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Williams****(10) Patent No.: US 8,053,196 B2  
(45) Date of Patent: \*Nov. 8, 2011****(54) CLINICAL ASSAYS FOR THROMBOSPONDIN  
FRAGMENTS IN THE DETECTION,  
DIAGNOSIS, AND EVALUATION OF CANCER**

## FOREIGN PATENT DOCUMENTS

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(US)****(\*) Notice:** Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 2084 days.

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See application file for complete search history.

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The invention relates to thrombospondin fragments found in plasma, their use or use of portions thereof in diagnostic methods, as method calibrators, method indicators, and as immunogens, and as analytes for methods with substantial clinical utility; and their detection in plasma or other bodily fluids for purpose of diagnostic methods, especially for cancer.

**104 Claims, 12 Drawing Sheets**

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Figure 1: Thrombospondin and fragments

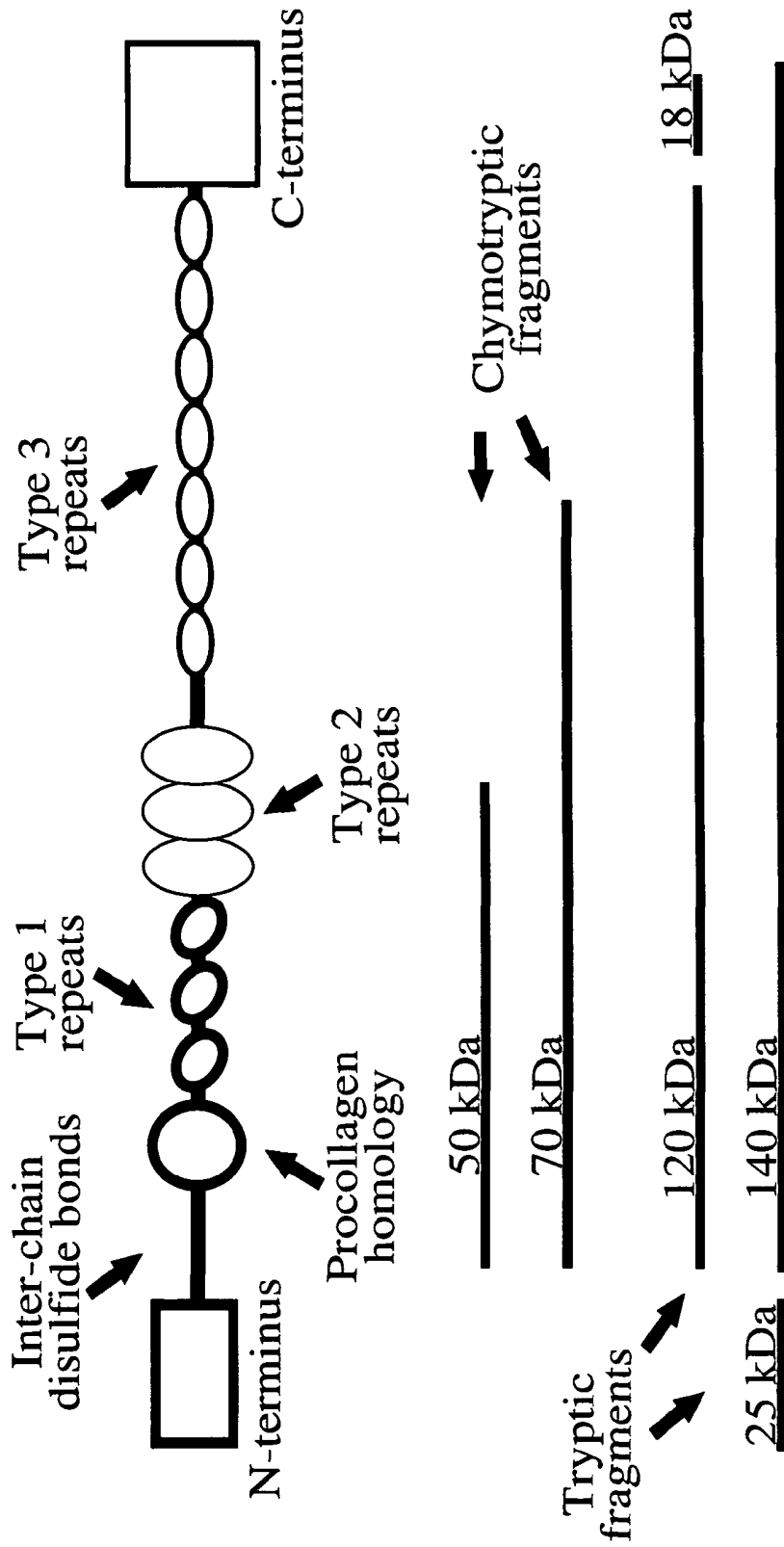
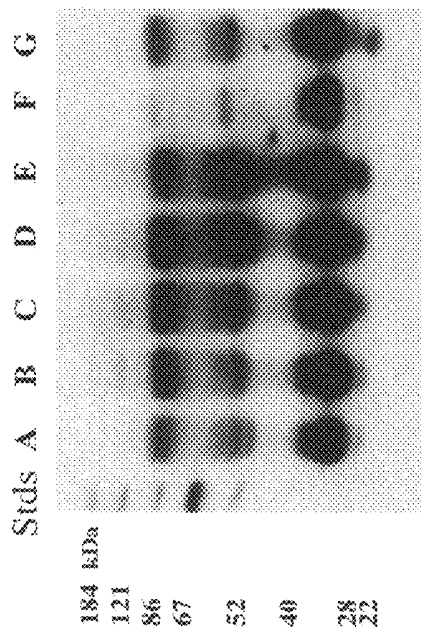




Figure 3



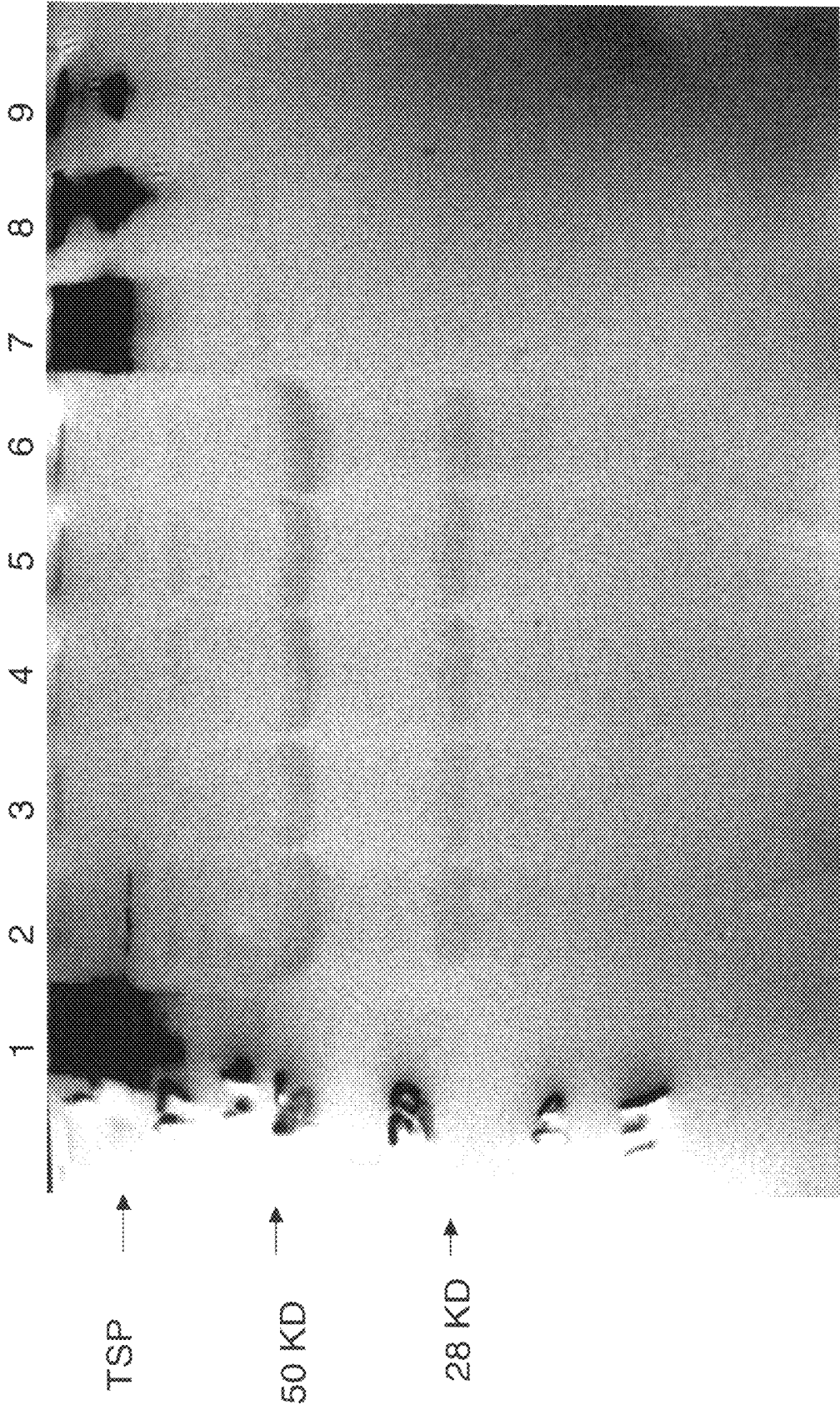
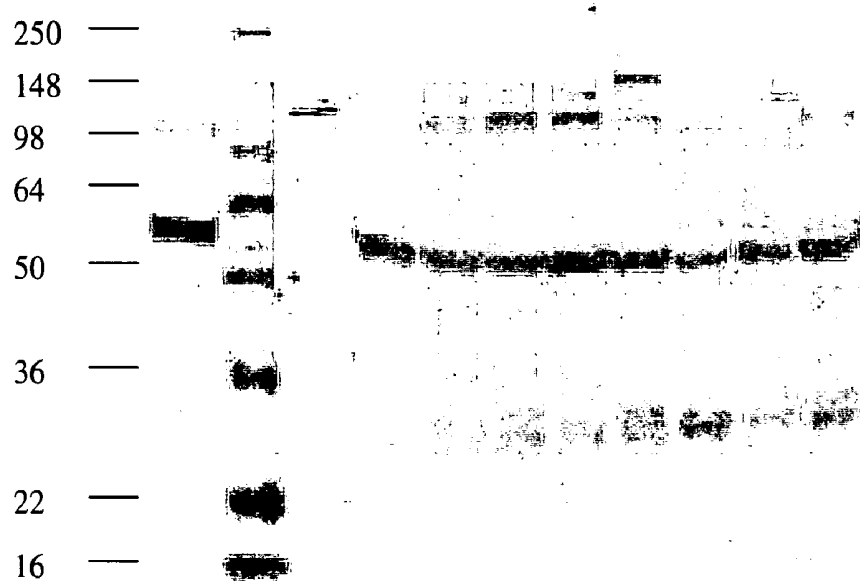


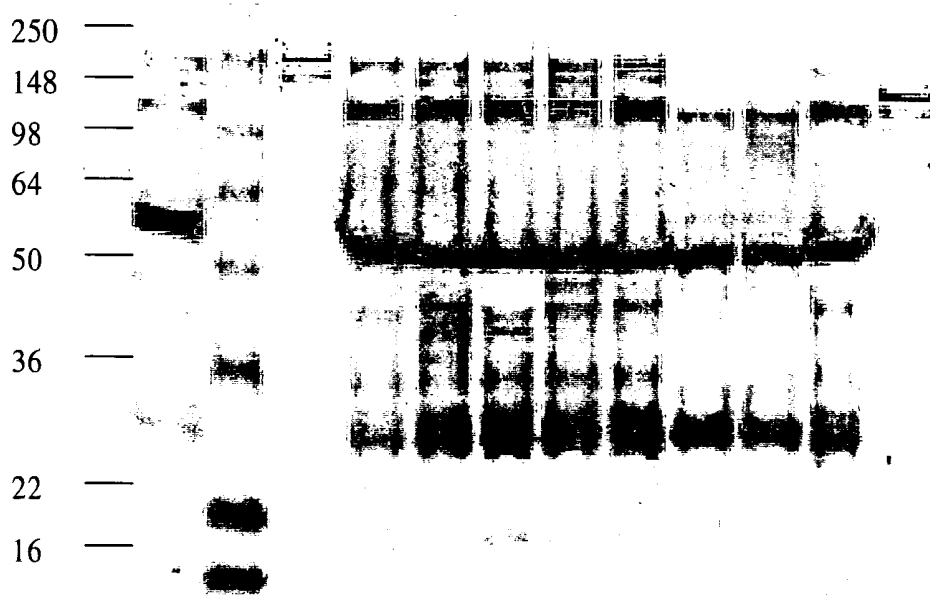
Figure 4

Figure 5

**Key**

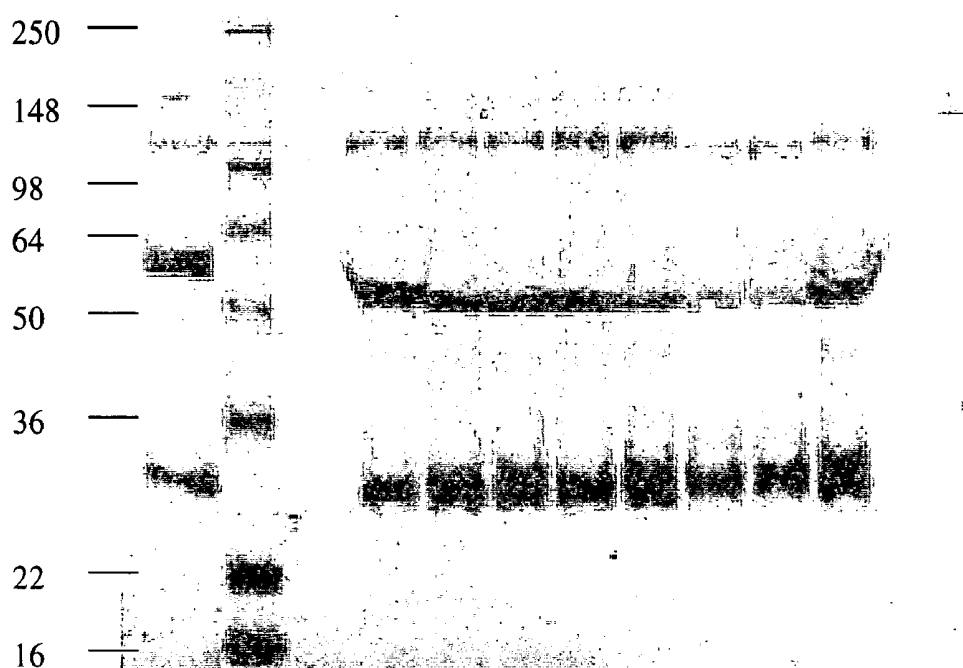
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- Lane 2: See Blue Plus  
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- Lane 4: MET-011
- Lane 5: MET-012
- Lane 6: MET-013
- Lane 7: MET-014
- Lane 8: MET-015
- Lane 9: MET-016
- Lane 10: MET-017
- Lane 11: Normal
- Lane 12: Empty

Figure 6



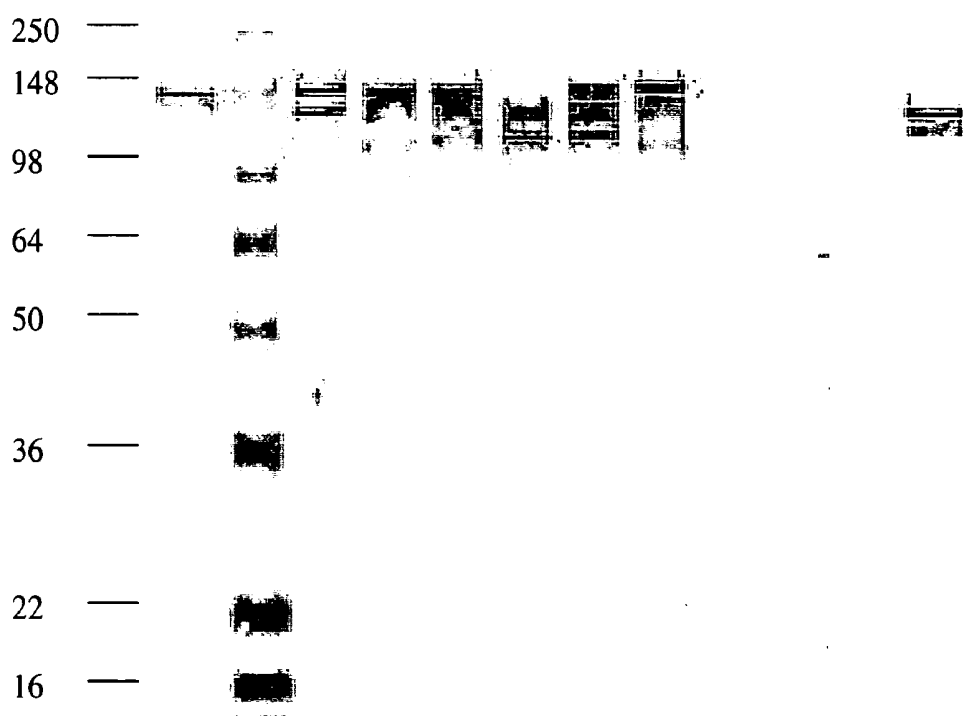
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Lane 5:	MET-012
Lane 6:	MET-013
Lane 7:	MET-014
Lane 8:	MET-015
Lane 9:	MET-016
Lane 10:	MET-017
Lane 11:	Normal
Lane 12:	50 ng LCP Lot 1

Figure 7

**Key**

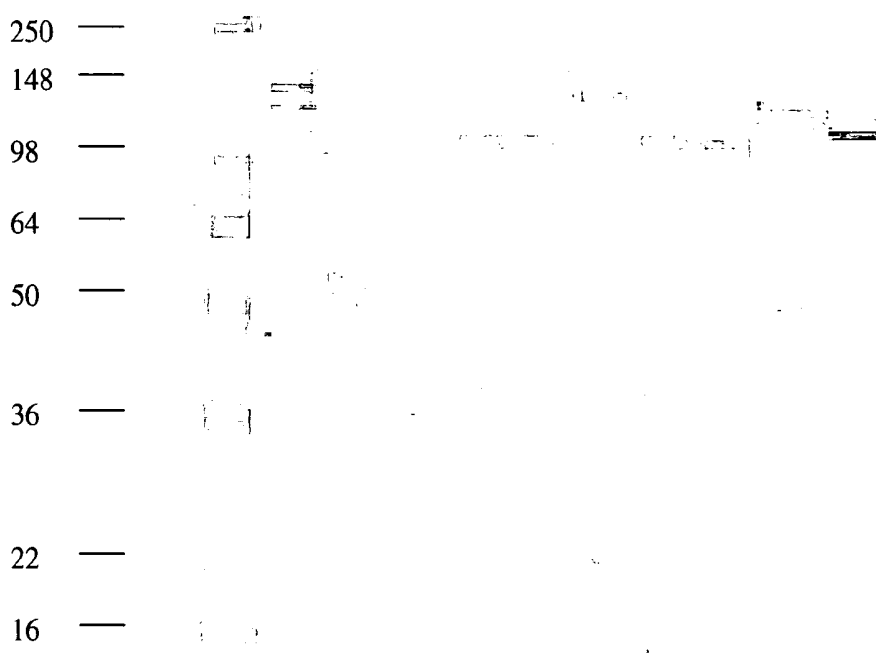
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- Lane 4: MET-011
- Lane 5: MET-012
- Lane 6: MET-013
- Lane 7: MET-014
- Lane 8: MET-015
- Lane 9: MET-016
- Lane 10: MET-017
- Lane 11: Normal
- Lane 12: 50 ng LCP Lot 1

Figure 8



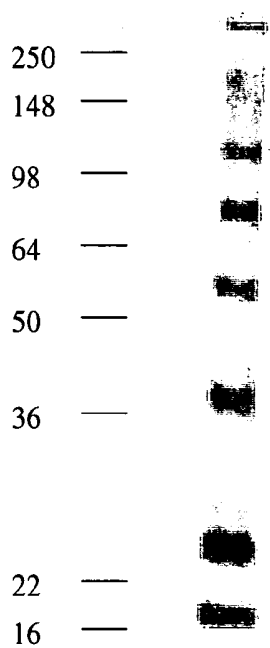
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Lane 6:	MET-013
Lane 7:	MET-014
Lane 8:	MET-015
Lane 9:	MET-016
Lane 10:	MET-017
Lane 11:	Normal
Lane 12:	50 ng LCP Lot 1

Figure 9



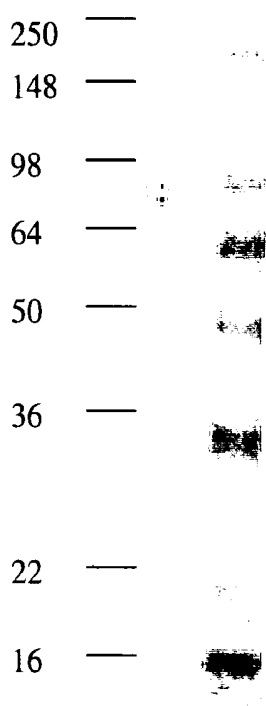
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Lane 2:	See Blue Plus Prestained Markers
Lane 3:	50 ng LCP Lot 2
Lane 4:	MET-011
Lane 5:	MET-012
Lane 6:	MET-013
Lane 7:	MET-014
Lane 8:	MET-015
Lane 9:	MET-016
Lane 10:	MET-017
Lane 11:	Normal
Lane 12:	50 ng LCP Lot 1

Figure 10



Key	
Lane 1:	Human Serum
Lane 2:	See Blue Plus Prestained Markers
Lane 3:	50 ng LCP Lot 2
Lane 4:	MET-011
Lane 5:	MET-012
Lane 6:	MET-013
Lane 7:	MET-014
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Lane 9:	MET-016
Lane 10:	MET-017
Lane 11:	Normal
Lane 12:	50 ng LCP Lot 1

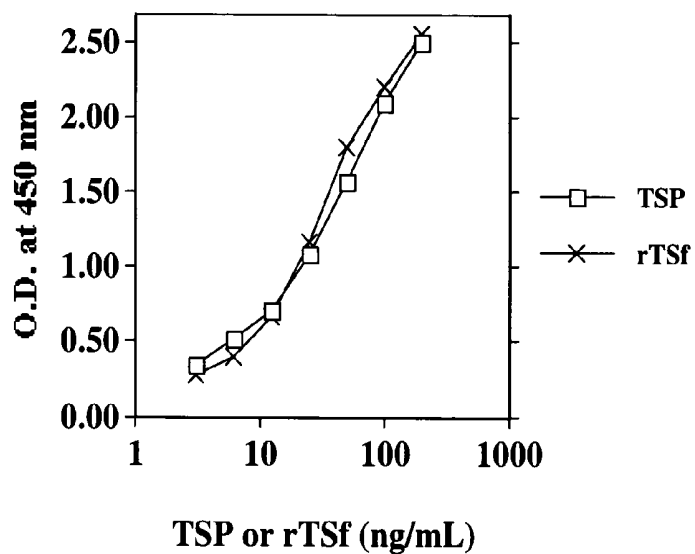
Figure 11



<b>Key</b>	
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Lane 2:	See Blue Plus Prestained Markers
Lane 3:	50 ng LCP Lot 2
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Lane 5:	MET-012
Lane 6:	MET-013
Lane 7:	MET-014
Lane 8:	MET-015
Lane 9:	MET-016
Lane 10:	MET-017
Lane 11:	Normal
Lane 12:	50 ng LCP Lot 1

Figure 12

ELISA of TSP vs recombinant TSP fragment (rTSf)



## CLINICAL ASSAYS FOR THROMBOSPONDIN FRAGMENTS IN THE DETECTION, DIAGNOSIS, AND EVALUATION OF CANCER

### CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. provisional application 60/405,494 filed Aug. 23, 2002; U.S. application Ser. No. 10/419,462, filed Apr. 21, 2003; and PCT application PCT/US03/26023, filed Aug. 20, 2003.

### FIELD OF THE INVENTION

The present invention relates to assays for blood levels of one or more thrombospondin fragments as a diagnostic test for cancers and other diseases, the use of such fragments and/or derivatives thereof to generate specific antibodies and other binding agents and/or to use as calibrators, competitors, and/or indicators in an assay, and to the fragments themselves. Specifically, the present invention relates to specific detection of high molecular weight fragments and forms of thrombospondin in cancer patients as compared to non-cancerous patients. The present invention also relates to methods of distinguishing between proper and improper sample collections of plasma as indicated by the detection of high molecular weight thrombospondin fragments and/or thrombospondin itself.

### BACKGROUND OF THE INVENTION

Thrombospondin (TSP), also known as TSP-1, is a multimeric glycoprotein comprised of identical monomers. The monomers migrate at an apparent molecular weight of approximately 185 kDa in SDS-polyacrylamide electrophoretic gels under reducing conditions. The predominant multimer is a trimer, which migrates at an apparent molecular weight of approximately 450 kDa on non-reducing gels. The molecular weights by sedimentation equilibrium are similar, at 135 kDa for monomers and 420 kDa for trimers. The predicted molecular weight from just the sequence of amino acid residues in the monomer is 127,524 Da, which does not include contributions from glycosylation and  $\beta$ -hydroxylation. The thrombospondin glycoprotein is produced by platelets and is released upon platelet activation from platelet  $\alpha$ -granules, along with many other proteins, such as platelet-derived growth factor,  $\beta$ -thromboglobulin, fibronectin, fibrinogen, and platelet factor-4 (see Chapter 1, "An introduction to the thrombospondins" in *The Thrombospondin Gene Family* by J C Adams, R P Tucker, & J Lawler, Springer-Verlag: N.Y., 1995, pp. 1-9, but especially p. 2; and Chapter 3, "The secondary and tertiary structure of the thrombospondins," *ibidem* pp. 43-56, especially Table 3.1). Thrombospondin is known to be involved in biological processes such as cell adhesion, proliferation and chemotaxis. It has also been reported that thrombospondin may be involved in the progression of malignant tumors. Furthermore, thrombospondin has been reported to be highly expressed in many human malignant tissues and in surrounding stroma and/or endothelium and has been reported to be present in higher than normal levels in the plasma of cancer patients. (e.g., Qian and Tuszyński, *Proc. Soc. Exp. Biol. Med.*, 212:199-207, 1996; de Fraipont F et al. *Trends Mol. Med.*, 7:401-407, 2001).

Despite the foregoing, as for any potential diagnostic test, it would be desirable to increase the specificity and sensitivity of such tests. To that end, the present inventor has discovered that thrombospondin is present in the blood and blood plasma

in relatively small amounts compared to fragments of thrombospondin, and this finding is true in the blood and blood plasma of cancer patients as well. This discovery provided a basis for the present inventions related to novel diagnostic assays that are more specific, more sensitive, more easily calibrated, and in some cases distinguish these thrombospondin fragments from each other and from thrombospondin itself. The present inventor has also identified specific high molecular weight thrombospondin fragments, forms, and/or cross-reacting material is useful in the detection of cancer. The present invention also relates to novel methods of distinguishing between a properly versus an improperly collected sample by detecting high molecular weight thrombospondin fragments, forms, cross-reactive material, and/or thrombospondin itself.

### BRIEF SUMMARY OF THE INVENTION

Important aspects of the invention are diagnostic methods and related kits that are based on the detection and quantification of thrombospondin fragments and/or thrombospondin in bodily fluids, especially plasma. Foremost among those diagnostic methods are those that detect or monitor the status of a cancer.

Aspects of the invention closely related to the diagnostic methods are thrombospondin fragments that are detected in the plasma, thrombospondin fragments that can be used to induce an antibody of interest for use in a diagnostic method or can be used in a competition-type or non-competitive diagnostic assay. Another important aspect of the invention includes the detection of high molecular weight thrombospondin fragments in cancerous versus non-cancerous plasma samples. Aspects of the invention also relate to methods of assaying proper sample collection by analysis of high molecular weight thrombospondin fragments and/or thrombospondin itself. The sample includes but is not limited to blood, serum or plasma.

#### Thrombospondin Fragments of the Invention

In one aspect, the invention is a purified thrombospondin fragment that has been extracted from a bodily fluid, especially plasma, said fragment being one within a molecular weight range selected from the group consisting of 80 to 148 kDa, 40 to 64 kDa, and 22 to 36 kDa, wherein the size in kDa is the apparent size on gel electrophoresis after disulfide bond reduction. Their uses include, but are not limited to, a) the induction of an antibody and/or other binding agent of interest, b) induction of an antibody and/or other binding agent for a diagnostic method, c) use in a competition-type diagnostic assay, d) as a reference molecule in an assay for a thrombospondin fragment or fragments or thrombospondin of human subjects, and e) the immunization of an animal. In a closely related aspect, the invention is a polypeptide or modified polypeptide, made by recombinant and/or chemical techniques, that has the identical primary structure as one of said purified thrombospondin fragments or a portion thereof. Such chemical techniques include, but are not limited to, glycosylation,  $\beta$ -hydroxylation, alkylation and reduction.

In particular embodiments, the fragment's molecular weight is one within a molecular weight range selected from the group consisting of 80 to 148 kDa, 40 to 64 kDa, and 22 to 36 kDa. Specific examples of fragment molecule weights are 85, 90, 50, and 30 kDa. Preferably, the fragment is one found in human plasma.

In a related aspect, the invention is a purified and/or synthetic thrombospondin fragment or portion thereof, said fragment being one that starts between amino acid I-165 (just after the N12/I peptide) and V-263 (the start of the procol-

lagen homology domain), inclusive (i.e., inclusive of I-165 and V-263), and ends between amino acid K412 (the end of the reported collagen type V-binding region) and I-530 (the end of the domain of type 1 repeats), inclusive. Preferred are such fragments that start between N-230 and G-253, inclusive (at or near the start of the domain of interchain disulfide bonds, I-241, which is the first residue downstream [meaning towards the C-terminus of the full protein] of a predicted cleavage site for chymotrypsin and/or a chymotrypsin-like protease), and end at between V-400 and S-428, inclusive (at or near a predicted chymotrypsin cleavage site, F-414, that falls two residues after the end of the collagen type V-binding region), said portion being at least 3 amino acyl acids in length (preferably at least 4 amino acyl residues in length, more preferably at least 6 amino acyl residues).

In a further related aspect, the invention is a purified and/or synthetic thrombospondin fragment or portion thereof, said fragment being one that starts between amino acid I-165 (just after the N12/I peptide) and V-263 (the start of the procollagen homology domain), inclusive, and ends between amino acid I-530 (the end of the type 1 repeats) and R-733 (the end of the first type 3 repeat), inclusive. Preferably such a fragment starts between N-230 and G-253, inclusive, and ends between D-527 and S-551, inclusive, which is at or near a predicted chymotrypsin cleavage site, F-539, in the first type 2 repeat; said portion being at least 3 amino acyl acids in length (preferably at least 4 amino acyl residues in length, more preferably at least 6 amino acyl residues).

In a still further related aspect, the invention is a purified and/or synthetic thrombospondin fragment or portion thereof, said fragment being one that starts between amino acid I-165 (just after the N12/I peptide) and V-263 (the start of the procollagen homology domain), inclusive, and ends between amino acid R-792 (the end of the third type 3 repeat) and Y-982 (the third of the predicted chymotrypsin cleavage sites in the C-terminal domain), inclusive. Preferably such a fragment starts between N-230 and G-253, inclusive, and ends between G-787 and V-811, inclusive, which is at or near a predicted chymotrypsin cleavage site, Y-799, in the fourth type 3 repeat; said portion being at least 3 amino acyl acids in length (preferably at least 4 amino acyl residues in length, more preferably at least 6 amino acyl residues). Protein molecular weights here were computed using standard computational aids (such aids are available, for example, at the web site of the Bioinformatics Organization, Inc., see Stothard, P. 2000. The sequence manipulation suite: JavaScript programs for analyzing and formatting protein and DNA sequences. *BioTechniques* 28: 1102-1104) and adjusted upwards to account for post-translational modifications. Predicted cleavage sites for chymotrypsin (and any closely related protease) were identified using tools available from the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (SIB) and were limited to predicted sites of at least 80% probability. The uses of said fragments and portions include, but are not limited to, the induction and/or screening of an antibody and/or another binding agent of interest in a diagnostic method and use in a diagnostic assay. In particular embodiments, the invention is one of the specified fragments, rather than a portion thereof. In additional embodiments, a fragment and/or a portion can incorporate or be linked to a label and/or a carrier.

Throughout, wherever reference is made to a fragment or a portion thereof (or an immunoreactive portion thereof), it is understood that the fragment is a preferred embodiment of the invention. It is also understood throughout this Application that immunogenic portions, immunoreactive portions, cross-reactive portions, and/or epitopes are generally six amino acyl

residues long or longer, but an occasional portion or epitope can be shorter. Such shorter portions or epitopes are also contemplated.

Six additional aspects are:

1) A purified and/or synthetic thrombospondin fragment, said fragment being at least 6 contiguous amino acyl residues in length, and wherein the fragment comprises a protease-resistant core domain or a part thereof, said domain or part thereof being selected from the group consisting of a domain of inter-chain disulfide bonds, an oligomerization domain, a procollagen-like domain, a type 1 repeat, a type 2 repeat, and a type 3 repeat, said part being at least 6 amino acyl residues in length.

2) A purified and/or synthetic thrombospondin fragment, said fragment being at least 6 contiguous amino acyl residues in length, and wherein the fragment comprises an amino acid sequence selected from the group consisting of TEENKE (SEQ ID NO:1), CLQDSIRKVTENKE (which includes an N-terminal Cys added to aid conjugation) (SEQ ID NO:2), LQDSIRKVTENKE (SEQ ID NO:3), EGEARE (SEQ ID NO:4), PQMNGKPCEGEARE (SEQ ID NO:5), EDTDLD (SEQ ID NO:6), YAGNGHICGEDTLD (SEQ ID NO:7), CNSPQMNGKPCEGEARE (SEQ ID NO:8), RKVTEENKELANELRRP (SEQ ID NO:9), CRKVTEENKELANELRRP (which includes an N-terminal Cys added to aid conjugation) (SEQ ID NO:10), PQMNGKPCEGEARE (SEQ ID NO:11), CEGEARE (SEQ ID NO:12), and RKVTEENKE (SEQ ID NO:13). (In particular embodiments the fragment comprises two, or even all of the foregoing sequences).

3) a purified and/or synthetic thrombospondin fragment, said fragment being at least 6 contiguous amino acyl residues in length, and wherein the fragment comprises a collagen type V binding domain or a portion thereof.

4) A purified and/or synthetic thrombospondin fragment, said fragment being at least 6 contiguous amino acyl residues in length, and wherein the fragment comprises an epitope for binding at least one of the following commercially available antibodies, each of which recognizes a ~450 kDa (non-reduced) protein that is specifically identified as thrombospondin (the TSP Ab numbering, e.g., "TSP Ab-2", comes from Lab Vision Corporation, Fremont, Calif.; clone designations refer to the hybridoma clone that produces a particular monoclonal antibody) It is also understood that said fragment includes a fragment that can be designed to bind a pre-existing monoclonal antibody, through the use of peptide scanning analysis, competition experiments, and other methods known in the art. It is also understood that the current invention includes, but is not limited to, uses of pre-existing antibodies independent of a purified and/or synthetic fragment, some of which uses are also listed below.

TSP Ab-2 (Clone D4.6): This antibody is stated to react against reduced and non-reduced protein, and its epitope has been reported to be in the calcium-binding domain of TSP (C-terminal 50 kDa piece of the 120 kDa fragment from protease digestion of Ca-replete TSP). The calcium-binding region is generally considered to be in the type 3 repeats (TSP residues 698-925). For example, it is expected that TSP Ab-2 will bind thrombospondin but not the 30 kDa circulating fragment. This antibody can be used to detect and/or quantify TSP and/or a circulating fragment; distinguish thrombospondin from a circulating fragment; and/or distinguish one or more fragments from each other. It has been reported to show no cross-reaction with fibronectin, fibrinogen, or von Willibrand factor. Its binding to thrombospondin is enhanced by EDTA i.e. at low [Ca<sup>2+</sup>]. Applicant's data indicates that this antibody also binds three major fragments (of apparent molecular weights of ~30 kDa, ~50 kDa, and ~115 kDa) of

TSP in human (and dog) plasma, and several minor fragments of TSP in human (and dog) plasma, as well as high molecular weight fragments or forms (above the ~115 kDa band) (See FIG. 5).

TSP Ab-4 (Clone A6.1): This antibody is stated to react against reduced and non-reduced protein, and its epitope has been reported to be in the collagen type V-binding domain. This antibody binds thrombospondin, and the applicant has discovered that it binds the three major TSP fragments in human plasma. Thus, this antibody can be used to detect and/or quantify TSP and/or a circulating fragment or fragments. In combination with another antibody or binding agent, it can be used in an assay to distinguish thrombospondin from a circulating fragment; and/or to distinguish one or more fragments from each other.

Applicant's data shows that this antibody also binds three major (~30 kDa, ~50 kDa, and ~115 kDa) and several minor fragments of TSP in human (and dog) plasma, as well as several molecular weight fragments or forms (above the ~115 kDa band) (See FIGS. 3, 4 and 6). This antibody, which is a mouse monoclonal, can be used in sandwich ELISAs for capture or detection and in competitive ELISAs (See FIG. 12). As an example meant to be illustrative and not restrictive, TSP Ab-4 is used to capture TSP and circulating fragments, and then the other antibody or binding agent is used for detection, but is able to distinguish TSP from a fragment or fragments, or one fragment from another. It is understood that TSP Ab-4 also binds thrombospondin and thrombospondin fragments from important non-human sources as well, including but not limited to the dog. Thus, the use of this antibody and/or a similar binding agent in an assay for a thrombospondin fragment or fragments in a sample from a non-human source, such as dog, is contemplated. This antibody shows no cross-reaction with fibronectin, fibrinogen, and von Willebrand factor. This antibody inhibits thrombospondin-collagen interaction, and its binding to thrombospondin is unaffected by glycosaminoglycans (e.g. hyaluronic acid, chondroitin sulfate, and heparin). Also, its binding is enhanced by EDTA i.e. at low conc. of Ca<sup>2+</sup>.

TSP Ab-5 (Clone B5.2): This antibody has been reported to react against reduced and non-reduced protein, and its epitope is in a 10 kDa fragment present at the junction of type 2 and type 3 repeats. The junctional region is listed elsewhere as residues 674-697, but this is only 24 residues and less than 10 kDa, so the epitope is less precisely mapped. It is expected that this antibody will bind TSP but not the 30 kDa circulating fragment; however, Applicant's data suggests that this antibody binds all three major fragments, including the ~30 kDa fragment(s) of TSP in human (and dog) plasma, and several minor fragments of TSP in human (and dog) plasma, as well as high molecular weight fragments or forms (above the ~115 kDa band) (See FIG. 7). Thus, this antibody can be used to detect and/or quantify TSP and/or a circulating fragment or fragments; distinguish thrombospondin from a circulating fragment; and/or distinguish one or more fragments from each other. It shows no cross-reaction with fibronectin, fibrinogen, and von Willebrand factor.

TSP Ab-9 (Clone MBC 200.1): This antibody has been reported to react against reduced and non-reduced protein, and its epitope is in the N-terminal heparin-binding domain of thrombospondin. Thus, it should bind to thrombospondin but not to major circulating fragments. In Western blotting, Ab-9 reacts with a 25 kDa peptide (heparin-binding domain) from thermolysin digests of thrombospondin that is not disulfide bonded to any other region of the thrombospondin molecule. Heparin efficiently inhibits the binding of Ab-9 to thrombospondin. Thus, this antibody can be used to detect and/or

quantify TSP; and/or distinguish thrombospondin from a circulating fragment or fragments. This antibody is not suitable for detecting all major fragments in the circulation.

TSP Ab-8 (rabbit ws3 antibody): Recognizes a ~450 kDa (non-reduced) or 180 kDa (reduced) protein, identified as TSP. This antibody, which is a rabbit polyclonal, can be used in sandwich ELISAs for capture or detection and in competitive ELISAs (see FIG. 12). Applicant has discovered that it binds the three major TSP fragments in human plasma (~30 kDa, ~50 kDa, and ~115 kDa), and minor fragments of TSP in human (and/or dog) plasma, as well as its binding to larger fragments or forms (above the ~115 kDa band) (See FIG. 9). Thus, this antibody can be used to detect and/or quantify TSP and/or a circulating fragment or fragments. In combination with another antibody or binding agent, it can be used in an assay to distinguish thrombospondin from a circulating fragment; and/or to distinguish one or more fragments from each other.

As an example meant to be illustrative and not restrictive, one takes the difference between (a) the result of an assay using an antibody or binding agent that binds TSP and circulating fragments in plasma, versus (b) the result of an assay using an antibody or binding agent that binds TSP but not fragments in plasma. The antibody or binding agent in (a) is selected from the group consisting of TSP Ab-4, TSP Ab-8, TSP Ab-11, and an antibody or binding agent that binds TSP and circulating fragments in plasma. The antibody or binding agent in (b) is selected from the group consisting of TSP Ab-3, TSP Ab-6, TSP Ab-9, and an antibody or binding agent that binds TSP but not circulating fragments. Said assay in (a) detects TSP plus fragments; said assay in (b) detects TSP; said difference, (a) minus (b), thereby gives a quantification of fragments without TSP. Likewise, differences can be taken between (c) the result of an assay using an antibody or binding agent that binds TSP and a subset of the circulating fragments in plasma and/or serum, versus the result of (a), above, to obtain a quantification of the fragment or fragments not detected in (c). Differences can also be taken of the result of (c) versus (b), above, to obtain a quantification of the fragment or fragments detected in (c) but without the signal from TSP. The antibody or binding agent in (c) is selected from the group consisting of TSP Ab-2, TSP Ab-5, TSP Ab-1, TSP Ab-7, and an antibody or binding agent that binds TSP and only a subset of the circulating fragments.

TSP Ab-11 (Clones D4.6+A6.1+MBC 200.1): The Ab-11 cocktail is designed for sensitive detection of thrombospondin by Western blotting. This antibody cocktail has been reported to show no cross-reaction with fibronectin, fibrinogen, or von Willebrand factor. Because it is a mixture of TSP Ab-2, TSP Ab-4, and TSP Ab-9, it detects TSP and the three major TSP fragments in human plasma. Thus, this antibody can be used to detect and/or quantify TSP and/or a circulating fragment or fragments. In combination with another antibody or binding agent, it can be used in an assay to distinguish thrombospondin from a circulating fragment; and/or to distinguish one or more fragments from each other. It can also be used in an assay for TSP and/or a TSP fragment or fragments in a sample from a non-human source, such as a dog.

Other antibodies that are useful, even though they have been disclosed only as binding non-reduced protein include, but are not limited to TSP Ab-1, TSP Ab-3, TSP Ab-6, and TSP Ab-7, which are described in more detail immediately below. The Applicant's results indicate that Ab-7 binds reduced material (See FIG. 8).

TSP Ab-1 (Clone A4.1): This antibody has been reported to bind the N-terminal half of the central stalk-like region of thrombospondin. This region is recovered as a 50 kDa frag-

ment after chymotryptic digestion of thrombospondin. Thus, Ab-1 may be used to detect and/or quantify TSP and/or a circulating fragment or fragments; distinguish thrombospondin from a circulating fragment or fragments; and/or distinguish one or more fragments from each other. TSP Ab-1 has been reported to show no cross-reaction with fibronectin, fibrinogen, and von Willebrand factor. It inhibits the adhesion of human melanoma G361 cells, keratinocytes, squamous carcinoma cells, and rat smooth muscle cells to thrombospondin. It does not inhibit aggregation of thrombin-induced platelets. This antibody is stated to block the anti-angiogenic activity of thrombospondin by inhibiting its binding to TSP-Receptor/CD36.

TSP Ab-3 (Clone C6.7): This antibody has been reported to bind the platelet or cell-binding domain at the extreme C-terminus of TSP and should therefore distinguish TSP from fragments, as well as a fragment or fragments with vs. without this epitope. Thus, this antibody can be used to detect and/or quantify TSP; and/or distinguish thrombospondin from a circulating fragment or fragments; and/or distinguish a fragment from another. This antibody should not be suitable for detecting all of the three major fragments in the circulation, although it may detect higher molecular weight fragments or forms. Heparin or EDTA may marginally affect binding of Ab-3 to thrombospondin. Ab-3 blocks thrombospondin-mediated agglutination of fixed red blood cells. It shows no effect on thrombospondin-mediated agglutination of fixed, activated platelets. It inhibits both thrombin- and A23187-induced aggregation of washed, live (not fixed) platelets without affecting the secretion of serotonin. Ab-3 inhibits adhesion of melanoma G361 cells to thrombospondin, and blocks the binding of C-terminal domain to Integrin-Associated Protein (LAP)/CD47.

TSP Ab-6 (Clone A2.5): This antibody has been reported to immunoprecipitate thrombospondin. This antibody has been reported to show no cross-reaction with fibronectin, fibrinogen, or von Willebrand factor. Its epitope has been reported to be localized in the heparin-binding domain of thrombospondin, and therefore, heparin efficiently inhibits the binding of Ab-6 to thrombospondin. Thus, this antibody can be used to detect and/or quantify TSP; and/or distinguish thrombospondin from a circulating fragment or fragments, as well as a fragment or fragments with vs. without this epitope. This antibody should not be suitable for detecting all of the three major fragments in the circulation, although it may detect higher molecular weight fragments or forms. Hyaluronic acid and chondroitin sulfate show no inhibition at low concentration and only partially inhibit over the concentration range at which heparin abolishes the binding. Thrombospondin binds with high affinity to a sulfated glycolipid or sulfatide found on red cell and platelet membranes. Ab-6 has been reported to block the binding of thrombospondin to sulfatides at low concentrations. Ab-6 has been reported to immunoprecipitate a 25 kDa peptide (heparin-binding domain) from chymotryptic digests of thrombospondin that is not disulfide bonded to any other region of the thrombospondin molecule. This antibody inhibits the hemagglutination of trypsinized, glutaraldehyde-fixed human erythrocytes by purified thrombospondin. It also inhibits the agglutination of fixed, activated platelets by thrombospondin. It does not inhibit either thrombin- or A23187-induced aggregation of washed, live platelets. Ab-6 does not bind to reduced and alkylated thrombospondin or thrombospondin transferred to nitrocellulose membrane after SDS-PAGE.

TSP Ab-7 (Clone HB8432): This antibody has been reported to bind type 2 repeats. Thus, Ab-7 may be used to detect and/or quantify TSP and/or a circulating fragment or

fragments; distinguish thrombospondin from a circulating fragment or fragments; and/or distinguish one or more fragments from each other. It shows no cross-reaction with fibronectin or any other serum or platelet proteins except thrombospondin. Its epitope has been reported to localize in the EGF-like repeats (type 2) in the stalk region of human thrombospondin (disulfide-bonded core remaining after trypsin digestion). Applicant's data suggests that Ab-7 may be used to distinguish between lower molecular weight fragments and higher molecular weight fragments (above ~115 kDa) of thrombospondin or TSP itself, as this antibody recognizes only the high molecular weight fragments or forms. In contrast, no fragments were recognized in normal human or dog plasma with this antibody (See FIG. 8).

All of the antibodies listed above can be purchased from Lab Vision Corporation, Fremont, Calif. See also the published literature such as, for TSP Ab-4, Galvin N J et al. Interaction of human thrombospondin with types I-V collagen: direct binding and electron microscopy. *J Cell Biol.* 1987 May; 104(5):1413-22). It is also understood that alternative antibodies may also be generated against any of the abovementioned epitopes.

5) A purified and/or synthetic thrombospondin fragment, said fragment being at least 6 contiguous amino acyl residues in length, and wherein the fragment does not comprise at least one fibrinogen-binding region selected from the group consisting of (1) a fibrinogen-binding domain within a 210 kDa fragment of TSP, where said 210 kDa fragment is composed of three 70 kDa fragments that contain the region of inter-chain disulfide bonds, the procollagen homology region, and the type 1 and type 2 repeats, (2) a fibrinogen-binding region in the amino-terminal domain of thrombospondin, (3) a fibrinogen-binding region in an 18 kDa amino-terminal heparin-binding domain of thrombospondin, and (4) a region corresponding to synthetic peptide N12/I encompassing amino acid residues 151-164 (I-151 to P-164) of the N-terminal domain of thrombospondin-1. In a particular embodiment, the fragment does not comprise any of the fibrinogen-binding regions in the group.

6) A purified and/or synthetic thrombospondin fragment, said fragment being at least 6 contiguous amino acyl residues in length, wherein the fragment (1) is recognized by the Ab-7 antibody; and (2) has a high molecular weight preferably above 80 kDa and/or above 115 kDa.

For certain applications of each of the 6 additional aspects, the molecular weight of the thrombospondin fragment exceeds an apparent molecular weight of 80 kDa and/or 115 kDa. For certain applications of each of the 6 additional aspects, the molecular weight of the thrombospondin fragment does not exceed 140 kDa; alternatively does not exceed 65 kDa; or alternatively does not exceed 35 kDa, wherein the size in kDa is the apparent size by gel electrophoresis after disulfide bond reduction. The fragments of the 6 additional aspects of the invention can be used to induce antibodies (and/or other binding molecules) of interest in the diagnostic methods or can be used in diagnostic assays, for example, as calibrators, indicators, and/or competitors. It is understood that a fragment can be derivatized, for example, to incorporate and/or be coupled to a label and/or a carrier.

A fragment that can be as little as 6 amino acyl residues in length is preferably immunoreactive. A typical method for immunizations comprises coupling the peptide to a carrier, such as keyhole limpet hemocyanin or ovalbumin. Said couplings to a carrier are also contemplated in the current invention.

The inclusion of the central protease-resistant core domain in the definition of the fragments follows from considerations

discussed elsewhere herein. This domain is considered to comprise locations in the mature thrombospondin protein selected from the group consisting of: a domain of interchain disulfide bonds (around Cys-252 and Cys-256, preferably residues 241-262); the procollagen homology domain (residues 263-360); the type 1 repeats (residues 361-530); the type 2 repeats (residues 531-673); there is a short segment (residues 674-697) between the type 2 repeat domain and the type 3 repeat domain; and then the type 3 repeats (residues 698-925); see FIG. 1 of this Application for examples of protease-resistant fragments that have been reported after artificial digestions in vitro; Chapter 2, "The primary structure of the thrombospondins" in *The Thrombospondin Gene Family* by J C Adams, R P Tucker, & J Lawler, Springer-Verlag: N.Y., 1995, pp. 11-42, particularly p. 12; and Chapter 6, "Mechanistic and functional aspects of the interactions of thrombospondins with cell surfaces," *ibidem*, pp. 105-157, particularly p. 115. Interchain disulfide bonds (in the region of residues 241-262) are often preserved in protease-resistant fragments. The term "mature", as used here to refer to the mature thrombospondin protein sequence, means without the 18- to 22-residue signal peptide sequence, here assumed to be 18 residues, following *The Thrombospondin Gene Family* by J C Adams et al. 1995; see the full human thrombospondin sequence given below in this text; see also FIG. 1 of this application, and the discussions thereof. Nevertheless, it is understood that GenBank file NM\_003246.1, also listed as GI:4507484, currently identifies nucleotide residues "112 . . . 204" as encoding the signal peptide, which implies a signal peptide of 31 amino acyl residues).

The identification of these peptides, TEENKE (SEQ ID NO:1), LQDSIRKVTEENKE (SEQ ID NO:3), EGEARE (SEQ ID NO:4), PQMNGKPCEGEARE (SEQ ID NO:5), EDTDLD (SEQ ID NO:6), YAGNGIICGEDTDLD (SEQ ID NO:7), CNSPSPQMNGKPCEGEARE (SEQ ID NO:8), RKVTEENKELANELRRP (SEQ ID NO:9), PQMNGKPCEGEARE (SEQ ID NO:11), CEGEARE (SEQ ID NO:12), and RKVTEENKE (SEQ ID NO:13) was achieved by computerized surveys of thrombospondin, the surveys done by request at commercial sources to identify immunogenic regions (epitopes), but these surveys identified many peptides with immunogenic regions, and so the surveys were followed by selection of relevant peptides and/or epitopes based on knowledge of circulating thrombospondin fragments. Other peptides and/or epitopes listed in this application were similarly identified.

A criterion that a fragment comprises an immunogenic and/or immunoreactive portion from a collagen type V binding domain follows from the published properties (e.g., Galvin N J et al. Interaction of human thrombospondin with types I-V collagen: direct binding and electron microscopy. *J Cell Biol.* May 1987; 104(5): 1413-22) of the commercially available TSP Ab-4 antibody used below to detect thrombospondin fragments of interest in the plasma. However, Applicant's data suggests that the actual epitopes to which these antibodies bind may be different than those that were asserted in the literature.

The collagen V-binding domain of thrombospondin has been mapped to the amino acid sequence corresponding to the region between valine(333) and lysine(412) (V-333 to K-412, using the single-letter symbols V and K for their respective amino acids), inclusive, of human thrombospondin-1 (Takagi T et al. A single chain 19 kDa fragment from bovine thrombospondin binds to type V collagen and heparin. *J Biol Chem* 268:15544-15549, 1993; as mentioned above, numbers here refer to the mature thrombospondin protein, that is, without the 18- to 22-residue signal peptide sequence, here assumed

to be 18 residues). This region would include a portion of the procollagen homology region of thrombospondin and all or nearly all of the first type 1 repeat of thrombospondin (see Chapter 2, "The primary structure of the thrombospondins" in *The Thrombospondin Gene Family* by J C Adams, R P Tucker, & J Lawler, Springer-Verlag: N.Y., 1995, pp. 11-42, but especially p. 24).

The criterion that the fragment comprises an epitope for binding the commercially available TSP Ab-4 antibody follows from the fact that the TSP Ab-4 antibody was used below to successfully detect thrombospondin fragments of interest in the plasma, including the plasma of cancer patients. Significantly, this TSP Ab-4 antibody is described as binding the collagen type V binding domain of thrombospondin.

For references regarding a fibrinogen-binding region within a 210 kDa fragment of TSP composed of three 70 kDa fragments that contain the region of interchain disulfide bonds, the procollagen homology region, and the type 1 and type 2 repeats, see p.24 of Adams et al. *The Thrombospondin Gene Family*; citation 53 therein, which is Lawler J et al. Thrombin and chymotrypsin interactions with thrombospondin. *Ann NY Acad Sci.* 1986; 485:273-87; and citations immediately below. Additional references for the fibrinogen-binding regions to be excluded include: for a region in an 18 kDa amino-terminal heparin-binding domain of thrombospondin (so-called TSP18), see Bonnefoy A et al.: A model of platelet aggregation involving multiple interactions of thrombospondin-1, fibrinogen, and GPIIb/IIIa receptor. *J Biol Chem.* Feb. 23, 2001; 276(8):5605-12. For a region corresponding to synthetic peptide N12/I encompassing amino acid residues 151-164 of the N-terminal domain of thrombospondin-1, see Voland C et al.: Platelet-osteosarcoma cell interaction is mediated through a specific fibrinogen-binding sequence located within the N-terminal domain of thrombospondin 1. *J Bone Miner Res.* Feb. 2000; 15(2):361-368. Citations for two fibrinogen-binding domains include p. 24 of Adams et al. *The Thrombospondin Gene Family* (and citations 51-54 therein), and for the role of the type 1 repeats include Panetti T S et al.: Interaction of recombinant procollagen and properdin modules of thrombospondin-1 with heparin and fibrinogen/fibrin. *J Biol Chem.* Jan. 1, 1999; 274(1):430-7.

Thrombospondin is a glycosylated protein. Therefore, depending on which portion of thrombospondin is considered, the thrombospondin fragments of the invention may be glycosylated or non-glycosylated. Potential sites for N-linked carbohydrate chains include N-230 (in the N-terminal domain), N-342 (in the procollagen homology domain), N-503 (in the type 1 repeat domain), N-690 (in the region between the type 2 and type 3 repeat domains), N-1033 (in the C-terminal domain), and N-1049 (in the C-terminal domain). It is also understood that specific C- and O-linked saccharide attachments occur, particularly in the type 1 repeat domain (see Hofsteenge J, Huwiler K G, Macek B, Hess D, Lawler J, Mosher D F, Peter-Katalinic J: C-mannosylation and O-fucosylation of the thrombospondin type 1 module. *J Biol Chem.* Mar. 2, 2001; 276(9):6485-6498). It is also understood that  $\beta$ -hydroxylation of thrombospondin can occur (such as N-592, which is in the type 2 repeat domain; see FIG. 2.2a in Adams J C et al. *The Thrombospondin Gene Family*, 1995, p. 16), and that any of these modifications can be incorporated, or not, into thrombospondin fragments and/or peptides of the current invention.

Nonglycosylated entities of particular interest are synthetic peptides.

In particular embodiments, the thrombospondin fragments of the invention are derivatized so that they comprise and/or are linked to a detectable label and/or a carrier. In particular

embodiments, the label is selected from the group consisting of a radioactive label, a fluorescent label, a chemical label, a colorimetric label, an enzymatic label, a non-fluorescent label, a non-radioactive label, a biotin moiety, and an avidin moiety. In particular embodiments, the carrier is selected from the group consisting of a bead, a microsphere, a coded microsphere, a solid matrix, a keyhole limpet hemocyanin, an albumin, linkage to a cross-linking agent, an epitope tag, and an epitope.

It is understood that a synthetic or purified thrombospondin fragment of the invention retains its identity as a fragment of the invention even if it has been derivatized by the addition of additional material, such as a detectable label, or through conjugation to another molecule, or by synthesizing it as part of a chimeric protein, to name just three of many possible examples.

#### Binding Agents

The detection of either thrombospondin fragments or thrombospondin usually requires the use of agents that will bind to them. Such agents may be multi-chain antibodies, single-chain antibodies, proteins that are not antibodies, non-protein molecules, or derivatives or combinations thereof. Polyclonal and monoclonal antibodies are normally immunoglobulins, i.e., multi-chain antibodies. In the case of immunoglobulin-G (IgG), each antibody molecule consists of a pair of heavy chains and a pair of light chains. The multichain antibodies are typically from mammalian or avian sources. Single-chain antibodies and non-antibodies are discussed below.

The term "antibodies" by itself, when not specified as being a single-chain antibody, refers to 4-chain antibodies, those with two heavy and two light polypeptide chains. By way of example, this includes but is not limited to the IgG classes of antibodies, but also other classes, such as ones that occur in higher multimers, such as IgM. IgA and IgY are also contemplated.

The term "protein" is intended to include not only molecules normally referred to as proteins but also those that may be referred to as polypeptides.

#### Methods of Detecting the Thrombospondin Fragments while Distinguishing, or not Distinguishing, from Thrombospondin Itself

In one such aspect, the invention includes an assay to detect a thrombospondin fragment of the invention wherein the assay distinguishes the thrombospondin fragment from thrombospondin itself. Thrombospondin fragments of particular interest are ones found in humans and are within a range selected from the group consisting of 80 to 140 kDa, 40 to 55 kDa and 20 to 30 kDa, wherein the size in kDa is that determined by gel electrophoresis after disulfide bond reduction. Preferably they are selected from the group consisting of a ~100 to 130 kDa fragment, ~85 kDa to 90 kDa fragment, an ~50 kDa fragment, and an ~30 kDa fragment. The assay may detect just one such fragment, or a combination of 2 or more.

In a preferred embodiment, a higher molecular weight fragment is detected in a sample, wherein the fragment has a molecular weight higher than ~115 kDa and may be detected by one of the following antibodies: TSP Ab-2, Ab-4, Ab-5, Ab-7 or Ab-8.

In another aspect, this invention contemplates a method of distinguishing between cancerous and non-cancerous plasma samples. This method utilizes an antibody to detect a high molecular weight thrombospondin fragment or fragments that are greater than ~115 kDa in cancerous plasma samples that are not detectable in non-cancerous plasma samples. In a preferred embodiment, the TSP Ab-2, Ab-4, Ab-5 or Ab-8 antibody is used for detection of the high molecular weight

fragments of thrombospondin. In a more preferred embodiment, the TSP Ab-7 antibody is used to recognize the high molecular weight fragments of thrombospondin. Other binding agents, including but not limited to an aptamer, are also contemplated. Automated, high throughput assays are also contemplated.

In a further embodiment, the invention includes a method of distinguishing between improperly collected plasma samples and properly collected plasma samples. This method includes analyzing various plasma samples with an antibody or other binding agent that recognizes high molecular weight thrombospondin fragments in improperly collected normal plasma samples and not in properly collected normal plasma samples. In a preferred embodiment, the antibody used for detection is TSP Ab-2, Ab-4, Ab-5, Ab-7, or Ab-8. Most preferable of these antibodies is Ab-7. Other binding agents, including but not limited to an aptamer, are also contemplated. Automated, high throughput assays are also contemplated.

In cases where the concentration of higher molecular weight forms of thrombospondin, including thrombospondin itself, is low in a sample (such as in the samples shown in FIGS. 3, 4 and 6), detection of fragments without necessarily excluding thrombospondin is an approach also contemplated by the current invention. Low concentrations of thrombospondin can be achieved in many cases by preventing or reducing platelet activation during sample collection and/or storage (see below for contemplated methods). This aspect of the current invention comprises several advantages over conventional detection methods that have used binding agents against the entire thrombospondin molecule (and these binding agents have been limited to antibodies). Said advantages include but are not limited to the use of binding agents that are directed specifically against the fragments of interest and not portions of the thrombospondin molecule outside of these fragments, the use of relevant peptides and/or thrombospondin fragments to generate said binding agents (such as antibodies), the use of relevant peptides and/or thrombospondin fragments as assay calibrators, and the use of relevant peptides and/or thrombospondin fragments as assay indicators.

Any of several acceptable approaches can be used for the assay of a thrombospondin fragment (or fragments) wherein the assay distinguishes it from thrombospondin, and more than one of these can be used in a given assay. In one approach, the assay comprises a step wherein the fragment is physically separated from the thrombospondin. Generally that approach is combined with a step in which the presence of the fragment or thrombospondin is shown by their reaction with a specific binding agent. Assay methods include but are not limited to those well-known in the art, such as ELISA, radioimmunoassay, Western blotting, immunohistochemistry, immunofluorescence, other immune-based methods, non-immune-based methods, quantitative methods, high throughput methods, automated methods, semi-quantitative methods and qualitative methods. In particular embodiments, the physical separation technique is selected from the group consisting of gel electrophoresis, dialysis, chromatography, size chromatography, affinity chromatography, immunoaffinity chromatography, adsorption, immunoadsorption, isoelectric focusing, mass spectrometry, centrifugation, sedimentation, floatation, precipitation, immunoprecipitation, and gel filtration.

In a second approach, the assay distinguishes the fragment (or fragments) based on one or more epitopes (here "epitope" meaning a target to which a binding agent, i.e., an antibody or a non-antibody, binds) in the fragment that are not present in thrombospondin, including but not limited to an epitope at an

end of a fragment and an epitope that is displayed by a fragment but is shielded in thrombospondin.

In a third approach, the assay distinguishes the fragment (or fragments) based on one or more epitopes in thrombospondin that are not present in the fragment. As an illustrative but not restrictive example, an epitope shared by thrombospondin and a thrombospondin fragment is used to obtain a quantitation of a total, thrombospondin plus thrombospondin fragment(s), from which is then subtracted a quantitation of thrombospondin obtained using an epitope present in thrombospondin but not present in a fragment. The difference between the two quantitations is a quantitation of the amount of fragment. As an example, epitopes in thrombospondin but not in at least one fragment from the group of an 80 to 140 kDa, a 40 to 55 kDa, or a 20 to 35 kDa fragment present in plasma can be selected from the group consisting of an epitope from outside the protease-resistant central core domain, an epitope in the N-terminal domain, an epitope in the N-terminal heparin-binding domain, a heparin-binding sequence in the N-terminal domain, a heparin-binding sequence in the N-terminal domain selected from the group consisting of residues 23-32 (RKGSGRRLVK; SEQ ID NO:59), residues 23-29 (RKGSGRR; SEQ ID NO:60), and residues 77-83 (RQMKKTR; SEQ ID NO:61) of the mature protein (see Chapter 2, "The primary structure of the thrombospondins" in *The Thrombospondin Gene Family* by J C Adams, R P Tucker, & J Lawler, Springer-Verlag: New York, 1995, pp. 11-42, but especially p. 13 & Table 2.1; Chapter 6, "Mechanistic and functional aspects of the interactions of thrombospondins with cell surfaces," *ibidem* pp. 105-157, but especially pp. 108 & 114; Lawler J et al. Expression and mutagenesis of thrombospondin. *Biochemistry*. 1992 Feb. 4; 31(4):1173-80; and Cardin A D & Weintraub H J. Molecular modeling of protein-glycosaminoglycan interactions. *Arteriosclerosis*. 1989 January-February; 9(1):21-32), a heparin-binding sequence in the N-terminal domain selected from the group consisting of residues 22-29 (ARKGSGRR; SEQ ID NO: 62), residues 79-84 (MKKTRG; SEQ ID NO: 63), and residues 178-189 (RLRIAKGGVNDN; SEQ ID NO: 64) of the mature protein (reviewed in the Discussion section of Voland C et al.: Platelet-osteosarcoma cell interaction is mediated through a specific fibrinogen-binding sequence located within the N-terminal domain of thrombospondin 1. *J Bone Miner Res*. 2000 February; 15(2):361-368), an epitope in the C-terminal domain, an epitope in the C-terminal cell-binding domain, a thrombospondin epitope not found in a plasma fragment, a thrombospondin epitope not found in a plasma fragment of 80 to 140 kDa, a thrombospondin epitope not found in a plasma fragment of 40 to 55 kDa, and a thrombospondin epitope not found in a plasma fragment of 20 to 35 kDa, where all kDa molecular weights are those after reduction. It is understood that the absence of a strong, functional heparin-binding domain from a thrombospondin fragment in plasma will be a factor allowing its accumulation in plasma (many heparin- or heparan-binding proteins are cleared from plasma very quickly; see for example, Wallinder L et al. Rapid removal to the liver of intravenously injected lipoprotein lipase. *Biochim Biophys Acta*. 1979 Oct. 26; 575(1):166-73).

The epitopes may be divided into three Groups. Group 1: An epitope shared by thrombospondin and a thrombospondin fragment present in plasma is preferably one that is contained within an amino acid sequence selected from the group consisting of TEENKE (SEQ ID NO:1), CLQDSIRKVTEENKE (which includes an N-terminal Cys added to aid conjugation), (SEQ ID NO:2), LQDSIRKVTEENKE (SEQ ID NO:3), EGEGARE (SEQ ID NO:4), PQMNGKPCGEARE (SEQ ID

NO:5), EDTDLD (SEQ ID NO:6), YAGNGIICGEDTDL (SEQ ID NO:7), CNSPSPQMNGKPCGEARE (SEQ ID NO:8), RKVTEENKELANELRRP (SEQ ID NO:9), CRKVTEENKELANELRRP (SEQ ID NO: 10), PQMNGKPCGEARE (SEQ ID NO:11), CEGEAR (SEQ ID NO:12), RKVTEENKE (SEQ ID NO:13), or a portion at least 3 amino acyl residues in length (preferably at least 4 amino acyl residues in length, more preferably at least 6 amino acyl residues) of such an amino acid sequence.

Group 2: An epitope in thrombospondin but not in an 80 to 140 kDa, 40 to 55 kDa, and/or 20 to 35 kDa fragment present in plasma is preferably one contained within an amino acid sequence selected from the group consisting of TERDDD (SEQ ID NO: 24), DFSGTFINTERDDD (SEQ ID NO: 25), ERKDHS (SEQ ID NO: 26), TRGTLLALERKDHS (SEQ ID NO: 27), CTRGTLLALERKDHS (SEQ ID NO: 28) (which includes an N-terminal Cys added to aid conjugation), DDKFQD (SEQ ID NO: 29), ANLIPPVPDDKFQD (SEQ ID NO: 30), CANLIPPVPDDKFQD (SEQ ID NO: 31) (which includes an N-terminal Cys added to aid conjugation), DCEKME (SEQ ID NO: 32), EDRAQLYIDCEKMEN (SEQ ID NO: 33) (although it is understood that this sequence and its fragments impinge on the sequence of the fibrinogen-binding N12/I peptide), CGTNRIPESGGDNSVFD (SEQ ID NO: 34), NRIPESGGDNSVFD (SEQ ID NO: 35), GWKDFTAYRWRLSHRPKTG (SEQ ID NO: 36), CGWKDFTAYRWRLSHRPKTG (SEQ ID NO: 37) (which includes an N-terminal Cys added to aid conjugation), or a portion at least 3 amino acyl residues in length (preferably at least 4 amino acyl residues in length, more preferably at least 6 amino acyl residues) of such an amino acid sequence.

Various modifications, such as a C-terminal Cys, can be added to a peptide of interest to allow easier conjugation to a carrier protein such as KLH, ovalbumin, and others. This is particularly true for the following peptides: RKVTEENKELANELRRP (SEQ ID NO: 9), LQDSIRKVTEENKE (SEQ ID NO: 3), TRGTLLALERKDHS (SEQ ID NO: 27), and ANLIPPVPDDKFQD (SEQ ID NO: 30), and these modifications provide alternative conjugation strategies for NRIPESGGDNSVFD (SEQ ID NO: 35) and others.

In approaches related to the above, the assay can distinguish fragments from each other, based on physical separation methods and/or on shared and/or non-shared binding agent targets. Thus, for example, size-exclusion chromatography and/or SDS-polyacrylamide gel electrophoresis can be used to separate the ~85 to 90, ~50-, and ~30 kDa fragments from each other, for separate quantitation (an example of this is shown in FIG. 3, with the quantitation presented in Table 2). Also, for example, an epitope (meaning a binding agent target) in the ~85 to 140 kDa fragment that is not contained in the ~50 kDa and/or the ~30 kDa fragments can be used to assay it separately, and/or can be used to subtract its contribution from a total to obtain results reflective of the smaller fragments.

Group 3: An additional epitope, useful as a binding agent target for distinguishing a fragment from full-length TSP, and/or distinguishing two fragments of different sizes is preferably one contained within an amino acid sequence selected from the group consisting of DDDDNDKIPDDRDN (SEQ ID NO: 14), DDDDNDKIPDDRDN[<sup>2</sup>NH<sub>2</sub>] (SEQ ID NO: 15), DDDDNDK (SEQ ID NO: 16), NLPNSGQEDYDKDG (SEQ ID NO: 17), CNLPNSGQEDYDKDG (SEQ ID NO: 18), EDYDKD (SEQ ID NO: 19), CPYNHNPDQADTDNNGEGD (SEQ ID NO: 20), CRLVPNPDQKDSGD (SEQ ID NO: 21), DQKDSGD (SEQ ID NO: 22), CPYVNPANQADHKDKGKGD (SEQ ID NO: 23), or a portion at least 3 amino acyl residues in length (preferably at least 4 amino

acyl residues in length, more preferably at least 6 amino acyl residues) of such an amino acid sequence.

It is also understood that some peptides that contain an epitope shared by thrombospondin and a first thrombospondin fragment present in plasma may contain an epitope that is not shared by a second thrombospondin fragment present in plasma. Said peptides are useful in many applications described herein, including but not limited to distinguishing thrombospondin from said second thrombospondin fragment, distinguishing said first from said second thrombospondin fragment, detecting and/or quantitating thrombospondin, detecting and/or quantitating said first thrombospondin fragment, detecting and/or quantitating said second thrombospondin fragment (in a combination assay described elsewhere herein), and producing a binding agent. Said peptides, which form a subset of Group 1, can be selected from the group consisting of EGEARE (SEQ ID NO: 4), PQMNGKPCGEARE (SEQ ID NO: 5), EDTDLD (SEQ ID NO: 6), YAGNGIICGEDTDL (SEQ ID NO: 7), CNSPSPQMNGKPCGEARE (SEQ ID NO: 8), PQMNGKPCGEARE (SEQ ID NO: 11), CEGEAR (SEQ ID NO: 12), or a portion at least 3 amino acyl residues in length (preferably at least 4 amino acyl residues in length, more preferably at least 6 amino acyl residues) of such an amino acid sequence.

It is also understood that the current invention also includes antibody and non-antibody molecules that bind these peptides, other peptides of thrombospondin specified herein, fragments thereof, and peptides that contain fragments thereof; as well as assays using a reagent from this list. It is understood that an antibody or a non-antibody that distinguishes thrombospondin from a fragment, or one fragment from another, can be employed to affinity-purify thrombospondin or a fragment.

In embodiments of particular interest, a sample of material (liquid tissue, solid tissue, urine, perspiration, cerebrospinal fluid, a body fluid, blood or a blood component, or stool; most preferably blood plasma) is taken or gathered from an organism (either a human or a non-human, preferably a mammal or a bird in the case of non-humans) and is subject to the assay. The inventions, disclosed herein not only apply to fragments of human thrombospondin, but also to fragments of non-human thrombospondin. For example, there is a need to detect the presence of or monitor the status of disease, such as a cancer, in livestock, racehorses, pets, and other economically and/or emotionally important animals. The current inventions meet these needs.

In one set of embodiments, the assay detects the presence of, or monitors the course of, diseases and conditions that can affect plasma levels of thrombospondin fragments. Such diseases include, but are not limited to, many that in the prior art were assumed to affect plasma levels of thrombospondin: a cancer, renal failure, renal disease, atopic dermatitis, vasculitis, acute vasculitis, renal allograft, allergic asthma, diabetes mellitus, myocardial infarction, liver disease, splenectomy, dermatomyositis, polyarteritis nodosa, systemic lupus erythematosus, lupus erythematosus, Kawasaki syndrome, non-specific vasculitis, juvenile rheumatoid arthritis, rheumatoid arthritis, vasculitis syndrome, Henoch-Schönlein purpura, thrombocytopenic purpura, purpura, an inflammatory condition, a condition associated with clotting, a condition associated with platelet activation, a condition associated with intravascular platelet activation, a condition associated with consumption of platelets, heparin-induced thrombocytopenia, disseminated intravascular coagulation, intravascular coagulation, extravascular coagulation, a condition associated with endothelial activation, a condition associated with

production and/or release of thrombospondin and/or a thrombospondin fragment, urticaria, hives, angioedema, a drug reaction, an antibiotic reaction, an aspartame reaction, atopic dermatitis, eczema, hypersensitivity, scleroderma, conditions associated with plugging of vessels, a condition associated with a cryofibrinogen, a condition associated with a cryoglobulin, and a condition associated with an anti-cardiolipin antibody.

In embodiments of particular interest, the assay for thrombospondin fragments is done to detect the presence of, or monitor the status of, a cancer in a human and/or in a non-human animal. In additional embodiments of interest, the assay is done to measure the degree of platelet activation.

In measurements of plasma levels of the fragments, it is preferred that the plasma is obtained by a method that prevents or reduces platelet activation and/or activation of a component of the clotting cascade during sample collection and/or storage; and/or by a method that prevents or reduces cleavage of thrombospondin into fragments (or fragments into smaller fragments) during sample collection and/or storage. Platelet activation and/or activation of a component of the clotting cascade during sample collection and/or storage can result in the release of thrombospondin, but also activation of proteases (including but not limited to a protease of the clotting cascade) that can cleave thrombospondin and some thrombospondin fragments, thereby complicating the assay. To prevent or reduce platelet activation during sample collection and/or storage, the method may be one that does not comprise the use of a tourniquet. Also to prevent or reduce platelet activation and/or activation of clotting during sample collection and/or storage, the method may, for example, comprise a step selected from the group consisting of: (1) use of a large-bore needle, (2) discarding of the initial portion of the collected blood, (3) use of a coated needle, (4) use of a coated tubing, (5) storage of sample between  $-1^{\circ}$  C. and  $5^{\circ}$  C., and (6) separation of plasma within 30 minutes of sample collection. Also to prevent or reduce platelet activation and/or protease activity during sample collection and/or storage, the method may comprise the use of an agent selected from the group consisting of a platelet inhibitor, a protease inhibitor, a serine protease inhibitor, an enzyme inhibitor, an inhibitor of an enzyme that is divalent cation dependent, a heparin, a heparin fragment, a heparan, an anti-coagulant, a COX inhibitor, an inhibitor of a cell-adhesion molecule, an inhibitor of a surface receptor, a glycoprotein inhibitor, an inhibitor of a glycoprotein IIb/IIIa receptor, a thrombin inhibitor, an inhibitor of degranulation, a chelator, a citrate compound, theophylline, adenosine, and dipyridamole (Diatube H vacutainers containing citrate, theophylline, adenosine, and dipyridamole are commercially available from Becton Dickinson; see Bergseth G et al. A novel enzyme immunoassay for plasma thrombospondin: comparison with beta-thromboglobulin as platelet activation marker in vitro and in vivo. *Thromb. Res.* 99:41-50, 2000; such tubes can be referred to as CTAD tubes). Devices that minimize platelet activation and/or protease activity in a sample are also contemplated and include, but are not limited to, a collection tube containing a cocktail of platelet and/or clotting inhibitors, a collection tube containing a protease inhibitor, a collection tube containing an inhibitor of a protease that is or is derived from a blood component, and a device that discards or allows the easy discarding of the initial portion of collected blood. These methods can also be applied to samples of other body fluids.

A related aspect of the invention is a combination diagnostic test (especially for cancer) comprising at least two types of diagnostic tests, one of said tests being the assay for a throm-

bospondin fragment (or fragments) or a portion (or portions) thereof in plasma, the other assay not being based on a thrombospondin fragment or portion. In one set of embodiments, the test not based on a thrombospondin fragment or portion thereof is selected from the group consisting of an imaging test, a radiographic test, a nuclear medicine test, a magnetic resonance imaging test, a blood test, a biopsy, a genetic test, a guaiac test, a test for fecal occult blood, and a test for fecal blood, a cancer test not based on a thrombospondin fragment or portion thereof, a disease test not based on a thrombospondin fragment or portion thereof, and an endoscopy. In particular embodiments of the foregoing methods, a thrombospondin fragment comprises a detectable label (at least during some part of the method).

Detection can, for example, be part of a screening process. Such a screening could include a comparison against a reference value, involve a comparison against a previous value from the same individual; and/or be done repeatedly and/or periodically (e.g., once a year, once every six months, or once every 2, 3, 4, 5 or 10 years.). It is understood that screening can be performed on humans and/or on non-human animals

The foregoing methods are assays to detect a thrombospondin fragment of the invention wherein the assay distinguishes, or does not distinguish, a thrombospondin fragment from thrombospondin, or one thrombospondin fragment from another thrombospondin fragment. In any case, such fragments can be referred to as "target" fragments for purposes of the assay. In many instances it is desirable to have the method also comprise a calibration step or procedure, in which known amounts of a thrombospondin fragment (such as a peptide) are subjected to the method. Such "calibration" fragments are optionally detectably labeled. It is possible to perform the assays in which the target and calibration fragments comprise different detectable labels (or where one is detectably labeled and the other is not).

It is understood that interference resulting from fibrinogen binding to an N-terminal domain of thrombospondin is unlikely to affect the detection of thrombospondin fragments related to the protease-resistant core domain (which lack the N-terminal domain). Nevertheless, assays of thrombospondin could be affected (thus, avoiding that region of the N-terminus when assaying thrombospondin and/or diluting, removing, inhibiting, and/or otherwise compensating for interfering molecules is contemplated).

Additional potentially interfering substances, inferred from reports that these molecules are present in plasma and that they bind TSP, are plasminogen, histidine rich proteins including histidine-rich glycoprotein, and fibronectin (See, for example, Walz D A et al., *Semin Thromb Hemost.* 13(3): 317-025 (1987); Vanguri V K et al., *Biochem J.* Apr. 15, 2000; 347(Pt 2):469-73). For binding of histidine-rich glycoprotein, two regions of thrombospondin have been implicated: type 1 repeats (Simantov et al. *J Clin Invest.* January 2001, 107(1): 45-52) and a TSP heparin binding domain (Vanguri V K et al., 2000). The heparin-binding domain of thrombospondin is expected to be absent from the circulating fragments.

To compensate for interfering substances in assays for thrombospondin fragments, diluting, removing, inhibiting, and/or otherwise compensating for interfering molecules is contemplated. As an illustrative, but not limiting, example, the inclusion of an inhibitor of thrombospondin-fibrinogen interactions is contemplated. Such an inhibitor is selected from the group consisting of synthetic peptide N12/I encompassing amino acid residues. 151-164 of the N-terminal domain of thrombospondin-1 (see Voland C et al.: Platelet-osteosarcoma cell interaction is mediated through a specific fibrinogen-binding sequence located within the N-terminal

domain of thrombospondin 1. *J Bone Miner Res.* February 2000; 15(2):361-8), and an antibody to the cyanogen bromide cleavage fragment composed of residues 241-476 of the carboxyl-terminal end of the alpha chain of fibrinogen (see Tuszynski G P et al.: The interaction of human platelet thrombospondin with fibrinogen. *Thrombospondin purification and specificity of interaction.* *J Biol Chem.* Oct. 5, 1985; 260(22): 12240-5).

#### Single Chain Antibodies and Non-Antibodies

Raising conventional antibodies (also referred to herein simply as "antibodies" as opposed to "single chain antibodies"; and an example of a conventional antibody is IgG, which is composed of two heavy chains and two light chains) is merely one of a number of methods that are generally based on the approach of random, semi-random, directed, combinatorial, and/or other means for the generation of large numbers of diverse peptides and/or non-peptides, that is then followed by a selection procedure to identify within this large number those peptides and/or non-peptides that bind to a target and/or an epitope within a target. Selection can then be followed by methods for improving the peptides and/or non-peptides to achieve better affinity and/or specificity. These diverse peptides and/or non-peptides may be conventional multi-chain antibodies (polyclonal or monoclonal), single-chain antibodies, or non-antibodies, including but not limited to peptides, products of phage display, aptamers, DNA, RNA, or modified DNA or RNA. Also contemplated are thrombospondin receptors and/or binding proteins (such as a CSVTG (SEQ ID NO:54) receptor, a CSVTG (SEQ ID NO:54) binding molecule, CD36, angiocidin, 26S proteasome non-ATPase regulatory subunit 4, and/or anti-secretory factor).

A well-known procedure for generation of large numbers of diverse peptides is through phage display, which is then followed by selection and can be further refined through other techniques such as molecular evolution (see, for example, Flores-Flores, C. et al, Development of human antibody fragments directed towards synaptic acetylcholinesterase using a semi-synthetic phage display library. *J Neural Transm Suppl.* 2002; (62):165-179; Qian, M. D, et al, Anti GPVI human antibodies neutralizing collagen-induced platelet aggregation isolated from a recombinant phage. *Human Antibodies.* 2002; 11(3):97-105). scFv constructs can be made by linking variable domains of heavy (VH) and light (VL) chains together via a polypeptide linker (for example, see Asvadi P et al. Expression and functional analysis of recombinant scFv and diabody fragments with specificity for human RhD. *J Mol Recognit* 15:321-330, 2002). Peptides generated then selected (and then possibly improved) via this approach have been used in ELISAs and ELISA-like assays of their targets (e.g., see Schlattner U et al. Isoenzyme-directed selection and characterization of anti-creatine kinase single chain Fv antibodies from a human phage display library. *Biochim Biophys Acta.* Dec. 12, 2002; 1579(2-3):124-32; Oelschlaeger P et al. Fluorophor-linked immunosorbent assay: a time- and cost-saving method for the characterization of antibody fragments using a fusion protein of a single-chain antibody fragment and enhanced green fluorescent protein. *Anal Biochem.* Oct. 1, 2002; 309(1):27; Nathan S et al. Phage display of recombinant antibodies toward Burkholderia pseudomallei exotoxin. *J Biochem Mol Biol Biophys.* February 2002; 6(1):45-53; Lu D et al. Fab-scFv fusion protein: an efficient approach to production of bispecific antibody fragments. *J Immunol Methods.* Sep. 15, 2002; 267(2):213-26; Zhang W et al. Production and characterization of human monoclonal anti-idiotypic antibodies to anti-dsDNA antibodies. *Lupus.* 2002; 11(6):362-9; Reiche N et al. Generation and characterization

of human monoclonal scFv antibodies against *Helicobacter pylori* antigens. *Infect Immun.* August 2002; 70(8):4158-64; Rau D et al. Single-chain Fv antibody-alkaline phosphatase fusion proteins produced by one-step cloning as rapid detection tools for ELISA. *J Immunoassay Immunochem.* 2002; 23(2): 129-43; and Zhou B et al. Human antibodies against spores of the genus *Bacillus*: a model study for detection of and protection against anthrax and the bioterrorist threat. *Proc Natl Acad Sci U S A.* Apr. 16, 2002; 99(8):5241-6; Baek H et al., An improved helper phage system for efficient isolation of specific antibody molecules in phage display. *Nucleic Acids Res.* Mar. 1, 2002; 30(5):e18.

scFv constructs can be based on antibodies, as in most of the references above, on T-cell receptors (e.g., Epel M et al. A functional recombinant single-chain T cell receptor fragment capable of selectively targeting antigen-presenting cells. *Cancer Immunol Immunother.* December 2002; 51(10):565-573), or on other sequences. Different phage coat proteins have been used to display the diverse peptides (see Gao C et al. A method for the generation of combinatorial antibody libraries using pIX phage display. *Proc Natl Acad Sci USA.* Oct. 1, 2002; 99(20):12612-6). For an example of fusion constructs, see Lu D et al. Fab-scFv fusion protein: an efficient approach to production of bispecific antibody fragments. *J Immunol Methods.* Sep. 15, 2002; 267(2):213-26.

For an example of molecular evolution to improve binding affinity, see Rau D et al. Cloning, functional expression and kinetic characterization of pesticide-selective Fab fragment variants derived by molecular evolution of variable antibody genes. *Anal Bioanal Chem.* January 2002; 372(2):261-7. Examples of other modifications "to improve affinity or avidity, respectively [include] by mutating crucial residues of complementarity determining regions or by increasing the number of binding sites making dimeric, trimeric or multimeric molecules." (the quote is from a review article, Pini A & Bracci L, Phage display of antibody fragments. *Curr Protein Pept Sci.* September 2000; 1(2):155-169). The initial set of diverse molecules can be enriched by using sequences from animals or humans exposed to or expressing antibodies against the target (see again Zhang W et al. *Lupus* 2002; and Reiche N et al. *Infect Immun* 2002).

Single chain antibodies can also be generated by using *Escherichia coli* (see Sinicola J R & Robinson A S, Rapid folding and polishing of single-chain antibodies from *Escherichia coli* inclusion bodies, *Protein Expr Purif.* November 2002; 26(2):301-308.)

Non-antibodies also include aptamers and non-antibodies that comprise aptamers. Aptamers are DNA or RNA molecules that have been selected (e.g., from random pools) on the basis of their ability to bind to another molecule (discussed for example at the web site of the Ellington lab, in the Institute of Cellular and Molecular Biology, at the University of Texas at Austin, wherein said molecule can be a nucleic acid, a small organic compound, or a protein, peptide, or modified peptide (such as thrombospondin or a portion thereof.). An aptamer beacon is an example of a non-antibody that comprises an aptamer (See Hamaguchi N et al. Aptamer beacons for the direct detection of proteins. *Anal. Biochem.* 2001 Jul. 15; 294(2):126-131.)

Angiocidin is a CSVTCG-specific tumor cell adhesion receptor, see patent application WO 0105968, also NCBI protein accession number CAC32386.1 and/or CAC32387.1 (corresponding to nucleotide accession numbers AX077201 and AX077202), the amino acid sequences specified by those two protein accession numbers as of the date of filing of this application being incorporated herein by reference. It is understood that anti-secretory factor cDNA contains essen-

tially identical nucleotide sequence (e.g., accession # U24704, 99% match by BLAST alignment) to that of angiocidin, as does the nucleotide sequence for the proteasome (prosome, macropain) 26S subunit, non-ATPase, 4 (PSMD4; e.g., accession # NM\_002810, also 99% match by BLAST). Anti-secretory factor has the same amino acid sequence as angiocidin, except that AX077201 has a 9-bp insert compared to AX077202, which would mean an additional three amino acyl residues in the encoded protein. Thus, the terms herein are used interchangeably. The NCBI summary for NM\_002810 is as follows: "The 26S proteasome is a multicatalytic proteinase complex with a highly ordered structure composed of 2 complexes, a 20S core and a 19S regulator. The 20S core is composed of 4 rings of 28 non-identical subunits; 2 rings are composed of 7 alpha subunits and 2 rings are composed of 7 beta subunits. The 19S regulator is composed of a base, which contains 6 ATPase subunits and 2 non-ATPase subunits, and a lid, which contains up to 10 non-ATPase subunits. Proteasomes are distributed throughout eukaryotic cells at a high concentration and cleave peptides in an ATP/ubiquitin-dependent process in a non-lysosomal pathway. An essential function of a modified proteasome, the immunoproteasome, is the processing of class I MHC peptides. This gene encodes one of the non-ATPase subunits of the 19S regulator lid. Two alternate transcripts encoding two different isoforms have been described. Pseudogenes have been identified on chromosomes 10 and 21. Transcript Variant: This variant (1) encodes the longer protein (isoform 1)." Other names for the protein from the protein accession file (NP\_002801.1) include "proteasome 26S non-ATPase subunit 4 isoform 1; antiseecretory factor 1; 26S protease subunit S5a; S5a/antiseecretory factor protein; multiubiquitin chain binding protein; 26S proteasome non-ATPase regulatory subunit 4".

#### High Molecular Weight Thrombospondin Fragments

An important aspect of the invention is the discovery of immunoreactive high molecular weight fragments of thrombospondin. These fragments not only useful in the detection of cancer but also in similar methods and applications described for the low molecular weight fragments as well.

In one aspect, the invention includes a purified high molecular weight thrombospondin fragment that has been extracted from a bodily fluid, wherein the purified thrombospondin fragment is immunoreactive with a TSP Ab-7 antibody. In one embodiment, the molecular weight of said fragment is less than 185 kDa. In another embodiment, the molecular weight is from 85 kDa to 185 kDa, or from 115 kDa to 185 kDa. In another embodiment, the bodily fluid is blood plasma taken from an animal and/or human suspected and/or known to have a cancer. In another embodiment, the purified thrombospondin fragment is immunoreactive with an antibody selected from the group consisting of TSP Ab-2, Ab-4, Ab-5 and Ab-8.

In a further embodiment, the immunoreactive fragment is detected by analysis using a binding agent, said binding agent selected from the group consisting of a polyclonal antibody, a monoclonal antibody, a single-chain antibody, a non-antibody, a protein, a product of phage display, an anti-secretory factor, an aptamer, a DNA, an RNA, a modified DNA, a modified RNA, a carbohydrate, a glycosaminoglycan, a heparin, a glycoprotein, a proteoglycan, and combinations and derivatives thereof. The fragment may comprise a detectable label, wherein said label is either an intrinsic or added moiety, wherein the detectable label is selected from the group consisting of a radioactive label, a fluorescent label, a chemical

label, a colorimetric label, an enzymatic label, a non-fluorescent label, a non-radioactive label, a biotin label, and an avidin moiety.

In another aspect, the invention includes a method to detect and/or quantify a high molecular weight thrombospondin fragment, said method comprising performing an immunoassay using an antibody selected from the group consisting of TSP Ab-2, Ab-4, Ab-5, Ab-7, Ab-8, and derivatives and combinations thereof. The immunoassay may be a Western Blot, an ELISA, a high throughput immunoassay, an automated immunoassay, a radioimmunoassay, immunohistochemistry, immunofluorescence, other immune-based methods, non-immune-based methods, quantitative methods, high throughput methods, automated methods, and semi-quantitative methods and qualitative methods.

In a preferred embodiment, a recombinant thrombospondin fragment is used as a standard for said immunoassay. The recombinant thrombospondin fragment is stable at 4° C., and does not contain hazardous, infectious agents. The recombinant fragment also does not vary from donor to donor, as it is not collected by a donor but rather produced in an organism such as bacteria.

In another aspect, the invention includes a method of distinguishing between a cancerous sample of bodily fluid and a non-cancerous sample of bodily fluid, said method comprising: 1) performing an assay on said samples using a binding agent; and 2) detecting thrombospondin and/or high molecular weight fragments of thrombospondin.

In another aspect, the invention includes a method of distinguishing between properly and improperly collected plasma samples, said method comprising: 1) performing an assay with a binding agent on said samples, wherein said samples include a normal human serum sample and at least one cancerous plasma sample; and 2) detecting the presence of thrombospondin and/or high molecular weight thrombospondin fragments. In one embodiment, the samples are drawn into a CTAD tube. In another embodiment, the high molecular weight thrombospondin fragments have a molecular weight from 85 kDa to 185 kDa, or from 115 kDa to 185 kDa.

In another aspect, the invention includes a method to detect and/or quantify a high molecular weight thrombospondin fragment or fragment portion thereof. In a preferred embodiment, the method distinguishes the thrombospondin fragment or fragment portion from thrombospondin based on one or more epitopes in the fragment or fragment portion that are not present in thrombospondin. In a further embodiment, the fragment or fragment portion is distinguished based on one or more epitopes in thrombospondin that are not present in the fragments, said method comprising the steps of: 1) utilizing an epitope shared by thrombospondin and the thrombospondin fragment or thrombospondin portion as a target for a binding molecule, such as an antibody, to obtain a quantitation of a total, thrombospondin plus either the thrombospondin fragment or thrombospondin portion, 2) utilizing an epitope present in thrombospondin but not present in the fragment or thrombospondin portion to obtain a quantitation of thrombospondin only; and 3) utilizing the difference between the quantitations obtained in steps (1) and (2) as a quantitation of the amount of fragment or thrombospondin portion. In one embodiment, the method is applied to a sample of material taken or gathered from an organism. In a preferred embodiment, the organism is a human.

In another preferred embodiment, the method is performed in order to detect the presence of, or monitors the course of, a disease or condition. In another embodiment, the disease or condition is selected from the group consisting of a cancer,

renal failure, renal disease, atopic dermatitis, vasculitis, acute vasculitis, renal allograft, asthma, diabetes mellitus, myocardial infarction, liver disease, splenectomy, dermatomyositis, polyarteritis nodosa, systemic lupus erythematosus, lupus erythematosus, Kawasaki syndrome, non-specific vasculitis, juvenile rheumatoid arthritis, rheumatoid arthritis, vasculitis syndrome, Henoch-Schönlein purpura, thrombocytopenic purpura, purpura, an inflammatory condition, a condition associated with clotting, a condition associated with platelet activation, a condition associated with intravascular platelet activation, a condition associated with consumption of platelets, heparin-induced thrombocytopenia, disseminated intravascular coagulation, intravascular coagulation, extravascular coagulation, a condition associated with endothelial activation, a condition associated with production and/or release of thrombospondin and/or a thrombospondin fragment, urticaria, hives, angioedema, a drug reaction, an antibiotic reaction, an aspartame reaction, atopic dermatitis, eczema, hypersensitivity, scleroderma, conditions associated with plugging of vessels, a condition associated with a cryofibrinogen, a condition associated with a cryoglobulin, and a condition associated with an anti-cardiolipin antibody.

In another preferred embodiment, the disease is a cancer selected from the group consisting of an adenoma, adenocarcinoma, carcinoma, lymphoma, leukemia, solid cancer, liquid cancer, metastatic cancer, pre-metastatic cancer, non-metastatic cancer, a cancer with vascular invasion, internal cancer, skin cancer, cancer of the respiratory system, cancer of the circulatory system, cancer of the musculoskeletal system, cancer of a muscle, cancer of a bone, cancer of a joint, cancer of a tendon or ligament, cancer of the digestive system, cancer of the liver or biliary system, cancer of the pancreas, cancer of the head, cancer of the neck, cancer of the endocrine system, cancer of the reproductive system, cancer of the male reproductive system, cancer of the female reproductive system, cancer of the genitourinary system, cancer of a kidney, cancer of the urinary tract, cancer of a sensory system, cancer of the nervous system, cancer of a lymphoid organ, blood cancer, cancer of a gland, cancer of a mammary gland, cancer of a prostate gland, cancer of an endometrial tissue, cancer of a mesodermal tissue, cancer of an ectodermal tissue, cancer of an endodermal tissue, a teratoma, a poorly-differentiated cancer, a well-differentiated cancer, and a moderately differentiated cancer.

In one embodiment, the method is performed to measure the degree of platelet activation. In another embodiment, the method is performed to measure the degree of secretion of thrombospondin and/or a thrombospondin fragment from a tissue selected from the group consisting of a cancer, a neoplasm, an activated endothelium, and a stroma. In another embodiment, the method is performed on plasma that was obtained by a method that prevents or reduces platelet activation and/or protease activity during sample collection and/or storage.

In yet another embodiment, the method further comprises a test not based on a thrombospondin fragment or portion thereof. This test may be an imaging test, a radiographic test, a nuclear medicine test, a magnetic resonance imaging test, a blood test, a biopsy, a histologic test, a cytologic test, an immunohistologic test, a genetic test, a guaiac test, a test for fecal occult blood, a test for fecal blood, a test for fecal DNA, a test for a fecal cancer marker, a cancer test not based on a thrombospondin fragment or portion thereof, a receptor test, an estrogen receptor test, a disease test not based on a thrombospondin fragment or portion thereof, an endoscopy, an upper gastrointestinal endoscopy, a lower gastrointestinal

endoscopy, a colonoscopy, a sigmoidoscopy, a gastroscopy, a laparoscopy, a laparotomy, a lymph node biopsy, a surgery, and a bronchoscopy.

The blood test is selected from the group consisting of a cancer antigen test, a cancer gene test, a cancer DNA test, a cancer mRNA test, a cancer RNA test, a cancer protein test, a cancer glycoprotein test, a cancer carbohydrate test, a cancer lipid test, a prostate specific antigen test, a test of carcinoembryonic antigen, a test of cancer antigen CA-125, a test of alpha-fetoprotein, a test of CA15-3, a test of CA19-9, a test of malignin, a test of anti-malignin antibody, a test of anti-secretory factor, a cancer antigen that contains a carbohydrate epitope, a cancer antigen that contains a protein or polypeptide epitope, a cancer antigen that contains a lipid epitope, a cancer antigen that contains a mixed epitope, CA 27.29, and episialin.

In another embodiment, the thrombospondin fragment or portion thereof comprises a detectable label, wherein the detectable label is a target and/or indicator fragment and wherein a known or unknown amount of an unlabeled or differently labeled fragment is also subjected to the method, said unlabeled or differently labeled fragment being a thrombospondin fragment or portion thereof.

In another aspect, the invention includes a method of producing antibodies against a high molecular weight thrombospondin fragment or a fragment portion thereof, said method comprising administering said fragment, fragment portion or immunogenic portion thereof to an organism capable of producing antibodies.

In yet another aspect, the invention includes a method of producing a peptide or non-peptide binding agent against a high molecular weight thrombospondin fragment or a fragment portion thereof, or epitope therein, said method comprising the steps of 1) a generating step (random, semi-random, directed, combinatorial, and/or other) to generate large numbers (>100) of diverse peptides and/or non-peptides; 2) a selection step to identify within this large number those peptides and/or non-peptides that bind to the thrombospondin fragment, fragment portion, and/or an epitope therein; and 3) optionally an improvement step for improving the peptide or non-peptide binding agent to achieve better affinity and/or specificity.

In another aspect, the invention includes a cell line capable of producing a binding agent produced by the above described methods. In another aspect, the invention includes a kit for the determination of the presence of, and/or the amount of, and/or the concentration of, a thrombospondin fragment in a material taken or gathered from an organism, said kit comprising a high molecular weight thrombospondin fragment or a fragment portion thereof. The invention also contemplates a kit for the determination of the presence of, and/or the amount of, and/or the concentration of, one or more thrombospondin fragments in a material taken or gathered from an organism, said kit comprising a binding agent capable of binding said one or more of said high molecular weight fragments. In a preferred embodiment, the binding agent is produced by the methods described. In another aspect of the invention, the high molecular weight thrombospondin fragment or portion thereof has been derivatized wherein the derivatization is selected from the group consisting of addition of a detectable label, incorporation of a detectable label, conjugation to another molecule, synthesis of the fragment as part of a chimeric protein, linkage to a carrier molecule or particle, linkage to a carrier, linkage to a bead, linkage to a solid matrix, linkage to keyhole limpet hemocyanin, linkage

to an albumin, linkage to an ovalbumin, linkage to a cross-linking agent, linkage to an epitope tag, and linkage to an epitope.

The invention also contemplates a kit for the determination of the presence of, and/or the amount of, high molecular weight thrombospondin fragments in a material taken or gathered from an organism, said kit comprising an antibody that will react with said thrombospondin fragments of interest but not with thrombospondin. The method may also comprise the step of binding a binding agent to the thrombospondin fragment or portion thereof.

The invention also contemplates a method to detect the presence and/or clinical course of a neoplastic disease in an individual, wherein the method comprises the steps of: (1) measuring the individual's plasma level of a high molecular weight thrombospondin fragment or fragments; (2) utilizing the result of step (1) in a diagnosis as to whether the individual has a neoplastic disease. In one embodiment, the individual referred to therein is a first individual and wherein the method further comprises the steps of: (3) measuring a second individual's plasma level of the thrombospondin fragment, said second individual considered to not have neoplastic disease; and (4) utilizing the result of step (3) is the diagnosis of whether the first individual has a neoplastic disease.

In one embodiment, the greater the extent to which the first individual's plasma high molecular weight thrombospondin fragment level exceeds the plasma thrombospondin level of the second individual, the more likely that the diagnosis will be that the first individual has a neoplastic disease and/or a neoplastic disease more advanced than that of the second person. In another embodiment, the method further comprises the steps of assaying the individual's plasma level of a thrombospondin fragment more than once, and utilizing the change in plasma level from an older to a more recent value to indicate appearance or progression or improvement wherein said appearance or progression is indicated by an increase in the plasma level and said improvement is indicated by a decrease in said plasma level.

In another aspect, the invention includes a method of producing a binding agent against a high molecular weight thrombospondin fragment, said fragment at least 6 amino acyl residues in length, said method comprising binding a phage to said thrombospondin fragment, and wherein the fragment may be further modified to have a modification selected from the group consisting of glycosylation,  $\beta$ -hydroxylation, and addition of groups or moieties to aid conjugation and/or stability. The binding agent may be an antibody induced by either thrombospondin, a modified form of thrombospondin, a fragment of thrombospondin, a fragment of a modified form of thrombospondin, or a modified fragment of thrombospondin.

In yet another aspect, the invention includes a method that distinguishes two thrombospondin fragments from each other, said fragments being a first fragment and a second fragment, respectively, said method comprising the steps of: (1) utilizing an epitope or binding target shared by said first fragment and said second fragment as a target for a binding agent to obtain a quantitation of a total of said first fragment plus said second fragment; (2) utilizing an epitope or binding target present in said first fragment but not present in said second fragment, to obtain a quantitation of said first fragment only; and (3) utilizing the difference between the quantitations in steps (1) and (2) as a quantitation of the amount of said second fragment. In a preferred embodiment, at least one of said first or second fragment is a high molecular weight fragment of thrombospondin.

The invention also contemplates an aptamer which recognizes a high molecular weight thrombospondin fragment, and can distinguish a high molecular weight thrombospondin fragment from a low molecular weight thrombospondin fragment. In a preferred embodiment, the aptamer distinguishes the same thrombospondin fragments as TSP Ab-7.

#### Methods of Producing Antibodies Against the Fragments of the Invention

In another general aspect, the invention includes a method of producing antibodies and/or other binding agents against an above-noted thrombospondin fragment and/or portion thereof, the method comprising administering such a fragment or portion to an organism (especially a mammal or a bird) capable of producing antibodies. These fragments include both the lower molecular weight fragments, as well as the higher molecular weight fragments. The lower molecular weight fragments include the three dominant forms that are ~30 kDa, ~50 kDa, and ~115 kDa. The higher molecular weight fragments include those that are more than ~115 kDa but less than 185 kDa. In a preferred embodiment, the higher molecular weight fragments are recognized by TSP Ab-7. It is understood that said antibodies may comprise monoclonal antibodies and/or polyclonal antibodies. For monoclonal antibodies it is understood that cells from the organism are typically used in the production of hybridomas. For production of antibodies, including monoclonal antibodies, it is understood that any of the thrombospondin fragments and/or portions can be conjugated to a carrier molecule, including but not limited to keyhole limpet hemocyanin and bovine serum albumin, to facilitate the antibody response.

A cell and a cell line for producing the aforementioned monoclonal antibodies are aspects of the invention. Examples of such cells include, but are not limited to, hybridomas, transfected cell lines, and infected cells.

#### Kits of the Invention

Kits related to the above inventions are themselves aspects of the invention. Such kits are, for example, those that facilitate the determination of the presence of, and/or the amount of, and/or the concentration of, a thrombospondin fragment or fragments in a material taken or gathered from an organism. Such kits optionally comprise a thrombospondin fragment or fragments, or a portion or portions thereof, of the invention. Such kits can comprise a binding agent or agents specific for a thrombospondin fragment, or portion thereof, of interest. They optionally comprise binding agents that will react with thrombospondin but not a fragment or fragments, and/or a portion or portions thereof, of interest. They optionally comprise binding agents that distinguish between thrombospondin and a fragment, and/or between one fragment and another. If intended for solid tissue, the kits may comprise a homogenizing means for extracting a fragment into a solution, which optionally may also be provided. Binding agents of the current invention can also be used for other well-known detection methods, including but not limited to immunohistochemistry.

Preferred binding agents are proteins, although non-proteins are also contemplated. Such proteins include both antibodies and nonantibodies.

Optionally, the kits comprise a means for separating or distinguishing a fragment or fragments (or portions thereof) from thrombospondin. The kits can also include a thrombospondin fragment, a peptide derived from such fragment, or a derivatized fragment or peptide, to facilitate detection and calibration.

In one set of embodiments, the kits are adapted for use in an automated assay, such as one using a clinical autoanalyzer.

Particular kit aspects of the invention can also be summarized as follows:

A kit for the determination of the presence of, and/or the amount of, and/or the concentration of, a thrombospondin fragment or fragments in a material taken or gathered from an organism, said kit comprising a thrombospondin fragment or portion thereof.

A kit for the determination of the presence of, and/or the amount of, and/or the concentration of, one or more thrombospondin fragments in a material taken or gathered from an organism, said kit comprising a binding agent capable of binding said one or more fragments.

Particular embodiments are:

Such kits wherein the binding agent comprises a protein.

Such kits wherein said protein comprises an antibody.

Such kits wherein the antibody is a monoclonal antibody or a polyclonal antibody.

Such kits wherein said protein comprises a fragment of an antibody.

Such kits wherein said protein comprises a single-chain antibody.

Such kits wherein said single chain antibody is derived from a phage display library.

Such kits wherein said protein is a non-antibody, the non-antibody being a protein that is neither a multi-chain antibody nor a single-chain antibody.

Such kits wherein said protein non-antibody is selected from the group consisting of a thrombospondin receptor, a thrombospondin receptor that binds within a protease-resistant core region, a thrombospondin receptor that binds a TSP fragment present in the plasma of a cancer patient, a CSVTCG (SEQ ID NO:54) receptor, a CSVTCG (SEQ ID NO:54) binding molecule, a CD36 (which reportedly binds CSVTCG (SEQ ID NO:54); see Carron J A et al., A CD36-binding peptide from thrombospondin-1 can stimulate resorption by osteoclasts in vitro. *Biochem Biophys Res Commun.* 2000 Apr. 21; 270(3):1124-7), angiocidin, anti-secretory factor, 26S proteasome non-ATPase regulatory subunit 4, fragments thereof that bind to their respective targets, and combinations, chimeras, and recombinant versions of said receptors and fragments.

Such kits wherein said binding agent comprises a non-protein.

Such kits wherein said binding agent comprises an aptamer.

Such kits wherein said binding agent comprises angiocidin, anti-secretory factor, and/or 26S proteasome non-ATPase regulatory subunit 4.

Other particular kit aspects of the invention can be summarized as follows:

A kit for the determination of the presence of, and/or the amount of, and/or the concentration of, one or more thrombospondin fragments in a material taken or gathered from an organism, said kit comprising a binding agent that will react with thrombospondin but not with a fragment of interest. Particular embodiments are:

Such kits wherein said binding agent comprises a protein;

Such kits wherein said protein comprises an antibody;

Such kits wherein said antibody is a monoclonal antibody or a polyclonal antibody;

Such kits wherein said protein comprises a fragment of an antibody;

Such kits wherein said protein comprises a single-chain antibody;

Such kits wherein said single chain antibody is derived from a phage display library;

Such kits wherein the protein is a non-antibody, the non-antibody being a protein that is neither an antibody nor a single-chain antibody;

Such kits wherein said non-antibody is selected from the group consisting of an integrin, an RGD receptor, an RFYV-VMWK (SEQ ID NO:55) receptor, an RFYVVM (SEQ ID NO:56) receptor, an FYVVMWK (SEQ ID NO:57) receptor, an IRVVM (SEQ ID NO:58) receptor, fragments thereof that bind to their respective targets, and combinations, chimeras, and recombinant versions of said receptors, integrins, and fragments; and

Such kits wherein said binding agent comprises an aptamer, meaning a DNA or RNA or related compound, that binds thrombospondin or a thrombospondin fragment.

Such kits wherein said binding agent comprises angiocidin, anti-secretory factor, and/or 26S proteasome non-ATPase regulatory subunit 4.

Several motifs within thrombospondin for binding to many of the receptors referred to above are shown in FIG. 2.2a of Adams, J. C., et al., *The thrombospondin Gene Family*, Springer Verlag, N.Y., 1995, p. 16. A CSVTCG receptor, a CSVTCG binding molecule, an angiocidin, an anti-secretory factor, a CD36, and/or fragments and derivatives thereof will be useful for assaying a thrombospondin fragment in a cancer patient.

Focus on Neoplastic Disease

The invention as it pertains to the detection or monitoring of neoplastic disease can also be summarized as the following:

A method to detect the presence of neoplastic disease in an individual, wherein the method comprises the steps of:

(1) measuring the individual's plasma level of a thrombospondin fragment;

(2) utilizing the result of step (1) in a diagnosis as to whether the individual has a neoplastic disease; said fragment being at least 6 contiguous amino acyl residues in length but less than 140 kDa.

A method of distinguishing between a cancerous and non-cancerous plasma sample based upon the presence of high molecular weight fragments of thrombospondin that are detectable with a TSP antibody such as TSP Ab-2, Ab-4, Ab-5, Ab-7 or Ab-8. Other binding agents, including but not limited to an aptamer, are also contemplated. Automated, high through-put assays are also contemplated.

Related is such a method, where the individual referred to therein is a first individual and wherein the method further comprises the steps of:

(3) measuring a second individual's plasma level of the thrombospondin fragment, said second individual considered to not have neoplastic disease;

(4) utilizing the result of step (3) is the diagnosis of whether the first individual has a neoplastic disease. For example, such a method wherein the greater the extent to which the first individual's plasma thrombospondin fragment level exceeds the plasma thrombospondin level of the second individual, the more likely that the diagnosis will be that the first individual has a neoplastic disease and/or a neoplastic disease more advanced than that of the second person. It is also understood that values from the first individual taken over time can be compared with one another, to assess the likelihood of the appearance of disease and/or progression and/or regression of disease. Particular embodiments are:

Such methods wherein the fragment is selected from the group consisting of an ~85 to 140 kDa fragment, and ~50 kDa fragment, and an ~30 kDa fragment, wherein the size in kDa is that determined by gel electrophoresis after disulfide bond reduction;

Such methods wherein the neoplastic disease is selected from the group consisting of an adenoma, adenocarcinoma, carcinoma, lymphoma, leukemia, and sarcoma;

Such methods wherein the neoplastic disease is an internal cancer;

Such methods wherein the neoplastic disease is selected from the group consisting of a cancer of the respiratory system, a cancer of the circulatory system, a cancer of the musculoskeletal system, a cancer of a muscle, a cancer of a bone, a cancer of a joint, a cancer of a tendon or ligament, a cancer of the digestive system, a cancer of the liver or biliary system, a cancer of the pancreas, a cancer of the head, a cancer of the neck, a cancer of the endocrine system, a cancer of the reproductive system, a cancer of the male reproductive system, a cancer of the female reproductive system, a cancer of the genitourinary system, a cancer of a kidney, a cancer of the urinary tract, a skin cancer, a cancer of other sensory organs (such as eye, ear, nose, tongue), a cancer of the nervous system, a cancer of a lymphoid organ, a blood cancer, a cancer of a gland, a cancer of a mammary gland, a cancer of a prostate gland, a cancer of endometrial tissue, a cancer of mesodermal tissue, a cancer of ectodermal tissue, and a teratoma;

Such methods wherein the neoplastic disease is selected from the group consisting of a cancer of solid tissue, a cancer of the blood or the lymphatic system, a non-metastatic cancer, a premetastatic cancer, a metastatic cancer, a poorly differentiated cancer, a well-differentiated cancer, and a moderately differentiated cancer.

Such methods wherein the measurement of a plasma thrombospondin fragment level comprises the use of a binding agent, said binding agent being capable of binding said thrombospondin fragment (Such binding agents are discussed above in the context of the kits of the invention); and

In particular embodiments, the thrombospondin fragment is separated from thrombospondin before said fragment is bound to the binding agent.

Such methods wherein said method comprises the use of a binding agent, comprising a binding agent capable of binding thrombospondin but not the thrombospondin fragment. Possible binding agents are discussed above in the context of kits of the invention.

In particular embodiments, the thrombospondin fragment is separated from thrombospondin before said fragment is bound to the binding agent.

Related inventions are:

A method of producing antibodies against a thrombospondin fragment, said method comprising administering said fragment to an organism capable of producing antibodies;

Said method of producing antibodies wherein said fragment is at least 6 amino acyl residues in length but less than 150 kDa or less than 185 kDa. A polyclonal antibody preparation produced by said method;

A monoclonal antibody produced by said method;

A cell line producing said monoclonal antibody; and

A method of producing a binding agent against a thrombospondin fragment, said method comprising the use of phage display.

Said method of producing a binding agent, wherein said method comprises the selection of a thrombospondin-binding or thrombospondin fragment-binding phage from a phage display.

Said method of producing a binding agent, wherein said fragment at least 6 amino acyl residues in length.

A method of distinguishing between properly and improperly collected plasma samples to be used for detecting thrombospondin fragments.

A method of generating other binding agents, including but not limited to an aptamer, against a thrombospondin fragment are also contemplated. Automated, high through-put assay methods are also contemplated.

Cancer Detection Method Comprising Measuring Platelet Activation

An additional general aspect of the invention is an assay for the presence of cancer in an organism, said method comprising measuring the extent of platelet activation. An additional general aspect of the invention is an assay for the presence of cancer in an organism, said method comprising measuring the extent of tumor stromal activity.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Schematic drawing of thrombospondin.

FIG. 2. Results of staining a gel with Coomassie Blue. Lanes, left to right are in the sequence: a lane with the molecular weight standards (Stds), followed by samples A to G.

FIG. 3. Results of Western Blot analysis using TSP Ab-4 antibody and fluorescence detection. Lanes, left to right are in the sequence: a lane with the molecular weight standards (Stds), followed by samples A to G, which correspond to aliquots of the same samples as in FIG. 2.

FIG. 4. Results of Western blot analysis using TSP Ab-4 antibody and colorimetric detection. Lanes, left to right are in the sequence: a lane with molecular weight standards, Lane 1 with purified human platelet TSP, Lanes 2-6 with plasma samples A through E (from subjects with cancer), Lanes 7-9 with dilutions of purified human platelet TSP. Samples were processed using urea denaturation before electrophoresis, followed by electrophoresis through a 12% acrylamide gel and enzymatic colorimetric detection after blotting.

FIG. 5. Results of Western Blot analysis using TSP Ab-2 antibody and biotinylation/avidin detection (Ab-2 had been biotinylated, and detection was accomplished with avidin-tagged horseradish peroxidase). Lanes, left to right are in the sequence: Lane 1, normal human serum at one-quarter strength (a separate sample from sample F); Lane 2, molecular weight standards; Lane 3, 50 ng of a recombinant human TSP fragment of ~140 kDa that was engineered to lack the N-terminal heparin-binding domain; Lanes 4-8, plasma samples from human patients with early cancer (samples A-E); Lanes 9-10, normal dog plasma drawn into CTAD tubes; Lane 11, normal human plasma drawn into a CTAD tube (a separate sample from sample G); Lane 12, empty.

FIG. 6. Results of Western Blot analysis using TSP Ab-4 antibody and biotinylation/avidin detection (Ab-4 had been biotinylated, and detection was accomplished with avidin-tagged horseradish peroxidase). Lanes, left to right are in the sequence: Lane 1, normal human serum at one-quarter strength; Lane 2, molecular weight standards; Lane 3, 50 ng human platelet TSP; Lanes 4-8, plasma samples from human

patients with early cancer; Lanes 9-10, normal dog plasma drawn into CTAD tubes; Lane 11, normal human plasma drawn into a CTAD tube; Lane 12, 50 ng of a recombinant human TSP fragment of ~140 kDa that was engineered to lack the N-terminal heparin-binding domain.

FIG. 7. Results of Western Blot analysis using TSP Ab-5 antibody and biotinylation/avidin detection (Ab-5 had been biotinylated, and detection was accomplished with avidin-tagged horseradish peroxidase). Lanes, left to right are in a sequence which corresponds to FIG. 6.

FIG. 8. Results of Western Blot analysis using TSP Ab-7 antibody and biotinylation/avidin detection (Ab-7 had been biotinylated, and detection was accomplished with avidin-tagged horseradish peroxidase). Lanes, left to right are in a sequence which corresponds to FIG. 6.

FIG. 9. Results of Western Blot analysis using TSP Ab-8 antibody and biotinylation/avidin detection (the polyclonal Ab-8 had been biotinylated, and detection was accomplished with avidin-tagged horseradish peroxidase). Lanes, left to right are in a sequence which corresponds to FIG. 6.

FIG. 10. Results of Western Blot analysis using TSP Ab-9 antibody and biotinylation/avidin detection (Ab-9 had been biotinylated, and detection was accomplished with avidin-tagged horseradish peroxidase). Lanes, left to right are in a sequence which corresponds to FIG. 6.

FIG. 11. Results of a Control Western Blot analysis using no primary antibody. Lanes, left to right are in a sequence which corresponds to FIG. 6.

FIG. 12. Represents a graphical display of ELISA analysis of TSP and a TSP fragment.

DETAILED DESCRIPTION OF THE INVENTION

The terms “thrombospondin” and “thrombospondin-1” are used interchangeably herein. It is understood that a single “band” on an electrophoresis gel may in fact reflect the presence of a collection of fragments that together form a population that, during gel electrophoresis under reducing conditions, electrophorese at similar rates.

The terms “test” and “assay” are also used interchangeably. A “purified” fragment is for example (1) one that is found in human plasma and that has been purified (for example has been isolated from gels on which the plasma has been electrophoresed). A purified fragment is not one that is in human plasma, or other part of a human, and that has not undergone at least some degree of purification.

A “synthesized fragment” is, for example, one that has been synthesized in a laboratory (e.g., by recombinant DNA technology or by chemical synthesis) so as to have the primary structure of such a fragment or a portion thereof.

The amino acid sequence of human thrombospondin-1 from GenBank is:   
 ACCESSION NM\_003246 (protein\_id=NP\_003237.1)   
 VERSION NM\_003246.1 GI:4507484

ACCESSION NM\_003246 (protein\_id=NP\_003237.1)   
 VERSION NM\_003246.1 GI:4507484

MGLAWGLGLVFLMHVCGTNRIPESGGDNSVFDIPELTGAARKGSGRRLVKGPDPSSPAFRIEDANLIPVPPDDKFDLVDVAVRAEK (SEQ ID NO: 38)   
 GPLLASLRLQMKKTRGTLALLERKDHSGQVFSVVSNGKAGTLDLSLTVQKQHVSVVEALLATGQWKSITLQVQEDRAQLYIDCE   
 KMENAEADVPIQSVFTRDLASIAARLRIAKGGVNDNFQGLQNVRFVFGTTPEDILRNKGCSSSTSVLLTLDNNVNGSSPAIRTNY   
 IGHKTKDLQAI CGISCDLSSMVLELRGLRTIVTTLQDSIRKVTEENKELANELRRPPLCYHNGVQYRNNEEWTVDSCTECHCQNS   
 VTICKKVCSPIMPCSNATVPDGECCPCWPSDSADDGWSPWSEWTS CSTSCNGIQQRGRSCDSLNNRCEGSSVQTRTCHIQCEDCK

- continued

RFKQDGGW<sup>31</sup>SHSWPSSCSVTCGDGVI<sup>32</sup>TRIRLCNSPSPQMNGKPCGEARETKACKKDACP INGGWGPWSPWDI CSVT CGGGVQKRS  
 RL<sup>33</sup>CNNPAPQFGGKDCVGDV<sup>34</sup>TENQICNKQDCPI<sup>35</sup>DGCLSNPCFAGVKCTSYPDG<sup>36</sup>SWKCGACPPGYSGNGIQCTDV<sup>37</sup>DECKEVPDACFNH  
 NGEHRCENTDPGYNCLPCPPRFTGSQPFQGGVEHATANKQVCKPRNPCTDGT<sup>38</sup>HDCNKNAKCN<sup>39</sup>YLGHYS<sup>40</sup>DPMYRCECKPGYAGNGII  
 CGEDTDLDGWP<sup>41</sup>ENENLVCVANATYHCKKDNCPNLPNSGQEDYDKDGIGDACDDDDNDKIP<sup>42</sup>DDRDNC<sup>43</sup>PFHYNPAQYDY<sup>44</sup>DRDDVGDRC  
 DNC<sup>45</sup>PYNHNPDQADTDNNGEGDACAADIDGDGILNERDNCQYVYNVDQ<sup>46</sup>RDTMDMGVGDQC<sup>47</sup>DNCPLEHNPDQLSDSDRIGDTC<sup>48</sup>DNNQ  
 DIDEDGHQNNLDNCPYVPNANQADHDKD<sup>49</sup>GKGDACDHDDNDGIPDDKDN<sup>50</sup>CRLVNPDPQKDSGDGGRGDACKDDFDHDSVPDID  
 PENVDI<sup>51</sup>SETDFRRFQMIPLDPKGT<sup>52</sup>SQNDPNWVVRHQKELVQTVNCDPGLAVGYDEFNAVDFSGTFFINTERDDDYAGFVFGYQSS  
 SRFYVVMWKQV<sup>53</sup>TQSYWDTNPTRAQGYSGLSVKVVN<sup>54</sup>STTGPEHLRNALWHTGNTPGQV<sup>55</sup>RTLWHDPRHIGWKDFTAYRWRLSHRPKT  
 GFIRVVMYEGKKIMADSGPIYDKTYAGGRLGLFVFSQEMVFFSDLKYE<sup>56</sup>CRDP

The underlined N in the first line of the sequence above refers to amino acid number 1 of the mature protein (i.e., without the 18- to 22-residue signal peptide sequence, here assumed to be 18 residues; see p. 13 and FIG. 1 in Adams J C et al. *The Thrombospondin Gene Family*, 1995).

Here is a partially annotated version of the human TSP-1 sequence from GenBank, broken into domains, and including indications of some of the functional regions that have been identified in the literature.

MGLAWGLGVLF<sup>39</sup>LMHVC<sup>40</sup>GT (SEQ ID NO: 39) [The signal peptide is considered to be 18-22 residues long (18 residues assumed here, following *The Thrombospondin Gene Family* by J C Adams et al. 1995)]

NRIPESGGDNSVDFIFELTGAA  
RKGSGRRLVKGPDPSSPAFRIEDANLIPPVPDDKFQ  
DLVD AVRAEKGFLLASL  
 RQMKKTRGTLALERKDHSGQVFSVVSNGKAGT  
LDLSLTVQGGKQHVVS VEEALLATGQWKSITL<sup>35</sup>FVQE-  
DRAQLY

IDCEKMENAE<sup>40</sup>LDVPIQSVFTRDLAS<sup>41</sup>IARLRIAKGGV  
 NDNFQGV<sup>42</sup>LQNVRFVFGTTPEDILRNKGC-  
 SSSTS<sup>43</sup>VLLTDNNV<sup>44</sup>VNGSSPAIR<sup>45</sup>TNY (SEQ ID NO: 40)  
 [N-terminal domain (1-240). The underlined N at the begin-  
 ning of this domain refers to amino acid number 1 of the  
 mature protein (i.e., without the 18- to 22-residue signal  
 peptide sequence, here assumed to be 18 residues; see p. 13  
 and FIG. 1 in Adams J C et al. *The Thrombospondin Gene*  
*Family*, 1995). Two apparent heparin-binding regions are  
 double-underlined. Finally, the last underlined region in this  
 domain corresponds to "synthetic peptide N12/I encompass-  
 ing amino acid residues 151-164 of the N-terminal domain of  
 TSP-1", which was reported to bind fibrinogen.]

IGHKTKDLQAICGISCDELSSM (SEQ ID NO: 41) [Do-  
 main of inter-chain disulfide bonds (241-262)]

VLELRGLRTIVTTLQDSIRK<sup>41</sup>VTEEN-  
 KELANELRRPPLCYHNGVQYRN<sup>42</sup>NEE-  
 WTVDSCTECHCQ NSVTICKK

VSCPIMPCSNATVPDGECCPRCWPSDSA [(SEQ ID NO: 42)  
 [Procollagen homology domain (263-360). Notice that  
 the collagen V-binding region (valine[333] to lysine[412]),  
 which is double underlined here, is partly in this domain and  
 partly in the first type 1 repeat, which immediately follows  
 this domain.]

DDGWS<sup>43</sup>PWSEWTS<sup>44</sup>CS<sup>45</sup>TSC<sup>46</sup>NGI<sup>47</sup>Q<sup>48</sup>QRGRSCDSLNNR  
 CEGSSVQ<sup>49</sup>TRTCHI<sup>50</sup>QEC<sup>51</sup>DKR<sup>52</sup>FKQ DGGWS<sup>53</sup>HWS<sup>54</sup>P-  
WSSCSVTCGDGVI<sup>55</sup>TRIRLCNSPSPQM-  
 NGKPCGEARETKACKKDACP INGGWGPWSPWD-  
 ICSVT CGGGVQKRSRLCNNPAPQFGGKDCVGDVTE  
 NQICNKQDCPI (SEQ ID NO: 43) [Domain of type 1  
 repeats (361-530). This domain consists of three type 1

repeats. The double-underlined segment at the beginning of this domain is the continuation of the collagen V-binding region (valine[333] to lysine[412].)]

20 DGCLSNPCFAGVKCTSYPDG<sup>21</sup>SWKCGAC-  
 PGYSGNGIQCTDV DECKEVPDACFNHGEHRCENT-  
 DPGYNCLPCPPRFTGSQPFQGGVEHATANKQVCKPR  
 NPCTDGT<sup>22</sup>HDCNKNAKCN<sup>23</sup>YLGHYS<sup>24</sup>DP-  
 MYRCECKPGYAGNGIICGE (SEQ ID NO: 44) [Domain of  
 type 2 repeats (531-673). This domain consists of three type  
 2 repeats.]

25 DTDLDGWPENENLVCVANATYHCKK (SEQ ID NO:  
 45) [Region between the type 2 and the type 3 repeat (674-  
 697)]

30 DNC<sup>31</sup>PNLPNSGQEDYDKDGIGDACDDDD-  
 NDKIPDDR (SEQ ID NO: 46) DNC<sup>32</sup>PFHYNPAQYDY-  
 DRDDVGDRC (SEQ ID NO: 47) DNC<sup>33</sup>PYNHN-  
 PDQADTDNNGEGDACAADIDGDGILNER (SEQ ID NO:  
 48) DNC<sup>34</sup>QYVYNVDQ<sup>35</sup>RDTMDMGVGDQC (SEQ ID NO:  
 49) DNC<sup>36</sup>PLEHNPDQLSDSDRIGDTC<sup>37</sup>DNNQ-  
 35 DIDEDGHQNNL (SEQ ID NO: 50) DNC<sup>38</sup>PYVPNAN-  
 QADHDKD<sup>39</sup>GKGDACDHDDNDGIPDDK (SEQ ID NO:  
 51) DNC<sup>40</sup>RLVNPDPQKDSGDGGRGDACKD-  
 DFDHDSVPDID (SEQ ID NO: 52) [Domain of type 3  
 repeats (698-925). This domain consists of seven type 3  
 repeats.]

40 DICPENVDISETDFRRFQMIPLDP-  
 KGT<sup>41</sup>SQNDPNWVVRHQKELVQTVNCD-  
 PGLAVGYDEFNA VDFSGTFFINTERDDDYAGFV<sup>42</sup>F-  
 GYQSSSRFYVVMWKQV<sup>43</sup>TQSYWDTNPTRAQGYSG  
 45 LSVKVVN STTGPEHLRNALWHTGNTPGQV<sup>44</sup>RTL-  
 WHDPRHIGWKDFTAYRWRLSHRPKTG-  
 FIRVVMYEGK KIMADSGPIYDKTYAGGRLGLFV<sup>45</sup>F-  
 SQEMVFFSDLKYE<sup>46</sup>CRDP (SEQ ID NO: 53) [C-terminal  
 domain (926-1152)]

50 It is understood that genetic variants of thrombospondin  
 exist, including but are not limited to human polymorphisms  
 (e.g., see dbSNP:229364, dbSNP:2228261, dbSNP:  
 2292305, dbSNP:2228262, and dbSNP:2228263 for variants  
 in the coding region; and dbSNP: 1051442, dbSNP:3743125,  
 55 dbSNP:3743124, dbSNP: 1051514, dbSNP: 1131745, and  
 dbSNP: 11282 for 3' UTR variants). The current invention  
 contemplates assays that detect polymorphic variants as well  
 as common types involving the coding region, either through  
 the use of an antibody or antibodies or other binding molecule  
 60 or molecules that recognize variant and common peptide  
 sequences, and/or through the use of sequences that are not  
 polymorphic. It is understood that A-505 [alanine(505)] in the  
 GenBank sequence NM\_003246 is instead given as a T  
 [threonine(505)] in FIG. 2.2a of Chapter 2, "The primary  
 structure of the thrombospondins" in *The Thrombospondin*  
*Gene Family* by J C Adams, R P Tucker, & J Lawler, Springer-  
 Verlag: N.Y., 1995, p. 16.

It is believed that the collagen type V binding domain corresponds to the region extending from valine(333) and lysine(412) of thrombospondin-1 (Takagi T et al. J Biol Chem 268:15544-15549, 1993; here, the residue numbers refer to the mature protein). Thus, the collagen type V-binding region would include a portion of the procollagen homology region of thrombospondin and all or nearly all of the first type 1 repeat of thrombospondin (see Chapter 2, "The primary structure of the thrombospondins" in *The Thrombospondin Gene Family* by J C Adams, R P Tucker, & J Lawler, Springer-Verlag: N.Y., 1995, pp. 11-42, but especially p. 24). See FIG. 1 of this application, as well as the annotated TSP sequence, above. As indicated on the FIG. 1 of this application, the leftmost rectangle represents the N-terminal domain (mature residues 1 to ~240), which contains heparin-binding sequence; the short vertical lines represent Cys(252) and Cys(256) of human thrombospondin-1, which are involved in inter-chain disulfide bonds, to form trimers; the first oval represents the procollagen homology domain (residues 263-360); the three slanted ovals represent the three type 1 repeats (residues 361-530), which resemble properdin and a malarial protein; the three tall ovals represent the three type 2 repeats (residues 531-673), which show similarities to the epidermal growth factor (EGF) repeat; there is a short sequence (residues 674-697) separating type 2 and type 3 repeats; the seven ovals represent the seven type 3 repeats (residues 698-925), which are rich in aspartic acid and resemble the calcium-binding pocket of parvalbumin or calmodulin; and right-hand square represents the C-terminal cell-binding domain (residues 926 to the end, that is, Proline-1152; see FIG. 2.2a in Adams J C et al. *The Thrombospondin Gene Family*, 1995, p. 16). The two chymotryptic fragments (70 kDa and 50 kDa), and to some extent the 120 kDa tryptic fragment, indicated schematically on FIG. 1, correspond to the protease-resistant central core domain of thrombospondin.

Examples of cancers that can be detected using assays for the thrombospondin fragments include but are not limited to: adenoma, adenocarcinoma, carcinoma, lymphoma, leukemia, sarcoma, solid cancer, liquid cancer, metastatic cancer, pre-metastatic cancer, non-metastatic cancer, a cancer with vascular invasion, internal cancer, skin cancer, cancer of the respiratory system, cancer of the circulatory system, cancer of the musculoskeletal system, cancer of a muscle, cancer of a bone, cancer of a joint, cancer of a tendon or ligament, cancer of the digestive system, cancer of the liver or biliary system, cancer of the pancreas, cancer of the head, cancer of the neck, cancer of the endocrine system, cancer of the reproductive system, cancer of the male reproductive system, cancer of the female reproductive system, cancer of the genitourinary system, cancer of a kidney, cancer of the urinary tract, cancer of a sensory system, cancer of the nervous system, cancer of a lymphoid organ, a blood cancer, cancer of a gland (for example but not limited to cancer of a mammary or a prostate gland), cancer of an endometrial tissue, cancer of a mesodermal tissue, cancer of an ectodermal tissue, cancer of an endodermal tissue, a teratoma, a poorly-differentiated cancer, a well-differentiated cancer, and a moderately differentiated cancer.

One of the options for tests for the presence of thrombospondin fragments is to fractionate the material (e.g., plasma) into fractions (e.g., positions on an electrophoresis gel, or chromatographic elution samples) collected by a technique capable of separating the fragments from thrombospondin (e.g., by electrophoresis, size-dependent chromatography, and/or affinity chromatography) and to detect the fragments in the fractions where such fragments would be expected to appear. Another of the various additional known options for

assays is to test the ability of plasma to inhibit the binding of thrombospondin fragments or portions thereof to compounds (e.g., antibodies) that specifically bind to them.

The thrombospondin fragments of interest in the diagnostic tests include, but are not limited to, ones that have apparent molecular weights of ~115, ~85 kDa (or ~90 kDa), ~50 kDa, and ~30 kDa as determined by SDS-PAGE electrophoresis after reduction (see FIGS. 3 and 4). There are also higher molecular weight fragments of interest that are between ~115 kDa and ~180 kDa. Thrombospondin itself can also be of interest. These higher molecular weight fragments are recognized in cancerous plasma samples by TSP Ab-7 (FIG. 8). They are also recognized in conjunction with the lower molecular weight fragments by TSP Ab-4, Ab-5, Ab-8, and Ab-2. Of note, detection of these fragments may also be used in distinguishing a cancerous versus a non-cancerous plasma sample; and a properly versus improperly collected plasma sample. For example, the higher molecular weight fragments of thrombospondin are extremely faint in the normal human plasma sample drawn into the CTAD tubes when probed with TSP Ab-7, as opposed to the improperly drawn samples. The higher molecular weight fragments of thrombospondin are extremely faint in the normal human plasma sample, as opposed to the cancerous plasma samples. Preferred conditions for determining the molecular weights are those referred to below as "Standard Gel Electrophoresis Protocol." The assignment of a number such as 50 kDa to the size of a fragment reflects its approximate molecular weight as determined using the Standard Gel Electrophoresis Protocol.

Based upon prior reports of epitope assignments for certain monoclonal antibodies, it could be concluded that the ~85 kDa, ~50 kDa, and ~30 kDa fragments identified herein all contain an immunogenic portion of "collagen type V-binding domain" of thrombospondin; however, Applicant's data suggests that some epitope assignments may contain inaccuracies. In a preferred aspect of the invention, the fragments are detected by antibody that binds to such a domain, as is believed to be the case for the TSP Ab-4 monoclonal antibody referred to below. Because the collagen V-binding domain is relatively small (~19 kDa; see Takagi et al. JBC 1993), it is concluded from the apparent molecular weights of these fragments, which are substantially greater than 19 kDa, that additional portions of the thrombospondin molecule must also be present in these fragments (multimers of the 19 kDa region are not a plausible explanation for the higher molecular weights, because the 19 kDa region does not comprise the region of inter-chain disulfide bonds, plus the fact that the gels in FIGS. 3 and 4 were run under reducing conditions). It is believed that additional portions come from the protease-resistant central core domain of thrombospondin, which can be selected from the group of thrombospondin domains consisting of the region of inter-chain disulfide bonds, the procollagen-like domain, a type 1 repeat, and to some extent a type 2 repeat and a type 3 repeat (see Prater C A et al. The properdin-like type 1 repeats of human thrombospondin contain a cell attachment site. J Cell Biol. 1991 March; 112(5): 1031-40; Schultz-Cherry S et al. The type 1 repeats of thrombospondin 1 activate latent transforming growth factor-beta. J Biol Chem. 1994 Oct. 28; 269(43):26783-8; FIG. 6.2 in Adams J C et al. *The Thrombospondin Gene Family*, 1995, p. 107; and chymotryptic and tryptic fragments of thrombospondin indicated schematically in FIG. 1 of this application). See also the sequence ranges given earlier in this Application. Note that several aforementioned peptides, such as, CNSPSPQMNGKPCEGEAR (SEQ ID NO:8) (residues 444-461), RKVTEENKELANELRPP (SEQ ID NO:9) (residues 281-297); PQMNGKPCEGEAR (SEQ ID NO:11) (residues

449-461); CEGEAR (SEQ ID NO:12) (residues 456-461; and RKVTEENKE (SEQ ID NO:13) (residues 281-289) are within the protease-resistant central core domain. An antibody against a region outside of a collagen V-binding domain, but present in a thrombospondin fragment present in a cancer patient, is also preferred.

In competition assays, a sample of material (e.g., plasma) that contains thrombospondin fragment(s) and/or thrombospondin is tested for its ability to interfere with the binding of one (or more) of the fragments to a fragment-specific binding agent, preferably an antibody, such as a monoclonal antibody. Under optimal conditions, the ability of the sample to interfere with the binding of the fragment increases monotonically in relation to the amount of similarly binding fragments in the sample. Thrombospondin will also interfere with the binding, but the present inventor has discovered that thrombospondin is present in plasma in significantly smaller amounts than the fragments. In addition, competition assays are easily standardized through the use of known quantities of fragments, synthetic or otherwise, and/or through the use of molecules, such as peptides, that contain an epitope recognized by the binding agent. In one scenario, assay detection is accomplished through the use of labeled fragments and/or peptides, and addition of a sample that contains a thrombospondin fragment or addition of known quantities of an unlabeled thrombospondin fragment (as a standard) results in competition with the binding of the labeled fragments and/or peptide to the binding agent. Loss of signal upon addition of known quantities of unlabeled or differently labeled thrombospondin fragments is used to standardize the assay.

In addition to an assay of thrombospondin fragments, other examples of platelet activation assays are well-known in the art.

#### Detection of Thrombospondin Fragments by Western Blot Analysis

The following protocol (Sections I, II, and III) is referred to herein as the "Standard Gel Electrophoresis Protocol" and is preferred for determining whether the size of a fragment is ~85 kDa, ~50 kDa, ~30 kDa or another size, including the higher molecular weight fragments. Nevertheless, suitable alternatives for fractionating and detecting molecules and molecular fragments are well-known in the art (see numerous methods articles and texts, as well as protocols from commercial sources) and are readily applied to the current situation with appropriate modifications.

##### I. Sample Preparation

Protease inhibitors added:

1  $\mu$ l of leupeptin solution (1 mg/ml in sterile water) is added per ml plasma

10  $\mu$ l of PMSF solution (1.74 mg/ml in isopropanol) is added per ml plasma

4 $\times$  sample buffer:

dH<sub>2</sub>O 4.0 ml/0.5M tris-HCl 1.0 ml/glycerol 0.8 ml/10% SDS 1.6 ml/2-mercaptoethanol 0.4 ml/0.05% bromophenol blue 0.2 ml

5  $\mu$ l plasma samples are diluted with 20  $\mu$ l distilled water, and 25  $\mu$ l 2 $\times$  sample buffer is added, followed by heating (to aid disulfide bond reduction).

10  $\mu$ l of each sample mixture is then run on the gel.

In an example of an alternative to the Standard Gel Electrophoresis Procedure, to aid reduction and denaturation, blood plasma is mixed with 5% fresh mercaptoethanol and 4-6 M fresh urea and boiled for at least 5 minutes in a fume hood.

##### II. Electrophoresis

Gel electrophoresis is done on SDS-polyacrylamide gels (4% stacking, 10% running gel) in tris/glycine/SDS buffer

(see running buffer below, pH 8.3) at 200 V/7-8 cm at 25 $^{\circ}$  C. for 34 minutes. Alternative electrophoretic set-ups and procedures are well-known in the art and can be used (e.g., using gels of about 8%-12% acrylamide; omission of the stacking gel), but should reliably separate 185 kDa, 85 kDa, 50 kDa, and 30 kDa (these are the approximate apparent weights on a reducing gel of thrombospondin and of the three major thrombospondin fragments in plasma). Molecular weight standards were: 184 kDa, 121 kDa, 86 kDa, 67 kDa, 52 kDa, 40 kDa, 28 kDa, and 22 kDa (FIG. 3). A slightly different set of molecular weight markers was used for FIGS. 5-11, as well as human platelet thrombospondin and a recombinant thrombospondin fragment. Other molecular weight markers are suitable as well, but should include markers near to 185 kDa (the approximate weight of thrombospondin on reducing gels) and near to 85, 50, and 30 kDa (the approximate weights on reducing gel of the major thrombospondin fragments present in plasma). Suitable molecular weight standards are purchasable from a variety of commercial sources, such as Invitrogen Life Technologies.

5 $\times$  running buffer pH 8.3: Tris Base 15 g/Glycine 72 g SDS 5 g/distilled water to 1 liter

The ~85 kDa thrombospondin fragment electrophoreses close to the 86 kD standard.

The ~50 kDa thrombospondin fragment electrophoreses close to the 52 kD standard.

The ~30 kDa thrombospondin fragment electrophoreses close to the 28 kDa standard.

##### III. Detection of the Fragments on the Gels

The fragments may be detected by the Western Blot procedure using antibodies that react with the ~115 kDa, ~55 kDa, and ~30 kDa fragments, as well as the high molecular weight fragments. As illustrative but not restrictive examples, TSP Ab-2, Ab-4, or Ab-5 monoclonal antibodies from Lab Vision Corporation (Fremont, Calif. can be used for this purpose (as primary antibody), as can polyclonal anti-TSP antibodies (such as Ab-8, a rabbit polyclonal antibody from Lab Vision). For more specific detection of higher molecular weight forms, TSP Ab-7 can be used. Following standard protocols, proteins from the polyacrylamide gel are transferred to a suitable membrane, unoccupied protein-binding sites of the membrane are then blocked (e.g., by incubation with skim milk), and the membrane is exposed to primary antibody. The presence of TSP antibodies that have bound to thrombospondin or thrombospondin fragments on the membrane can be detected by reacting those antibodies with fluorophore-labeled antibodies against mouse IgG (secondary antibody, i.e., that themselves react with the TSP Ab-4 antibodies), followed by subsequent fluorescence-based scanning of the membrane. Detection of polyclonal anti-TSP antibodies is performed similarly, using appropriate secondary antibodies. Other systems for detection of primary antibody are well-known in the art, including but not limited to other systems for labeling a secondary antibody, such as conjugation to an enzyme, such as horseradish peroxidase. Biotin-avidin systems are also well-known in the art, as are radioactive labeling methods.

Determination of Albumin Concentration in Plasma Samples for Purposes of Normalizing the Western Blot Results.

Gels are run under the same conditions as for the Western Blot, but then stained with Coomassie Blue. The major band (which is near the 67 kDa standard) is albumin, which is quantified by densitometric scanning.

Illustrative, but not Restrictive, Examples of Quantitative Assays for TSf (i.e., a Thrombospondin Fragment or Fragments):

Enzyme-linked immunosorbent assays (ELISA) and related approaches are well-known in the art (for an example of an ELISA of thrombospondin, but not directed towards thrombospondin fragments, see Tuszynski, G. P., Switalska, H. I., and Knudsen, K.: *Modern Methods in Pharmacology in* 5 *Methods of Studying Platelet-Secreted Proteins and the Platelet Cytoskeleton*," Vol. 4, Alan R. Liss, Inc., N.Y., p.267-286, 1987; for an example of an ELISA using antibodies that the Applicant has shown to react with a TSP fragment, see FIG. 12). Two types of ELISAs are competitive ELISAs, 10 which require only one anti-TSf antibody, and sandwich ELISAs, which can require two anti-TSf antibodies. Essentially identical assays are also contemplated, in which a binding agent other than an antibody is used.

For a competitive ELISA or ELISA-like assay, two sets of 15 wells can be used, one a set of reaction wells and the other a set of pre-mix wells. In the reaction wells, antigen is bound to a surface, such as a plate or a bead (for simplicity, the rest of this description refers to such a surface as a plate or a well, but it is understood that other surfaces can also be used). Here, the antigen would be based on a thrombospondin fragment 20 present in a cancer patient. Said antigen could take a form selected from the group consisting of thrombospondin (TSP) itself, a TSP fragment found in a cancer patient, a TSP fragment that contains a TSP fragment found in a cancer patient, a TSP fragment that is contained within a TSP fragment found in a cancer patient, a peptide that contains an epitope from a TSP fragment in a cancer patient (where said peptide can be synthetic), and a derivatized peptide and/or fragment. The essential requirement for the fragment, protein or peptide coated on the walls is that it can compete with the TSP 25 fragment of interest (for example a fragment in a patient's plasma) for binding to a binding agent, such as an antibody, used in the ELISA. As an illustration, TSP itself can be used, as stated above. TSP can be prepared by activating platelets in vitro (which then release TSP-1), followed by purification of this TSP from the platelet-conditioned medium; if standard 96-well microtiter plates are used, 75 ng of TSP-1 in 200  $\mu$ L of phosphate-buffered saline can be added per well. Corresponding amounts (molar or mass) of TSP fragments and/or 30 peptides can be used instead, and are preferable, based on ease of preparation and standardization. After binding the antigen to the immobilized surface, additional binding sites on the surface are blocked by standard protocols (for example, incubation with bovine serum albumin then Tween, both in phosphate-buffered saline).

The pre-mix wells are prepared with no antigen, but then blocked (e.g., with BSA then Tween). These pre-mix wells can be used as convenient reaction vessels for the initial binding of anti-TSf antibody with either known amounts of antigen in 35 solution (for a standard curve) or unknown amounts of antigens in a sample to be tested (see the next two paragraphs).

In order to generate a standard curve, to the pre-mix wells are added different concentrations of a standard antigen in 40 solution. The standard antigen might (as described elsewhere herein) be selected so as to quantify the amount of thrombospondin fragments of the invention, the amount of a subset of thrombospondin fragment or fragments, the amount of thrombospondin, or their combined total. The antigen may be synthetic, isolated from a cancer patient, isolated from an individual without cancer, or isolated from any other appropriate source, including but not limited to recombinant material. As indicated above, the immobilized antigen in the reaction wells and the antigen in solution in the pre-mix wells do not have to be the same, but they should both react with—and 45 thereby eventually compete for—the binding agent (such as a primary antibody) used in the assay. As an illustrative

example, if TSP-1 itself is the standard antigen in solution in the pre-mix wells, 0, 2, 5, 10, 20, 40, 60, and 80 ng can be added per well, in PBS-Tween, in volume of 110  $\mu$ L per microtiter well. Corresponding amounts (molar or mass) of 5 TSP fragments or peptides can be used instead, and are preferable, based on their ease of preparation and standardization. These wells will be used to generate a standard curve.

Unknowns (i.e., samples in which it is desired to quantify the concentration of a TSP fragment) are also added, to separate pre-mix wells. For plasma samples, it is typical to dilute them beforehand, say, with PBS-Tween. This can be important, to bring the amount of TSf down into the range of the standard curve, and also to dilute potentially interfering substances in plasma (one such interfering substance may be fibrinogen, which can bind TSP and some TSP fragments). Total volume should be the same as for the soluble antigen standards. Diluted binding agent, such as an antibody (e.g., in 10 110  $\mu$ L), that reacts against a TSP fragment found in a cancer patient is then added. Note that the antigen immobilized in the reaction wells and the antigen in solution in the pre-mix wells must be chosen to also react against this binding agent. An incubation is performed, to allow antigen-antibody binding (or target-binding agent binding) to occur in the pre-mix wells.

An aliquot (e.g., 200  $\mu$ L) of liquid from each pre-mix well (standards and unknowns) is then transferred to an antigen-coated reaction well, followed by an incubation (as a blank, some wells can receive buffer only, such as PBS-Tween). After this incubation, liquid is removed from the antigen-coated reaction wells, and the wells are washed. If a primary antibody is used as the binding agent, enzyme-conjugated secondary antibody (specific against the primary antibody) is then added to the wells, followed by an incubation to allow it to bind to whatever primary antibody has bound to the immobilized TSf on the plate. This step is followed by detection (for 15 example, if the secondary antibody is conjugated to alkaline phosphatase, detection can be accomplished with Sigma phosphatase substrate followed by absorbance readings at 405 nm). A standard curve is plotted, and quantities of a TSf in the unknown samples are calculated based on the standard curve. Note that higher amounts of TSf in the sample will result in less primary antibody bound to the immobilized antigen on the well, and hence less signal from the secondary antibody. Similar detection methods are used if the binding agent is a non-antibody.

Sandwich ELISAs and ELISA-like assays are also contemplated. In this case, a first anti-TSf antibody (or a first non-antibody binding agent that binds TSf) is immobilized on a plate, a bead, or another surface, and it is used to capture the TSf in a standard or unknown sample. The first antibody (for capture) is often polyclonal, but this is not a requirement. Detection of captured material is then accomplished with a second anti-TSf antibody. The second antibody (for detection) is often monoclonal, but this is not a requirement (in 20 FIG. 12, the ELISA used monoclonal Ab-4 for capture and polyclonal Ab-8 for detection). As is well-known in the art, the first and second antibodies should not substantially interfere with each other's access to the captured material. Assay read-out can be accomplished with an enzyme-linked antibody specific to the second anti-TSf antibody. Again, if the first (capturing) binding agent and/or the second (detecting) binding agent is a non-antibody, similar methods are used.

Many variants well-known in the art are contemplated for these competitive and sandwich ELISAs and ELISA-like assays. For example, non-enzymatic methods, such as radioactive, fluorescent, biotin-avidin-based methods, and other methods for assay read-out (to quantify the second anti-TSf

antibody) are contemplated. Also, automated assays, such as ones performed on a clinical autoanalyzer, are contemplated. Also, various anti-TSf antibodies are contemplated, including but not limited to polyclonal antibodies, monoclonal antibodies, anti-peptide antibodies, antibodies against a TSP fragment present in a cancer patient, antibodies against a TSP fragment generated in vitro, and antibodies against a TSP fragment generated in vitro by proteolysis. Single-chain antibodies are also contemplated, as are non-antibodies.

For the sandwich ELISA, one option is the use of color-coded microbeads (microspheres) with immobilized anti-TSf antibody to capture, then a fluorescent second anti-TSf antibody to detect. The detection apparatus reads each bead, one at a time, assaying for bead color as well as the signal from the second anti-TSf antibody. The advantage here is that several different analytes can be assayed at once, such as one group of beads for full-length TSP (or an epitope outside of what circulates in substantial concentration in a cancer patient) and another group of beads, of a different color, for a TSP fragment. Or, one group of beads to assay an epitope present in the larger molecular weight forms that is not present in the ~85-, ~50 kDa or ~30 kDa fragments, and another group of beads to assay an epitope present in the ~85 kDa fragment but not the ~50 kDa or ~30 kDa fragments (this is followed by a numerical calculation to yield the amounts of higher molecular weight forms, and of the ~85 kDa fragment, and of the ~50 kDa and 30 kDa fragments separately). An example of this use of color-coded beads can be found at the web site for Linco Research, Inc.

Another option for analyzing multiple analytes is SearchLight™ Proteome Arrays, which are multiplexed sandwich ELISAs, currently adapted for the quantitative measurement of two to 16 proteins per well. It is understood herein that the method can also be used for protein fragments, such as one or more thrombospondin fragments. Using a spotting technique, 2 to 16 target-specific antibodies are bound to each well of a microplate, although it is understood that this number can be expanded with improved spotting techniques and/or larger wells. Following a standard sandwich ELISA procedure, luminescent signals are imaged with a cooled CCD (charged coupled device) camera. The image is then analyzed using Array Vision™ software. The amount of signal generated at each spot is related to the amount of target protein in the original standard or sample. Values for specific proteins and/or protein fragments can be calculated based on the position of the spots and comparison of density values for unknowns to density values for known standards (and TSP fragments or peptides can be used as standards). The SearchLight™ technology is available through Pierce Boston Technology Center, including customized arrays using proprietary reagents from outside Pierce or assay targets not currently available at Pierce. Other technologies for multiplex assaying are also contemplated.

Other assay methods are also contemplated. They include but are not limited to immunoblotting, dot-blotting and immunoturbidimetric assays (for a detailed example of this last approach with another plasma protein, see Levine, D. M. and Williams, K. J.: Automated measurement of mouse apolipoprotein B: convenient screening tool for mouse models of atherosclerosis. *Clin. Chem.* 43:669-674, 1997), as well as blotting and/or turbidimetric assays that use binding agents in general. Other competitive assays are also contemplated, such as ones in which material in a standard and an unknown competes with one or more labeled peptides, one or more labeled TSP fragments, and/or labeled TSP for binding to an agent that binds TSf, such as an anti-TSf antibody (the label is then used for detection and hence quantification). One

example of this approach is to immobilize an anti-TSf antibody, and then add sample mixed with labeled peptide, labeled TSP fragments, or labeled TSP, so that sample and labeled material compete for binding to the immobilized antibody (note that this approach requires only one anti-TSf antibody). Binding of labeled material is then quantified. It is understood that any of these assays, including immune-based and non-immune-based assays, can be automated. It is also understood that potentially interfering substances in unknown samples can be diluted, removed, inhibited, avoided (for example, in the case of fibrinogen, by using epitopes away from a fibrinogen-binding region of TSP), and/or compensated for.

Use of Thrombospondin Fragments as Immunogens to Generate Fragment-Specific Antibodies:

A purified preparation of fragments (e.g., by elution of fragments from the gel, by immunoprecipitation or antibody column or other immune-based purification methods, by recombinant DNA techniques, by chemical synthesis, or by a combination of synthesis and/or purification methods) is injected into a rabbit or rabbits with any of the standard adjuvants used with peptide immunogens and antibodies are collected using protocols well known in the art. For small peptides, linkage to a carrier, such as keyhole limpet hemocyanin or bovine serum albumin, is well-known in the art. Injection into other animals is also well-known, including but not limited to a goat, sheep, chicken, turkey, donkey, dog, cat, rat, and mouse. Monoclonal antibodies can be prepared using antibody-producing cells obtained from any immunized animal, but the technology is most easily available for the mouse (for example, antibody-producing cells from an immunized animal are fused with an immortal cell, then clones of fused cells are screened for their production of antibody against one or more thrombospondin fragments of interest).

It is understood that the methods disclosed herein are readily applied to other members of the thrombospondin gene family, including but not limited to TSP-2 (for a description of the thrombospondin gene family, see *The Thrombospondin Gene Family* by J C Adams, R P Tucker, & J Lawler, Springer-Verlag: N.Y., 1995; de Fraipont F et al. *Trends Mol. Med.*, 7:401-407, 2001; and elsewhere). It is also understood that the methods disclosed herein are readily applied to other animal species of economic and/or emotional importance, including but not limited to pets, animals used in breeding, racehorses, and racing dogs.

## EXAMPLES

### Western Blot Analysis of Plasma Samples from Cancer Patients

Electrophoresis was done according to the Standard Gel Electrophoresis Protocol described above.

Table I shows plasma and serum samples obtained for analysis.

TABLE I

Sample	Plasma/ Serum	Cancer	Stage/Grade	Age/ Sex	Comment
A	plasma	colon T2	I/G2	57/F	Ascending
B	plasma	colon T3	II/G2	71/M	Ascending
C	plasma	prostate	II/Gleason 6	71/M	DRE-abnormal
D	plasma	prostate	II/Gleason 5	63/M	DRE-abnormal
E	plasma	lung T2	IB/G2	67/M	Squamous

TABLE 1-continued

Sam- ple	Plasma/ Serum	Cancer	Stage/Grade	Age/ Sex	Comment
F	serum				TSP is released from platelets during clotting, and proteases are activated during clotting.
G	plasma	no cancer	N/A	F	lichen planus, a non-cancerous inflammatory disorder

The results are shown in FIGS. 2 and 3, and the quantitative data are summarized in Table 2.

TABLE 2

Quantitation of thrombospondin fragments, normalized for sample loading							
Approx MW (kDa)	A Colon-1	B Colon-2	C Prostate-1	D Prostate-2	E Lung	F Serum	G No cancer
85	0.572 108.8%	0.847 161.1%	1.175 223.6%	1.292 245.7%	1.142 217.4%	1.434 272.9%	0.526 100.0%
50	0.534 89.7%	0.666 111.8%	1.037 174.0%	1.416 237.7%	1.809 303.6%	2.722 456.9%	0.596 100.0%
30	1.210 85.0%	1.401 98.4%	1.687 118.5%	1.593 111.9%	1.988 139.6%	7.351 516.3%	1.424 100.0%
Total Ab4 signal	2.316 91.0%	2.914 114.5%	3.898 153.2%	4.301 169.0%	4.939 194.1%	11.507 452.1%	2.545 100.0%
Albumin signal above bkg	24020	26723	25187	27073	23888	4359	26110

Numbers refer to the strengths of TSP signal from the Western blot (FIG. 3), normalized to the albumin signal from Coomassie staining (FIG. 2 and final row of numbers in this Table). Percentages indicate the ratio to the no-cancer sample (sample G).

The results summarized in Table 2 represent data generated by densitometric scanning of the photographic film generated by fluorescent staining of the TSP Ab-4 Western Blot (See FIG. 3). Thus, for very dark signals, such as the band or group of bands around 30 kDa, the fact that the signals on film saturate when very strong means that increases compared to the no-cancer control sample are seriously under-estimated. This is not particularly evident in the serum sample, which served as the positive control for increased signal, owing to platelet activation (much less serum was loaded, as is evident from the albumin signal; so even though it generated a strong normalized signal, it did not saturate the film nearly as much).

To obtain the data for Table 2, the signal (above background) for the Western Blot was determined and that signal was normalized to the albumin signal (above background) for the gel shown in FIG. 2. Table 2 shows the normalized signal (e.g., 0.572) with the percentage (e.g., 108.8%) underneath the normalized signal being the percentage of the "no-cancer" signal.

The molecular weight standards used were 184 kDa, 121 kDa, 86 kDa, 67 kDa, 52 kDa, 40 kDa, 28 kDa and 22 kDa. Based on the given molecular weights and the relative positions of the standard bands versus the TSP Ab-4 bands and groups of bands, it was concluded that the TSP Ab-4 signals were in three general bands or groups of bands, at approximately 85, 50, and 30 kDa (see FIG. 3). Notice, for example, the relative strength of signals at around 185 kDa (thrombospondin monomer) versus around 85, 50, and 30 kDa (fragments). It is clear that there is overwhelmingly more signal from the fragments than from thrombospondin itself. Thus, detection of specific fragments, as disclosed in the current inventions, is preferred over detection of the TSP molecule itself, or general detection of epitopes that occur throughout

the whole TSP molecule, or detection of epitopes outside of those contained within the specific fragments demonstrated herein.

The plasma samples from cancer patients (lanes A-E) came from Golden West Biologicals, Inc. of Temecula, Calif. The serum sample (lane F) was from a non-cancerous individual. The no-cancer control plasma (lane G) came from an individual with lichen planus, a non-cancerous but inflammatory skin condition.

The serum sample (Lane F) was prepared by deliberately clotting the blood. Protease inhibitors were not added to sample F until after clotting had been completed and the serum had been harvested. Ideally for the current invention, however, blood is not allowed to clot during sample collection

(activation of platelets during clotting causes release of thrombospondin, which was used here on purpose to increase the signal from sample F), and protease inhibitors are added promptly during sample collection (not done for sample F because the clotting cascade involves activation of proteases).

The predominance of thrombospondin fragments, as opposed to thrombospondin itself, in sample F is consistent with a) platelet activation and release of thrombospondin, plus b) activation of proteases of the clotting cascade, which evidently cleaved the newly released thrombospondin. A similar result is seen in the first lanes of FIGS. 5, 6, and 7.

Plasma samples from Golden West Biologicals were samples from individuals with relatively early disease. The first colon cancer sample (lane A) was from an individual with stage I, grade G2 disease. All other cancer samples (lanes B-E) came from individuals with stage II disease (except for lane E, which was stage IB). Plasma from patients with such relatively early stage cancers would be expected to have a lower concentration of thrombospondin fragments than plasma from patients with more advanced cancers. Nevertheless, the robustness of the technique is demonstrated by the fact that (1) increased levels were found with the earlier stage cancers, and (2) gel scanning was done under conditions in which portions of the detecting film were saturated or nearly saturated.

All cancer samples show an increased signal from the 85 kDa band (or group of similarly electrophoresing bands). All but the stage I sample show increased signal from the 50 kDa band (or group of bands), as well as increased total Ab-4 signal. All but the two early colon cancer samples show increased signal from the 30 kDa band (or group of bands). Thus, the detection and quantitation of specific thrombospondin fragments works to distinguish even relatively early cancer patients from a no-cancer control who has a non-cancer-

ous disease. These thrombospondin fragments are well-suited for diagnostic assays because (a) they have specific molecular weights (or molecular weight ranges); and (b) they contain specific epitopes. The present results provide validation for other fragment-based approaches, including (but not limited to) non-competitive ELISA and ELISA-like assays, and competition assays. Of note are higher molecular weight fragments, above the ~85 kDa band but below 185 kDa. These fragments appear fainter on the Western blot than do the three fragments emphasized above. These higher molecular weight fragments are most evident in samples B through E, and not in sample G (see those lanes in FIG. 3, particularly the region near the level of the 121 kDa standard).

FIG. 4 shows the results of an independent gel analysis of the samples. The samples were denatured then run on a 12% gel, transblotted, and then stained with the same TSP Ab-4 that we used before. Unlike the blot shown in FIG. 3, the denaturation step here included urea, and detection used an enzymatic colorimetric method that is based on horseradish peroxidase conjugates and the BioRad Opti-4CN substrate kit, not fluorescence as before. Along the left edge of lane 1, there are from top to bottom, the following handwritten numbers evident: 1, 97, 66, 45, 30, 20, and 14, respectively. With the exception of 1, the numbers correspond to the positions where standard proteins of corresponding molecular weights (in kDa) had electrophoresed.

In FIG. 4, Lanes 2 through 6 correspond to patients A through E, respectively, in Table 1. Lanes 1 and 7 through 9 show the electrophoresis patterns of purified thrombospondin. The results confirm that:

a) there is very little, if any, detectable TSP in the plasma samples (the first plasma lane shows some TSP at its appropriate monomeric molecular weight, but this is certainly spillover from the vastly overloaded first sample lane);

b) there are indeed TSP fragments in the plasma samples; and

c) the fragments have molecular weights of about 28, 50, and a faint band around 90 kDa. In this blot, the TSP bands are very sharp, implying well-defined molecular weight fragments (presumably a purely technical improvement, owing to better denaturation in the presence of urea). As in FIG. 3, there are a number of less prominent fragment bands at other molecular weights. It is understood that a thrombospondin fragment in any of these bands can also be assayed and used in diagnosis and screening and in kits.

#### Additional Western Blot Analysis of Plasma from Cancer Patients

##### I. Sample Preparation

1X sample buffer was made by using the following formula:	
Invitrogen Tris-Glycin 2X sample buffer	5 ml
BME	0.625 ml
Urea	2.4-3.6
dH <sub>2</sub> O	to 10 mLs

##### II. Electrophoresis

Electrophoresis was performed similarly to the Standard Gel Electrophoresis described above. The first lane in each blot is normal human serum, used at 1/4 the amount as the plasma samples.

The second lane contains the molecular weight standards (250, 148 which is yellowish, 98, 64, 50, 36, 22 which is purple, and 16 kDa).

The third lane is human platelet TSP (except in the Ab-2 blot, in which a recombinant TSP fragment that is shorter than TSP was used).

Lanes 4-8 contain plasma from human cancer patients (these are the same plasma samples A through E as were used in FIGS. 2-4).

Lanes 9 & 10 contain plasma that had been collected into CTAD tubes from two dogs.

Lane 11 contains a normal human plasma sample that had been drawn into CTAD tubes.

Lane 12 contains the recombinant TSP fragment (except in the Ab-2 blot, in which lane 12 is empty). As noted elsewhere, this recombinant TSP fragment was engineered to lack the N-terminal heparin-binding domain. It has a molecular weight of approximately 140 kDa, and was produced in insect cells.

The TSP and the recombinant TSP fragment had been diluted to 50 ng/uL in PBS. Human serum was diluted so the volume added to sample buffer was 1/4 the amount of plasma added. 5 uL was then added to 15 uL PBS, and 20 uL of prestained marker was added to 20 uL of sample buffer. Then, 5 uL of each plasma sample, 5 uL of diluted human serum, and 5 uL of TSP or the recombinant TSP fragment (diluted to 50 ng/uL) was added to 45 uL 1x sample buffer and boiled for at least 5 minutes. Plasma samples were previously treated with protease inhibitors, aliquoted into cryovials, and stored at -40° C. 10 uL of each sample was then loaded onto a 12% gel. 10 uL of See Blue Plus 2 prestained markers were prepared and loaded in 1e to lane 2 of each gel.

The gel was run at 130V for 80 minutes and then transferred onto a nitrocellulose membrane using 30V for 90 minutes. After transfer, the membrane was blocked with 50 mLs 1x PBS/Casein overnight at 4° C., and then washed with 200 mLs of PBST for 10 minutes. Biotinylated monoclonal antibodies (Ab 7-Bt, Ab 2-Bt, Ab 4-Bt, Ab 9-Bt) were diluted 1:500 (the dilution of the polyclonal Ab 8-Bt was also 1:500) in 1x PBS/Casein (20 mLs), added to the blot, and incubated for 2 hours at room temperature. The blot was then washed 3 times with 200 mLs of PBST, 10 minutes each time. Detection was performed with SA-HRP diluted 1:20,000 in 1x PBS/Casein (30 mLs), and incubated for 1 hour at room temperature. The blot was then washed again 3 times with 200 mLs of PBST, 10 minutes each time, and then developed with TMB for membranes for 4 minutes. The TMB reaction was stopped by incubating in dH<sub>2</sub>O for 2 minutes.

##### III. Detection of Fragments

In human cancer plasma samples probed with Ab-4, there are three major fragments, with apparent molecular weights of ~30 kDa, ~55 kDa, and ~85 kDa, as well as other bands between the ~30 and ~55 kDa bands, at least one band between the ~55 and ~115 kDa bands, and bands above ~115 kDa (FIGS. 3, 4 and 6).

As shown in FIG. 8, Ab-7 shows a strong detection of only the largest TSP fragment(s) or form(s) in serum and in the human cancer plasma samples. It also detects human platelet TSP (FIG. 8, lane 3, top band) and the recombinant TSP fragment (FIG. 8, lane 12). It does not substantially recognize anything in normal dog plasma drawn into CTAD tubes (FIG. 8, lanes 9 & 10). Most interestingly, it does not substantially recognize anything in the normal human plasma sample drawn into CTAD tubes (FIG. 8, lane 11). Thus, Ab-7 can be used to distinguish cancerous from normal plasma, and/or properly collected plasma from less properly collected plasma. Ab-7 confirms, and magnifies, the effect seen with Ab-2, -4, -5, and -8, (FIGS. 5, 6, 7, and 9, respectively) namely, that the largest TSP fragment(s) or form(s) are fainter in the normal human plasma sample than in the cancerous

human plasma samples (a similar result was seen in FIG. 3; in that Western, the apparent molecular weights were somewhat different, particularly for the highest of the three major fragments and the fainter signals above it). Therefore, Ab-7 is a highly specific antibody that distinguishes fragments from each other, fragments from TSP, and plasma samples from each other. The top band above the ~115 kDa band in the cancer plasma samples (FIG. 8, lanes 4, 5, 7, and 8; this top band is absent or faint in lane 6) runs with or slightly below TSP (TSP is the top dark band in FIG. 8, lane 3) but above the large recombinant TSP fragment (FIG. 8, lane 12).

It is important to note that Ab-2, Ab-4, Ab-5, and Ab-8 each detect higher and lower molecular weight bands as well (FIGS. 5, 6, 7, and 9, respectively). Thus, these antibodies can be used for detection and/or quantitation of these TSP fragments. Ab-5 does not seem to strongly recognize TSP or the large recombinant TSP fragment. Ab-9 is thought to bind the N-terminal heparin-binding domain of TSP. The Ab-9 blot shows faint signals at ~30 kDa and ~55 kDa (FIG. 10). It is understood that Ab-2, Ab-4, Ab-5, and Ab-8 each recognize TSP fragments in non-human plasma as well (lanes 9 & 10, FIGS. 5, 6, 7 and 9, respectively), which will be useful in screening, diagnosis, and/or monitoring of non-human animals.

In other words, Ab-7 appears to recognize higher molecular weight forms (some, if not all, of which run differently from human platelet TSP) that are very faint or absent from the normal human plasma that had been drawn into CTAD tubes and promptly centrifuged and separated. These higher molecular weight forms are also apparent on the Ab-2, 4, -5, and -8 blots. Identification and quantification of these higher molecular weight forms will be useful in distinguishing cancerous from non-cancerous; and/or properly collected from improperly collected samples.

In addition, the data from Examples 1 and 2 cast suspicion on the conventional wisdom about the locations of the epitopes within the TSP molecule that are recognized by these antibodies. For example, one would not expect that a 30 kDa fragment would be recognized by an antibody that binds to the collagen type V-binding domain (which is where Ab-4 is believed to bind; this collagen-binding domain is believed to be in the procollagen homology domain and the first type 1 repeat), and by an antibody that binds to the Ca-binding domains (which is where Ab-2 is believed to bind; the Ca-binding domains are believed to be in the type 3 repeats). These regions should be more than 30 kDa apart in TSP. Furthermore, the type 2 repeats, which is where Ab-7 is believed to bind, lie between these two regions, yet Ab-7 does not recognize the 30 kDa fragment (FIG. 8). Explanations include, but are not limited to, incorrect determinations of binding epitopes and the existence of multiple fragments of similar molecular weights.

Furthermore, these data are also consistent with the conclusion that TSP fragments that arise from tumors and/or tumor stroma and/or activated platelets in cancer are different

from TSP fragments that arise in the absence of cancer. Such a difference could include (but not be limited to) a difference in glycosylation, tertiary structure, intra-chain bonds, inter-chain bonds, post-translational processing, and/or binding to a binding agent, such as Ab-7.

#### ELISA Analysis of Plasma Using TSP Ab-4 and Ab-8

The ELISA was performed similarly to the method previously described. The ELISA was performed by coating Ab-4 onto an ELISA plate, then blocking the plate. The indicated concentrations of TSP and the recombinant TSP fragment were added and detection was accomplished with Ab-8. Assay read-out was performed using an anti-rabbit antibody that was conjugated to HRP and shows minimal reaction with mouse IgG. FIG. 12 represents a graphical display of the data from Table 2, showing ELISA read-outs of human platelet TSP versus a recombinant TSP fragment engineered to lack the N-terminal heparin-binding domain. This experiment shows (1) a working ELISA; (2) a good dynamic range; (3) an ability to recognize a TSP fragment; and (4) essentially identical read-outs from TSP and from the recombinant TSP fragment. It is understood that the recombinant TSP fragment is stable, including during storage at 4° C., which makes it an attractive standard for use in ELISAs and other assays for TSP and/or TSP fragments and forms (such as those seen on the Western blots with Ab-2, -4, -5, -7, or -8). Because it is recombinant, it can be produced with uniformity from batch to batch, and there should be no risk of contamination with blood-borne pathogens; these features are also attractive. Other TSP fragments, including but not limited to recombinant fragments, are also contemplated for use as standards and immunogens. Other antibodies are also contemplated. As an example, not meant as restrictive, this recombinant TSP fragment has been reported to bind TSP Ab-1. i.e., clone A4.1).

TABLE 2

ng/mL	Lot 1	Lot 2
200.0	2.563	2.499
100.0	2.204	2.093
50.00	1.805	1.574
25.00	1.168	1.081
12.50	0.662	0.713
6.250	0.400	0.514
3.125	0.279	0.345
blank	0.166	0.166

#### SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 64

<210> SEQ ID NO 1

<211> LENGTH: 6

<212> TYPE: PRT

<213> ORGANISM: Artificial

<220> FEATURE:

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<223> OTHER INFORMATION: Thrombospondin Region

<400> SEQUENCE: 1

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<210> SEQ ID NO 2

<211> LENGTH: 15

<212> TYPE: PRT

<213> ORGANISM: Artificial

<220> FEATURE:

<223> OTHER INFORMATION: thrombospondin Region which includes an N-terminal CYS added to aid conjugation

<400> SEQUENCE: 2

Cys Leu Gln Asp Ser Ile Arg Lys Val Thr Glu Glu Asn Lys Glu  
1                   5                   10                   15

<210> SEQ ID NO 3

<211> LENGTH: 14

<212> TYPE: PRT

<213> ORGANISM: Artificial

<220> FEATURE:

<223> OTHER INFORMATION: Thrombospondin Region

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Leu Gln Asp Ser Ile Arg Lys Val Thr Glu Glu Asn Lys Glu  
1                   5                   10

<210> SEQ ID NO 4

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<212> TYPE: PRT

<213> ORGANISM: Artificial

<220> FEATURE:

<223> OTHER INFORMATION: Thmbospondin Region

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Glu Gly Glu Ala Arg Glu  
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<210> SEQ ID NO 5

<211> LENGTH: 14

<212> TYPE: PRT

<213> ORGANISM: Artificial

<220> FEATURE:

<223> OTHER INFORMATION: Thrombospondin Region

<400> SEQUENCE: 5

Pro Gln Met Asn Gly Lys Pro Cys Glu Gly Glu Ala Arg Glu  
1                   5                   10

<210> SEQ ID NO 6

<211> LENGTH: 6

<212> TYPE: PRT

<213> ORGANISM: Artificial

<220> FEATURE:

<223> OTHER INFORMATION: Thrombospondin Region

<400> SEQUENCE: 6

Glu Asp Thr Asp Leu Asp  
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<211> LENGTH: 15

<212> TYPE: PRT

<213> ORGANISM: Artificial

<220> FEATURE:

<223> OTHER INFORMATION: Thrombospondin

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&lt;400&gt; SEQUENCE: 7

Tyr Ala Gly Asn Gly Ile Ile Cys Gly Glu Asp Thr Asp Leu Asp  
 1                    5                    10                    15

&lt;210&gt; SEQ ID NO 8

&lt;211&gt; LENGTH: 18

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Thrombospondin Region

&lt;400&gt; SEQUENCE: 8

Cys Asn Ser Pro Ser Pro Gln Met Asn Gly Lys Pro Cys Glu Gly Glu  
 1                    5                    10                    15

Ala Arg

&lt;210&gt; SEQ ID NO 9

&lt;211&gt; LENGTH: 17

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Thrombospondin Region

&lt;400&gt; SEQUENCE: 9

Arg Lys Val Thr Glu Glu Asn Lys Glu Leu Ala Asn Glu Leu Arg Arg  
 1                    5                    10                    15

Pro

&lt;210&gt; SEQ ID NO 10

&lt;211&gt; LENGTH: 18

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Thrombospondin Region which includes an N-terminal CYS added to aid conjugation

&lt;400&gt; SEQUENCE: 10

Cys Arg Lys Val Thr Glu Glu Asn Lys Glu Leu Ala Asn Glu Leu Arg  
 1                    5                    10                    15

Arg Pro

&lt;210&gt; SEQ ID NO 11

&lt;211&gt; LENGTH: 13

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Thrombospondin Region

&lt;400&gt; SEQUENCE: 11

Pro Gln Met Asn Gly Lys Pro Cys Glu Gly Glu Ala Arg  
 1                    5                    10

&lt;210&gt; SEQ ID NO 12

&lt;211&gt; LENGTH: 6

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Thrombospondin Region

&lt;400&gt; SEQUENCE: 12

Cys Glu Gly Glu Ala Arg  
 1                    5

&lt;210&gt; SEQ ID NO 13

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<211> LENGTH: 9  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Thrombospondin Region

<400> SEQUENCE: 13

Arg Lys Val Thr Glu Glu Asn Lys Glu  
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<210> SEQ ID NO 14  
 <211> LENGTH: 15  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Thrombospondin Region

<400> SEQUENCE: 14

Asp Asp Asp Asp Asn Asp Lys Ile Pro Asp Asp Arg Asp Asn Cys  
 1 5 10 15

<210> SEQ ID NO 15  
 <211> LENGTH: 15  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Thrombospondin Region additional NH2 Group

<400> SEQUENCE: 15

Asp Asp Asp Asp Asn Asp Lys Ile Pro Asp Asp Arg Asp Asn Cys  
 1 5 10 15

<210> SEQ ID NO 16  
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 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Thrombospondin Region

<400> SEQUENCE: 16

Asp Asp Asp Asp Asn Asp Lys  
 1 5

<210> SEQ ID NO 17  
 <211> LENGTH: 14  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Thrombospondin Region

<400> SEQUENCE: 17

Asn Leu Pro Asn Ser Gly Gln Glu Asp Tyr Asp Lys Asp Gly  
 1 5 10

<210> SEQ ID NO 18  
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 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Thrombospondin Region plus N-terminal CYS to aid conjugation

<400> SEQUENCE: 18

Cys Asn Leu Pro Asn Ser Gly Gln Glu Asp Tyr Asp Lys Asp Gly  
 1 5 10 15

<210> SEQ ID NO 19  
 <211> LENGTH: 6

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<212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: thrombospondin Region

<400> SEQUENCE: 19

Glu Asp Tyr Asp Lys Asp  
 1 5

<210> SEQ ID NO 20  
 <211> LENGTH: 19  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Thrombospondin Region

<400> SEQUENCE: 20

Cys Pro Tyr Asn His Asn Pro Asp Gln Ala Asp Thr Asp Asn Asn Gly  
 1 5 10 15

Glu Gly Asp

<210> SEQ ID NO 21  
 <211> LENGTH: 15  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Thrombospondin Region

<400> SEQUENCE: 21

Cys Arg Leu Val Pro Asn Pro Asp Gln Lys Asp Ser Asp Gly Asp  
 1 5 10 15

<210> SEQ ID NO 22  
 <211> LENGTH: 8  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Thrombospondin region

<400> SEQUENCE: 22

Asp Gln Lys Asp Ser Asp Gly Asp  
 1 5

<210> SEQ ID NO 23  
 <211> LENGTH: 20  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Thrombospondin Region

<400> SEQUENCE: 23

Cys Pro Tyr Val Pro Asn Ala Asn Gln Ala Asp His Asp Lys Asp Gly  
 1 5 10 15

Lys Gly Asp Ala  
 20

<210> SEQ ID NO 24  
 <211> LENGTH: 6  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: thrombospondin Region

<400> SEQUENCE: 24

Thr Glu Arg Asp Asp Asp  
 1 5

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<210> SEQ ID NO 25  
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 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Thrombospondin Region

<400> SEQUENCE: 25

Asp Phe Ser Gly Thr Phe Phe Ile Asn Thr Glu Arg Asp Asp Asp  
 1 5 10 15

<210> SEQ ID NO 26  
 <211> LENGTH: 6  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Thrombospondin Region

<400> SEQUENCE: 26

Glu Arg Lys Asp His Ser  
 1 5

<210> SEQ ID NO 27  
 <211> LENGTH: 14  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Thrombospondin Region

<400> SEQUENCE: 27

Thr Arg Gly Thr Leu Leu Ala Leu Glu Arg Lys Asp His Ser  
 1 5 10

<210> SEQ ID NO 28  
 <211> LENGTH: 15  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Thrombospondin Region plus N-terminal CYS

<400> SEQUENCE: 28

Cys Thr Arg Gly Thr Leu Leu Ala Leu Glu Arg Lys Asp His Ser  
 1 5 10 15

<210> SEQ ID NO 29  
 <211> LENGTH: 6  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Thrombospondin Region

<400> SEQUENCE: 29

Asp Asp Lys Phe Gln Asp  
 1 5

<210> SEQ ID NO 30  
 <211> LENGTH: 14  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Thrombospondin Region

<400> SEQUENCE: 30

Ala Asn Leu Ile Pro Pro Val Pro Asp Asp Lys Phe Gln Asp  
 1 5 10

<210> SEQ ID NO 31

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<211> LENGTH: 15  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Thrombospondin Region plus N-terminal CYS

<400> SEQUENCE: 31

Cys Ala Asn Leu Ile Pro Pro Val Pro Asp Asp Lys Phe Gln Asp  
 1 5 10 15

<210> SEQ ID NO 32  
 <211> LENGTH: 6  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Thrombospondin region

<400> SEQUENCE: 32

Asp Cys Glu Lys Met Glu  
 1 5

<210> SEQ ID NO 33  
 <211> LENGTH: 15  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Thrombospondin Region

<400> SEQUENCE: 33

Glu Asp Arg Ala Gln Leu Tyr Ile Asp Cys Glu Lys Met Glu Asn  
 1 5 10 15

<210> SEQ ID NO 34  
 <211> LENGTH: 17  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Thrombospondin Region

<400> SEQUENCE: 34

Cys Gly Thr Asn Arg Ile Pro Glu Ser Gly Gly Asp Asn Ser Val Phe  
 1 5 10 15

Asp

<210> SEQ ID NO 35  
 <211> LENGTH: 14  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Thrombospondin region

<400> SEQUENCE: 35

Asn Arg Ile Pro Glu Ser Gly Gly Asp Asn Ser Val Phe Asp  
 1 5 10

<210> SEQ ID NO 36  
 <211> LENGTH: 19  
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 <220> FEATURE:  
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<400> SEQUENCE: 36

Gly Trp Lys Asp Phe Thr Ala Tyr Arg Trp Arg Leu Ser His Arg Pro  
 1 5 10 15

Lys Thr Gly

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<210> SEQ ID NO 37  
 <211> LENGTH: 20  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Thrombospondin region plus N-terminal CYS

<400> SEQUENCE: 37

Cys Gly Trp Lys Asp Phe Thr Ala Tyr Arg Trp Arg Leu Ser His Arg  
 1 5 10 15

Pro Lys Thr Gly  
 20

<210> SEQ ID NO 38  
 <211> LENGTH: 1170  
 <212> TYPE: PRT  
 <213> ORGANISM: Human

<400> SEQUENCE: 38

Met Gly Leu Ala Trp Gly Leu Gly Val Leu Phe Leu Met His Val Cys  
 1 5 10 15

Gly Thr Asn Arg Ile Pro Glu Ser Gly Gly Asp Asn Ser Val Phe Asp  
 20 25 30

Ile Phe Glu Leu Thr Gly Ala Ala Arg Lys Gly Ser Gly Arg Arg Leu  
 35 40 45

Val Lys Gly Pro Asp Pro Ser Ser Pro Ala Phe Arg Ile Glu Asp Ala  
 50 55 60

Asn Leu Ile Pro Pro Val Pro Asp Asp Lys Phe Gln Asp Leu Val Asp  
 65 70 75 80

Ala Val Arg Ala Glu Lys Gly Phe Leu Leu Leu Ala Ser Leu Arg Gln  
 85 90 95

Met Lys Lys Thr Arg Gly Thr Leu Leu Ala Leu Glu Arg Lys Asp His  
 100 105 110

Ser Gly Gln Val Phe Ser Val Val Ser Asn Gly Lys Ala Gly Thr Leu  
 115 120 125

Asp Leu Ser Leu Thr Val Gln Gly Lys Gln His Val Val Ser Val Glu  
 130 135 140

Glu Ala Leu Leu Ala Thr Gly Gln Trp Lys Ser Ile Thr Leu Phe Val  
 145 150 155 160

Gln Glu Asp Arg Ala Gln Leu Tyr Ile Asp Cys Glu Lys Met Glu Asn  
 165 170 175

Ala Glu Leu Asp Val Pro Ile Gln Ser Val Phe Thr Arg Asp Leu Ala  
 180 185 190

Ser Ile Ala Arg Leu Arg Ile Ala Lys Gly Gly Val Asn Asp Asn Phe  
 195 200 205

Gln Gly Val Leu Gln Asn Val Arg Phe Val Phe Gly Thr Thr Pro Glu  
 210 215 220

Asp Ile Leu Arg Asn Lys Gly Cys Ser Ser Ser Thr Ser Val Leu Leu  
 225 230 235 240

Thr Leu Asp Asn Asn Val Val Asn Gly Ser Ser Pro Ala Ile Arg Thr  
 245 250 255

Asn Tyr Ile Gly His Lys Thr Lys Asp Leu Gln Ala Ile Cys Gly Ile  
 260 265 270

Ser Cys Asp Glu Leu Ser Ser Met Val Leu Glu Leu Arg Gly Leu Arg  
 275 280 285

Thr Ile Val Thr Thr Leu Gln Asp Ser Ile Arg Lys Val Thr Glu Glu  
 290 295 300

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Asn Lys Glu Leu Ala Asn Glu Leu Arg Arg Pro Pro Leu Cys Tyr His  
 305 310 315 320

Asn Gly Val Gln Tyr Arg Asn Asn Glu Glu Trp Thr Val Asp Ser Cys  
 325 330 335

Thr Glu Cys His Cys Gln Asn Ser Val Thr Ile Cys Lys Lys Val Ser  
 340 345 350

Cys Pro Ile Met Pro Cys Ser Asn Ala Thr Val Pro Asp Gly Glu Cys  
 355 360 365

Cys Pro Arg Cys Trp Pro Ser Asp Ser Ala Asp Asp Gly Trp Ser Pro  
 370 375 380

Trp Ser Glu Trp Thr Ser Cys Ser Thr Ser Cys Gly Asn Gly Ile Gln  
 385 390 395 400

Gln Arg Gly Arg Ser Cys Asp Ser Leu Asn Asn Arg Cys Glu Gly Ser  
 405 410 415

Ser Val Gln Thr Arg Thr Cys His Ile Gln Glu Cys Asp Lys Arg Phe  
 420 425 430

Lys Gln Asp Gly Gly Trp Ser His Trp Ser Pro Trp Ser Ser Cys Ser  
 435 440 445

Val Thr Cys Gly Asp Gly Val Ile Thr Arg Ile Arg Leu Cys Asn Ser  
 450 455 460

Pro Ser Pro Gln Met Asn Gly Lys Pro Cys Glu Gly Glu Ala Arg Glu  
 465 470 475 480

Thr Lys Ala Cys Lys Lys Asp Ala Cys Pro Ile Asn Gly Gly Trp Gly  
 485 490 495

Pro Trp Ser Pro Trp Asp Ile Cys Ser Val Thr Cys Gly Gly Gly Val  
 500 505 510

Gln Lys Arg Ser Arg Leu Cys Asn Asn Pro Ala Pro Gln Phe Gly Gly  
 515 520 525

Lys Asp Cys Val Gly Asp Val Thr Glu Asn Gln Ile Cys Asn Lys Gln  
 530 535 540

Asp Cys Pro Ile Asp Gly Cys Leu Ser Asn Pro Cys Phe Ala Gly Val  
 545 550 555 560

Lys Cys Thr Ser Tyr Pro Asp Gly Ser Trp Lys Cys Gly Ala Cys Pro  
 565 570 575

Pro Gly Tyr Ser Gly Asn Gly Ile Gln Cys Thr Asp Val Asp Glu Cys  
 580 585 590

Lys Glu Val Pro Asp Ala Cys Phe Asn His Asn Gly Glu His Arg Cys  
 595 600 605

Glu Asn Thr Asp Pro Gly Tyr Asn Cys Leu Pro Cys Pro Pro Arg Phe  
 610 615 620

Thr Gly Ser Gln Pro Phe Gly Gln Gly Val Glu His Ala Thr Ala Asn  
 625 630 635 640

Lys Gln Val Cys Lys Pro Arg Asn Pro Cys Thr Asp Gly Thr His Asp  
 645 650 655

Cys Asn Lys Asn Ala Lys Cys Asn Tyr Leu Gly His Tyr Ser Asp Pro  
 660 665 670

Met Tyr Arg Cys Glu Cys Lys Pro Gly Tyr Ala Gly Asn Gly Ile Ile  
 675 680 685

Cys Gly Glu Asp Thr Asp Leu Asp Gly Trp Pro Asn Glu Asn Leu Val  
 690 695 700

Cys Val Ala Asn Ala Thr Tyr His Cys Lys Lys Asp Asn Cys Pro Asn  
 705 710 715 720

Leu Pro Asn Ser Gly Gln Glu Asp Tyr Asp Lys Asp Gly Ile Gly Asp

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725					730					735					
Ala	Cys	Asp	Asp	Asp	Asp	Asp	Asn	Asp	Lys	Ile	Pro	Asp	Asp	Arg	Asp
			740					745					750		
Asn	Cys	Pro	Phe	His	Tyr	Asn	Pro	Ala	Gln	Tyr	Asp	Tyr	Asp	Arg	Asp
		755					760					765			
Asp	Val	Gly	Asp	Arg	Cys	Asp	Asn	Cys	Pro	Tyr	Asn	His	Asn	Pro	Asp
	770					775					780				
Gln	Ala	Asp	Thr	Asp	Asn	Asn	Gly	Glu	Gly	Asp	Ala	Cys	Ala	Ala	Asp
785					790					795					800
Ile	Asp	Gly	Asp	Gly	Ile	Leu	Asn	Glu	Arg	Asp	Asn	Cys	Gln	Tyr	Val
				805					810						815
Tyr	Asn	Val	Asp	Gln	Arg	Asp	Thr	Asp	Met	Asp	Gly	Val	Gly	Asp	Gln
			820					825					830		
Cys	Asp	Asn	Cys	Pro	Leu	Glu	His	Asn	Pro	Asp	Gln	Leu	Asp	Ser	Asp
		835						840					845		
Ser	Asp	Arg	Ile	Gly	Asp	Thr	Cys	Asp	Asn	Asn	Gln	Asp	Ile	Asp	Glu
	850					855						860			
Asp	Gly	His	Gln	Asn	Asn	Leu	Asp	Asn	Cys	Pro	Tyr	Val	Pro	Asn	Ala
865					870					875					880
Asn	Gln	Ala	Asp	His	Asp	Lys	Asp	Gly	Lys	Gly	Asp	Ala	Cys	Asp	His
				885					890						895
Asp	Asp	Asp	Asn	Asp	Gly	Ile	Pro	Asp	Asp	Lys	Asp	Asn	Cys	Arg	Leu
			900					905						910	
Val	Pro	Asn	Pro	Asp	Gln	Lys	Asp	Ser	Asp	Gly	Asp	Gly	Arg	Gly	Asp
		915					920						925		
Ala	Cys	Lys	Asp	Asp	Phe	Asp	His	Asp	Ser	Val	Pro	Asp	Ile	Asp	Asp
	930					935					940				
Ile	Cys	Pro	Glu	Asn	Val	Asp	Ile	Ser	Glu	Thr	Asp	Phe	Arg	Arg	Phe
945				950						955					960
Gln	Met	Ile	Pro	Leu	Asp	Pro	Lys	Gly	Thr	Ser	Gln	Asn	Asp	Pro	Asn
				965					970						975
Trp	Val	Val	Arg	His	Gln	Gly	Lys	Glu	Leu	Val	Gln	Thr	Val	Asn	Cys
			980					985						990	
Asp	Pro	Gly	Leu	Ala	Val	Gly	Tyr	Asp	Glu	Phe	Asn	Ala	Val	Asp	Phe
		995					1000						1005		
Ser	Gly	Thr	Phe	Phe	Ile	Asn	Thr	Glu	Arg	Asp	Asp	Asp	Tyr	Ala	
	1010					1015						1020			
Gly	Phe	Val	Phe	Gly	Tyr	Gln	Ser	Ser	Ser	Arg	Phe	Tyr	Val	Val	
	1025					1030						1035			
Met	Trp	Lys	Gln	Val	Thr	Gln	Ser	Tyr	Trp	Asp	Thr	Asn	Pro	Thr	
	1040					1045						1050			
Arg	Ala	Gln	Gly	Tyr	Ser	Gly	Leu	Ser	Val	Lys	Val	Val	Asn	Ser	
	1055					1060						1065			
Thr	Thr	Gly	Pro	Gly	Glu	His	Leu	Arg	Asn	Ala	Leu	Trp	His	Thr	
	1070					1075						1080			
Gly	Asn	Thr	Pro	Gly	Gln	Val	Arg	Thr	Leu	Trp	His	Asp	Pro	Arg	
	1085					1090						1095			
His	Ile	Gly	Trp	Lys	Asp	Phe	Thr	Ala	Tyr	Arg	Trp	Arg	Leu	Ser	
	1100					1105						1110			
His	Arg	Pro	Lys	Thr	Gly	Phe	Ile	Arg	Val	Val	Met	Tyr	Glu	Gly	
	1115					1120						1125			
Lys	Lys	Ile	Met	Ala	Asp	Ser	Gly	Pro	Ile	Tyr	Asp	Lys	Thr	Tyr	
	1130					1135						1140			

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Ala Gly Gly Arg Leu Gly Leu Phe Val Phe Ser Gln Glu Met Val  
1145 1150 1155

Phe Phe Ser Asp Leu Lys Tyr Glu Cys Arg Asp Pro  
1160 1165 1170

<210> SEQ ID NO 39  
<211> LENGTH: 18  
<212> TYPE: PRT  
<213> ORGANISM: Artificial  
<220> FEATURE:  
<223> OTHER INFORMATION: Thrombospondin Region

<400> SEQUENCE: 39

Met Gly Leu Ala Trp Gly Leu Gly Val Leu Phe Leu Met His Val Cys  
1 5 10 15

Gly Thr

<210> SEQ ID NO 40  
<211> LENGTH: 240  
<212> TYPE: PRT  
<213> ORGANISM: Artificial  
<220> FEATURE:  
<223> OTHER INFORMATION: Thrombospondin Region plus N-terminal domain

<400> SEQUENCE: 40

Asn Arg Ile Pro Glu Ser Gly Gly Asp Asn Ser Val Phe Asp Ile Phe  
1 5 10 15

Glu Leu Thr Gly Ala Ala Arg Lys Gly Ser Gly Arg Arg Leu Val Lys  
20 25 30

Gly Pro Asp Pro Ser Ser Pro Ala Phe Arg Ile Glu Asp Ala Asn Leu  
35 40 45

Ile Pro Pro Val Pro Asp Asp Lys Phe Gln Asp Leu Val Asp Ala Val  
50 55 60

Arg Ala Glu Lys Gly Phe Leu Leu Leu Ala Ser Leu Arg Gln Met Lys  
65 70 75 80

Lys Thr Arg Gly Thr Leu Leu Ala Leu Glu Arg Lys Asp His Ser Gly  
85 90 95

Gln Val Phe Ser Val Val Ser Asn Gly Lys Ala Gly Thr Leu Asp Leu  
100 105 110

Ser Leu Thr Val Gln Gly Lys Gln His Val Val Ser Val Glu Glu Ala  
115 120 125

Leu Leu Ala Thr Gly Gln Trp Lys Ser Ile Thr Leu Phe Val Gln Glu  
130 135 140

Asp Arg Ala Gln Leu Tyr Ile Asp Cys Glu Lys Met Glu Asn Ala Glu  
145 150 155 160

Leu Asp Val Pro Ile Gln Ser Val Phe Thr Arg Asp Leu Ala Ser Ile  
165 170 175

Ala Arg Leu Arg Ile Ala Lys Gly Gly Val Asn Asp Asn Phe Gln Gly  
180 185 190

Val Leu Gln Asn Val Arg Phe Val Phe Gly Thr Thr Pro Glu Asp Ile  
195 200 205

Leu Arg Asn Lys Gly Cys Ser Ser Ser Thr Ser Val Leu Leu Thr Leu  
210 215 220

Asp Asn Asn Val Val Asn Gly Ser Ser Pro Ala Ile Arg Thr Asn Tyr  
225 230 235 240

<210> SEQ ID NO 41  
<211> LENGTH: 22  
<212> TYPE: PRT

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&lt;213&gt; ORGANISM: Artificial

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Thrombospondin region plus domain of inter-chain disulfide bonds

&lt;400&gt; SEQUENCE: 41

Ile Gly His Lys Thr Lys Asp Leu Gln Ala Ile Cys Gly Ile Ser Cys  
1 5 10 15Asp Glu Leu Ser Ser Met  
20

&lt;210&gt; SEQ ID NO 42

&lt;211&gt; LENGTH: 98

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Thrombospondin region plus procollagen homology domain

&lt;400&gt; SEQUENCE: 42

Val Leu Glu Leu Arg Gly Leu Arg Thr Ile Val Thr Thr Leu Gln Asp  
1 5 10 15Ser Ile Arg Lys Val Thr Glu Glu Asn Lys Glu Leu Ala Asn Glu Leu  
20 25 30Arg Arg Pro Pro Leu Cys Tyr His Asn Gly Val Gln Tyr Arg Asn Asn  
35 40 45Glu Glu Trp Thr Val Asp Ser Cys Thr Glu Cys His Cys Gln Asn Ser  
50 55 60Val Thr Ile Cys Lys Lys Val Ser Cys Pro Ile Met Pro Cys Ser Asn  
65 70 75 80Ala Thr Val Pro Asp Gly Glu Cys Cys Pro Arg Cys Trp Pro Ser Asp  
85 90 95

Ser Ala

&lt;210&gt; SEQ ID NO 43

&lt;211&gt; LENGTH: 170

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Thrombospondin region plus domain of type 1 repeats

&lt;400&gt; SEQUENCE: 43

Asp Asp Gly Trp Ser Pro Trp Ser Glu Trp Thr Ser Cys Ser Thr Ser  
1 5 10 15Cys Gly Asn Gly Ile Gln Gln Arg Gly Arg Ser Cys Asp Ser Leu Asn  
20 25 30Asn Arg Cys Glu Gly Ser Ser Val Gln Thr Arg Thr Cys His Ile Gln  
35 40 45Glu Cys Asp Lys Arg Phe Lys Gln Asp Gly Gly Trp Ser His Trp Ser  
50 55 60Pro Trp Ser Ser Cys Ser Val Thr Cys Gly Asp Gly Val Ile Thr Arg  
65 70 75 80Ile Arg Leu Cys Asn Ser Pro Ser Pro Gln Met Asn Gly Lys Pro Cys  
85 90 95Glu Gly Glu Ala Arg Glu Thr Lys Ala Cys Lys Lys Asp Ala Cys Pro  
100 105 110Ile Asn Gly Gly Trp Gly Pro Trp Ser Pro Trp Asp Ile Cys Ser Val  
115 120 125

Thr Cys Gly Gly Gly Val Gln Lys Arg Ser Arg Leu Cys Asn Asn Pro



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35

<210> SEQ ID NO 47  
 <211> LENGTH: 23  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Thrombospondin Region

<400> SEQUENCE: 47

Asp Asn Cys Pro Phe His Tyr Asn Pro Ala Gln Tyr Asp Tyr Asp Arg  
 1 5 10 15

Asp Asp Val Gly Asp Arg Cys  
 20

<210> SEQ ID NO 48  
 <211> LENGTH: 36  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Thrombospondin Region

<400> SEQUENCE: 48

Asp Asn Cys Pro Tyr Asn His Asn Pro Asp Gln Ala Asp Thr Asp Asn  
 1 5 10 15

Asn Gly Glu Gly Asp Ala Cys Ala Ala Asp Ile Asp Gly Asp Gly Ile  
 20 25 30

Leu Asn Glu Arg  
 35

<210> SEQ ID NO 49  
 <211> LENGTH: 23  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: thrombospondin region

<400> SEQUENCE: 49

Asp Asn Cys Gln Tyr Val Tyr Asn Val Asp Gln Arg Asp Thr Asp Met  
 1 5 10 15

Asp Gly Val Gly Asp Gln Cys  
 20

<210> SEQ ID NO 50  
 <211> LENGTH: 38  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Thrombospondin Region

<400> SEQUENCE: 50

Asp Asn Cys Pro Leu Glu His Asn Pro Asp Gln Leu Asp Ser Asp Ser  
 1 5 10 15

Asp Arg Ile Gly Asp Thr Cys Asp Asn Asn Gln Asp Ile Asp Glu Asp  
 20 25 30

Gly His Gln Asn Asn Leu  
 35

<210> SEQ ID NO 51  
 <211> LENGTH: 36  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Thrombospondin region

-continued

&lt;400&gt; SEQUENCE: 51

Asp Asn Cys Pro Tyr Val Pro Asn Ala Asn Gln Ala Asp His Asp Lys  
 1 5 10 15

Asp Gly Lys Gly Asp Ala Cys Asp His Asp Asp Asp Asn Asp Gly Ile  
 20 25 30

Pro Asp Asp Lys  
 35

&lt;210&gt; SEQ ID NO 52

&lt;211&gt; LENGTH: 36

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Thrombospondin region plus domain of type 3 repeats

&lt;400&gt; SEQUENCE: 52

Asp Asn Cys Arg Leu Val Pro Asn Pro Asp Gln Lys Asp Ser Asp Gly  
 1 5 10 15

Asp Gly Arg Gly Asp Ala Cys Lys Asp Asp Phe Asp His Asp Ser Val  
 20 25 30

Pro Asp Ile Asp  
 35

&lt;210&gt; SEQ ID NO 53

&lt;211&gt; LENGTH: 227

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Thrombospondin Region plus C-terminal domain

&lt;400&gt; SEQUENCE: 53

Asp Ile Cys Pro Glu Asn Val Asp Ile Ser Glu Thr Asp Phe Arg Arg  
 1 5 10 15

Phe Gln Met Ile Pro Leu Asp Pro Lys Gly Thr Ser Gln Asn Asp Pro  
 20 25 30

Asn Trp Val Val Arg His Gln Gly Lys Glu Leu Val Gln Thr Val Asn  
 35 40 45

Cys Asp Pro Gly Leu Ala Val Gly Tyr Asp Glu Phe Asn Ala Val Asp  
 50 55 60

Phe Ser Gly Thr Phe Phe Ile Asn Thr Glu Arg Asp Asp Asp Tyr Ala  
 65 70 75 80

Gly Phe Val Phe Gly Tyr Gln Ser Ser Ser Arg Phe Tyr Val Val Met  
 85 90 95

Trp Lys Gln Val Thr Gln Ser Tyr Trp Asp Thr Asn Pro Thr Arg Ala  
 100 105 110

Gln Gly Tyr Ser Gly Leu Ser Val Lys Val Val Asn Ser Thr Thr Gly  
 115 120 125

Pro Gly Glu His Leu Arg Asn Ala Leu Trp His Thr Gly Asn Thr Pro  
 130 135 140

Gly Gln Val Arg Thr Leu Trp His Asp Pro Arg His Ile Gly Trp Lys  
 145 150 155 160

Asp Phe Thr Ala Tyr Arg Trp Arg Leu Ser His Arg Pro Lys Thr Gly  
 165 170 175

Phe Ile Arg Val Val Met Tyr Glu Gly Lys Lys Ile Met Ala Asp Ser  
 180 185 190

Gly Pro Ile Tyr Asp Lys Thr Tyr Ala Gly Gly Arg Leu Gly Leu Phe  
 195 200 205

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Val Phe Ser Gln Glu Met Val Phe Phe Ser Asp Leu Lys Tyr Glu Cys  
 210 215 220

Arg Asp Pro  
 225

<210> SEQ ID NO 54  
 <211> LENGTH: 6  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Thrombospondin region

<400> SEQUENCE: 54

Cys Ser Val Thr Cys Gly  
 1 5

<210> SEQ ID NO 55  
 <211> LENGTH: 8  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Thrombospondin Region

<400> SEQUENCE: 55

Arg Phe Tyr Val Val Met Trp Lys  
 1 5

<210> SEQ ID NO 56  
 <211> LENGTH: 6  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Thrombospondin region

<400> SEQUENCE: 56

Arg Phe Tyr Val Val Met  
 1 5

<210> SEQ ID NO 57  
 <211> LENGTH: 7  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Thrombospondin Region

<400> SEQUENCE: 57

Phe Tyr Val Val Met Trp Lys  
 1 5

<210> SEQ ID NO 58  
 <211> LENGTH: 5  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Thrombospondin Region

<400> SEQUENCE: 58

Ile Arg Val Val Met  
 1 5

<210> SEQ ID NO 59  
 <211> LENGTH: 10  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Thrombospondin Region

<400> SEQUENCE: 59

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Arg Lys Gly Ser Gly Arg Arg Leu Val Lys  
1                   5                   10

<210> SEQ ID NO 60  
<211> LENGTH: 7  
<212> TYPE: PRT  
<213> ORGANISM: Artificial  
<220> FEATURE:  
<223> OTHER INFORMATION: thrombospondin Region

<400> SEQUENCE: 60

Arg Lys Gly Ser Gly Arg Arg  
1                   5

<210> SEQ ID NO 61  
<211> LENGTH: 7  
<212> TYPE: PRT  
<213> ORGANISM: Artificial  
<220> FEATURE:  
<223> OTHER INFORMATION: Thrombospondin region

<400> SEQUENCE: 61

Arg Gln Met Lys Lys Thr Arg  
1                   5

<210> SEQ ID NO 62  
<211> LENGTH: 8  
<212> TYPE: PRT  
<213> ORGANISM: Artificial  
<220> FEATURE:  
<223> OTHER INFORMATION: Thrombospondin Region

<400> SEQUENCE: 62

Ala Arg Lys Gly Ser Gly Arg Arg  
1                   5

<210> SEQ ID NO 63  
<211> LENGTH: 6  
<212> TYPE: PRT  
<213> ORGANISM: Artificial  
<220> FEATURE:  
<223> OTHER INFORMATION: Thrombospondin Region

<400> SEQUENCE: 63

Met Lys Lys Thr Arg Gly  
1                   5

<210> SEQ ID NO 64  
<211> LENGTH: 12  
<212> TYPE: PRT  
<213> ORGANISM: Artificial  
<220> FEATURE:  
<223> OTHER INFORMATION: Thrombospondin Region

<400> SEQUENCE: 64

Arg Leu Arg Ile Ala Lys Gly Gly Val Asn Asp Asn  
1                   5                   10

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The invention claimed is:

1. A method to detect the presence and/or clinical course of a neoplastic disease in an individual, wherein the method comprises the steps of:

- (1) measuring a first individual's plasma level of a thrombospondin fragment or fragments, the plasma level of said fragment or fragments in the first individual being the first individual's plasma fragment level;
- (2) measuring, in a second individual, the plasma level of the same thrombospondin fragment or fragments measured for the first individual, said second individual considered to not have neoplastic disease, the plasma level of said fragment or fragments in the second individual being the second individual's plasma fragment level;
- (3) utilizing the results of steps (1) and (2) in a diagnosis as to whether the first individual has a neoplastic disease such that the greater the extent to which the first individual's plasma fragment level exceeds the second individual's plasma fragment level the more likely that the diagnosis will be that a neoplastic disease is present in the first individual; said fragment or fragments being at least 6 continuous amino acyl residues in length but of a molecular weight of 140 kDa or less; wherein the size in kDa is that determined by gel electrophoresis after disulfide bond reduction.

2. A method of claim 1 further comprising the steps of assaying the first individual's plasma level of a thrombospondin fragment or fragments more than once, and utilizing a change in said plasma level from an older to a more recent value to indicate appearance or progression or improvement of a neoplastic disease wherein said appearance or progression is indicated by an increase in the plasma level and said improvement is indicated by a decrease in said plasma level.

3. A method of claim 1 or 2 wherein the measurement of a plasma level of a thrombospondin fragment or fragments comprises the use of a binding agent, said binding agent capable of binding said fragment or fragments.

4. A method of claim 3 wherein the thrombospondin fragment or fragments are separated from thrombospondin before said fragment or fragments are bound to the binding agent.

5. A method to detect the presence and/or clinical course of a neoplastic disease in an individual, wherein the method comprises the steps of:

- (1) measuring a first individual's plasma level of a thrombospondin fragment or fragments, the plasma level of said fragment or fragments in the first individual being the first individual's plasma fragment level;
- (2) measuring, in a second individual, the plasma level of the same thrombospondin fragment or fragments measured for the first individual, said second individual considered to not have neoplastic disease, the plasma level of said fragment or fragments in the second individual being the second individual's plasma fragment level;
- (3) utilizing the results of steps (1) and (2) in a diagnosis as to whether the first individual has a neoplastic disease; said fragment or fragments being within a molecular weight range selected from the group consisting of 80 to 140 kDa, 40 to 60 kDa, and 20 to 35 kDa such that the greater the extent to which the first individual's plasma fragment level exceeds the second individual's plasma fragment level the more likely that the diagnosis will be that a neoplastic disease is present in the first individual, wherein the size in kDa is that determined by gel electrophoresis after disulfide bond reduction.

6. A method of claim 5 further comprising the steps of assaying the first individual's plasma level of a thrombospondin fragment or fragments more than once, and utilizing a

change in said plasma level from an older to a more recent value to indicate appearance or progression or improvement of a neoplastic disease wherein said appearance or progression is indicated by an increase in the plasma level and said improvement is indicated by a decrease in said plasma level.

7. A method of claim 5 or 6, wherein the measurement of the level of a plasma thrombospondin fragment or fragments comprises the use of a binding agent, said binding agent capable of binding said fragment or fragments.

8. A method of claim 7 wherein the thrombospondin fragment or fragments are separated from thrombospondin before said fragment or fragment are bound to the binding agent.

9. A method of claim 1 or 2 wherein the molecular weight of each of the fragment or fragments is at least 20 kDa, wherein the size in kDa is that determined by gel electrophoresis after disulfide bond reduction.

10. A method of claim 3 wherein the molecular weight of each of the fragment or fragments is at least 20 kDa, wherein the size in kDa is that determined by gel electrophoresis after disulfide bond reduction.

11. A method of claim 4 wherein the molecular weight of each of the fragment or fragments is at least 20 kDa, wherein the size in kDa is that determined by gel electrophoresis after disulfide bond reduction.

12. A method of claim 3, wherein the binding agent is an antibody.

13. A method of claim 4, wherein the binding agent is an antibody.

14. A method of claim 10, wherein the binding agent is an antibody.

15. A method of claim 11, wherein the binding agent is an antibody.

16. A method of claim 5 or 6 wherein the molecular weight of the fragment or each of the fragments is within a molecular weight range selected from the group consisting of 85 to 140 kDa, 47 to 53 kDa, and 27 to 33 kDa, wherein the size in kDa is that determined by gel electrophoresis after disulfide bond reduction.

17. A method of claim 5 or 6 wherein the molecular weight of the fragment or fragments is within a range of 80 to 140 kDa wherein the size in kDa is that determined by gel electrophoresis after disulfide bond reduction.

18. A method of claim 7, wherein the binding agent is an antibody.

19. A method of claim 8, wherein the binding agent is an antibody.

20. A method to detect the presence and/or clinical course of a neoplastic disease in an individual, wherein the method comprises the steps of:

- (1) measuring a first individual's plasma level of a thrombospondin fragment or fragments, the plasma level of said fragment or fragments in the first individual being the first individual's plasma fragment level;
- (2) measuring, in a second individual, the plasma level of the same thrombospondin fragment or fragments measured for the first individual, said second individual considered to not have neoplastic disease, the plasma level of said fragment or fragments in the second individual being the second individual's plasma fragment level;
- (3) utilizing the results of steps (1) and (2) in a diagnosis as to whether the first individual has a neoplastic disease such that the greater the extent to which the first individual's plasma fragment level exceeds the second individual's plasma fragment level the more likely that the diagnosis will be that a neoplastic disease is present in the first individual; wherein said fragment or fragments either start between amino acyl residues I-165 and

V-263, inclusive, and end between amino acyl residues R-792 and inclusive, or is a portion of the range I-165 to Y-982, said portion being at least 150 amino acyl residues in size and wherein I-165, V-263, R-792 and Y-982 refer to residues 183, 281, 810, and 1000, respectively of SEQ ID NO:38.

21. A method of claim 20 further comprising the steps of assaying the first individual's plasma level of a thrombospondin fragment or fragments more than once, and utilizing a change in said plasma level from an older to a more recent value to indicate appearance or progression or improvement of a neoplastic disease wherein said appearance or progression is indicated by an increase in the plasma level and said improvement is indicated by a decrease in said plasma level.

22. A method of claim 20 or 21 wherein the measurement of a plasma level of a thrombospondin fragment or fragments comprises the use of a binding agent, said binding agent capable of binding said fragment or fragments.

23. A method of claim 22 wherein the thrombospondin fragment or fragments are separated from thrombospondin before said fragment or fragments are bound to the binding agent.

24. A method of claim 20 wherein said fragment or fragments further comprising an amino acyl sequence corresponding to SEQ ID NO: 1.

25. A method of claim 20 or 21 wherein the molecular weight of the portion is at least 20 kDa, wherein the size in kDa is that determined by gel electrophoresis after disulfide bond reduction.

26. A method of claim 22 wherein the molecular weight of the portion is at least 20 kDa, wherein the size in kDa is that determined by gel electrophoresis after disulfide bond reduction.

27. A method of claim 23 wherein the molecular weight of the portion is at least 20 kDa wherein the size in kDa is that determined by gel electrophoresis after disulfide bond reduction.

28. A method of claim 22, wherein the binding agent is an antibody.

29. A method of claim 23, wherein the binding agent is an antibody.

30. A method of claim 26, wherein the binding agent is an antibody.

31. A method of claim 27, wherein the binding agent is an antibody.

32. A method to detect the presence and/or clinical course of a neoplastic disease in an individual, wherein the method comprises the steps of:

- (1) measuring a first individual's plasma level of a thrombospondin fragment or fragments, the plasma level of said fragment or fragments in the first individual being the first individual's plasma fragment level;
- (2) measuring, in a second individual, the plasma level of the same thrombospondin fragment or fragments measured for the first individual, said second individual considered to not have neoplastic disease, the plasma level of said fragment or fragments in the second individual being the second individual's plasma fragment level;
- (3) utilizing the results of steps (1) and (2) in a diagnosis as to whether the first individual has a neoplastic disease such that the greater the extent to which the first individual's plasma fragment level exceeds the second individual's plasma fragment level the more likely that the diagnosis will be that a neoplastic disease is present in the first individual; the molecular weight of said fragment or any of said fragments not exceeding 140 kDa, the molecular weight of said fragment or fragments

being at least 20 kDa, wherein the size in kDa is that determined by gel electrophoresis after disulfide bond reduction, and wherein the fragment or each of said fragments comprises a portion of thrombospondin selected from the group consisting of

a collagen type V binding domain, and a domain or a part thereof within the protease-resistant core of thrombospondin, said domain being selected from the group consisting of a domain of inter-chain disulfide bonds, an oligomerization domain, a procollagen-like domain, a type 1 repeat, a type 2 repeat, and a type 3 repeat.

33. A method of claim 32 further comprising the steps of assaying the first individual's plasma level of a thrombospondin fragment or fragments more than once, and utilizing a change in said plasma level from an older to a more recent value to indicate appearance or progression or improvement of a neoplastic disease wherein said appearance or progression is indicated by an increase in the plasma level and said improvement is indicated by a decrease in said plasma level.

34. A method of claim 32 or 33, wherein the measurement of a plasma level of a thrombospondin fragment or fragments comprises the use of a binding agent, said binding agent capable of binding said fragment or fragments.

35. A method of claim 34 wherein the thrombospondin fragment or fragments are separated from thrombospondin before said fragment or fragments are bound to the binding agent.

36. A method of claim 32 wherein said fragment or fragments further comprising an amino acyl sequence corresponding to SEQ ID NO: 1.

37. A method of claim 34, wherein the binding agent is an antibody.

38. A method of claim 35, wherein the binding agent is an antibody.

39. A method to detect the presence and/or clinical course of a neoplastic disease in an individual, wherein the method comprises the steps of:

- (1) measuring a first individual's plasma level of a thrombospondin fragment or fragments, the plasma level of said fragment or fragments in the first individual being the first individual's plasma fragment level;
- (2) measuring, in a second individual, the plasma level of the same thrombospondin fragment or fragments measured for the first individual, said second individual considered to not have neoplastic disease, the plasma level of said fragment or fragments in the second individual being the second individual's plasma fragment level;
- (3) utilizing the results of steps (1) and (2) in a diagnosis as to whether the first individual has a neoplastic disease such that the greater the extent to which the first individual's plasma fragment level exceeds the second individual's plasma fragment level the more likely that the diagnosis will be that a neoplastic disease is present in the first individual; wherein said plasma level is measured using a binding agent that is capable of binding to said fragment or fragments provided that said binding agent does not bind a region selected from the group consisting of the fibrinogen-binding region in the amino-terminal domain of thrombospondin, and a heparin-binding sequence in the amino-terminal domain of thrombospondin; wherein the molecular weight of each of the fragment or fragments is at least 20 kDa but not more than 140 kDa, wherein the size in kDa is that determined by gel electrophoresis after disulfide bond reduction.

40. A method of claim 39 further comprising the steps of assaying the first individual's plasma level of a thrombospondin fragment or fragments more than once, and utilizing a change in said plasma level from an older to a more recent value to indicate appearance or progression or improvement of a neoplastic disease wherein said appearance or progression is indicated by an increase in the plasma level and said improvement is indicated by a decrease in said plasma level.

41. A method of claim 39 or 40, wherein the thrombospondin fragment or fragments are separated from thrombospondin before said fragment or fragments are bound to the binding agent.

42. A method of claim 39 or 40 wherein the binding agent is an antibody.

43. A method of claim 41, wherein the binding agent is an antibody.

44. A method to detect the presence and/or clinical course of a neoplastic disease in an individual, wherein the method comprises the steps of:

(1) measuring a first individual's plasma level of a thrombospondin fragment or fragments, the plasma level of said fragment or fragments in the first individual being the first individual's plasma fragment level;

(2) measuring, in a second individual, the plasma level of the same thrombospondin fragment or fragments measured for the first individual, said second individual considered to not have neoplastic disease, the plasma level of said fragment or fragments in the second individual being the second individual's plasma fragment level;

(3) utilizing the results of steps (1) and (2) in a diagnosis as to whether the first individual has a neoplastic disease such that the greater the extent to which the first individual's plasma fragment level exceeds the second individual's plasma fragment level the more likely that the diagnosis will be that a neoplastic disease is present in the first individual; wherein said method comprises the use of a binding agent that binds to an epitope within a plasma fragment in the molecular weight range selected from the group consisting of 80 to 140 kDa, 40 to 60 kDa, and 20 to 35 kDa, wherein the size in kDa is that determined by gel electrophoresis after disulfide bond reduction.

45. A method of claim 44 further comprising the steps of assaying the first individual's plasma level of a thrombospondin fragment or fragments more than once, and utilizing a change in said plasma level from an older to a more recent value to indicate appearance or progression or improvement of a neoplastic disease wherein said appearance or progression is indicated by an increase in the plasma level and said improvement is indicated by a decrease in said plasma level.

46. A method of claim 44 or 45 wherein the thrombospondin fragment or fragments are separated from thrombospondin before said fragment or fragments are bound to the binding agent.

47. A method of claim 44 or 45, wherein the binding agent is an antibody.

48. A method of claim 46, wherein the binding agent is an antibody.

49. A method to detect the presence and/or clinical course of a neoplastic disease in an individual, wherein the method comprises the steps of:

1) utilizing a first binding agent to obtain a quantification, for the plasma of a first individual, of a total, thrombospondin plus either the thrombospondin fragment or fragments;

2) utilizing a second binding agent to obtain a quantification, for the plasma of said first individual, of thrombospondin only;

3) utilizing the difference between the quantifications obtained in steps (1) and (2) as a quantitation, for the plasma of said first individual, of the amount of thrombospondin fragment or fragments;

4) utilizing the first binding agent to obtain a quantification, for the plasma of a second individual, of a total, thrombospondin plus either the thrombospondin fragment or fragments, said second individual considered not to have neoplastic disease;

5) utilizing the second binding agent to obtain a quantification, for the plasma of said second individual, of thrombospondin only;

6) utilizing the difference between the quantifications obtained in steps (4) and (5) as a quantitation, for the plasma of said second individual, of the amount of thrombospondin fragment or fragments; and

7) utilizing the results of steps (3) and (6) in a diagnosis as to whether the first individual has a neoplastic disease such that the greater the extent to which the first individual's plasma level of said thrombospondin fragment or fragments exceeds the second individual's plasma level of said thrombospondin fragment or fragments, the more likely that the diagnosis will be that a neoplastic disease is present in said first individual;

wherein the first binding agent binds to an epitope shared by thrombospondin and the thrombospondin fragment or fragments, and wherein the second binding agent binds to an epitope present in thrombospondin but not present in the fragment or fragments.

50. A method of claim 49 wherein said fragment or fragments are at least 6 continuous amino acyl residues in length but of a molecular weight of 140 kDa or less; wherein the size in kDa is that determined by gel electrophoresis after disulfide bond reduction.

51. A method of claim 49 wherein said fragment or fragments are within a molecular weight range selected from the group consisting of 80 to 140 kDa, 40 to 60 kDa, and 20 to 35 kDa, wherein the size in kDa is that determined by gel electrophoresis after disulfide bond reduction.

52. A method of claim 49 wherein said fragment or fragments either start between amino acyl residues I-165 and V-263, inclusive, and end between amino acyl residues R-792 and Y-982, inclusive, or is a portion of the range I-165 to Y-982, said portion being at least 150 amino acyl residues in size and wherein I-165, V-263, R-792 and Y-982 refer to residues 183, 281, 810, and 1000, respectively of SEQ ID NO:38.

53. A method of claim 49 wherein the molecular weight of said fragment or any of said fragments not exceeding 140 kDa, the molecular weight of said fragment or fragments being at least 20 kDa, wherein the size in kDa is that determined by gel electrophoresis after disulfide bond reduction, and wherein the fragment or each of said fragments comprises a portion of thrombospondin selected from the group consisting of

a collagen type V binding domain, and

a domain or a part thereof within the protease-resistant core of thrombospondin, said domain being selected from the group consisting of a domain of inter-chain disulfide bonds, an oligomerization domain, a procollagen-like domain, a type 1 repeat, a type 2 repeat, and a type 3 repeat.

54. A method of claim 49 wherein said first binding agent does not bind a region selected from the group consisting of

the fibrinogen-binding region in the amino-terminal domain of thrombospondin, and a heparin-binding sequence in the amino-terminal domain of thrombospondin; wherein the molecular weight of each of the fragment or fragments is at least 20 kDa but not more than 140 kDa, wherein the size in kDa is that determined by gel electrophoresis after disulfide bond reduction.

55. A method of claim 49 wherein said first binding agent binds to an epitope within a plasma fragment in the molecular weight range selected from the group consisting of 80 to 140 kDa, 40 to 60 kDa, and 20 to 35 kDa, wherein the size in kDa is that determined by gel electrophoresis after disulfide bond reduction.

56. A method of claim 49, 50, 51, 52, 53, 54 or 55 wherein one or both of said first and second binding agents is an antibody.

57. A method of claim 1 wherein the first individual is suspected of having, or known to have, a neoplastic disease.

58. A method of claim 57 further comprising the steps of assaying the first individual's plasma level of a thrombospondin fragment or fragments more than once, and utilizing a change in said plasma level from an older to a more recent value to indicate appearance or progression or improvement of a neoplastic disease wherein said appearance or progression is indicated by an increase in the plasma level and said improvement is indicated by a decrease in said plasma level.

59. A method of claim 57 or 58 wherein the measurement of a plasma level of a thrombospondin fragment or fragments comprises the use of a binding agent, said binding agent capable of binding said fragment or fragments.

60. A method of claim 59 wherein the thrombospondin fragment or fragments are separated from thrombospondin before said fragment or fragments are bound to the binding agent.

61. A method of claims 57 or 58 wherein the molecular weight of each of the fragment or fragments is at least 20 kDa, wherein the size in kDa is that determined by gel electrophoresis after disulfide bond reduction.

62. A method of claim 59 wherein the molecular weight of each of the fragment or fragments is at least 20 kDa, wherein the size in kDa is that determined by gel electrophoresis after disulfide bond reduction.

63. A method of claim 60 wherein the molecular weight of each of the fragment or fragments is at least 20 kDa, wherein the size in kDa is that determined by gel electrophoresis after disulfide bond reduction.

64. A method of claim 59, wherein the binding agent is an antibody.

65. A method of claim 60, wherein the binding agent is an antibody.

66. A method of claim 62, wherein the binding agent is an antibody.

67. A method of claim 63, wherein the binding agent is an antibody.

68. A method of claim 5 wherein the first individual is suspected of having, or known to have, a neoplastic disease.

69. A method of claim 68 further comprising the steps of assaying the individual's plasma level of a thrombospondin fragment or fragments more than once, and utilizing a change in said plasma level from an older to a more recent value to indicate appearance or progression or improvement of a neoplastic disease wherein said appearance or progression is indicated by an increase in the plasma level and said improvement is indicated by a decrease in said plasma level.

70. A method of claim 68 or 69 wherein the measurement of a plasma level of a thrombospondin fragment or fragments

comprises the use of a binding agent, said binding agent capable of binding said fragment or fragments.

71. A method of claim 70 wherein the thrombospondin fragment or fragments are separated from thrombospondin before said fragment or fragments are bound to the binding agent.

72. A method of claim 70, wherein the binding agent is an antibody.

73. A method of claim 71, wherein the binding agent is an antibody.

74. A method of claim 20 wherein the first individual is suspected of having, or known to have, a neoplastic disease.

75. A method of claim 74 further comprising the steps of assaying the first individual's plasma level of a thrombospondin fragment or fragments more than once, and utilizing a change in said plasma level from an older to a more recent value to indicate appearance or progression or improvement of a neoplastic disease wherein said appearance or progression is indicated by an increase in the plasma level and said improvement is indicated by a decrease in said plasma level.

76. A method of claim 74 or 75 wherein the measurement of a plasma level of a thrombospondin fragment or fragments comprises the use of a binding agent, said binding agent capable of binding said fragment or fragments.

77. A method of claim 76 wherein the thrombospondin fragment or fragments are separated from thrombospondin before said fragment or fragments are bound to the binding agent.

78. A method of claim 74 or 75 wherein the molecular weight of each of the fragment or fragments is at least 20 kDa, wherein the size in kDa is that determined by gel electrophoresis after disulfide bond reduction.

79. A method of claim 76 wherein the molecular weight of each of the fragment or fragments is at least 20 kDa, wherein the size in kDa is that determined by gel electrophoresis after disulfide bond reduction.

80. A method of claim 77 wherein the molecular weight of each of the fragment or fragments is at least 20 kDa, wherein the size in kDa is that determined by gel electrophoresis after disulfide bond reduction.

81. A method of claim 76, wherein the binding agent is an antibody.

82. A method of claim 77, wherein the binding agent is an antibody.

83. A method of claim 79, wherein the binding agent is an antibody.

84. A method of claim 80, wherein the binding agent is an antibody.

85. A method of claim 32 wherein the first individual is suspected of having, or known to have, a neoplastic disease.

86. A method of claim 85 further comprising the steps of assaying the first individual's plasma level of a thrombospondin fragment or fragments more than once, and utilizing a change in said plasma level from an older to a more recent value to indicate appearance or progression or improvement of a neoplastic disease wherein said appearance or progression is indicated by an increase in the plasma level and said improvement is indicated by a decrease in said plasma level.

87. A method of claim 85 or 86 wherein the measurement of a plasma level of a thrombospondin fragment or fragments comprises the use of a binding agent, said binding agent capable of binding said fragment or fragments.

88. A method of claim 87 wherein the thrombospondin fragment or fragments are separated from thrombospondin before said fragment or fragments are bound to the binding agent.

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89. A method of claim 87, wherein the binding agent is an antibody.

90. A method of claim 88, wherein the binding agent is an antibody.

91. A method of claim 39 wherein the first individual is suspected of having, or known to have, a neoplastic disease.

92. A method of claim 91 further comprising the steps of assaying the first individual's plasma level of a thrombospondin fragment or fragments more than once, and utilizing a change in said plasma level from an older to a more recent value to indicate appearance or progression or improvement of a neoplastic disease wherein said appearance or progression is indicated by an increase in the plasma level and said improvement is indicated by a decrease in said plasma level.

93. A method of any one of claim 91 or 92 wherein the binding agent is an antibody.

94. A method of claim 44 wherein the first individual is suspected of having, or known to have, a neoplastic disease.

95. A method of claim 94 further comprising the steps of assaying the first individual's plasma level of a thrombospondin fragment or fragments more than once, and utilizing a change in said plasma level from an older to a more recent value to indicate appearance or progression or improvement of a neoplastic disease wherein said appearance or progression is indicated by an increase in the plasma level and said improvement is indicated by a decrease in said plasma level.

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96. A method of any one of claim 94 or 95, wherein the binding agent is an antibody.

97. A method of any one of claim 1 or 2, wherein the neoplastic disease is colon cancer.

98. A method of claim 9 wherein the neoplastic disease is colon cancer.

99. A method of any one of claim 57 or 58, wherein the neoplastic disease is colon cancer.

100. A method of claim 61 wherein the neoplastic disease is colon cancer.

101. A method of any one of claim 1 or 2, wherein the neoplastic disease is selected from the group consisting of lung cancer and prostate cancer.

102. A method of claim 9 wherein the neoplastic disease is selected from the group consisting of lung cancer and prostate cancer.

103. A method of any one of claim 57 or 58, wherein the neoplastic disease is selected from the group consisting of lung cancer and prostate cancer.

104. A method of claim 61 wherein the neoplastic disease is selected from the group consisting of lung cancer and prostate cancer.

\* \* \* \* \*

专利名称(译)	血小板反应蛋白片段在癌症检测，诊断和评估中的临床分析		
公开(公告)号	<a href="#">US8053196</a>	公开(公告)日	2011-11-08
申请号	US10/782968	申请日	2004-02-20
[标]申请(专利权)人(译)	WILLIAMS KEVIN J		
申请(专利权)人(译)	WILLIAMS KEVIN J.		
当前申请(专利权)人(译)	W2 HOLDINGS , INC.		
[标]发明人	WILLIAMS KEVIN J		
发明人	WILLIAMS, KEVIN J.		
IPC分类号	G01N33/53 G01N1/00 G01N1/18 C12Q1/00 G01N33/48 G01N33/00 C07K14/78 G01N33/68		
CPC分类号	C07K14/78 G01N33/6887 G01N33/57488 G01N33/6893 Y10T436/25375 Y10T436/25 G01N2333/4728		
优先权	60/405494 2002-08-23 US		
其他公开文献	US20050065324A1		
外部链接	<a href="#">Espacenet</a> <a href="#">USPTO</a>		

摘要(译)

本发明涉及在血浆中发现的血小板反应蛋白片段，它们在诊断方法中的用途或用途，作为方法校准物，方法指示物和免疫原，以及用作具有实质临床效用的方法的分析物；以及它们在血浆或其他体液中的检测，用于诊断方法，特别是用于癌症。

