a form that is observed in the insect stage (31)

In the present study, six human proteins (CD180 antigen, ceruloplasmin, fibrinogen β chain, fibrinogen β chain isoform 2 preprotein, isoform gamma-A of fibrinogen γ chain, and serum paraoxonase) were detected in more than half of Chagas disease patients. Among them, fibrinogen was the most frequently detected. The fibrinogen-like domain, which consists of approximately 200 amino acid residues and has high similarity to the C-terminal halves of fibrinogen β and γ chains, has been found in a growing number of proteins (32). Several fibrinogen-related proteins have been reported in various species (32), with all of these fibrinogen-related proteins containing a shared C-terminal fibrinogen-like domain; therefore, these proteins likely are produced by cross-reactivity between species (32, 33).

Another IC-associated antigen is CD180. This protein is expressed on the B lymphocytes; ligating CD180 with its antibody triggers B cell activation and proliferation (34-36). Furthermore, Chaplin *et al* (2011) found that inoculation of mice with a high dose of anti-CD180 induces extremely rapid and robust polyclonal IgG production, even in the absence of CD40 signaling or T cells (37). Those authors also reported that antigen delivery by coupling with anti-CD180 antibody yielded increased antigen-specific

IgG response compared to immunization with antigen alone. Given that human CD180 was cross-reactive with a *T. cruzi* antigen, *T. cruzi* infection might potentiate polyclonal IgG production through CD180 ligation by a cross-reactive antibody.

Paraoxonase and ceruloplasmin were frequently detected, with identification in all and 70% of the patients, respectively; however, the relation of these antigens to the pathogenesis of Chagas disease is not clear.

When we consider the relationships between clinical Chagas symptoms and their IC-forming antigens, a human (host) antigen, isoform short of complement factor H-related protein 2, was identified significantly more frequently in patients with the indeterminate form than in those with complication-positive chronic Chagas.

Though numerous secreted parasite proteins (38, 39) and immunogenic proteins (40, 41) have been found in the Chagas patient plasma, we could not find any of them in the circulating ICs. The reason could be explained as: (i) these proteins were highly resistant to trypsin digestion and/or ionization in our experimental condition; (ii) these proteins are actually not presented in circulating ICs; and (iii) their plasma levels are under limit detection in our method. Another limitation in our preliminary screening is that our detected ICs were not confirmed by an *in vitro* incubation of patients' serum and parasite extracts. In addition, sugar epitopes could be responsible for the formation of ICs containing non-specific proteins and antibodies, indicated by a large variation in detected proteins between patients' sera.

In conclusion, this report is the first to comprehensively identify the constituent *T. cruzi* and human antigens of circulating ICs detected in Chagas disease patients. We identified 39 *T. cruzi* antigens and 113

human auto-antigens; these markers were not found in healthy donors, demonstrating that these antigens were specific to Chagas disease patients. Among these markers, two parasite antigens (surface protease GP63, glucose-6-isomerase) and six human antigens (CD180 antigen, ceruloplasmin, fibrinogen beta chain, fibrinogen beta chain isoform 2 preprotein, isoform gamma-A of fibrinogen γ chain, serum paraoxonase), respectively, were found in more than half of all the Chagas disease patients. Theses antigens are candidates for further investigation of the pathology of this infectious disease, serving as potential leads for novel diagnostic and treatment strategies.

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## CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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**Table 1**. Summary of *T. cruzi* antigens in circulating Immune Complexes (ICs) isolated from Chagas disease patients. Proteins found when using both Protein A and Protein G beads were shown in Italic.

**Protein A**

| Accession | Description | Determinate (n = 11) | Indeterminate (n = 9) | Total (n = 20) |
|---|---|---|---|---|
| 407844964 | *surface protease GP63, putative, partial* | 4 | 7 | 11 |
| 407415774 | hypothetical protein MOQ_002231 | 5 | 0 | 5 |
| 407851634 | hypothetical protein TCSYLVIO_003482 | 5 | 0 | 5 |
| 407846725 | hypothetical protein TCSYLVIO_006245 | 1 | 3 | 4 |
| 256033096 | *glucose 6-phosphate isomerase* | 3 | 0 | 3 |
| 71657357 | hypothetical protein | 0 | 3 | 3 |
| 407405480 | hypothetical protein MOQ_005739 | 3 | 0 | 3 |
| 407408781 | hypothetical protein MOQ_004062 | 0 | 2 | 2 |
| 407406970 | *hypothetical protein MOQ_005166* | 2 | 0 | 2 |
| 407392283 | hypothetical protein MOQ_009997 | 2 | 0 | 2 |
| 407850005 | hypothetical protein TCSYLVIO_004377 | 1 | 1 | 2 |

**Protein G**

| Accession | Description | Determinate (n = 11) | Indeterminate (n = 9) | Total (n = 20) |
|---|---|---|---|---|
| 407426680 | hypothetical protein MOQ_000078 | 4 | 5 | 9 |
| 256033096 | *glucose 6-phosphate isomerase* | 3 | 5 | 8 |
| 407848130 | hypothetical protein TCSYLVIO_005346 | 3 | 3 | 6 |
| 71417633 | trans-sialidase | 1 | 5 | 6 |
| 407407748 | hypothetical protein MOQ_004731 | 2 | 4 | 6 |
| 71422660 | hypothetical protein | 3 | 2 | 5 |
| 407844964 | *surface protease GP63, putative, partial* | 4 | 0 | 4 |
| 71419822 | hypothetical protein | 0 | 4 | 4 |
| 71668079 | hypothetical protein | 2 | 2 | 4 |
| 71654189 | puromycin-sensitive aminopeptidase-like protein | 1 | 3 | 4 |

| | | | | |
|---|---|---|---|---|
| 71425706 | hypothetical protein | 2 | 1 | 3 |
| 407410040 | NADH dehydrogenase, putative | 1 | 2 | 3 |
| 71412578 | mucin-associated surface protein (MASP) | 1 | 2 | 3 |
| 407853597 | silent information regulator 2, putative | 1 | 2 | 3 |
| 407400614 | hypothetical protein MOQ_007533, partial | 1 | 2 | 3 |
| 71421733 | hypothetical protein | 1 | 1 | 2 |
| 407406970 | *hypothetical protein MOQ_005166* | 0 | 2 | 2 |
| 407393873 | trans-sialidase, putative, partial | 1 | 1 | 2 |
| 71656596 | hypothetical protein | 1 | 1 | 2 |
| 407864530 | ribosomal RNA methyltransferase, putative | 0 | 2 | 2 |
| 71414244 | hypothetical protein | 1 | 1 | 2 |
| 407411418 | mitochondrial ATP-dependent zinc metallopeptidase | 2 | 0 | 2 |
| 71665558 | hypothetical protein | 1 | 1 | 2 |
| 407846909 | hypothetical protein TCSYLVIO_006119 | 0 | 2 | 2 |
| 407420114 | hypothetical protein MOQ_001321 | 0 | 2 | 2 |
| 407853301 | hypothetical protein TCSYLVIO_002548 | 2 | 0 | 2 |
| 407410296 | hypothetical protein MOQ_003360 | 2 | 0 | 2 |
| 407852699 | hypothetical protein TCSYLVIO_002860 | 1 | 1 | 2 |
| 70879807 | hypothetical protein, conserved | 2 | 0 | 2 |
| 407405746 | argonaute-like protein, putative,PIWI-like protein 1, putative | 2 | 0 | 2 |
| 71413875 | 5'-3' exonuclease XRNC | 2 | 0 | 2 |

**Table 2.** Summary of autoantigens in circulating Immune Complexes (ICs) isolated from Chagas disease patients. Proteins found when using both Protein A and Protein G beads were shown in Italic.

**Protein A**

| Accession | Description | Determinate (n = 11) | Indeterminate (n = 9) | Total (n = 20) |
|---|---|---|---|---|
| IPI00965713.3 | *Fibrinogen beta chain isoform 2 preproprotein* | 8 | 9 | 17 |
| IPI00219713.1 | *Isoform Gamma-A of Fibrinogen gamma chain* | 7 | 9 | 16 |
| IPI00793108.2 | *98 kDa protein* | 4 | 4 | 8 |
| IPI00877625.1 | Uncharacterized protein | 3 | 2 | 5 |
| IPI00982758.1 | Uncharacterized protein | 5 | 0 | 5 |
| IPI00975939.1 | *SAA2-SAA2 protein* | 2 | 2 | 4 |
| IPI01010362.1 | cDNA FLJ54464, highly similar to Signal transducer and activator of transcription 5A | 4 | 0 | 4 |
| IPI00760925.2 | Isoform 3 of Myosin-XVIIIa | 4 | 0 | 4 |
| IPI00448925.6 | *44 kDa protein* | 1 | 2 | 3 |
| IPI00942353.1 | *74 kDa protein* | 3 | 0 | 3 |
| IPI00022429.3 | *Alpha-1-acid glycoprotein 1* | 1 | 2 | 3 |
| IPI00947307.1 | *cDNA FLJ58075, highly similar to Ceruloplasmin* | 1 | 2 | 3 |
| IPI00794469.1 | *Isoform 4 of Voltage-dependent calcium channel subunit alpha-2/delta-2* | 3 | 0 | 3 |
| IPI00019399.2 | *Serum amyloid A-4 protein* | 1 | 2 | 3 |
| IPI00924913.1 | Uncharacterized protein | 0 | 3 | 3 |
| IPI00902580.1 | cDNA FLJ11050 fis, clone PLACE1004564, highly similar to Cleavage and polyadenylation specificity factor 100 kDa subunit | 0 | 3 | 3 |
| IPI01014438.2 | Uncharacterized protein | 1 | 2 | 3 |
| IPI01010386.1 | C4 complement C4d region (Fragment) | 2 | 1 | 3 |
| IPI00782966.1 | Zinc finger protein 106 homolog | 3 | 0 | 3 |
| IPI00166938.1 | Isoform 3 of BEN domain-containing protein 6 | 3 | 0 | 3 |
| IPI00741335.4 | putative TAF11-like protein ENSP00000332601-like | 3 | 0 | 3 |

| | | | | |
|---|---|---|---|---|
| IPI00827532.1 | *Anti-folate binding protein (Fragment)* | 1 | 1 | 2 |
| IPI00298497.3 | *Fibrinogen beta chain* | 0 | 2 | 2 |
| IPI00298731.2 | *Serine/threonine-protein phosphatase 1 regulatory subunit 10* | 0 | 2 | 2 |
| IPI00978302.1 | *Uncharacterized protein* | 1 | 1 | 2 |
| IPI00828061.1 | Anti-mucin1 heavy chain variable region (Fragment) | 1 | 1 | 2 |
| IPI01010467.1 | Uncharacterized protein | 0 | 2 | 2 |
| IPI00032220.3 | Angiotensinogen | 2 | 0 | 2 |
| IPI00375317.2 | Isoform 2 of Protein angel homolog 2 | 0 | 2 | 2 |
| IPI00944623.1 | Isoform 3 of Golgin subfamily A member 3 | 0 | 2 | 2 |
| IPI00794668.3 | Isoform 2 of Centrosomal protein of 290 kDa | 2 | 0 | 2 |
| IPI00854834.2 | echinoderm microtubule-associated protein-like 4 isoform b | 0 | 2 | 2 |
| IPI00418130.2 | Isoform 3 of Mediator of RNA polymerase II transcription subunit 8 | 1 | 1 | 2 |
| IPI00884981.2 | Isoform 2 of Pregnancy zone protein | 1 | 1 | 2 |
| IPI00894122.1 | Uncharacterized protein | 1 | 1 | 2 |
| IPI00027547.2 | Dermcidin | 2 | 0 | 2 |
| IPI00297462.6 | Uncharacterized protein C1orf65 | 2 | 0 | 2 |
| IPI00963845.1 | Uncharacterized protein | 2 | 0 | 2 |
| IPI00446834.2 | Isoform 2 of Sulfotransferase 1A3/1A4 | 2 | 0 | 2 |
| IPI00022731.1 | Apolipoprotein C-IV | 2 | 0 | 2 |
| IPI00165579.6 | Isoform 2 of Cytosolic non-specific dipeptidase | 2 | 0 | 2 |

**Protein G**

| Accession | Description | Determinate (n = 11) | Indeterminate (n = 9) | Total (n = 20) |
|---|---|---|---|---|
| IPI00218732.4 | Serum paraoxonase/arylesterase 1 | 11 | 9 | 20 |
| IPI00219713.1 | *Isoform Gamma-A of Fibrinogen gamma chain* | 8 | 9 | 17 |
| IPI00298497.3 | *Fibrinogen beta chain* | 7 | 9 | 16 |
| IPI00947307.1 | *cDNA FLJ58075, highly similar to Ceruloplasmin* | 6 | 8 | 14 |
| IPI00645038.1 | Uncharacterized protein | 5 | 7 | 12 |
| IPI00023722.2 | CD180 antigen | 3 | 7 | 10 |

| | | | | |
|---|---|---|---|---|
| IPI00793108.2 | *98 kDa protein* | 6 | 3 | 9 |
| IPI00298731.2 | *Serine/threonine-protein phosphatase 1 regulatory subunit 10* | 4 | 5 | 9 |
| IPI00019399.2 | *Serum amyloid A-4 protein* | 4 | 5 | 9 |
| IPI00879937.1 | Uncharacterized protein | 3 | 6 | 9 |
| IPI00514530.5 | Uncharacterized protein | 4 | 4 | 8 |
| IPI00877852.2 | inter-alpha-trypsin inhibitor heavy chain H1 isoform c | 3 | 5 | 8 |
| IPI00003478.2 | Dual specificity protein phosphatase 5 | 3 | 4 | 7 |
| IPI01011344.1 | Uncharacterized protein | 3 | 4 | 7 |
| IPI00968182.1 | Uncharacterized protein | 3 | 4 | 7 |
| IPI01021041.1 | Protein | 3 | 4 | 7 |
| IPI00018244.1 | MCM3-associated gene antisense protein | 1 | 5 | 6 |
| IPI00022488.1 | Hemopexin or Vh1-D-J3-region (Fragment) | 2 | 4 | 6 |
| IPI00794469.1 | *Isoform 4 of Voltage-dependent calcium channel subunit alpha-2/delta-2* | 3 | 2 | 5 |
| IPI01014975.1 | Uncharacterized protein | 2 | 3 | 5 |
| IPI00218949.1 | Isoform Short of Complement factor H-related protein 2 | 0 | 5 | 5 |
| IPI00555812.5 | vitamin D-binding protein isoform 1 precursor | 2 | 3 | 5 |
| IPI01010642.1 | Uncharacterized protein | 3 | 2 | 5 |
| IPI01015306.2 | Uncharacterized protein | 4 | 1 | 5 |
| IPI00942257.3 | Uncharacterized protein | 4 | 1 | 5 |
| IPI00942353.1 | *74 kDa protein* | 3 | 1 | 4 |
| IPI00022429.3 | *Alpha-1-acid glycoprotein 1* | 3 | 1 | 4 |
| IPI00827532.1 | *Anti-folate binding protein (Fragment)* | 4 | 0 | 4 |
| IPI00451401.3 | Isoform 2 of Triosephosphate isomerase | 2 | 2 | 4 |
| IPI00917183.1 | Uncharacterized protein | 2 | 2 | 4 |
| IPI00001567.1 | PR domain zinc finger protein 14 | 2 | 2 | 4 |
| IPI00022463.2 | Serotransferrin | 2 | 2 | 4 |
| IPI01015050.2 | Uncharacterized protein | 1 | 3 | 4 |
| IPI00032291.2 | Complement C5 | 1 | 3 | 4 |
| IPI00922613.1 | Isoform 6 of Filamin A-interacting protein 1-like | 2 | 2 | 4 |
| IPI00007193.7 | Isoform 2 of Ankyrin repeat domain-containing protein 26 | 2 | 2 | 4 |
| IPI00448925.6 | *44 kDa protein* | 1 | 2 | 3 |
| IPI00965713.3 | *fibrinogen beta chain isoform 2 preproprotein* | 1 | 2 | 3 |

| | | | | |
|---|---|---|---|---|
| IPI00978302.1 | *Uncharacterized protein* | 0 | 3 | 3 |
| IPI01015781.1 | Uncharacterized protein | 1 | 2 | 3 |
| IPI00552578.2 | Serum amyloid A protein | 0 | 3 | 3 |
| IPI00641737.2 | Haptoglobin | 1 | 2 | 3 |
| IPI00983835.1 | Uncharacterized protein | 3 | 0 | 3 |
| IPI00004489.1 | Adenylyltransferase and sulfurtransferase MOCS3 | 2 | 1 | 3 |
| IPI00642751.1 | Uncharacterized protein | 1 | 2 | 3 |
| IPI00020996.5 | Insulin-like growth factor-binding protein complex acid labile subunit | 0 | 3 | 3 |
| IPI00926149.1 | Isoform 1 of Zinc finger homeobox protein 2 | 1 | 2 | 3 |
| IPI00978863.1 | Uncharacterized protein | 0 | 3 | 3 |
| IPI01024846.1 | 20 kDa protein | 0 | 3 | 3 |
| IPI00798006.2 | Protein | 3 | 0 | 3 |
| IPI00844211.2 | Uncharacterized protein | 3 | 0 | 3 |
| IPI00026314.1 | Isoform 1 of Gelsolin | 2 | 1 | 3 |
| IPI00022479.6 | Uncharacterized protein | 1 | 2 | 3 |
| IPI00339224.3 | Isoform 4 of Fibronectin | 1 | 2 | 3 |
| IPI00377087.4 | Uncharacterized protein | 2 | 1 | 3 |
| IPI00945190.2 | SPATA21 protein | 3 | 0 | 3 |
| IPI00975939.1 | *SAA2-SAA2 protein* | 0 | 2 | 2 |
| IPI00019502.3 | Isoform 1 of Myosin-9 | 0 | 2 | 2 |
| IPI00434711.1 | Putative uncharacterized protein FP6679 | 1 | 1 | 2 |
| IPI00006146.4 | serum amyloid A2 isoform a | 0 | 2 | 2 |
| IPI00976712.1 | Uncharacterized protein | 1 | 1 | 2 |
| IPI00335581.5 | Isoform 1 of E3 ubiquitin-protein ligase UBR3 | 2 | 0 | 2 |
| IPI00946590.1 | 26 kDa protein | 2 | 0 | 2 |
| IPI00297550.8 | Coagulation factor XIII A chain | 0 | 2 | 2 |
| IPI00553169.6 | Uncharacterized protein | 0 | 2 | 2 |
| IPI01015522.1 | cDNA FLJ55253, highly similar to Actin, cytoplasmic 1 | 1 | 1 | 2 |
| IPI00792677.2 | cDNA FLJ60097, highly similar to Tubulin alpha-ubiquitous chain | 0 | 2 | 2 |
| IPI00171410.1 | Isoform 1 of Uncharacterized protein C3orf21 | 0 | 2 | 2 |
| IPI00807498.1 | CCDC6 protein (Fragment) | 1 | 1 | 2 |
| IPI00967146.1 | Uncharacterized protein | 0 | 2 | 2 |

| | | | | |
|---|---|---|---|---|
| IPI00290755.6 | Protein FAM81A | 0 | 2 | 2 |
| IPI00644372.3 | Isoform 4 of Transmembrane channel-like protein 5 | 1 | 1 | 2 |
| IPI00942787.1 | 42 kDa protein | 1 | 1 | 2 |
| IPI00973032.1 | V1-17 protein | 1 | 1 | 2 |
| IPI00736778.4 | cDNA FLJ50187 | 0 | 2 | 2 |
| IPI00023529.1 | Cyclin-dependent kinase 6 | 1 | 1 | 2 |
| IPI00796316.5 | Uncharacterized protein | 2 | 0 | 2 |
| IPI00480042.3 | Isoform 2 of Abnormal spindle-like microcephaly-associated protein | 1 | 1 | 2 |
| IPI00216345.2 | Leucine-rich repeat neuronal protein 4 | 2 | 0 | 2 |
| IPI00976079.1 | Uncharacterized protein | 1 | 1 | 2 |
| IPI01009693.1 | Uncharacterized protein | 1 | 1 | 2 |
| IPI00385079.1 | MSTP151 | 0 | 2 | 2 |
| IPI00242956.5 | IgGFc-binding protein | 0 | 2 | 2 |
| IPI00896559.1 | follistatin-related protein 5 isoform c | 0 | 2 | 2 |
| IPI00021885.1 | Isoform 1 of Fibrinogen alpha chain | 2 | 0 | 2 |
| IPI00853553.2 | Uncharacterized protein | 2 | 0 | 2 |