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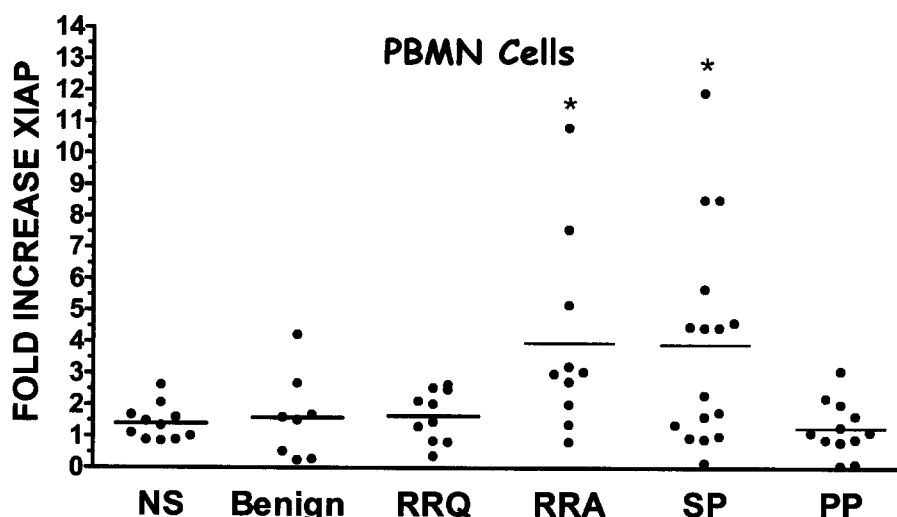
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[Continued on next page]

(54) Title: METHOD FOR DIFFERENTIATING BETWEEN MULTIPLE SCLEROSIS SUBTYPES



(57) Abstract: A method for differentiating between multiple sclerosis subtypes by analysing the gene or protein expression of inhibitor of apoptosis protein (IAP) family members: X-linked inhibitor of apoptosis protein (XIAP), human inhibitor of apoptosis protein -1 (HIAP-1), human inhibitor of apoptosis protein -2 (HIAP-2), survivin and neuronal apoptosis inhibitor protein (NAIP). The method comprises a) determining by qRT-PCR or western blotting an amount of an IAP gene or protein expressed in a blood sample, a peripheral blood mononuclear cell sample or a resting T lymphocyte sample; and b) correlating the amount of the IAP gene or protein expression level with the presence of Relapsing-Remitting (RR), Secondary-Progressive (SP), Primary-Progressive (PP), Relapsing-Remitting Quiescent (RRQ) and/or Relapsing-Remitting Active (RRA) multiple sclerosis subtypes.

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METHOD FOR DIFFERENTIATING BETWEEN MULTIPLE SCLEROSIS SUBTYPES

5 FIELD OF THE INVENTION

The present invention concerns methods for differentiating between multiple sclerosis subtypes, and more particularly to methods of using different IAP expression levels as markers for the multiple sclerosis subtypes.

10 BACKGROUND OF THE INVENTION

Multiple sclerosis (MS) is a progressive neurological disorder characterized by an autoimmune mediated attack against the myelin sheath in the CNS resulting in inflammation, demyelination, gliosis and ultimately axonal degeneration [Bruck and Stadelmann 2003]. The clinical course of MS has been divided into four major categories: 15 RR, SP, PP and benign. Patients who have clinical relapses every few months or years with intervening periods of clinical stability define RRMS. RRMS is twice as common in females than males in the second or third decade of life [Noseworthy *et al.* 2000]. Although the majority of MS patients are initially diagnosed with RRMS, over time increasing numbers of these individuals convert to SPMS characterized by a gradual 20 decline in neurological function [Trojano *et al.* 2003]. Approximately 15% of MS patients have PPMS characterized by an absence of clinical relapses and an unrelenting deterioration of neurological function from disease onset [McDonnell and Hawkins 2002]. PPMS is characterized by relatively late-onset (mean age ~ 39 years) although it has been suggested based on clinical and MRI data that the pre-clinical phase of PPMS occurs 25 during the same time frame as in RRMS. In PPMS as in RRMS the CNS lesions have the same age of onset, but it takes 10 years for PPMS patients to develop symptoms [McDonnell *et al.* 2003]. MRI with the contrast agent gadolinium is often employed during or following an initial attack to identify lesions within the CNS that are consistent with a diagnosis of MS. MRI is also used at protracted intervals in MS patients to identify new 30 areas of demyelinated plaques within the brain and spinal cord [Calabresi 2004]. Moreover, MRI activity correlates with immune cell perturbations in early possible MS. For example, Rinaldi *et al.* (2006) demonstrated that distinct changes in peripheral lymphocyte subsets occur over the course of 1 year, which differentiate MRI active, and MRI inactive

patients following a clinically isolated syndrome. Yet results obtained by MRI do not correlate with clinical disability and are therefore not recommended as therapeutic end-points for new MS therapeutics [Siva 2006]. In addition to RRMS, SPMS and PPMS, there is a benign form of the disease that affects approximately 15% of RRMS patients.

5 Benign MS is arbitrarily defined in RRMS patients who after more than 10 to 15 years following initial diagnosis are still mobile and show only mild deficits ($EDSS \leq 4$). Typically, these patients show little or no progression after their initial attack. Moreover, those patients with an EDSS score of ≤ 2 and disease duration of more than 10 to 15 years tend to maintain a low EDSS disability score for an additional 10 years. Benign MS requires no
10 therapeutic intervention, however, it is not possible to diagnose this form of MS until at least 5 to 10 years from MS onset [Hawkins and McDonnell 1999; Pittock *et al.* 2004]. Unfortunately, there are no diagnostic tests that would allow a clinician to predict whether a newly diagnosed MS patient will follow a benign or aggressive disease course.

15 MS is considered to be a T cell-mediated autoimmune disease of the brain and spinal cord [Traugott *et al.* 1983; Vizler *et al.* 1999]. While there appears to be a localized CNS immune response, peripheral immune cell abnormalities appear to correlate with central disease activity [Hafler and Weiner 1989] and may precede MRI activity. Apoptosis is an important mechanism in immune system regulation, responsible for elimination of
20 autoreactive T-lymphocytes (T cells), B-lymphocytes (B cells) and monocytes from the circulation and prevention of their entry into the CNS [Mahoney and Rosen 2005; Todaro *et al.* 2004]. It has been hypothesized that a genetic predisposition exists in MS patients whereby a failure of autoreactive T cells and B cells as well as activated macrophages to undergo apoptosis contributes to the pathogenesis of MS [Bernard and Derosbo 1992;
25 Pender 1998; Pender and Rist 2001]. Consistent with this hypothesis expression of members of the IAP family of anti-apoptotic proteins are elevated in mitogen (PHA) stimulated T cells derived from the CSF or blood of MS patients relative to healthy or neurological control subjects [Segal and Cross 2000; Seki *et al.* 1988; Semra *et al.* 2002; Sharief *et al.* 2002b; Sharief and Semra 2001; Tsukamoto *et al.* 1986]. The IAP family of
30 anti-apoptotic genes encodes proteins that directly bind to and inactivate initiator and effector caspases, a group of cysteinyl proteases that mediate the initiation and execution of apoptosis [Holcik *et al.* 2001; Salvesen and Duckett 2002]. First discovered in baculovirus, the IAPs are well conserved in eukaryotes, ranging from yeast to humans and to date, eight human IAPs have been identified [Holcik *et al.* 2001; Nachmias *et al.*
35 2004]. Importantly, IAPs are the only intrinsic inhibitors of caspases. The IAPs are typified

by the presence of a variable number of highly conserved domains about 70 amino acids in length, known as BIR domains, which are critical for anti-apoptotic activity. For example, while XIAP, HIAP-1 and HIAP-2 contain three BIR domains, survivin possesses only one BIR domain. Although highly similar, the individual BIR domains are not functionally equivalent. The BIR2 domain and the preceding linker region of XIAP, HIAP-1, and HIAP-2 facilitate the interaction with and suppression of caspases 3 and 7; the two most potent effector caspases [Eckelman and Salvesen 2006; Nachmias *et al.* 2004; Robertson *et al.* 2000]. Inhibition of the initiator caspase 9 is accomplished by the BIR3 domain. While XIAP, HIAP-1 and HIAP-2 possess BIR domains 2 and 3 capable of binding caspases 7 and 9, only XIAP contains critical domain residues capable of direct caspase inhibition [Eckelman and Salvesen 2006]. In addition to BIR domains, XIAP, HIAP-1 and HIAP-2 possess a carboxy-terminal RING zinc finger motif that has E3 ubiquitin ligase activity targeting caspases for degradation by proteosomes [Holcik *et al.* 2001]. In addition, it has been shown recently that IAPs are themselves controlled by ubiquitin-mediated degradation. For example, HIAP-1 is a direct target for HIAP-2-mediated ubiquitination and proteosomal degradation [Conze *et al.* 2005]. The RING domain of XIAP also mediates polyubiquitination of TAK-1, an enzyme responsible for activation of the pro-apoptotic kinase, JNK [Kaur *et al.* 2005]. In this fashion, XIAP is able to target TAK-1 for degradation by the proteosome thereby preventing JNK-mediated apoptosis. Structurally similar, both HIAP-1 and HIAP-2 possess a CARD, a highly conserved domain noted to promote homodimerization and oligomerization with other CARD containing proteins. While elevated expression of XIAP, HIAP-1, HIAP-2 and survivin in mitogen-stimulated T cells from patients with active RRMS correlates with clinical features of disease activity, deficits in Fas mediated cell death [Comi *et al.* 2000] and T cell resistance to apoptosis [Sharief *et al.* 2002b; Sharief and Semra 2001], a systematic examination of the expression patterns of these genes in whole blood, PBMC and resting T cells in patients with various forms of MS has yet to be done. Given that the failed apoptosis of autoreactive T cells has been implicated in MS pathogenesis and that MS is a clinically heterogeneous disorder [Chofflon 2005], it would be advantageous if specific patterns of IAP expression in different immune cell subtypes could be measured and correlated with distinct forms of the disease. Furthermore, it would be highly advantageous to develop a reliable, rapid and inexpensive diagnostic test for multiple sclerosis subtypes based on specific patterns of basal IAP gene expression in peripheral immune cells. Finally, a diagnostic test would allow clinicians to decide whether interferon drug treatment is appropriate for a specific disease subtype.

SUMMARY OF THE INVENTION

We have unexpectedly discovered that human patients suffering from the major categories (or subtypes) of multiple sclerosis (MS) have basal IAP gene expression profiles that correlate with the disease severity. Specifically, we have discovered that XIAP and HIAP-2 are differentially expressed in high levels and are associated with aggressive forms of the disease including active relapsing remitting MS and secondary progressive MS. Advantageously, the gene expression profiles of XIAP and HIAP-2 are useful as diagnostic markers that may aid early diagnosis of the aggressive forms of MS.

Furthermore, the unique IAP gene expression profiles allow differentiation between the various subtypes of MS. With early diagnosis, patients can be treated more rapidly than previously allowable thereby ensuring optimal therapeutic response. Moreover, the differences in IAP expression profiles are predictive of interferon- β (IFN- β) responsiveness, which will allow clinicians to appropriately select patients for whom the use of IFN- β drugs will provide maximum benefit and avoid the use of IFN- β drugs, which are expensive, on patients who suffer from benign and primary progressive MS for which little or no therapeutic benefit will be afforded. Moreover, in patients suffering from PPMS, we have also discovered that survivin expression is elevated in the resting T cells compared to XIAP, HIAP 1 and HIAP-2. Surprisingly, in mRNA extracted from whole blood, NAIP mRNA levels were elevated in all forms of MS except those patients with benign MS or normal subjects.

According to one embodiment of the present invention, there is provided a method for differentiating between multiple sclerosis subtypes in a patient, the method comprising:

- a) determining an amount of an IAP gene expression level in a blood sample obtained from the patient; and
- b) correlating the amount of the IAP gene expression level in the blood sample with the presence of a multiple sclerosis subtype in the patient.

In one aspect, typically the IAP gene expression level is compared those of control subjects. An increase in the IAP gene expression level relative to those of the control subjects indicates that the patient is suffering from the MS subtype. The control subjects are those having benign MS or are healthy normal subjects. The MS subtype is benign MS, quiescent relapsing remitting MS, active relapsing remitting MS, primary progressive MS or secondary progressive MS. The IAP gene expression level is determined by

measuring the levels of transcribed IAP mRNA in the blood sample. The level of IAP mRNA is measured using quantitative real time polymerase chain reaction (qRT-PCR).

5 In one aspect of the present invention, the IAP gene expression level is determined by measuring the level of IAP protein in the blood sample. The IAP protein level is measured using an immunoassay.

In another aspect, the IAP gene encodes an IAP protein selected from NAIP, XIAP, HIAP-1, HIAP-2 or survivin.

10

In another aspect, typically peripheral blood mononuclear (PBMN) cells are isolated from the blood sample. The IAP gene expression level is measured in the PBMNs. The IAP gene expression level is compared to those of control subjects, an increase in the IAP gene expression level relative to those of the control subjects indicates that the patient is
15 suffering from the MS subtype. The increased gene expression level includes increased levels of XIAP mRNA and HIAP-2 mRNA. The MS subtype is active relapsing remitting MS. The MS subtype is secondary progressive MS. Typically, the control subjects are those having benign MS, or are healthy normal subjects.

20 In another aspect, typically T cells are isolated from the blood sample. The IAP gene expression level is measured in the T cells. The T cells are resting T cells.

In one aspect, typically, the IAP gene expression level is compared to those of control subjects, an increase in the IAP gene expression level relative to those of the control
25 subjects indicates that the patient is suffering from the MS subtype. The increased gene expression level includes increased levels of HIAP-1 mRNA or HIAP-2 mRNA. The increased levels of only HIAP-1 mRNA indicate that the patient is suffering from quiescent relapsing remitting MS. The increased levels of HIAP-1 mRNA and HIAP-2 mRNA levels indicate that the patient is suffering from active relapsing remitting MS. The increased
30 levels of only HIAP-2 mRNA indicate that the patient is suffering from secondary progressive MS. The increased gene expression level includes increased levels of survivin mRNA. The increased level of survivin mRNA indicates that the patient is suffering from primary progressive MS. The control subjects are those having benign MS, or are healthy normal subjects. The level of HIAP-2 expression correlates to the degree of disability
35 measured by EDSS scores.

In another aspect, the blood sample is whole blood. The IAP gene expression is measured in the whole blood. The IAP gene expression level is compared to those of control subjects, an increase in the IAP gene expression level relative to those of the control subjects indicates that the patient is suffering from the MS subtype. The increased gene expression level includes increased levels of NAIP mRNA in patients who are suffering from relapsing remitting MS, quiescent relapsing remitting MS, primary progressive MS and secondary progressive MS. The control subjects are those having benign MS or are healthy normal subjects.

10 According to another embodiment of the present invention, there is provided a method for differentiating active relapsing remitting multiple sclerosis in a patient, the method comprising:

a) determining an amount of XIAP and HIAP-2 gene expression levels in peripheral blood mononuclear (PBMN) cells obtained from the patient; and

15 b) comparing the amount of the XIAP and HIAP-2 gene expression levels in the PBMNs with the XIAP and HIAP-2 gene expression levels of control subjects, an increase in the XIAP and HIAP-2 gene expression levels being an indication that the patient is suffering from active relapsing remitting multiple sclerosis.

20 According to another embodiment of the present invention, there is provided a method for differentiating secondary progressive multiple sclerosis in a patient, the method comprising:

a) determining an amount of XIAP and HIAP-2 gene expression levels in peripheral blood mononuclear (PBMN) cells obtained from the patient; and

25 b) comparing the amount of the XIAP and HIAP-2 gene expression levels in the PBMNs with the XIAP and HIAP-2 gene expression levels of control subjects, an increase in the XIAP and HIAP-2 gene expression levels being an indication that the patient is suffering from secondary progressive multiple sclerosis.

30 According to another embodiment of the present invention, there is provided a method for differentiating quiescent relapsing remitting multiple sclerosis in a patient, the method comprising:

a) determining an amount of HIAP-1 gene expression level in T cells obtained from the patient; and

b) comparing the amount of the HIAP-1 gene expression level in the T cells with the HIAP-1 gene expression levels of control subjects, an increase in the HIAP-1 gene expression levels being an indication that the patient is suffering from quiescent relapsing remitting multiple sclerosis.

5

According to another embodiment of the present invention, there is provided a method for differentiating active relapsing remitting multiple sclerosis in a patient, the method comprising:

a) determining an amount of HIAP-1 and HIAP-2 gene expression levels in T cells
10 obtained from the patient; and

b) comparing the amount of the HIAP-1 and HIAP-2 gene expression levels in the T cells with the HIAP-1 and HIAP-2 gene expression levels of control subjects, an increase in the HIAP-1 and HIAP-2 gene expression levels being an indication that the patient is suffering from active relapsing remitting multiple sclerosis.

15

According to an alternative embodiment of the present invention, there is provided a method for differentiating between quiescent and active relapsing remitting multiple sclerosis in a patient, the method comprising:

a) determining an amount of HIAP-1 and HIAP-2 gene expression levels in T cells
20 obtained from the patient; and

b) comparing the amount of the HIAP-1 and HIAP-2 gene expression levels in the T cells with the HIAP-1 and HIAP-2 gene expression levels of control subjects, either an increase in only HIAP-1 gene expression levels being an indication that the patient is suffering from quiescent relapsing remitting multiple sclerosis, or an increase in both .the
25 HIAP-1 and HIAP-2 gene expression levels being an indication that the patient is suffering from active relapsing remitting multiple sclerosis.

According to another alternative embodiment of the present invention, there is provided a method for differentiating secondary progressive multiple sclerosis in a patient, the
30 method comprising:

a) determining an amount of HIAP-2 gene expression level in T cells obtained from the patient; and

b) comparing the amount of the HIAP-2 gene expression level in the T cells with the HIAP-2 gene expression levels of control subjects, an increase in the HIAP-2 gene

expression levels being an indication that the patient is suffering from secondary progressive multiple sclerosis.

According to another embodiment of the present invention, there is provided a method for
5 differentiating primary progressive multiple sclerosis in a patient, the method comprising:

a) determining an amount of survivin gene expression level in T cells obtained from the patient; and

b) comparing the amount of the survivin gene expression level in the T cells with the survivin gene expression levels of control subjects, an increase in the survivin gene
10 expression levels being an indication that the patient is suffering from primary progressive multiple sclerosis.

According to another embodiment of the present invention, there is provided a method for differentiating between benign and either relapsing remitting MS, primary progressive MS
15 or secondary progressive MS in a patient, the method comprising:

a) determining an amount of NAIP gene expression levels in whole blood obtained from the patient; and

b) comparing the amount of NAIP gene expression levels with the amount of NAIP gene expression levels in control subjects, an increase in the NAIP gene expression levels
20 being an indication that the patient is suffering from either relapsing remitting MS, primary progressive MS or secondary progressive MS and not benign MS.

According to another embodiment of the present invention, there is provided a method of testing a patient's suitability for interferon- β treatment of multiple sclerosis, the method
25 comprising:

a) determining an amount of an IAP gene expression level in a blood sample obtained from the patient suspected of having an aggressive subtype of multiple sclerosis; and

b) comparing the amount of the IAP gene expression level to the IAP gene
30 expression levels of control subjects, an increase in IAP gene expression levels being an indication that the patient is suffering from an aggressive subtype of multiple sclerosis, the patient being suitable for treatment with interferon- β .

According to another embodiment of the present invention, there is provided a method of identifying whether a patient is at risk of developing secondary progressive multiple sclerosis from active relapsing remitting multiple sclerosis, the method comprising:

- a) obtaining a blood sample for the patient; and
- 5 b) comparing XIAP mRNA levels in PBMNs isolated from the sample, and HIAP-1 and HIAP-2 mRNA levels in T cells isolated from the sample, with those of a control subject, an increase in the XIAP mRNA levels in the PBMNs and an increase the HIAP-2 mRNA levels in the T cells, and normal HIAP-1 levels in the T cells, being an indication that the patient is at risk of developing secondary progressive MS. The method further
- 10 including detecting a normalization of HIAP-1 in T cells.

According to an alternative embodiment of the present invention, there is provided use of IAP mRNA expression or IAP protein expression as a biomarker for the differentiation of multiple sclerosis subtypes in a subject, an increased level of expression compared to

15 control subjects being an indication that the patient has a multiple sclerosis subtype.

According to another embodiment of the present invention, there is provided a method for monitoring the progress of a multiple sclerosis therapy of a patient, the method comprising:

- 20 a) determining an amount of an IAP gene expression level in a first blood sample obtained from the patient at first time period;
- b) determining an amount of the IAP gene expression level in a second blood sample obtained from the patient at a second time period; and
- b) comparing in the IAP gene expression levels, a decrease in the IAP gene
- 25 expression level at the second time period being an indication that the patient is responding to the multiple sclerosis therapy.

According to an alternative embodiment of the present invention, there is provided a diagnostic kit for differentiating a patient suspected of having a subtype of multiple

30 sclerosis, the kit comprising:

- a) a vessel or vessels for receiving a blood sample from the subject;
- b) an agent that specifically detects IAP protein or amplifies IAP mRNA; and
- c) printed instructions for detecting the IAP protein or the amplified IAP mRNA in
- the sample.

35

In one aspect, typically, the agent for amplifying the IAP mRNA are the primers and probes selected from Table 2. The IAP protein is detected using immunoassays. The immunoassay is an ELISA.

5 According to another embodiment of the present invention, there is provided a method of differentiating between multiple sclerosis subtypes in a patient blood sample, the method comprising:

a) determining an amount of an IAP gene expression level in the blood sample;
and

10 b) correlating the amount of the IAP gene expression level in the blood sample with the presence of a multiple sclerosis subtype.

According to an alternative embodiment of the present invention, there is provided a method for the differentiation of a multiple sclerosis subtype in a subject, the method
15 comprising:

a) obtaining a peripheral blood sample from the subject; and

b) detecting an increased level of IAP mRNA in the sample compared to the level in a healthy subject, wherein the increased level of the IAP mRNA is diagnostic of the multiple sclerosis subtype in the subject.

20

Examples of IAPs that are useful in practicing the methods of the invention include, but are not limited to, human and mouse survivin, NAIP, XIAP, HIAP-1 (cIAP2), and HIAP-2 (cIAP1), which are disclosed United States patent numbers 5,919,912; 6,156,535; 6,656,704; 6,020,127; 6,656,684; 6,429,011; 6,994,957; US2002/0137028.

25

BRIEF DESCRIPTION OF THE DRAWINGS

Further aspects and advantages of the present invention will become better understood with reference to the description in association with the following Figures, wherein:

30 **Figures 1A to 1C** are graphs illustrating the relative quantification of XIAP mRNA expression in RNA extracted from (A) whole blood, (B) PBMC cells and (C) T cells employing qRT-PCR. The graphs depict the expression of XIAP mRNA relative to the expression of the endogenous control gene β_2 microglobulin ($2^{-\Delta\Delta CT}$) in normal subjects (NS) and patients with benign, Relapsing-Remitting (RR), Secondary-Progressive (SP) or
35 Primary-Progressive (PP) MS patients. Quiescent (Q)=MS symptoms in remission at time

of blood draw and the patient has not experienced any relapses in the 2 years prior to blood draw; Active (A) = MS symptoms are active at time of blood draw and the patient has experienced at least one relapse in the 2 years prior to blood draw. (A) XIAP gene expression in whole blood was not affected in MS relative to NS. (B) XIAP gene

5 expression is increased in PBMN cell RNA of RRA and SPMS patients relative to NS.

Note also that XIAP mRNA levels are higher in patients whose symptoms are active (RRA) rather than quiescent (RRQ). (C) XIAP gene expression in T cells is similar among NS and all subtypes of MS patients. * $p < .05$.

10 **Figures 2A to 2C** are graphs illustrating the relative quantification of HIAP-1 mRNA expression in RNA extracted from (A) whole blood, (B) PBMN cells and (C) T cells employing qRT-PCR. The graphs depict the expression of HIAP-1 mRNA relative to the expression of the endogenous control gene β_2 microglobulin ($2^{-\Delta\Delta CT}$) in normal subjects (NS) and patients with benign, Relapsing-Remitting (RR), Secondary-Progressive (SP) or

15 Primary-Progressive (PP) MS patients. Quiescent (Q)=MS symptoms in remission at time of blood draw and the patient has not experienced any relapses in the 2 years prior to blood draw; Active (A) = MS symptoms are active at time of blood draw and the patient has experienced at least one relapse in the 2 years prior to blood draw. (A) HIAP-1 gene expression in whole blood was not affected in MS relative to NS. (B) There appears to be

20 no change in HIAP-1 gene expression in PBMN cells of MS patients relative to NS. (C) In contrast to XIAP gene expression levels in T cells, HIAP-1 gene expression is increased in T cells of RRMS patients irrespective of disease activity (RRQ and RRA) relative to NS and patients with benign and PPMS. * $p < .05$.

25 **Figures 3A to 3C** are graphs illustrating the relative quantification of HIAP-2 mRNA expression in RNA extracted from (A) whole blood, (B) PBMN cells and (C) T cells employing qRT-PCR. The graphs depict the expression of HIAP-2 mRNA relative to the expression of the endogenous control gene β_2 microglobulin ($2^{-\Delta\Delta CT}$) in normal subjects (NS) and patients with benign, Relapsing-Remitting (RR), Secondary-Progressive (SP) or

30 Primary-Progressive (PP) MS patients. Quiescent (Q)=MS symptoms in remission at time of blood draw and the patient has not experienced any relapses in the 2 years prior to blood draw; Active (A) = MS symptoms are active at time of blood draw and the patient has experienced at least one relapse in the 2 years prior to blood draw. (A) HIAP-2 gene expression in whole blood was not affected in MS relative to NS. (B) HIAP-2 gene

35 expression is increased in PBMN cell RNA of RRA and SPMS patients relative to NS. (C)

The increased HIAP-2 expression in PBMN cells is most likely due to increased HIAP-2 T cell gene expression. HIAP-2 is increased in T cells of RRA and SPMS patients relative to NS. * $p < .05$.

- 5 **Figures 4A to 4C** are graphs illustrating the relative quantification of survivin mRNA expression in RNA extracted from (A) whole blood, (B) PBMN cells and (C) T cells employing qRT-PCR. The graphs depict the expression of survivin mRNA relative to the expression of the endogenous control gene β_2 microglobulin ($2^{-\Delta\Delta CT}$) in normal subjects (NS) and patients with benign, Relapsing-Remitting (RR), Secondary-Progressive (SP) or
- 10 Primary-Progressive (PP) MS patients. Quiescent (Q)=MS symptoms in remission at time of blood draw and the patient has not experienced any relapses in the 2 years prior to blood draw; Active (A) = MS symptoms are active at time of blood draw and the patient has experienced at least one relapse in the 2 years prior to blood draw. (A) Survivin gene expression in whole blood was not affected in MS relative to NS. (B) Survivin gene
- 15 expression was not affected in PBMN cells of MS patients relative to NS. (C) Survivin gene expression was increased in T cells of patients with PPMS relative to NS and all other MS groups.

- Figures 5A and 5B** illustrate the IAP protein levels in patients with different types of MS and normal control subjects. A. XIAP protein levels in patients with different types of MS and normal control subjects. In PBMN cells there was a difference in XIAP protein levels between the various groups with increased levels of protein detected in RRA and SP MS patients relative to all other groups. These findings were matched by a concordant increase in XIAP PBMN cell mRNA levels in patients with RRA and SP MS relative to all
- 20 other groups (see Figure 1B). B. Optical density analysis confirmed the increase of XIAP protein in patients with RRA and SPMS (* $p < 0.05$).
- 25

- Figures 6A to 6C** illustrate the HIAP-1 protein levels in patients with different types of MS and normal control subjects. (A) HIAP-1 protein levels in T cells from patients with RR, SP and PP MS appeared to be increased relative to subjects with benign MS and normal control subjects. (B) The variability of HIAP-1 protein expression in PPMS patients was reminiscent of the changes observed in mRNA (see Figure 2C). (C) Optical density analysis confirmed the increase of HIAP-1 in RRMS relative to normal subjects, benign patients and patients with either SPMS or PPMS (* $p < 0.05$).
- 30

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Figures 7A to 7C illustrates the relative quantification of NAIP mRNA expression in RNA extracted from (A) whole blood, (B) PBMN cells and (C) T cells employing qRT-PCR. This graph depicts the expression of NAIP mRNA relative to the expression of the endogenous control gene β_2 microglobulin ($2^{-\Delta\Delta CT}$) in normal subjects (NS) and patients with benign, Relapsing-Remitting (RR), Secondary-Progressive (SP) or Primary-Progressive (PP) MS patients. Quiescent (Q)=MS symptoms in remission at time of blood draw and the patient has not experienced any relapses in the 2 years prior to blood draw; Active (A) = MS symptoms are active at time of blood draw and the patient has experienced at least one relapse in the 2 years prior to blood draw. (A) NAIP gene expression in whole blood was increased in RRQ, RRA and SPMS relative to normal subjects. (B) NAIP gene expression was not affected in PBMN cells of MS patients relative to normal control subjects. (C) NAIP gene expression appeared to be increased in a subset of PPMS patients relative to normal control subjects ($P>.05$).

DETAILED DESCRIPTION OF THE INVENTION

Definitions

Unless otherwise stated, the following terms apply:

The singular forms “a”, “an” and “the” include corresponding plural references unless the context clearly dictates otherwise.

As used herein, the term “comprising” is intended to mean that the list of elements following the word “comprising” are required or mandatory but that other elements are optional and may or may not be present.

As used herein, the term “consisting of” is intended to mean including and limited to whatever follows the phrase “consisting of”. Thus the phrase “consisting of” indicates that the listed elements are required or mandatory and that no other elements may be present.

As used herein the terms “apoptosis” is intended to mean the process of cell death in which a dying cell displays a set of well-characterized biochemical indicia that include cell membrane blebbing, cell soma shrinkage, chromatin condensation, and DNA laddering.

As used herein, the term “cell” is intended to mean a single-cellular organism, a cell from a multi-cellular organism or it may be a cell contained in a multi-cellular organism.

As used herein, the term "subject" or "patient" is intended to mean humans and non-human mammals such as primates, cats, dogs, swine, cattle, sheep, goats, horses, rabbits, rats, mice and the like. In one example, the subject is a human.

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As used herein, the term "IAP gene" is intended to mean a gene encoding a polypeptide having at least one BIR domain and which is capable of modulating (inhibiting or enhancing) apoptosis in a cell or tissue. The IAP gene is a gene having about 50% or greater nucleotide sequence identity to at least one of survivin, NAIP, HIAP-1 (cIAP2), HIAP-2 (cIAP1), and XIAP. The region of sequence over which identity is measured is a region encoding at least one BIR domain and a ring zinc finger domain. Mammalian IAP genes include nucleotide sequences isolated from any mammalian source. In one example, the mammal is a human.

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As used herein, the term "protein", "polypeptide" or "polypeptide fragment" is intended to mean any chain of more than two amino acids, regardless of post-translational modification, for example, glycosylation or phosphorylation, constituting all or part of a naturally occurring polypeptide or peptide, or constituting a non-naturally occurring polypeptide or peptide.

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As used herein, the term "IAP protein" or "IAP polypeptide" is intended to mean a polypeptide or protein, or fragment thereof, encoded by an IAP gene. Examples of IAP polypeptides include, but are not limited, to survivin, NAIP, HIAP-1 (cIAP2), HIAP-2 (cIAP1), and XIAP.

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As used herein, the term "blood sample" is intended to mean whole blood taken from the periphery of the subject from which immune cells, for example, peripheral blood mononuclear (PBMN) cells; and T cells can be isolated.

As used herein, the term "IAP mRNA expression" is intended to mean expression of IAP genes which encode survivin, NAIP, XIAP, HIAP-1 and HIAP-2.

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As used herein the term "IAP gene expression level" is intended to mean a measurable amount of the IAP gene expressed in a PBMN or a T cell, such as for example, an amount of IAP mRNA or an amount of an IAP protein.

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As used herein, the term "biomarker" is intended to mean a detectable level of either an IAP mRNA or an IAP protein in such a pattern characterizing a specific MS subtype.

5 As used herein, the term "multiple sclerosis subtype" is intended to mean one or more of four categories of multiple sclerosis and includes relapsing-remitting (RRMS), secondary-progressive (SPMS), primary progressive (PPMS) and benign. The term "relapsing-remitting multiple sclerosis" can be further categorized into quiescent relapsing-remitting multiple sclerosis (RRQ) and active relapsing-remitting multiple sclerosis (RRA).

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As used herein, the term "aggressive form of multiple sclerosis" is intended to mean RRA and SPMS.

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As used herein, the term "control subjects" is intended to mean subjects who are healthy normal, or who have been diagnosed as having benign MS.

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We have unexpectedly discovered distinct patterns of IAP mRNA and IAP protein level expression in whole blood, peripheral blood mononuclear cells (PBMN) and resting T cells from healthy subjects and patients with benign MS, quiescent RRMS (RRQ), active RRMS (RRA), SPMS or PPMS. Taken together, these results demonstrate that patterns of IAP expression in whole blood, PBMN and resting T cells can be used to differentiate between, and are diagnostic of, sub-types of MS. Thus, we can now design new (non-MRI based) diagnostic tests for MS based on the detection of the IAP gene expression levels and that these genes may serve as surrogate biomarkers for therapeutics based on the treatment of MS by the induction of apoptosis in auto-reactive immune cells.

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Accordingly in an embodiment of the present invention, there is provided a method for differentiating between multiple sclerosis subtypes in a patient, the method comprising: a) determining an amount of an IAP gene expression level in a blood sample obtained from the patient; and b) correlating the amount of the IAP gene expression level in the blood sample with the presence of a multiple sclerosis subtype in the patient. For example, the IAP gene expression level is compared to those of control subjects in which an increase in the IAP gene expression level relative to those of the control subjects indicates that the patient is suffering from one of the MS subtypes. The MS subtypes include benign MS, quiescent relapsing remitting MS, active relapsing remitting MS, primary progressive MS

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or secondary progressive MS. The control subjects are those subjects who have been previously diagnosed as having benign MS or are healthy normal subjects.

The invention provides for quantitative detection and determination of the IAP gene
5 expression levels by measuring either the levels of transcribed IAP mRNA or the level of
IAP protein in the blood sample. One skilled in the art will recognize that many techniques
are available to measure the levels of the aforesaid IAP gene expression levels. The IAP
genes encode an IAP protein selected from NAIP, XIAP, HIAP-1, HIAP-2 or survivin. In
one example, IAP mRNA is measured using quantitative real time polymerase chain
10 reaction (qRT-PCR), which is described in more detail below, whereas IAP protein level is
measured using an immunoassay.

Immunoassays for example include, but are not limited to, competitive and non-
competitive assay systems using techniques such as western blots, radioimmunoassays,
15 ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays,
immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions,
immunodiffusion assays, fluorescent immunoassays and the like. Such assays are routine
and well known in the art (see, e.g., Ausubel et al, eds, 1994, Current Protocols in
Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York, which is incorporated by
20 reference herein in its entirety). Exemplary immunoassays are described briefly below (but
are not intended by way of limitation).

Immunoprecipitation protocols generally comprise lysing a population of cells in a lysis
buffer such as RIPA buffer (1% NP-40 or Triton X-100, 1% sodium deoxycholate, 0.1%
25 SDS, 0.15 M NaCl, 0.01 M sodium phosphate at pH 7.2, 1% Trasyolol) supplemented with
protein phosphatase and/or protease inhibitors (e.g., EDTA, PMSF, aprotinin, sodium
vanadate), adding an antibody of interest to the cell lysate, incubating for a period of time
(e.g., 1-4 hours) at 4degreesC., adding protein A and/or protein G sepharose beads to the
cell lysate, incubating for about an hour or more at 4 degrees C., washing the beads in
30 lysis buffer and re-suspending the beads in SDS/sample buffer. The ability of the antibody
to immunoprecipitate a particular antigen can be assessed by, e.g., western blot analysis.
One skilled in the art would be knowledgeable as to the parameters that can be modified
to increase the binding of the antibody to an antigen and decrease the background (e.g.,
pre-clearing the cell lysate with sepharose beads). For further discussion regarding
35 immunoprecipitation protocols see, e.g., Ausubel et al, eds, 1994, Current Protocols in

Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York at 10.16.1.

Western blot analysis generally comprises preparing protein samples, electrophoresis of the protein samples in a polyacrylamide gel (e.g., 8%-20% SDS-PAGE depending on the molecular weight of the antigen), transferring the protein sample from the polyacrylamide gel to a membrane such as nitrocellulose, PVDF or nylon, blocking the membrane in blocking solution (e.g., PBS with 3% BSA or non-fat milk), washing the membrane in washing buffer (e.g., PBS-Tween 20), blocking the membrane with primary antibody (the antibody of interest) diluted in blocking buffer, washing the membrane in washing buffer, blocking the membrane with a secondary antibody (which recognizes the primary antibody, e.g., an anti-human antibody) conjugated to an enzymatic substrate (e.g., horseradish peroxidase or alkaline phosphatase) or radioactive molecule (e.g., ³²P or ¹²⁵I) diluted in blocking buffer, washing the membrane in wash buffer, and detecting the presence of the antigen. One skilled in the art would be knowledgeable as to the parameters that can be modified to increase the signal detected and to reduce the background noise. For further discussion regarding western blot protocols see, e.g., Ausubel et al, eds, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York at 10.8.1.

ELISAs comprise preparing antigen (i.e. an IAP polypeptide biomarker), coating the well of a 96 well microtiter plate with the antigen, adding the antibody of interest conjugated to a detectable compound such as an enzymatic substrate (e.g., horseradish peroxidase or alkaline phosphatase) to the well and incubating for a period of time, and detecting the presence of the antigen. In ELISAs the antibody of interest does not have to be conjugated to a detectable compound; instead, a second antibody (which recognizes the antibody of interest) conjugated to a detectable compound may be added to the well. Further, instead of coating the well with the antigen, the antibody may be coated to the well. In this case, a second antibody conjugated to a detectable compound may be added following the addition of the antigen of interest to the coated well. One skilled in the art would be knowledgeable as to the parameters that can be modified to increase the signal detected as well as other variations of ELISAs known in the art. For further discussion regarding ELISAs see, e.g., Ausubel et al, eds, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York at 11.2.1.

In one example, peripheral blood mononuclear (PBMN) cells and T cells are typically isolated from the blood samples of the patients and are analyzed for IAP gene expression levels as is described in more detail below. The IAP gene expression levels are compared to those of the control subjects, an increase in the IAP gene expression level relative to those of the control subjects indicates that the patient is suffering from one of the MS subtypes described above.

Detection of certain IAP gene biomarkers are diagnostic of the specific subtype of MS. For example, in MS patients with RRA (but not RRQ) and SPMS, XIAP mRNA and protein levels were selectively elevated in PBMN cells relative to normal control subjects or patients with benign or PPMS. HIAP-2 mRNA was elevated in PBMN cells of patients with RRA and SPMS relative to normal control subjects and patients with a benign MS disease course. In T cells, HIAP-2 mRNA levels were elevated in patients with RRMS irrespective of disease activity and SPMS relative to normal control subjects. The expression of HIAP-1 was selectively elevated in resting T cells from RRMS patients irrespective of disease activity relative to all other experimental groups. In PPMS patients, survivin expression was elevated in T cells relative to normal control subjects, RRMS and SPMS patients.

Distinguishing between specific MS subtypes

As illustrated in Table 3 below, the diagnostic methods of the invention can be used to differentiate between specific MS subtypes. For example, in one alternative aspect of the invention, there is provided a method for distinguishing between quiescent and active forms of relapsing remitting multiple sclerosis in a patient. The method comprises determining the amount of XIAP and HIAP-2 gene expression levels in peripheral blood mononuclear (PBMN) cells obtained from the patient. The amount of XIAP and HIAP-2 gene expression levels are compared to XIAP and HIAP-2 gene expression levels of control subjects, an increase in the gene expression levels coupled with clinical presentation being an indication that the patient is suffering from active relapsing remitting multiple sclerosis. In addition, there is also provided a method for distinguishing secondary progressive multiple sclerosis in a patient. In this case, the method comprises determining an amount of XIAP and HIAP-2 gene expression levels in peripheral blood mononuclear (PBMN) cells obtained from the patient. The amount of the XIAP and HIAP-2 gene expression levels are compared to these gene expression levels of control subjects, an increase in the XIAP and HIAP-2 gene expression levels being an indication that the patient is suffering from secondary progressive multiple sclerosis.

In another alternative aspect, there is provided a method for differentiating quiescent relapsing remitting multiple sclerosis in a patient. In this case, the method comprises determining an amount of HIAP-1 gene expression level is determined in T cells obtained
5 from the patient. The amount of the HIAP-1 gene expression level in the T cells is compared to the HIAP-1 gene expression levels of control subjects, an increase in the HIAP-1 gene expression levels being an indication that the patient is suffering from quiescent relapsing remitting multiple sclerosis.

10 In addition, there is provided a method for differentiating active relapsing remitting multiple sclerosis in a patient. In this case, the method comprises determining an amount of HIAP-1 and HIAP-2 gene expression levels in T cells obtained from the patient. The amount of the HIAP-1 and HIAP-2 gene expression levels in the T cells is compared to the HIAP-1 and HIAP-2 gene expression levels of control subjects, an increase in the HIAP-1 and
15 HIAP-2 gene expression levels being an indication that the patient is suffering from active relapsing remitting multiple sclerosis.

In certain circumstances, where a clinician suspects that a patient has RRMS, but is uncertain as to whether the RRMS is quiescent or active, there is provided a method for
20 differentiating between quiescent and active relapsing remitting multiple sclerosis in a patient. This method comprises determining an amount of HIAP-1 and HIAP-2 gene expression levels in T cells obtained from the patient. The amount of the HIAP-1 and HIAP-2 gene expression levels in the T cells is compared to the HIAP-1 and HIAP-2 gene expression levels of control subjects. An increase in only HIAP-1 gene expression levels
25 is an indication that the patient is suffering from quiescent relapsing remitting multiple sclerosis, whereas an increase in both the HIAP-1 and HIAP-2 gene expression levels is an indication that the patient is suffering from active relapsing remitting multiple sclerosis.

In another alternative aspect of the present invention, there is provided a method for
30 differentiating secondary progressive multiple sclerosis in a patient. In this case, the method comprises determining an amount of HIAP-2 gene expression level in T cells obtained from the patient. The amount of the HIAP-2 gene expression level in the T cells is compared to the HIAP-2 gene expression levels of control subjects, an increase in the HIAP-2 gene expression levels being an indication that the patient is suffering from
35 secondary progressive multiple sclerosis.

In another alternative aspect of the present invention, there is provided a method for differentiating primary progressive multiple sclerosis in a patient. In this case, the method comprises determining an amount of survivin gene expression level in T cells obtained
5 from the patient. The amount of the survivin gene expression level in the T cells is compared to the survivin gene expression levels of control subjects, an increase in the survivin gene expression levels being an indication that the patient is suffering from primary progressive multiple sclerosis.

10 Currently, benign MS cannot be distinguished from other types of MS, including relapsing remitting MS relapsing remitting MS, primary progressive MS or secondary progressive MS. In patients who first present themselves to a clinician, no diagnostic tests are available to distinguish the benign MS from quiescent relapsing remitting MS. It would
15 therefore be advantageous to distinguish between benign MS and the other types of MS to prevent un-needed treatment of the benign MS patients with interferon drugs. We have shown that NAIP expression is the only biomarker in which there is an increase in whole blood. RRQ, RRA and SPMS relative to normal healthy subjects. There was no difference in benign or PPMS whole blood NAIP. In T cells, a subset of PPMS patients showed an increase in NAIP mRNA expression.

20 Thus, according to another aspect of the present invention, there is provided a method for differentiating between benign and either relapsing remitting MS, primary progressive MS or secondary progressive MS in a patient. In this case, the method comprises determining an amount of NAIP gene expression levels in whole blood obtained from the patient. The NAIP gene expression levels are then compared to the amount of NAIP gene expression
25 levels in control subjects, an increase in the NAIP gene expression levels being an indication that the patient is suffering from either relapsing remitting MS, primary progressive MS or secondary progressive MS and not benign MS.

Determination of a patient's suitability for interferon drug treatment

30 One advantage of the methods of the present invention concern a clinician's ability to appropriately treat MS sufferers with interferon drugs, such as Rebif 22, Rebif 44, Avonex, and Betaseron. In instances where such treatment is inappropriate, such as in patients suffering from benign MS or PPMS, the clinician may now first screen patients to eliminate those for which treatment would be inappropriate. Thus, according to an alternative
35 aspect of the present invention, there is provided a method of testing a patient's suitability

for interferon- β treatment of multiple sclerosis. This method comprises determining an amount of an IAP gene expression level in a blood sample obtained from the patient suspected of having an aggressive subtype of multiple sclerosis. The amount of the IAP gene expression level can then be compared to the IAP gene expression levels of control subjects, an increase in IAP gene expression levels being an indication that the patient is suffering from an aggressive subtype of multiple sclerosis, the patient being suitable for treatment with interferon- β .

For those patients for which interferon therapy is appropriate, one aspect of the invention would be a method for monitoring the progress of a multiple sclerosis therapy of a patient. This method comprises determining an amount of an IAP gene expression level in a first blood sample obtained from the patient at first time period, followed by determining an amount of the IAP gene expression level in a second blood sample obtained from the patient at a second time period. The difference between the two time periods who be determined by the type of therapy used by the clinician and how quickly the patient is progressing. The IAP gene expression levels would be compared and a decrease in the IAP gene expression level at the second time period being an indication that the patient is responding to the multiple sclerosis therapy. The therapy could be continued until the IAP gene expression is significantly diminished or essentially eliminated.

For those patients who present clinical symptoms of active relapsing remitting MS, it is advantageous to determine whether the patient is at risk of developing secondary progressive MS. Thus, another alternative embodiment of the present invention provides a method of identifying whether a patient is at risk of developing secondary progressive multiple sclerosis from active relapsing remitting multiple sclerosis, the method comprising: a) obtaining a blood sample for the patient; and b) comparing XIAP mRNA levels in PBMNs, and HIAP-1 and HIAP-2 mRNA levels in T cells, with those of a control subject, elevated XIAP mRNA levels in the PBMNs, elevated HIAP-2 mRNA levels in the T cells, and normal HIAP-1 levels in the T cells, being an indication that the patient is at risk of developing secondary progressive MS.

Furthermore, the aforesaid alternative method may further include detecting a normalization of HIAP-1 in T cells. Such a change would suggest initiation of more aggressive treatments such as Tysabri®.

II: Diagnostic kits

Generally, speaking a clinician's office may be adapted to aid quick and reliable diagnosis of a patient suspected of having one of the above mentioned subtypes of MS. Thus, according to one embodiment of the invention, there is provided a diagnostic kit for diagnosing a patient suspected of having a subtype of multiple sclerosis. The kit
5 comprises a vessel or vessels for receiving a blood sample taken from the subject, an agent that specifically detects IAP protein or amplifies IAP mRNA; and printed instructions for detecting the IAP protein or the amplified IAP mRNA in the sample.

For example, the kits can be used to detect any one or more of the IAP gene expression
10 levels, such as IAP mRNA or IAP polypeptide described herein, which biomarkers are differentially present in samples of a patient and normal subjects. The kits of the invention have many applications. For example, the kits can be used to differentiate if a subject has benign MS, quiescent or active relapsing remitting MS, primary progressive MS or secondary progressive MS. In another example, the kits can be used to identify
15 compounds that modulate expression of one or more of the markers in in vitro or in vivo animal models to determine the effects of treatment.

The IAP protein is detected using immunoassays as described above.

In one example, a kit comprises (a) an antibody that specifically binds to an IAP
20 polypeptide; and (b) a detection agent. Such kits can be prepared from the materials described in US patent number 5,919,912.

In the case of IAP mRNA, the agent for amplifying the IAP mRNA are the primers and probes selected from Table 2 below. In some instances, the kit may further comprise
25 instructions for suitable operation parameters in the form of a label or a separate insert.

The invention also includes a diagnostic kit which includes a substantially isolated antibody specifically immunoreactive with IAP polypeptide antigens, and means for detecting the binding of the polypeptide antigen to the antibody. In example, the antibody
30 is attached to a solid support. In a specific example, the antibody may be a monoclonal antibody. The detecting means of the kit may include a second, labeled monoclonal antibody. Alternatively, or in addition, the detecting means may include a labeled, competing antigen.

35 Optionally, the kit may further comprise a standard or control information so that a test

sample can be compared with a control information standard to determine if the test amount of a marker detected in a sample is indicative of a MS subtype in a patient clinically diagnosed with MS.

- 5 Optionally, the kit can further comprise instructions for suitable operational parameters in the form of a label or a separate insert. For example, the kit may have standard instructions informing a consumer how to wash a probe after a sample is contacted on the probe. In another example, the kit may have instructions for pre-fractionating a sample to reduce complexity of IAP proteins in the sample. In another example, the kit may have
- 10 instructions for automating the fractionation or other processes.

The methods described above and below may also be carried out on patient blood samples, which may have been obtained and stored according to methods known to those skilled in the art of blood sample handling and storage. Thus it is within the scope

15 of the present invention to provide a method of differentiating between multiple sclerosis subtypes in a patient blood sample. In this case, the method comprises determining an amount of an IAP gene expression level in the blood sample, as described above, and then correlating the amount of the IAP gene expression level in the blood sample with the presence of a multiple sclerosis subtype.

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Materials and Methods

The following examples are offered by way of illustration, not by way of limitation. While specific examples have been provided, the above description is illustrative and not restrictive. Any one or more of the features of the previously described embodiments can

25 be combined in any manner with one or more features of any other embodiments in the present invention. Furthermore, many variations of the invention will become apparent to those skilled in the art upon review of the specification. The scope of the invention should, therefore, be determined not with reference to the above description, but instead should be determined with reference to the appended claims along with their full scope of

30 equivalents.

Unless otherwise stated, the following abbreviations are used throughout:

CNS=central nervous system;

35 CSF= cerebral spinal fluid;

EDSS= Expanded Disability Status Scale;

HIAP-1= human inhibitor of apoptosis protein-1;

HIAP-2= human inhibitor of apoptosis protein-2;

IAP = inhibitor of apoptosis protein;

5 MRI= Magnetic Resonance Imaging;

MS= multiple sclerosis;

NAIP = neuronal apoptosis inhibitor protein;

PBMN cells = peripheral blood mononuclear cells;

RR= relapsing-remitting;

10 RRQ= relapsing-remitting quiescent disease activity;

RRA= relapsing-remitting active
disease;

RRMS= relapsing remitting Multiple Sclerosis,

SP= secondary-progressive;

15 SPMS= secondary-progressive Multiple Sclerosis;

PP= primary progressive;

PPMS=primary-progressive Multiple Sclerosis;

qRT-PCR= quantitative reverse transcriptase polymerase chain reaction; and

XIAP= X-linked inhibitor of apoptosis protein.

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1. Patient Selection and Blood Sampling

Qualified clinical personnel obtained blood samples from MS patients with Benign (n=12),
RRMS (n=24), SPMS (n=22) and PPMS (n=19) during the patients regularly scheduled
25 visit to the Dalhousie Multiple Sclerosis Research Unit (DMSRU). Research and clinical
personnel recruited 14 healthy volunteer subjects from the community. All subjects gave
informed consent prior to blood draw. A practicing neurologist with a special interest in MS
(VB) at the DMSRU prior to study entry diagnosed all patients with definite MS according
to established diagnostic criteria [McDonald *et al.* 2001] and determined each individual
30 patient's level of disability and disease progression employing the EDSS. Benign MS was
characterized in patients that had disease duration of more than 10 years and an EDSS of
2.0 or less or in patients with disease duration of greater than 15 years and an EDSS 3.0
or less. Relapsing-remitting MS quiescent disease state (RRQ) was characterized in
patients that did not experience any relapses within the last 2 years and did not
35 experience EDSS progression over the same time period. Relapsing-remitting MS active

disease state (RRA) was characterized in patients that had experienced 1 or more relapses in the last year or gadolinium positive (Gd⁺) lesions on recent MRI. Patients were diagnosed as having secondary-progressive MS (SPMS) following a previous diagnosis of RRMS but were currently experiencing progression of disability with or without concurrent relapses. In comparison to RRMS most SPMS patients have an EDSS greater than 4 and often greater than 6 as compared to the usual EDSS course associated with RRMS. Primary progressive MS (PPMS) was diagnosed in patients that displayed a progressive, non-phasic neurological syndrome. Most patients presented as a spinal cord syndrome or a cerebellar dysfunction coupled with Gd⁺ lesions disseminated in space and time by MRI criteria with continued clinical progression for at least one year, according to the revised McDonald diagnostic criteria [Polman *et al.* 2005]. Patients and normal control subjects were included if they were 18 years of age or older, had no other major medical illness and gave written informed consent. Subjects did not receive corticosteroid or immune suppressive therapy within 3 months prior to blood draw. Subject profiles are presented in Table 1

Table 1

MS Subtype	Gender	Ave. Age ± S.D.	Ave. EDSS ± S.D.	Ave. Disease Duration (years)	# Patients On DMT	Ave. Years on DMT ± S.D.
NS	10 F; 9 M	39.7 ± 11.8	n/a	n/a	n/a	n/a
Benign	14 F; 4 M	54.1 ± 6.4	1.8 ± 1.0	23.7 ± 6.1	0	6.3 ± 2.1
RRQ	15 F; 1 M	48.6 ± 7.9	2.5 ± 1.3	12.9 ± 6.9	12	3.0 ± 2.2
RRA	11 F; 4 M	42.1 ± 7.8	2.9 ± 1.5	9.3 ± 9.3	6	2.6 ± 2.8
SP	18 F; 6 M	55.3 ± 9.4	6.0 ± 1.3	22.6 ± 9.0	12	5.2 ± 2.3
PP	20 F; 11 M	57.7 ± 9.5	5.9 ± 1.9	17.8 ± 12.3	0	n/a

NS= normal control subjects; Benign= Benign MS; RRQ = RRMS quiescent disease activity RRA = RRMS active disease activity; SP= SPMS; PP= PPMS; F= female; M= male; Ave.= average; S.D.= standard deviation; DMT= disease modifying therapy (Rebif 22, Rebif 44, Avonex, Betaseron, Copaxone).

Thirty-seven milliliters of blood was collected in four 8 ml BD Vacutainer® sodium citrate Ficoll gradient tubes and two 2.5 ml Paxgene (Qiagen) whole blood RNA tubes. Our investigation sought to determine not only if IAP levels were differentially affected in

specific subtypes of MS, but also whether these changes in IAP expression could be detected in whole blood or peripheral blood mononuclear (PBMN) cells in addition to resting T lymphocytes. The blood samples were immediately transported at room temperature to the Good Laboratory Practice (GLP) Accredited hematology laboratory at the Queen Elizabeth Hospital, centrifuged at high speeds, and the PBMN cells and T lymphocytes isolated from whole blood employing negative selection and stored at -80°C until RNA and protein extraction.

II. RNA Expression, Cell Purification, RNA Amplification and Statistical Analyses

A. Whole Blood

Blood was collected in two 2.5 ml Paxgene RNA tubes, as last blood-draw following collection in the 4 Ficoll-Gradient blood tubes (below). Tubes were inverted several times to mix and left at room temperature (RT) for 24 hours. Following the 24-hour RT incubation period tubes were centrifuged at 3400 x g in a swinging bucket centrifuge for 10 minutes to pellet nucleic acids. The pellets were washed, resuspended, and incubated in optimized buffers containing Proteinase K to digest proteins followed by a second centrifugation step to remove residual cell debris. The supernatant was transferred to a fresh microcentrifuge tube, ethanol added and the lysate applied to a Paxgene RNA spin column. The column was washed and treated with RNase-free DNase (Qiagen) and then pure RNA eluted according to the manufacturer's protocols (Qiagen). Total RNA yields were measured by ultraviolet (UV) absorbance and overall RNA quality was assessed by gel electrophoresis and staining for visualization of RNA band integrity. RNA samples were diluted in RNase free water to give a final concentration of 10 ng/μl, prior to analysis of IAP gene expression by qRT-PCR.

B. Isolation of RNA from Peripheral blood mononuclear cells (PBMN) and T lymphocytes

Whole blood is made up of a heterogeneous population of erythrocytes, granulocytes, and other peripheral blood mononuclear (PBMN) cells that make it difficult to detect differential IAP gene and protein expression that may occur in only a subset of immune cell types. As such, in addition to blood collection in Paxgene whole-blood RNA tubes, thirty-two ml of blood was collected in four 8 ml Ficoll-gradient tubes from each subject to isolate mononuclear cells, including T and B lymphocytes as well as monocytes/macrophages. Two of the tubes for each subject were used to isolate PBMN cells and two of the tubes were used to isolate resting (i.e. unactivated; not mitogen activated) T lymphocytes. For

the isolation of highly purified T lymphocytes (2×10^5) from whole blood of MS patients by negative selection (StemCell Technologies, Vancouver, BC) four hundred microliters (μL) of RosetteSepT (Stem Cell Technologies) was added to two of the four Ficoll-gradient tubes (Becton, Dickinson and Company; BD Vacutainer® CPT™ Cell Preparation Tube with Sodium Citrate) and incubated at room temperature for 20 minutes. Negative selection was chosen over positive selection in order to minimize T cell activation. For the isolation of PBMN cells, RosetteSep T was withheld. All tubes were inverted twice to mix and centrifuged at 1650-1800 x g for 25 minutes in a swinging bucket centrifuge. The T cells and PBMN cells were pipetted off into separate 15 ml conical centrifugation tubes, treated with ammonium chloride (StemCell Technologies, Vancouver, B.C.) for 10 minutes on ice to remove any remaining red blood cells, and centrifuged at 1000 x g for 10 minutes in a swinging bucket centrifuge to pellet the cells. The red blood cells (RBC) and polynuclear cells, including the neutrophils, the most abundant of the white blood cells [Biswas *et al.* 2003; Stroncek *et al.* 1997] were destroyed. Cell pellets were washed twice in phosphate buffered saline/fetal bovine serum and stored at -80°C until RNA and protein extraction. In most cases, pending difficulties in blood withdrawal (i.e. vein collapsed) or tube breakage, two T cell pellets and two PBMN cell pellets were obtained for each subject. Total RNA was extracted from one T cell pellet and one PBMN cell pellet from each patient using the Qiagen RNeasy kit (Mississauga, Ontario) according to the manufacturer's instructions. All sample preparations included RNase-free DNase treatment. Total RNA yields were measured by UV absorbance. RNA samples were diluted in RNase free water to give a final concentration of 10 ng/ μL .

III. Quantitative RT-PCR

Quantitative RT-PCR was performed to confirm changes in NAIP, XIAP, HIAP-1, HIAP-2 and survivin gene expression in patients with different subtypes of MS relative to normal control subjects. Aegera Therapeutics Inc. provided Taqman probes and Taqman primers for the IAPs. Total RNA (50 ng) isolated from whole blood, PBMN or T cells was reverse transcribed to yield first-strand cDNA and amplified using the Taqman one-step EZ RT-PCR Core reagents kit (Applied Biosystems, Foster City, CA, USA). For detection of each IAP, forward and reverse primers were used at a concentration of 600 nmol/L and all the Taqman fluorogenic probes at a concentration of 200 nmol/L. The expression of beta $\beta 2$ microglobulin was used as an endogenous control reference (Applied Biosystems, Foster City, CA, USA). $\beta 2$ microglobulin was amplified using Taqman $\beta 2$ Microglobulin Control Reagents Kit (Applied Biosystems, Foster City, CA, USA). The Taqman probe and primer

sequences for the IAPs are presented in Table 2. All amplifications were done in triplicate within the same 96 well plate and threshold cycle (CT) scores were averaged for subsequent calculations of relative expression values. The CT scores represent the cycle number at which the fluorescence signal (ΔR_n) crossed an arbitrary (user-defined)

5 threshold. Data was extracted and amplification plots generated with MJ Research Inc. (USA) software. Quantification of IAP gene expression relative to $\beta 2$ microglobulin was calculated according to the experimental protocol's $2^{-\Delta\Delta CT}$ method [Livak and Schmittgen 2001]. After the PCR amplification was finished the data were analyzed using the software provided by MJ Research and then the analyzed data was exported into an
10 Excel/SPSS spreadsheet for further statistical analysis. Results were expressed in Fold increase or Relative to a "calibrator." The calibrator sample was the RNA sample from a normal age-matched female subject. For amplicons designed and optimized according to the Applied Biosystems guidelines (amplicon size < 150 bp), the efficiency is close to one. Therefore, the amount of target, normalized to an endogenous reference (i.e., $\beta 2$

15 microglobulin) and relative to a calibrator (i.e., normal control RNA), is given by $2^{-\Delta\Delta CT}$, where CT = Cycle Threshold (fractional cycle number at which the amount of amplified target reaches a fixed threshold), $\Delta CT = CT_X - CT_R$ (difference in threshold cycles for target and reference) and $\Delta\Delta CT = \Delta CT(\text{target}) - \Delta CT(\text{calibrator value})$. $\Delta\Delta CT$ scores for the IAP mRNAs for each sample were subjected to separate one-way analysis of variance
20 (ANOVA) assessing the level of expression of each of the genes among patients with different types of MS (Benign, RR, SP, PP) and normal controls. The $\Delta\Delta CT$ scores for the IAP mRNAs for RRMS patients were assessed according to disease activity (RRQ or RRA) at the time of blood draw. Graphpad Prism 4 (San Diego, California, USA) and SPSS 11.0 (SPSS, Inc., Chicago, IL, USA) software were used for all statistical analyses.
25 Individual group differences following significant ANOVA ($\alpha=0.05$) results were analyzed using Tukey's honestly significant differences (HSD) or least significant differences (LSD) where appropriate. Parametrical analysis using the Pearson product moment correlation coefficient was employed to assess the relationship between IAP levels and EDSS scores of MS patients. Non-parametrical analysis using the Spearman rank correlation coefficient
30 was employed to assess the relationship between MS disease subtype and EDSS scores. Normal subjects were included in the analysis with an EDSS score of 0 indicative of normal neurological function.

Table 2

Human**IAP****Forward and Reverse Primer and Probe Sequences**

- XIAP** *Forward* 5'-GGT GAT AAA GTA AAG TGC TTT CAC TGT-3' (SEQ ID NO: 1)
 Reverse 5'-TCA GTA GTT CTT ACC AGA CAC TCC TCA A-3' (SEQ ID NO: 2)
 Probe 5'-(FAM)CAA CAT GCT AAA TGG TAT CCA GGG TGC AAA TAT C(TAMRA)-3'
 (SEQ ID NO: 3)
- HIAP-1** *Forward* 5'-TGG AGA TGA TCC ATG GGT TCA-3' (SEQ ID NO: 4)
 Reverse 5'-GAA CTC CTG TCC TTT AAT TCT TAT CAA GT-3' (SEQ ID NO: 5)
 Probe 5'-(FAM)CTC ACA CCT TGG AAA CCA CTT GGC ATG(TAMRA)-3'
 (SEQ ID NO: 6)
- HIAP-2** *Forward* 5'-TCT GGA GAT GAT CCA TGG GTA GA-3' (SEQ ID NO: 7)
 Reverse 5'-TGG CCT TTC ATT CGT ATC AAG A-3' (SEQ ID NO: 8)
 Probe 5'-(FAM)CTC ACA CCT TGG AAA CCA CTT GGC ATG(TAMRA)-3'
 (SEQ ID NO: 9)
- Survivin** *Forward* 5'-TCT GCT TCA AAG AGC TGG AA-3' (SEQ ID NO: 10)
 Reverse 5'-GAA AGG AAA GCG CAA CC G-3' (SEQ ID NO: 11)
 Probe 5'-(FAM)AGC CAG ATG ACG ACC CCA TAG AGG AAC ATA(TAMRA)-3'
 (SEQ ID NO: 12)
- NAIP** *Forward* 5'-GCC ATT TTA TGT CCA AGG GAT ATC-3' (SEQ ID NO: 13)
 Reverse 5'-CTT CCC AAT TTC CTA AAC ACT CA-3' (SEQ ID NO: 14)
 Probe 5'-(FAM)CTG TAC CGT GTC CTG TTT ACC TGT AAA GAC AAA GC(TAMRA)-3'
 (SEQ ID NO: 15)

IV. Protein purification, protein expression and quantification

- Results from our gene profiling studies were complimented and cross-validated by
 5 determining whether there were corresponding increases in the encoded proteins. In brief,
 T cells were isolated by negative selection from 8 mL of blood as described above. T cells
 and PBMN cells were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer with
 complete protease inhibitor (Roche) and incubated on ice for 10 minutes. Cell lysates
 were centrifuged at 12000 rpm in an eppendorf microcentrifuge at 4°C for 20 minutes.

The supernatant was extracted and transferred to a new tube. Small aliquots (2 μ L) were used to determine protein concentrations of the individual patient samples using the BCA protein assay method (Pierce, Rockford, IL, USA). Supernatants were diluted 1:1 with 2X sodium dodecyl sulfate (SDS) sample buffer with reducing agent dithiothreitol (DTT)

5 (Sigma, D-9779) and kept at -80°C until analyses.

Twenty μ g of protein was loaded on a 12% polyacrylamide gel, separated by SDS-PAGE and transferred at 100 volts for 120 minutes to an Immobilon-P (polyvinylidene fluoride) membrane (Millipore, Biorad Laboratories, Mississauga, Ontario, Canada). Membranes
10 were blocked for 2 hours at room temperature (RT) in 5% skim milk powder in tris-buffered saline (TBS)/0.05% polyoxyethylenesorbitan monolaurate (Tween-20) (Sigma, Canada). Membranes were probed with XIAP (1:250; BD Pharminogen), HIAP-1 (1:250; BD Pharminogen), HIAP-2 (1:250; BD Pharminogen) or survivin (1:250; Novus Biologicals) overnight at 4°C. Membranes were washed in TBS/0.05% Tween-20 and reprobed with
15 an IgG anti-mouse peroxidase (1:10 000) labeled antibody (Vector laboratories). Chemiluminescence was detected by autoradiography with Hyperfilm ECL™ high performance chemiluminescence film (Amersham Pharmacia Biotech, England) and bands were quantified by analysis of scanned images by Kodak 1D Scientific Imaging software (Kodak, USA). Several film exposures were obtained for each protein
20 investigated to make certain that optical density signals were within a linear range, permitting accurate quantification. Blots were then stripped (Reblot, Chemicon) and reprobed for the endogenous control protein β -Actin (1:10 000; Sigma, Canada) to ensure equal protein loading employing a peroxidase anti-mouse secondary antibody (1:10 000 Vector laboratories). Chemiluminescence was detected as described above.

25 The optical density scores were subjected to a one-way ANOVA assessing the relative protein expression of each of the IAPs in the different MS patient groups (Benign, RRQ, RRA, SP, PP) and normal controls. The optical density scores for the IAPs for RRMS patients were assessed according to disease activity (active or quiescent) at the time of
30 blood draw. The inclusion of disease activity was used to assess the protein expression profiles of the IAPs from PBMN cells or T cells from RRMS patients to determine whether the expression of specific proteins was related to disease course. Individual group differences following significant ANOVA ($\alpha=0.05$) results were analyzed using Tukey's honestly significant differences (HSD) or least significant difference (LSD) multiple
35 comparisons where appropriate. It should be mentioned that although the aim of this

investigation was to analyze 3 different RNA preparations from the same subject (i.e. whole blood, PBMN cells and T cells) along with the corresponding protein (PBMN cells and T cells) most, but not all (~70%), patients are represented in the three 3 types of RNA samples and 2 types of protein samples due most commonly to low RNA and protein yields, especially with respect to T cell samples.

Results

Patient Demographics and Past Clinical History

The Dalhousie Multiple Sclerosis Research Unit (DMSRU) is the sole regional specialty clinic for MS in Nova Scotia and maintains clinical records and a database on all patients seen since 1979. The DMSRU database provides a detailed clinical history of the majority of MS patients in Nova Scotia (Bhan et al., 2005) permitting clinical features and biochemical markers (mRNA, proteins) derived from blood samples to be correlated. Patients in the present study first presented to the DMSRU as early as 1980. All patients included in this study, presented to the DMSRU with a RR, RR progressive or primary progressive disease activity. At this time, patients with RR progressive or primary progressive disease activity displayed higher scores on Kurtzke pyramidal ($F(2, 94)=38.74, p<.0001$) and bowel and bladder scales ($F(2, 94)=9.432, p<.0001$), while patients with primary progressive disease activity also displayed elevated cerebellar symptom contributions ($F(2, 94)=3.162, p<.05$) relative to RR disease course.

The presentation of neurological symptoms of patients included in the present study were obtained from the DMSRU database and revealed several specific clinical characteristics that distinguished patients that come to display a progressive MS disease course (SPMS and PPMS) from patients with present-day benign, RRQ or RRA MS. Clinical histories of the various MS patients revealed that patients that presented to the clinic with pyramidal ($F(4, 95)=8.576, p<.0001$) or bowel/bladder ($F(4, 95)=3.53, p=.01$) dysfunction as measured by the Kurtzke pyramidal or bowel and bladder score (0-6), indicative of motor cortical and spinal cord deficits, would later develop a progressive disease course (SP or PPMS). The frequency of initial symptom presentation in patients that would be at the present date diagnosed with benign, RRQ, RRA, SP or PP are as follows: Benign: frequent presentation with brainstem (45%), cerebellar dysfunction (15 %), spinal cord (95%), cerebral-pyramidal symptoms (0 %) and optic neuritis (25%); RRQ: frequent presentation with brainstem (20%), cerebellar dysfunction (20 %), spinal cord (100%), cerebral-pyramidal symptoms (0 %) and optic neuritis (20%); RRA: frequent presentation

- with brainstem (53%), cerebellar dysfunction (0 %), spinal cord (66%), cerebral-pyramidal symptoms (6 %) and optic neuritis (43%); SPMS: frequent presentation with brainstem (42%), cerebellar dysfunction (29 %), spinal cord (100%), cerebral-pyramidal symptoms (9 %) and optic neuritis (33%); PPMS: frequent presentation with brainstem (33%),
- 5 cerebellar dysfunction (19 %), spinal cord (96%), cerebral-pyramidal symptoms (4 %) and optic neuritis (30%).

Expanded Disability Status Scale (EDSS)

- There was a strong correlation ($r = 0.833$, $p < 0.0001$) between EDSS score and MS
- 10 disease subtype. Patients with SPMS (range 2.5 to 7.5) or PPMS (range 2 to 8.5) displayed higher EDSS scores than patients with benign MS (range 0 to 3) or RRMS that were categorized as either quiescent (range 1 to 6) or active (range 1.5 to 6).

qRT-PCR

15 A. Whole blood

- There were no differences in XIAP (Figure 1A), HIAP-1 (Figure 2A), HIAP-2 (Figure 3A), or survivin (Figure 4A) mRNA levels in RNA extracted from whole blood (ANOVA, $p > 0.05$) among normal control subjects (NS), or patients with benign, RRQ, RRA, SP or PPMS [XIAP $F(4, 66) = 1.552$, $p = 0.1974$; HIAP-1 $F(4, 70) > 1$, $p = 0.605$, HIAP-2 $F(4, 69) = 1.421$, $p = 0.236$, and survivin $F(4, 61) = 1.448$, $p = 0.229$, respectively].
- 20

B. PBMN Cells

- XIAP mRNA expression was increased approximately four and a half-fold in PBMN cells from patients with RRA ($n=10$) or SPMS ($n=16$) compared to NS ($n=11$), or patients with
- 25 benign MS ($n=8$), RRQ ($n=10$) or PPMS ($n=12$) [$F(5, 61) = 4.180$, $p < 0.01$]. There were no differences in PBMN cell XIAP expression between NS or patients with Benign, RRQ or PPMS (Figure 1B). HIAP-1 expression in PBMN cells was similar in NS ($n=8$) as well as in patients with Benign ($n=10$), RR ($n=16$), SP ($n=16$) and PPMS ($n=12$) [$F(4, 57) = 1.605$, $p = 0.186$] (Figure 2B). HIAP-2 expression was increased approximately two-fold in PBMN
- 30 cells from RRA ($n=8$) and SPMS patients ($n=14$) compared to NS ($n=10$), and patients with benign ($n=9$) MS [$F(5, 57) = 5.083$, $p < 0.001$]. There were no differences in HIAP-2 PBMN mRNA levels among RRQ ($n=9$) or PPMS ($n=12$) patients (Figure 3B). There was a weak positive correlation ($r = 0.310$, $p < .05$) between EDSS scores and HIAP-2 mRNA levels in PBMN cells. Survivin PBMN cell mRNA levels were the same in NS ($n=6$),
- 35 patients with Benign ($n=8$), RR ($n=16$), SP ($n=12$), and PPMS ($n=6$) [$F(4, 47) = 1.655$,

p=0.178] (Figure 4B). NAIP expression in PBMN cells was similar in normal control subjects (n=7), or patients with Benign MS patients (n=10), qRRMS quiescent (n=7), aRRMS (n=8), SPMS (n=14) or PPMS patients (n=9) [F (5,50) < 1, p= .545], (Figure 7B).

5 C. Resting T cells

XIAP mRNA expression in resting T cells was similar in NS (n=13) and patients with Benign (n=10), RR (n=17), SP (n=20) and PPMS (n=17) [F (4,72)=1.433, p=0.232] (Figure 1C). In contrast, HIAP-1 mRNA expression was elevated approximately three-fold in resting T cells from patients with RRMS irrespective of disease activity (n=19) compared to NS (n=11) and patients with benign or PPMS [F (4,77)=3.871, p<0.01] (Figure 2C). There were no differences in T cell HIAP-1 mRNA levels between patients with RRMS and SPMS (n=22) (see Figure 2C). In patients with RR (n=24) and SPMS (n=20) HIAP-2 mRNA expression was increased approximately two and a half-fold compared to NS (n=12) [F (4,81)=2.528, p<0.01]. There were no differences in T cell HIAP-2 mRNA expression between patients with benign MS (n=12) or PPMS (n=19) (Figure 3C). Survivin T cell mRNA expression was increased almost four and a half fold in PPMS (n=18) relative to NS (n=10), patients with benign (n=7), RR (n=15) and SPMS (n=17) [F (4,62)=4.271, p<0.01]. There was a positive correlation ($r = 0.376$; $p<0.05$) between the degree of disability as reflected by EDSS scores and survivin mRNA expression in T cells, (Figure 4C). NAIP was also differentially increased in resting T cells of a small subset of PPMS patients. At this juncture, however, it is not clear what the increase in NAIP represents.

Table 3 summarizes the relationships between IAP expression patterns, immune cell subtype and multiple sclerosis subtype. The arrows (↑) indicate an increase in the IAP expression relative to control subjects.

Table 3

MS Subtype	Whole blood	PBMN cells	Resting T cells
Benign	No change	No change	No change
RRMSQ	↑ NAIP	No change	↑ HIAP-1
RRMSA	↑ NAIP	↑ XIAP , ↑ HIAP-2	↑ HIAP-1 , ↑ HIAP-2
SPMS	↑ NAIP	↑ XIAP , ↑ HIAP-2	↑ HIAP-2
PPMS	No change	No change	↑ Survivin

Expression patterns of the IAPs in whole blood, PBMNs and T cells distinguish subtypes of MS. IAPs were not elevated in patients with benign MS. Patients with RRMS, RRMS or SPMS displayed elevated levels of NAIP in whole blood. Patients with relapse remitting MS that were quiescent (RRMSQ) displayed elevated HIAP-1 in resting T cells. By contrast, patients with relapse remitting MS that were in the active state (RRMSA) displayed elevated XIAP and HIAP-2 in PBMNs and elevated HIAP-1 and HIAP-2 in resting T cells. The same changes occurred in patients with secondary progressive MS (SPMS) with the exception that only HIAP-2 was elevated in resting T cells. Lastly, patients with primary progressive MS (PPMS) only displayed elevated survivin expression in resting T cells. Survivin up-regulation is often observed in many forms of cancer suggestive of increased resistance to apoptosis and elevated high proliferative status.

Western blotting

XIAP PBMN cell optical density (55 kDa band) was increased approximately two and a half-fold in PBMN cells from patients with RRA (n=12) or SPMS (n=15) compared to NS (n=13), or patients with PPMS (n=10) [$F(5,65)=5.207$, $p<.001$]. There were no differences in PBMN cell XIAP expression between NS or patients with Benign, RRQ or PPMS (Figure 5). Quantification of the HIAP-1 band (72 kDa) revealed a 1.5 fold elevation of this protein from extracts from T cells of patients with RRMS irrespective of disease activity (combined RRQ and RRA) relative to other groups [$F(94, 56)=6.144$, $p<0.001$] (Figure 6). The results obtained by western blotting were therefore consistent with those obtained by qRT-PCR.

Discussion

The present invention demonstrates that distinct patterns of elevated IAP expression (XIAP, HIAP-1, HIAP-2, survivin) in PBMN cells and resting T cells are associated with different types of MS. This finding is consistent with the proposal that MS is not a single clinical entity but rather a heterogeneous disorder. Our findings suggest that distinct apoptotic mechanisms are activated in different populations of immune cells in patients with various forms of MS. Furthermore, our results are consistent with the hypothesis that increased IAP expression may contribute to the pathogenesis of MS by delaying the removal of auto-reactive immune cells by apoptosis [Sharief and Semra 2001; Semra *et al.* 2002] thereby preventing the death of myelin-reactive immune cells resulting in inflammation and demyelination in the brain and spinal cord.

In the present investigation, XIAP mRNA and protein was differentially increased in PBMN cell fractions with no notable differences among groups of MS patients in whole blood or T cells. Increased PBMN cell expression of XIAP was noted in RRMS patients with a more active disease course as well as among patients with SPMS. Monocytes comprise about 3-8% of the leukocytes in the blood. It is therefore tempting to speculate that monocytes contribute to the elevation of XIAP mRNA and protein observed in patients with aggressive forms of MS. XIAP is expressed in normal monocytes but relatively undetectable in granulocytes. Moreover, exaggerated monocyte XIAP expression is associated with acute myeloid leukemia that has a poor clinical outcome [Tamm *et al.* 2000]. Neutrophils are the primary immune cell subtype in whole blood comprising up to approximately 70% of leukocytes present in whole blood. Neutrophils can regulate suppressor T cell responses to myelin [Zehntner *et al.* 2005b], have been associated with demyelinated plaques in a mouse model of experimental autoimmune encephalomyelitis [Tsunoda *et al.* 2000] and promote demyelination [Gaupp *et al.* 2003]. Calpain-mediated degradation of XIAP is thought to play a critical role in controlling the number of circulating neutrophils [Kobayashi *et al.* 2002] while the ability of the bacterium *Anaplasma phagocytophilum* to delay neutrophil apoptosis is associated with reduced degradation of XIAP [Ge and Rikihisa 2006]. XIAP was not increased in whole blood of MS patients relative to normal control subjects although several patients with benign, RR, SP and PP MS demonstrated values that were greater than controls. These results suggest that at least as far as XIAP, HIAP-1 and HIAP-2 are concerned; the resistance of neutrophils to apoptosis does not play a major role in MS. The relative lack of XIAP mRNA induction in whole blood was not due to an overall decrease in T cell numbers or a reduced percentage of T cells in whole blood in MS patients as levels of XIAP mRNA in subsequent resting T cell fractions from the same patients were unremarkable. The lack of increased XIAP mRNA expression in T cells in patients with aggressive forms of MS contrasts with the report by Sharief and Semra [2001] in which elevated XIAP protein levels were observed in mitogen activated T cells isolated from RRMS patients relative to normal and neurological control subjects. One explanation for this discrepancy between the results of the present study and those of Sharief and Semra [2001] is that we examined levels of XIAP mRNA in resting T cells while levels of XIAP protein in mitogen activated T cells were examined in the former. In MS, T cells may be primed by PHA stimulation resulting in elevated XIAP expression.

The increase in XIAP, HIAP-1 and HIAP-2 in mitogen activated immune cells [Sharief *et*

a/. 2002b], while supporting a role for impaired activation-induced apoptosis in the immune cells of MS patients, may simply be an exaggeration of the immune cells sensitized to myelin antigens and thus more likely to display elevated IAP levels upon stimulation with PHA. Consistent with this notion, our inability to detect elevated XIAP levels in resting T cells from MS patients suggests that mitogen stimulation artificially activates a survival pathway by mechanisms not relevant to MS. In further support of this hypothesis, mitogen-stimulated apoptosis in non-myelin reactive CD4+ and CD8+ T cells has been reported to be comparable in MS patients and normal control subjects while activation of myelin-reactive CD4+ and CD8+ T cells was associated with decreased Fas associated apoptosis in MS patients [Saresella *et al.* 2005; Zang *et al.* 1999]. The demonstration that XIAP is increased in resting PBMN cells in the present study suggests that these cells have been stimulated with MS relevant antigens in the peripheral blood circulation such as myelin basic protein or myelin oligodendrocyte glycoprotein, inducing differentiation and prolonging their lifespan. Although monocytes, B lymphocytes and T lymphocytes compose the cell types present in the PBMN cell fraction, the increase in XIAP expression was not present in T cell fractions, thus implicating either B lymphocytes or monocytes as the major contributor to increases in this apoptotic gene. While evidence for increased XIAP levels in B lymphocytes in MS is lacking, the contribution of XIAP expression in B lymphocytes to the overall increase in PBMN cell XIAP in patients with RRMS and SPMS cannot be dismissed entirely. In humans XIAP, HIAP-1 and HIAP-2 mRNAs are strongly expressed in B lymphocytes of normal subjects as well as in patients with B-cell chronic lymphocytic leukemia as measured by RNase protection assay [Munzert *et al.* 2002]. Still, monocytes have an important role in the pathogenesis of MS and are one of the primary cell types responsible for tissue damage [van der Goes *et al.* 2005]. Yet, it has previously been demonstrated in monocytes derived from normal subjects that differentiation induction and clonal expansion led to an upregulation of XIAP protein, an effect not observed in neutrophils, contributing to increased longevity of cells derived from the monocyte lineage while treatment with XIAP antisense oligonucleotides resulted in significant loss of cell viability [Miranda *et al.* 2003]. XIAP, HIAP-1 and HIAP-2 mRNA is expressed in neutrophils albeit the relative protein expression is low. Yet stimulation of neutrophils with lipopolysaccharide (LPS) of gram-negative bacteria induces upregulation of HIAP-1 and HIAP-2 mRNA. Moreover, in septic patients characterized by infection of the bloodstream by toxin-producing bacteria, delayed neutrophil apoptosis has been associated with increased XIAP mRNA expression with no changes observed in HIAP-1 or HIAP-2 mRNA expression in patients possibly contributing to the persistence of

an inflammatory reaction [O'Neill *et al.* 2004]. The lack of XIAP, HIAP-1 and HIAP-2 in neutrophils of MS patients relative to mitogen-induced IAP and reduced neutrophil apoptosis in septic patients demonstrates the diversity of the immune cells involved in differential immune perturbations and outlines the specificity of inflammatory events underlying MS pathology. Taken together, while neutrophils may characterize more acute peripheral inflammatory conditions, increased expression of monocytes displaying exaggerated XIAP mRNA levels suggest that these cell types play a major role in more aggressive types of MS, characterized by widespread tissue damage and marked neurological disability.

Expression of HIAP-1 and HIAP-2 appear to be more tissue specific than XIAP [Liston *et al.* 2003; Vischioni *et al.* 2006]. Highest levels of HIAP-1 and HIAP-2 are found in tissues of lymphoid origin including the thymus, peripheral lymphocytes and spleen [Vischioni *et al.* 2006]. Both HIAP-1 and HIAP-2 have CARD domains and are involved in the inactivation of caspase 9 [Reed *et al.* 2003]. Through homotypic protein-protein interactions CARD domains are thought to influence function of inflammatory and apoptotic signaling pathways [Reed *et al.* 2003]. HIAP-1 and HIAP-2 mRNA is increased in T cells in MS patients that are experiencing relapses/remissions and/or disease progression, respectively suggesting a prominent role for inflammatory T cells in the pathogenesis of RRMS disease progression reminiscent of data previously reported by Sharief *et al.* in mitogen stimulated T cells. Moreover, it has been revealed that the phenotypic profile of immune cell types differs in patients in early MS at least as far as in patients that have experienced a clinically isolated syndrome. Patients with active MRI activity in the proceeding year displayed distinct changes in peripheral lymphocyte subsets relative to MRI-inactive patients that were predictive of MRI activity in early disease.. Interestingly, it has been reported that infiltrating and clonally expanded autoreactive T cells persisted for longer than 7 years in the CSF and blood of patients with RRMS, irrespective of interferon- β (IFN- β) treatment history, which does not eliminate autoreactive T cells, contributing to the propensity of increased clinical exacerbations and possibly the eventual progression of neural disability [Skulina *et al.* 2004]. While clonal expansion of auto-reactive T cells have been identified in the blood and CSF of normal subjects, these cell populations are susceptible to apoptosis [Skulina *et al.* 2004], while myelin-primed T cells in MS are resistant to programmed cell death proliferating and inducing CNS damage [Pender 1998]. In the present investigation, increased expression of the anti-apoptotic genes HIAP-1 and HIAP-2 in RRMS and SPMS would render the

cells apoptosis-resistant, increasing their life-span and contributing to myelin destruction at the very least during the relapse phase. The sole increase in HIAP-1 mRNA levels in un-stimulated T cells is consistent with a critical role for this population of immune cells in MS. The differential expression of IAPs suggests cell-type specific apoptotic pathways and redundancy in the immune system such that defects in one pathway will not abolish apoptosis altogether.

The gene expression pattern of HIAP-2 within immune cells isolated from MS patients differed slightly from the pattern of expression observed for HIAP-1. Elevated PBMN cell HIAP-2 expression was found in patients with RRA and SPMS likely reflecting a contribution from T cells. HIAP-2 expression was increased in T cells in patients with RRA and SPMS, but not subjects with benign or PPMS, suggesting that the increased expression of this anti-apoptotic gene in T cells is associated with a clinical course characterized by either relapses and remission or an unrelenting disease progression. Benign MS patients can convert to RRA and SPMS with time, and increased IAP levels within specific immune cell populations may have prognostic value indicative of a change in MS disease course prior to the reappearance of symptoms. In summary, XIAP, HIAP-1 and HIAP-2 displayed cell specific induction patterns among patients with different subtypes and severity of RRMS that may perpetuate the continuous phasic presentation of inflammation and resolution within the CNS.

Survivin is a unique member of the IAP family implicated in both cell proliferation and apoptosis [Ambrosini *et al.* 1997]. The survivin pathway interfaces with both the cell-death machinery and mechanisms of cell-cycle progression and microtubule stability, specifically expressed during the G2/M phase of the cell cycle. Survivin expression is undetectable in most normal adult tissues [Fukuda and Pelus 2001] including PBMN cells, T cells and whole-blood cell lysates, but is over expressed in virtually every human tumor that has been studied [Ouhitit 2007]. Survivin expression was relatively undetectable in whole blood, PBMN cells or resting T lymphocytes in NS and patients with benign, RR and SPMS, reminiscent of the findings of Sharief and Semra [Livak and Schmittgen 2001]. Although several RRMS and SPMS patients with elevated EDSS scores demonstrated increased survivin levels in whole blood and PBMN cells suggestive of active inflammatory disease these increases were not associated with a specific MS disease subtype. It should be mentioned that although survivin mRNA levels were low or undetectable in most normal and benign MS blood samples, detectable levels of survivin expression have been

previously reported in normal peripheral lymphocytes [Shinozawa *et al.* 2000] that are upregulated during clonal expansion of these cells as well as non-malignant T cells increasing their resistance to apoptosis [Sharief and Semra 2001]. In comparison, mitogen activation of T cells in culture elevated survivin expression may be associated with clonal expansion as reported by Sharief and Semra [Sharief *et al.* 2002a] and therefore may simply reflect cell proliferation and not apoptotic status. Survivin expression prior to T cell activation was undetectable, while increased survivin expression was found in memory T cells following stimulation with PHA in lymphocytes isolated from normal donors [Kornacker *et al.* 2001]. Indeed, survivin is now believed to regulate chromosome segregation during mitosis [Fukuda and Pelus 2001]. In the present study, the scattered increase in survivin expression in whole-blood and PBMN cells may be indicative of cells undergoing division and proliferation at the time of blood draw, although the correlation of survivin levels with EDSS disability may be of pathogenic significance in MS, at the very least allowing prolonged immune cell viability, proliferation and ultimately contributing to CNS damage and clinical disability.

It should be underscored; however, that in the present study, PPMS patients displayed survivin levels approximately 5 fold higher in resting T cells relative to NS, benign, RR and SPMS. Primary progressive MS has been associated with a specific polymorphism of the cytotoxic T-lymphocyte antigen 4 gene, that down-regulates T- cell function [Maurer *et al.* 2002]. In contrast, a small portion of T cells with elevated survivin would be expected to reduce T-cell elimination producing a more prolonged disease time-course. It is notable that survivin, a marker and potential target for the treatment of cancer therapy is upregulated in a type of MS for which there is no effective treatment. As a result, survivin may hold diagnostic utility for distinguishing SPMS and PPMS in patients for which a clear clinical history is unavailable.

Neuronal apoptosis inhibitory protein (NAIP) like XIAP, human IAP-1 (HIAP-1) and human IAP-2 (HIAP-2) possesses 3 BIR domains, while survivin possesses 1 BIR domain, that support the suppression of caspase 3 and 7; the two most potent effector caspases. NAIP, unlike the other IAPs, has a NACHT domain and proteins of the NACHT family may serve as critical pathogen sensing and signal transducing molecules within the innate immune system. In contrast to XIAP, HIAP-1, or HIAP-2 expression in whole blood, NAIP mRNA expression (Figure 7A) was increased approximately two to three-fold elevation of NAIP mRNA in the whole blood from patients with qRRMS (n= 9), aRRMS (n=11), SPMS

(n=14), PPMS (n= 8) relative to patients with Benign MS (n=8) or healthy subjects (n=14) [F (5, 59)=1.947, p=0.1]. There was a moderate positive correlation ($r = 0.368$, $p < .01$) between EDSS disability and NAIP mRNA expression in whole blood. There were no difference between whole blood NAIP expression between normal subjects (n=14) or patients with Benign MS (n=8). Neutrophils are the primary immune cell subtype in the PBMN fraction that comprise up to approximately 70% of leukocytes present in whole blood. Neutrophils can regulate suppressor T cell responses to myelin, have been associated with demyelinated plaques in a mouse model of experimental autoimmune encephalomyelitis and promote demyelination. Calpain-mediated degradation of XIAP is though to play a critical role in controlling the number of circulating neutrophils while the ability of the bacterium *Anaplasma phagocitophilum* to delay neutrophil apoptosis is associated with reduced degradation of XIAP. However, XIAP is not increased in whole blood.

One unexpected observation was the differential expression of NAIP within whole blood. Regarding NAIP expression in patients within the different sub-types of MS, NAIP was differentially increased, unlike the other IAPs in which the increase was restricted to a specific cell fraction, in both whole blood of patients with RRMS and SPMS. Interestingly, NAIP is increased in whole blood but not PBMN cell mRNA of patients with RR and SPMS. The difference in the expression of NAIP mRNA in whole blood compared to PBMN cell fractions likely reflects the increased number of granulocytes or dendritic cells in whole blood, cells in low levels or relatively absent in PBMN cell fractions. NAIP possess a NACHT NTPase domain that codes for proteins involved in intracellular pathogen sensing and regulation of the innate immune response and inflammation. It is of note that NACHT gene mutations have been associated with inflammatory bowel disease, suggesting a prominent role of NACHT proteins in deterring pathogens from the gut. The NACHT domain is also present in those proteins involved in Major Histocompatibility Complex (MHC) type II antigen transcription. MHC II antigens are present on dendritic cells, macrophages and neutrophils, the major antigen presenting cells (APC) involved in the innate immune response, representing the initial line of defense against foreign pathogens, present in whole blood. In RR and SP MS patients experiencing relapses, NAIP may increase the lifespan of APC such as neutrophils. Gene and protein expression of HIAP-1, HIAP-2 and XIAP are found in neutrophils, although at relatively low levels compared to NAIP and survivin. Monocytes in whole blood can differentiate into phagocytotic macrophages or dendritic cells (DCs). DCs are antigen-presenting

leukocytes that function to initiate the immune response by activating T-lymphocytes and stimulating the secretion of cytokines. In this regard, DCs stimulate an immune response that may be prolonged by the extended survival of DCs by blocking DC apoptosis. In normal subjects, less than 0.1% of circulating leukocytes are immature DCs. Mature DCs are localized in lymphoid tissues. However, among individuals with RR, SP and PP MS circulating dendritic cells secreting pro-inflammatory cytokines are increased relative to normal control subjects that are thought to reflect a prominent role of DCs in MS disease pathogenesis. Under basal states of inactivation, NAIP is strongly expressed in immature DCs, while HIAP-1, HIAP-2 are rather weakly expressed, and XIAP and survivin are not expressed at all. Upon activation with antigen and subsequent maturation, NAIP expression was down regulated while levels of XIAP, HIAP-1 and HIAP-2 gene expression increased. Survivin levels remained undetectable. Bcl-2 and Bcl-xL, members of the Bcl-2 family of anti-apoptotic genes, were not or rather weakly expressed, in DCs, respectively. Although Bcl-2 protein levels are high in resting T lymphocytes, the expression of Bcl-2 protein was similarly expressed in basal and cultured T cells activated with PHA among normal subjects and corresponding patients with RRMS. In humans, NAIP mRNA levels are low while XIAP, HIAP-1 and HIAP-2 mRNAs are strongly expressed in B lymphocytes of normal subjects. NAIP gene expression appears to be regulated by different factors than HIAP-1, HIAP-2, XIAP, survivin and the Bcl-2 family. The increase of NAIP expression in whole blood likely reflects increased survival of immune cells involved in inflammatory processes and myelin damage as reflected by increased EDSS scores. In the mouse, NAIP is highly expressed in macrophages. NAIP is increased in macrophages in mice in response to infection with an intracellular pathogen (*Legionella pneumophila*) that causes Legionnaire's disease, an acute form of pneumonia, in humans. While the success of *Legionella pneumophila* infection is dependant upon macrophage apoptosis, inducible NAIP expression within macrophages serves a protective role by inhibiting caspase 3 activities thus increasing the lifespan of the cell. Monocytes, macrophages, neutrophils and dendritic cells secrete pro-inflammatory cytokines that are associated with the inflammatory relapse phase of relapsing-remitting forms of MS. It is not clear at this juncture whether the increase NAIP expression in sub-types of MS with a prominent inflammatory response dictates specific immune cell contributions to the heterogeneous subtypes of MS, defining inflammatory and non-inflammatory conditions. Yet, neutrophil apoptosis regulation during an immune-mediated inflammatory response plays a key role in its resolution. Neutrophils are activated following release of pro-inflammatory cytokines, including IL-1, IL-6, tumor necrosis factor α and interferon γ that

also serve to inhibit apoptotic death. Increase of NAIP in whole blood RNA from RR and SPMS patients, suggests that over expression of NAIP may prolong neutrophils-induced inflammatory response which may be the basis for the correlation of NAIP in whole blood with EDSS scores, akin to the response of macrophages to infection with *Legionella*
5 *pneumophila* associated with the relapse phase of the disease underlying extensive myelin damage. Increased expression of NAIP in inflammatory neutrophils would prevent their apoptotic deletion and delay resolution of acute MS symptomatology.

Conclusions

10 Taken together, the present findings indicate that specific patterns of IAP expression in distinct populations of immune cells have potential diagnostic and prognostic value for MS. To recapitulate, high levels of XIAP and HIAP-2 expression in PBMC cells and HIAP-2 in T cells may be associated with aggressive forms of this disorder (RRA and SPMS) characterized by inflammation that are best managed through early intervention. In
15 contrast, XIAP, HIAP-1 and HIAP-2 expression in immune cell subsets is not elevated in benign and primary progressive forms of MS that do not require treatment or do not respond to current disease modifying therapies, respectively. In patients with PPMS, elevated expression of survivin may provide a novel therapeutic target for this devastating form of MS in which there are no current treatments. In addition to the prognostic value of
20 IAP expression within distinct cell populations, our findings suggest that apoptotic dysregulation of potentially autoreactive immune cells in MS occurs through multiple immune pathways involving cells of both the innate (PBMC cells) and the adaptive (T cells) immune systems. The over expression of IAPs increase the resistance of specific cell types to apoptosis that contribute to the various subtypes of MS. It is of note that pre-
25 clinical studies with the XIAP anti-sense molecule (data not shown) demonstrate that decreasing levels of XIAP in the immune system reduce symptom severity in an animal model of MS [Zehntner *et al.* 2005a]. Preliminary studies in cancer patients suggest that this compound has clinical efficacy suggesting that such a compound may represent a novel treatment for other diseases where impaired apoptosis has been implicated such as
30 MS. In conclusion, basal expression patterns of several IAPs in specific immune cell populations not only reflect the diverse range of apoptotic mechanisms involved in MS pathogenesis but may have diagnostic utility in distinguishing between various subtypes of MS and serve as potential drug targets for different forms of this autoimmune disorder.

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Other Embodiments

From the foregoing description, it will be apparent to one of ordinary skill in the art that variations and modifications may be made to the invention described herein to adapt it to various usages and conditions. Such embodiments are also within the scope of the present invention.

All publications mentioned in this specification are hereby incorporated by reference.

CLAIMS

We claim:

- 5 1. A method for differentiating between multiple sclerosis subtypes in a patient, the method comprising:
 - a) determining an amount of an IAP gene expression level in a blood sample obtained from the patient; and
 - b) correlating the amount of the IAP gene expression level in the blood sample
10 with the presence of a multiple sclerosis subtype in the patient.
2. The method, according to claim 1, in which the IAP gene expression level is compared to those of control subjects.
- 15 3. The method, according to claim 2, in which an increase in the IAP gene expression level relative to those of the control subjects indicates that the patient is suffering from the MS subtype.
4. The method, according to claim 2, in which the control subjects are those having
20 benign MS or are healthy normal subjects.
5. The method, according to claim 1, in which the MS subtype is benign MS, quiescent relapsing remitting MS, active relapsing remitting MS, primary progressive MS or secondary progressive MS.
25
6. The method, according to claim 1, in which the IAP gene expression level is determined by measuring the levels of transcribed IAP mRNA in the blood sample.
7. The method, according to claim 6, in which the level of IAP mRNA is measured
30 using quantitative real time polymerase chain reaction (qRT-PCR).
8. The method, according to claim 1, in which the IAP gene expression level is determined by measuring the level of IAP protein in the blood sample.

9. The method, according to claim 8, in which the IAP protein level is measured using an immunoassay.

10. The method, according to claim 1, in which the IAP gene encodes an IAP protein
5 selected from NAIP, XIAP, HIAP-1, HIAP-2 or survivin.

11. The method, according to claim 1, in which peripheral blood mononuclear (PBMN) cells are isolated from the blood sample.

10 12. The method, according to claim 11, in which the IAP gene expression level is measured in the PBMNs.

13. The method, according to claim 12, in the IAP gene expression level is compared those of control subjects, an increase in the IAP gene expression level relative to those of
15 the control subjects indicates that the patient is suffering from the MS subtype.

14. The method, according to claim 13, in which the increased gene expression level includes increased levels of XIAP mRNA and HIAP-2 mRNA.

20 15. The method, according to claim 13, in which the MS subtype is active relapsing remitting MS.

16. The method, according to claim 13, in which the MS subtype is secondary progressive MS.

25 17. The method, according to claim 13, in which the control subjects are those having benign MS or are healthy normal subjects.

18. The method, according to claim 1, in which T cells are isolated from the blood
30 sample.

19. The method, according to claim 18, in which the IAP gene expression level is measured in the T cells.

35 20. The method, according to claim 19, in which the T cells are resting T cells.

21. The method, according to claim 19, in which the IAP gene expression level is compared to those of control subjects, an increase in the IAP gene expression level relative to those of the control subjects indicates that the patient is suffering from the MS subtype.

22. The method, according to claim 21, in which the increased gene expression level includes increased levels of HIAP-1 mRNA or HIAP-2 mRNA.

23. The method, according to claim 22, in which the increased levels of only HIAP-1 mRNA indicate that the patient is suffering from quiescent relapsing remitting MS.

24. The method, according to claim 22, in which the increased levels of HIAP-1 mRNA and HIAP-2 mRNA levels indicate that the patient is suffering from active relapsing remitting MS.

25. The method, according to claim 22, in which the increased levels of only HIAP-2 mRNA indicate that the patient is suffering from secondary progressive MS.

26. The method, according to claim 21, in which the increased gene expression level includes increased levels of survivin mRNA.

27. The method, according to claim 26, in which the increased level of survivin mRNA indicates that the patient is suffering from primary progressive MS.

28. The method, according to claim 21, in which the control subjects are those having benign MS or are healthy normal subjects.

29. The method, according to claim 14, in which the level of HIAP-2 expression correlates to the degree of disability measured by EDSS scores.

30. The method, according to claim 1, in which the blood sample is whole blood.

31. The method, according to claim 30, in which the IAP gene expression is measured in the whole blood.

32. The method, according to claim 31, in which the IAP gene expression level is compared to those of control subjects, an increase in the IAP gene expression level relative to those of the control subjects indicates that the patient is suffering from the MS subtype.

33. The method, according to claim 32, in which the increased gene expression level includes increased levels of NAIP mRNA in patients who are suffering from relapsing remitting MS, quiescent relapsing remitting MS, primary progressive MS and secondary progressive MS.

34. The method, according to claim 32, in which the control subjects are those having benign MS or are healthy normal subjects.

35. A method for differentiating active relapsing remitting multiple sclerosis in a patient, the method comprising:

a) determining an amount of XIAP and HIAP-2 gene expression levels in peripheral blood mononuclear (PBMN) cells obtained from the patient; and

b) comparing the amount of the XIAP and HIAP-2 gene expression levels in the PBMNs with the XIAP and HIAP-2 gene expression levels of control subjects, an increase in the XIAP and HIAP-2 gene expression levels being an indication that the patient is suffering from active relapsing remitting multiple sclerosis.

36. A method for differentiating secondary progressive multiple sclerosis in a patient, the method comprising:

a) determining an amount of XIAP and HIAP-2 gene expression levels in peripheral blood mononuclear (PBMN) cells obtained from the patient; and

b) comparing the amount of the XIAP and HIAP-2 gene expression levels in the PBMNs with the XIAP and HIAP-2 gene expression levels of control subjects, an increase in the XIAP and HIAP-2 gene expression levels being an indication that the patient is suffering from secondary progressive multiple sclerosis.

37. A method for differentiating quiescent relapsing remitting multiple sclerosis in a patient, the method comprising:

a) determining an amount of HIAP-1 gene expression level in T cells obtained from the patient; and

b) comparing the amount of the HIAP-1 gene expression level in the T cells with the HIAP-1 gene expression levels of control subjects, an increase in the HIAP-1 gene expression levels being an indication that the patient is suffering from quiescent relapsing remitting multiple sclerosis.

38. A method for differentiating active relapsing remitting multiple sclerosis in a patient, the method comprising:

10 a) determining an amount of HIAP-1 and HIAP-2 gene expression levels in T cells obtained from the patient; and

b) comparing the amount of the HIAP-1 and HIAP-2 gene expression levels in the T cells with the HIAP-1 and HIAP-2 gene expression levels of control subjects, an increase in the HIAP-1 and HIAP-2 gene expression levels being an indication that the patient is suffering from active relapsing remitting multiple sclerosis.

39. A method for differentiating between quiescent and active relapsing remitting multiple sclerosis in a patient, the method comprising:

20 a) determining an amount of HIAP-1 and HIAP-2 gene expression levels in T cells obtained from the patient; and

b) comparing the amount of the HIAP-1 and HIAP-2 gene expression levels in the T cells with the HIAP-1 and HIAP-2 gene expression levels of control subjects, either an increase in only HIAP-1 gene expression levels being an indication that the patient is suffering from quiescent relapsing remitting multiple sclerosis, or an increase in both .the HIAP-1 and HIAP-2 gene expression levels being an indication that the patient is suffering from active relapsing remitting multiple sclerosis.

40. A method for diagnosing secondary progressive multiple sclerosis in a patient, the method comprising:

30 a) determining an amount of HIAP-2 gene expression level in T cells obtained from the patient; and

b) comparing the amount of the HIAP-2 gene expression level in the T cells with the HIAP-2 gene expression levels of control subjects, an increase in the HIAP-2 gene expression levels being an indication that the patient is suffering from secondary progressive multiple sclerosis.

41. A method for diagnosing primary progressive multiple sclerosis in a patient, the method comprising:

5 a) determining an amount of survivin gene expression level in T cells obtained from the patient; and

b) comparing the amount of the survivin gene expression level in the T cells with the survivin gene expression levels of control subjects, an increase in the survivin gene expression levels being an indication that the patient is suffering from primary progressive multiple sclerosis.

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42. A method for differentiating between benign and either relapsing remitting MS, primary progressive MS or secondary progressive MS in a patient, the method comprising:

a) determining an amount of NAIP gene expression levels in whole blood obtained from the patient; and

15 b) comparing the amount of NAIP gene expression levels with the amount of NAIP gene expression levels in control subjects, an increase in the NAIP gene expression levels being an indication that the patient is suffering from either relapsing remitting MS, primary progressive MS or secondary progressive MS and not benign MS.

20 43. A method of testing a patient's suitability for interferon- β treatment of multiple sclerosis, the method comprising:

a) determining an amount of an IAP gene expression level in a blood sample obtained from the patient suspected of having an aggressive subtype of multiple sclerosis; and

25 b) comparing the amount of the IAP gene expression level to the IAP gene expression levels of control subjects, an increase in IAP gene expression levels being an indication that the patient is suffering from an aggressive subtype of multiple sclerosis, the patient being suitable for treatment with interferon- β .

30 44. A method of identifying whether a patient is at risk of developing secondary progressive multiple sclerosis from active relapsing remitting multiple sclerosis, the method comprising:

a) obtaining a blood sample for the patient; and

35 b) comparing XIAP mRNA levels in PBMCs isolated from the sample, and HIAP-1 and HIAP-2 mRNA levels in T cells isolated from the sample, with those of a control

subject, an increase in the XIAP mRNA levels in the PBMNs and an increase the HIAP-2 mRNA levels in the T cells, and normal HIAP-1 levels in the T cells, being an indication that the patient is at risk of developing secondary progressive MS.

5 45. The method, according to claim 44, further including detecting a normalization of HIAP-1 in T cells.

46. Use of IAP mRNA expression or IAP protein expression as a biomarker for the diagnosis of multiple sclerosis subtypes in a subject, an increased level of expression
10 compared to control subjects being an indication that the patient has a multiple sclerosis subtype.

47. A method for monitoring the progress of a multiple sclerosis therapy of a patient, the method comprising:

15 a) determining an amount of an IAP gene expression level in a first blood sample obtained from the patient at first time period;
b) determining an amount of the IAP gene expression level in a second blood sample obtained from the patient at a second time period; and
c) comparing in the IAP gene expression levels, a decrease in the IAP gene
20 expression level at the second time period being an indication that the patient is responding to the multiple sclerosis therapy.

48. A diagnostic for diagnosing a patient suspected of having a subtype of multiple sclerosis, the kit comprising:

25 a) a vessel or vessels for receiving a blood sample from the subject;
b) an agent that specifically detects IAP protein or amplifies IAP mRNA; and
c) printed instructions for detecting the IAP protein or the amplified IAP mRNA in the sample.

30 49. The kit, according to claim 48, in which the agent for amplifying the IAP mRNA are the primers and probes selected from Table 2.

50. The kit, according to claim 48, in which the IAP protein is detected using immunoassays.

35

51. The kit, according to claim 50, in which the immunoassay is an ELISA.

52. A method of differentiating between multiple sclerosis subtypes in a patient blood sample, the method comprising:

- 5 a) determining an amount of an IAP gene expression level in the blood sample;
and
 b) correlating the amount of the IAP gene expression level in the blood sample
with the presence of a multiple sclerosis subtype.

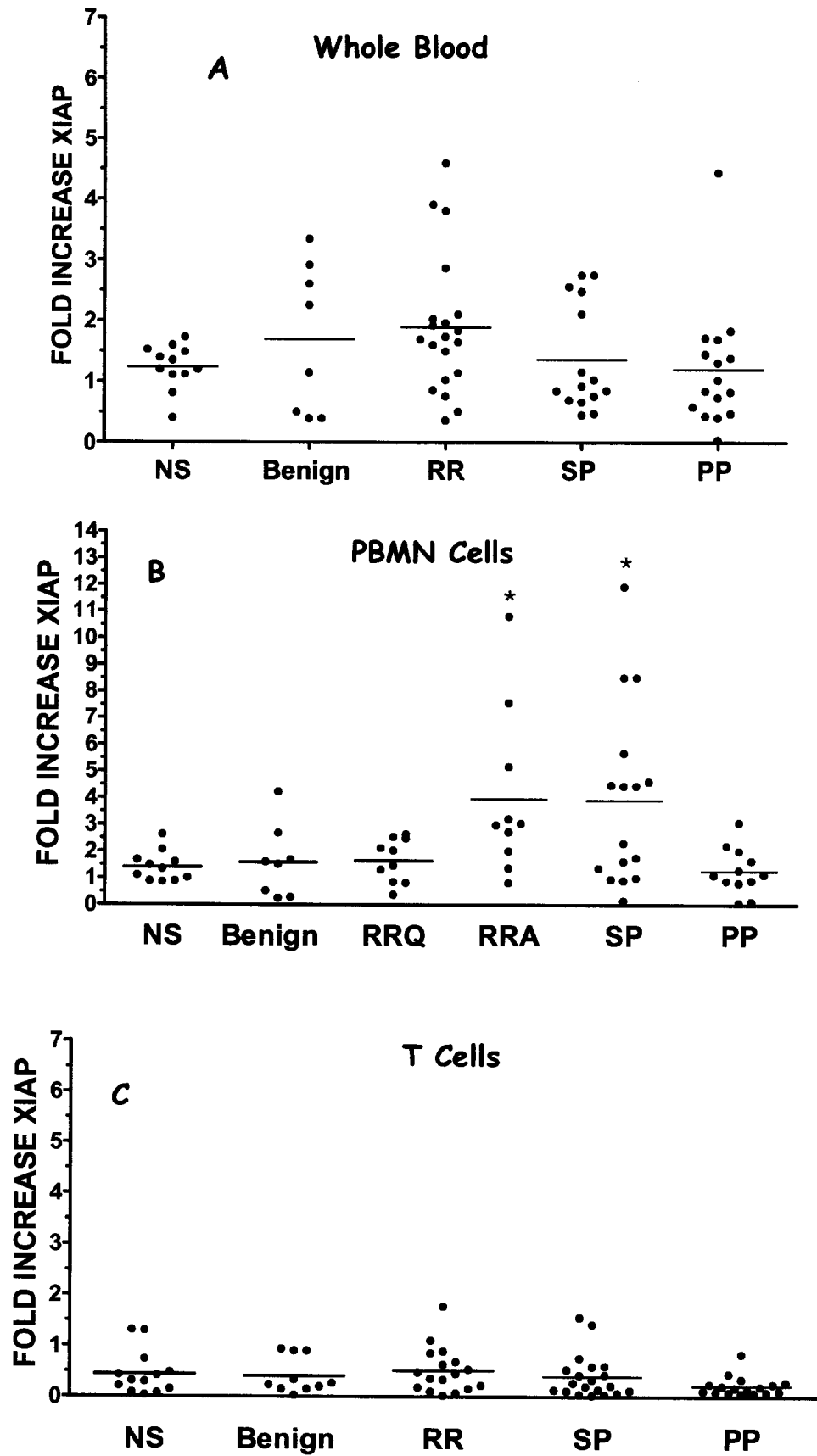


FIGURE 1

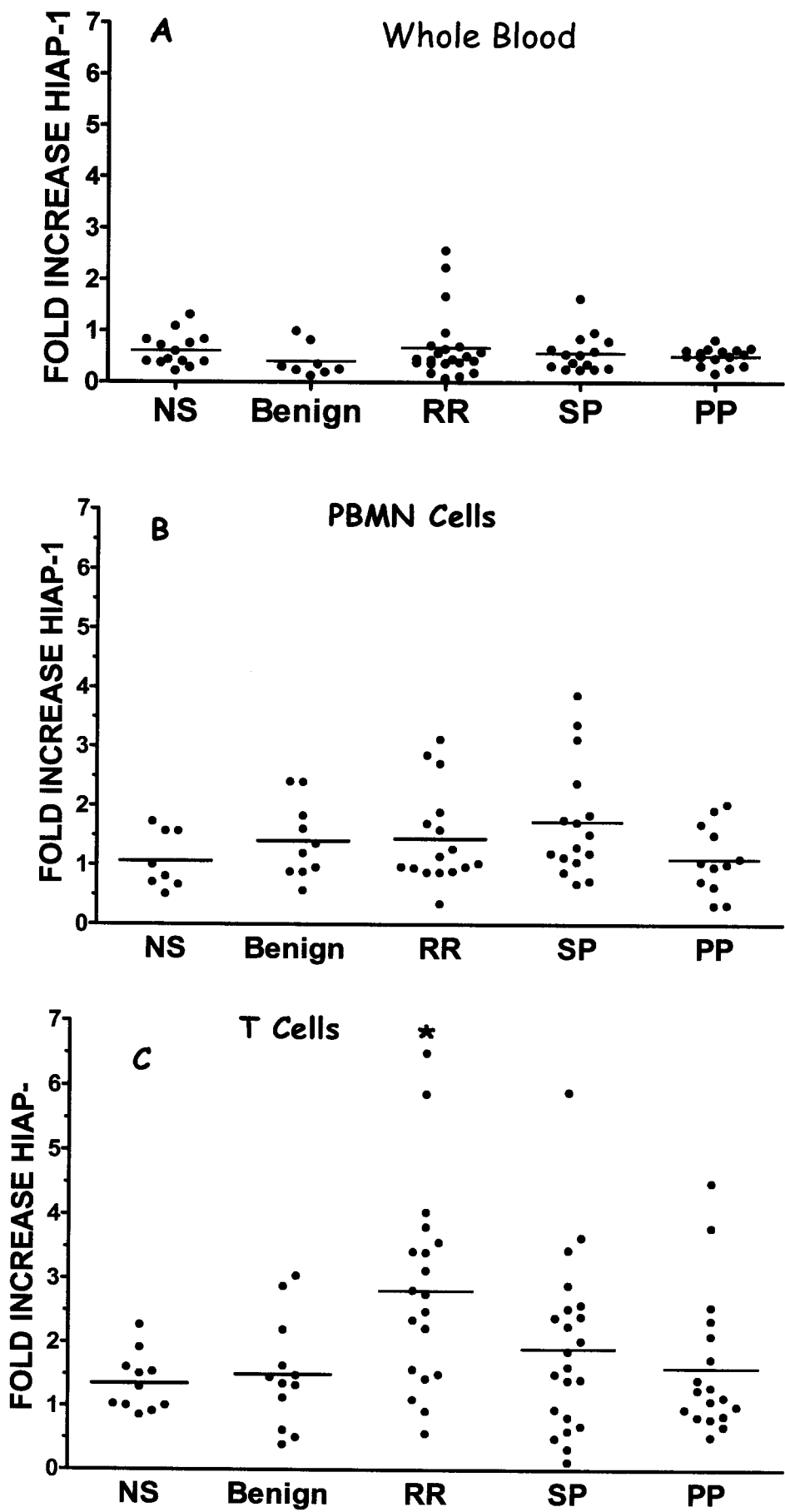


FIGURE 2

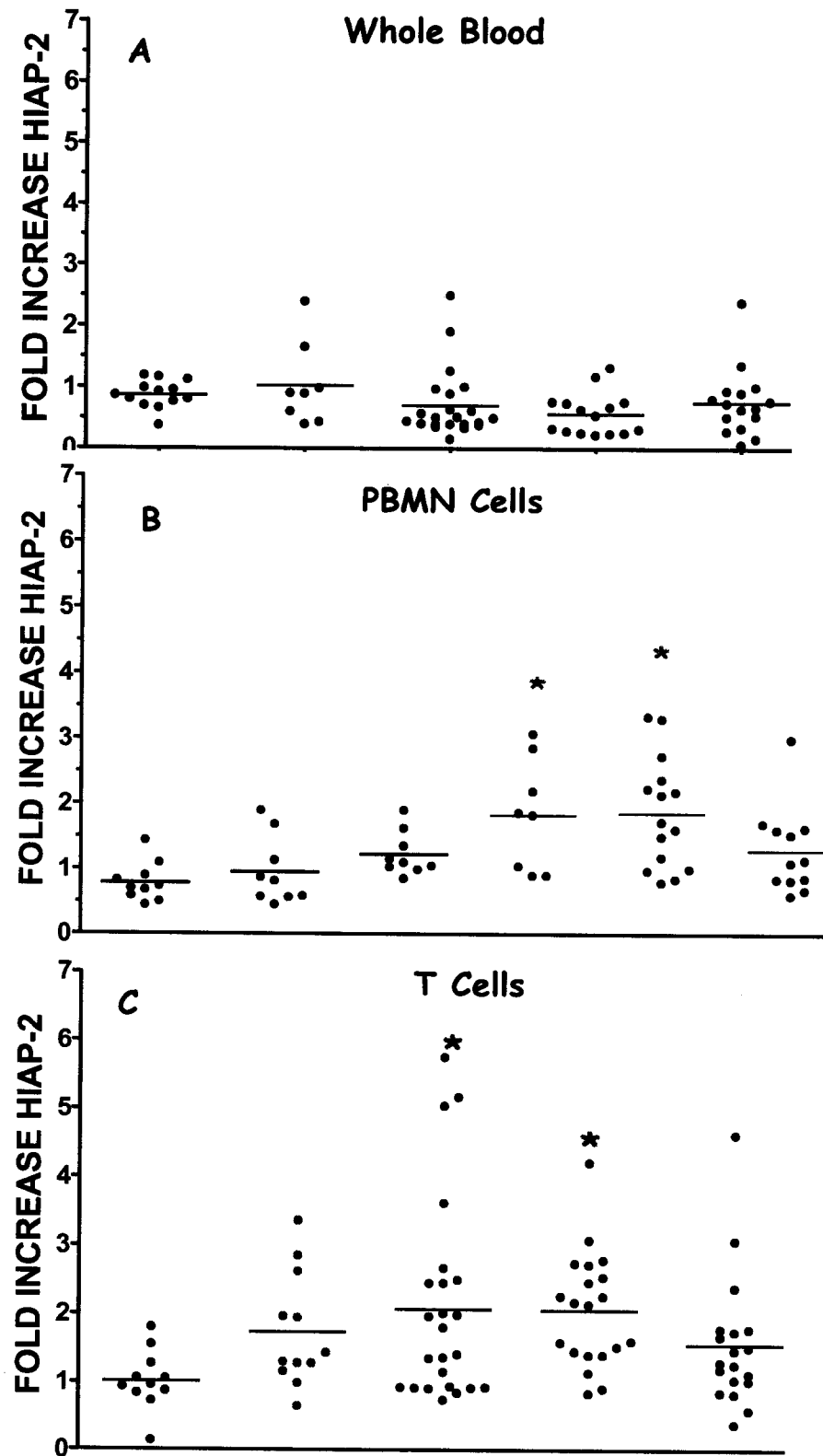


FIGURE 3

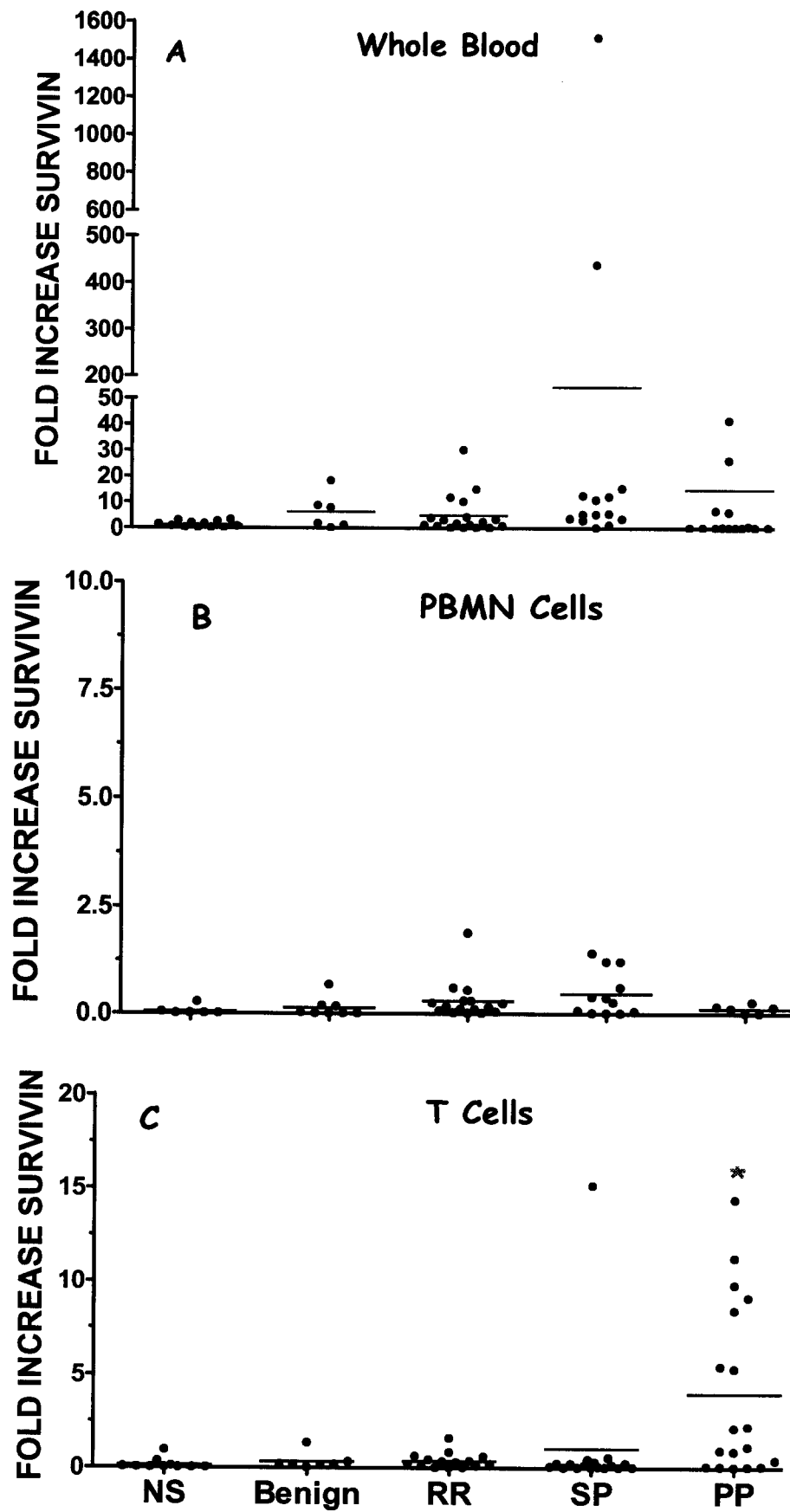


FIGURE 4

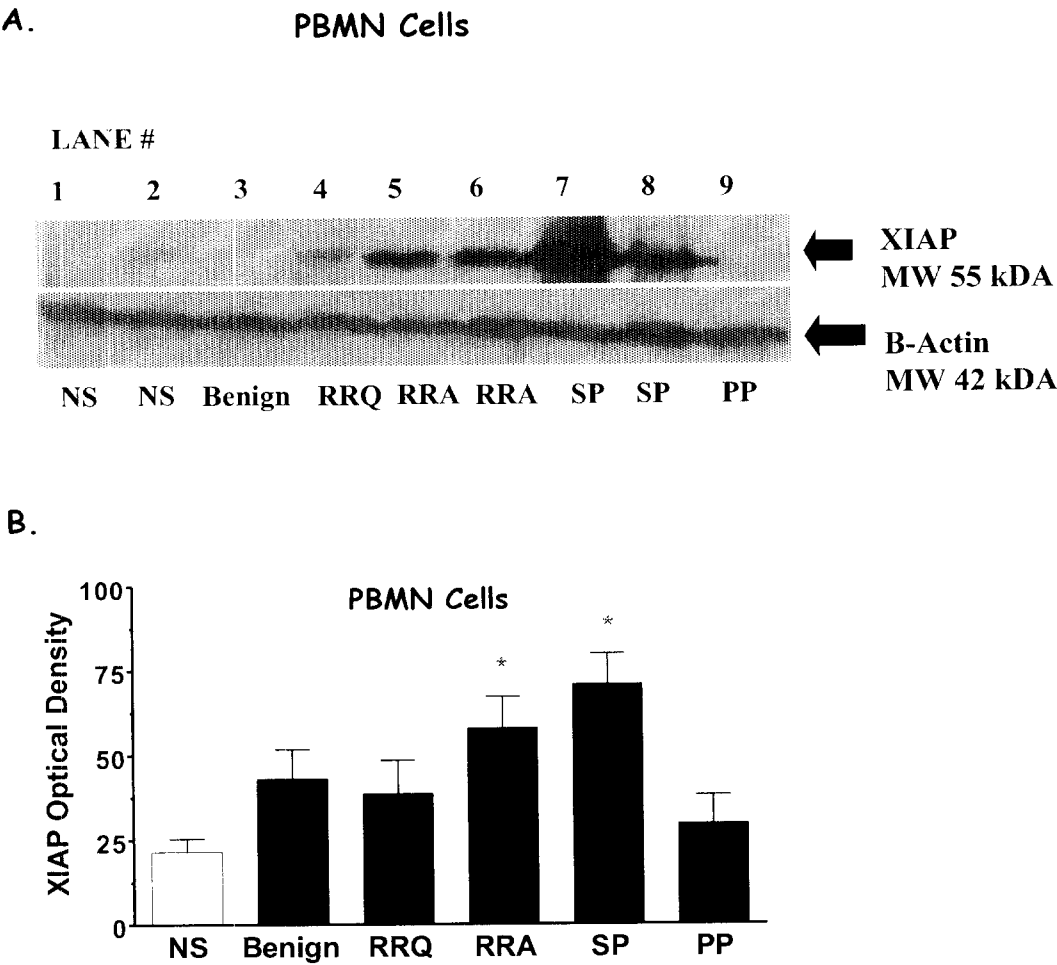


Figure 5

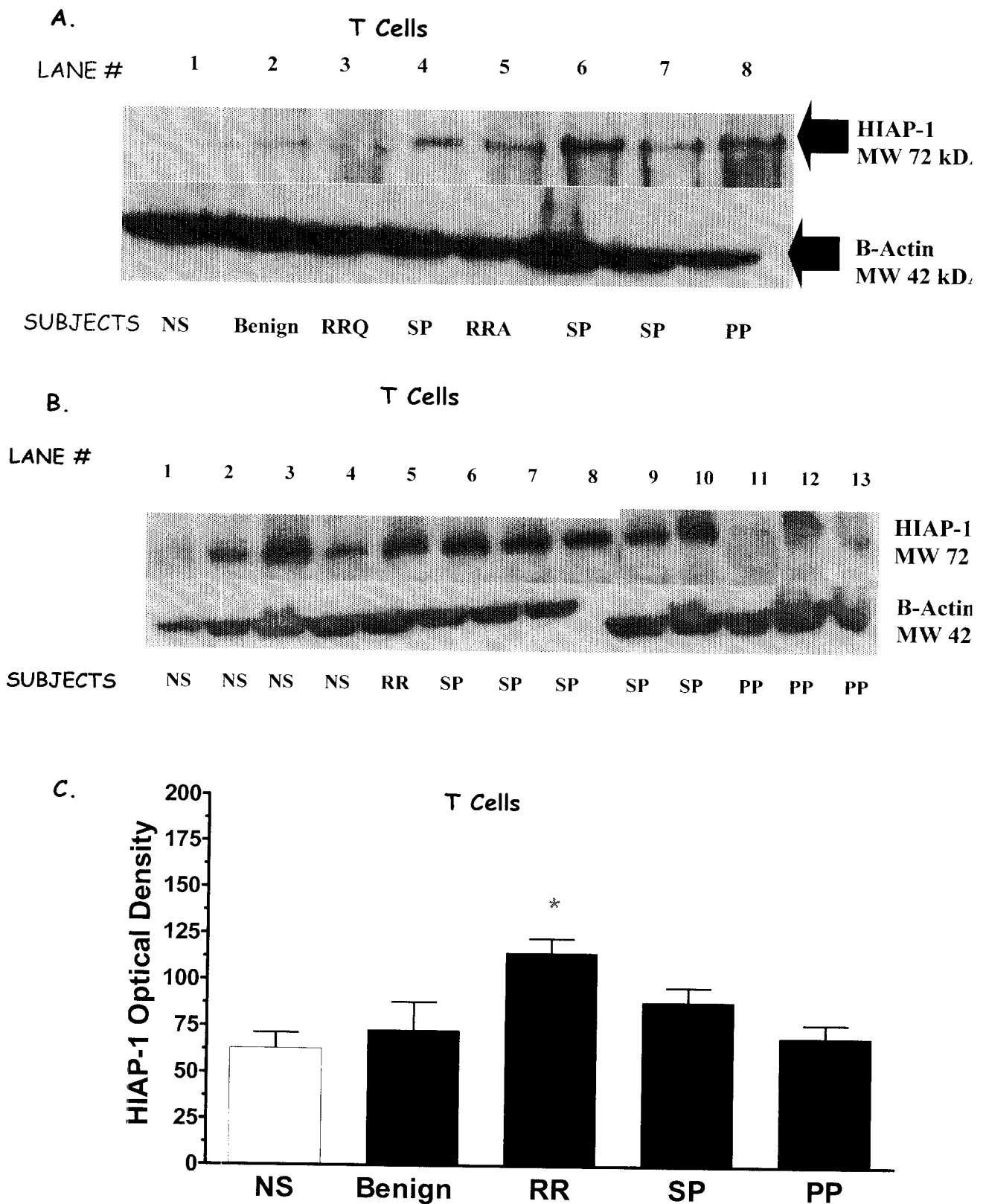


Figure 6

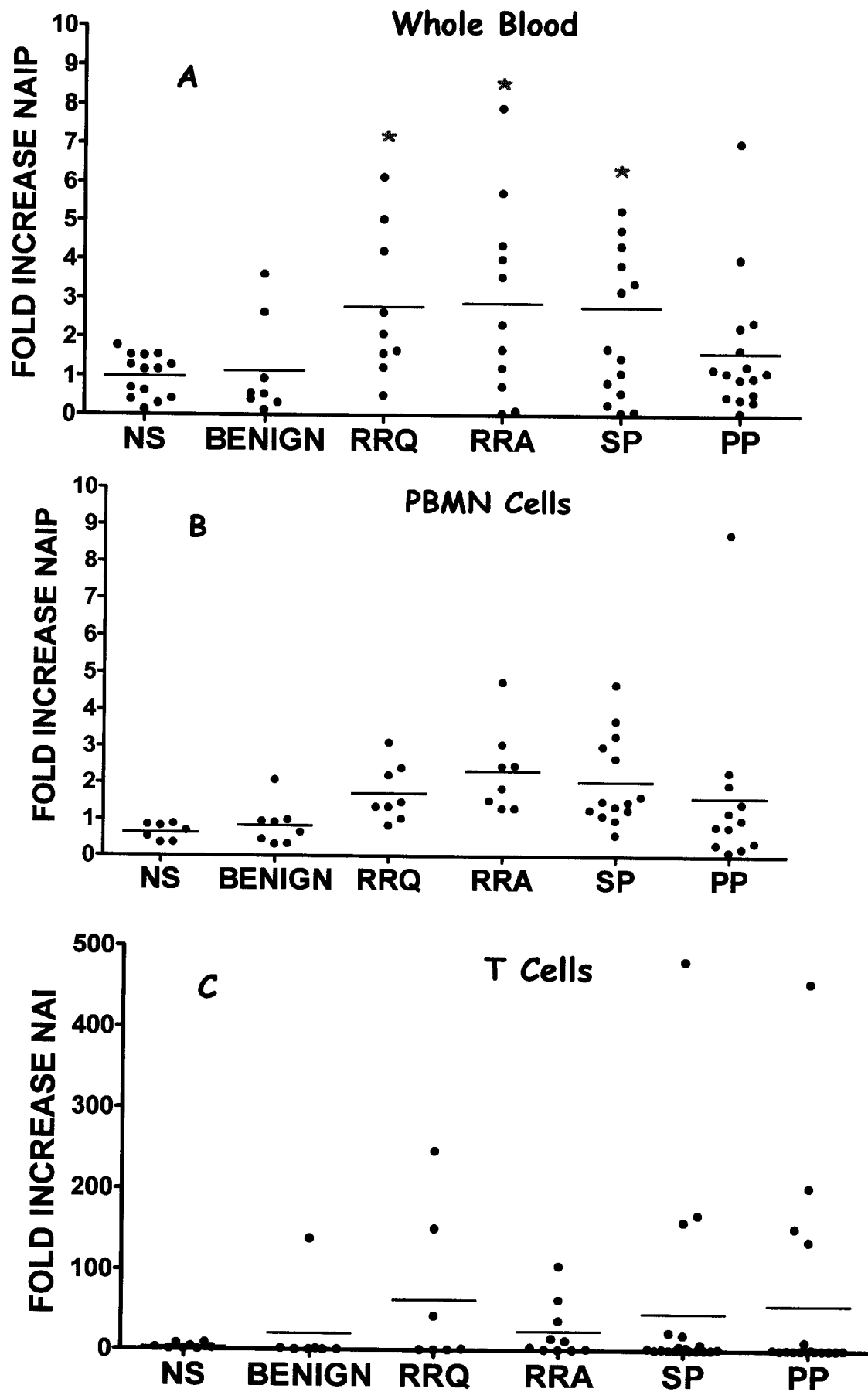


Figure 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2007/000183

A. CLASSIFICATION OF SUBJECT MATTER

IPC: *C12Q 1/68* (2006.01) , *C12N 15/12* (2006.01) , *C12P 19/34* (2006.01) , *C12Q 1/00* (2006.01) ,
G01N 33/53 (2006.01) , *C07K 16/18* (2006.01), *C07K 14/81* (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: *C12Q 1/68* (2006.01) , *C12N 15/12* (2006.01) , *C12P 19/34* (2006.01) , *C12Q 1/00* (2006.01) ,
G01N 33/53 (2006.01) , *C07K 16/18* (2006.01), *C07K 14/81* (2006.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)

Databases: PUBMED, SCOPUS, DELPHION, CANADIAN PATENT DATABASE

Keywords: multiple sclerosis, inhibitor of apoptosis protein (IAP), survivin, NAIP, HIAP-1, cIA2, HIAP-2, cIAP2, XIAP, BIR domain

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SEMRA YK ET AL. Disease activity in multiple sclerosis correlates with T lymphocyte expression of the inhibitor of apoptosis proteins, JOURNAL OF NEUROIMMUNOLOGY. January 2002 vol. 122, pages 159 - 166	1 - 25, 28 - 42, 44 - 46 and 48 - 52
A	SHARIEF MK ET AL. Upregulation of the inhibitor of apoptosis proteins in activated T lymphocytes from patients with multiple sclerosis, JOURNAL OF NEUROIMMUNOLOGY. October 2001 vol. 119, pages 350 - 357	1 - 25, 28 - 42, 44 - 46 and 48 - 52

☒ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents :	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

June 5, 2007 (05-04-2007)

Date of mailing of the international search report

5 June 2007 (05-06-2007)

Name and mailing address of the ISA/CA
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Authorized officer

Antonio Candeliere 819- 934-7935

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2007/000183

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SHARIEF MK ET AL. Heightened expression of survivin in activated T lymphocytes from patients with multiple sclerosis. JOURNAL OF NEUROIMMUNOLOGY, October 2001 vol. 119, pages 358 – 364	10, 26, 27 and 41
A	SHARIEF MK ET AL. Reduced expression of the inhibitor of apoptosis proteins in T cells from patients with multiple sclerosis following interferon- β therapy. JOURNAL OF NEUROIMMUNOLOGY, August 2002 vol. 129, pages 224 - 231	43 and 47

专利名称(译)	区分多发性硬化亚型的方法		
公开(公告)号	EP1991693A1	公开(公告)日	2008-11-19
申请号	EP2007701772	申请日	2007-02-06
申请(专利权)人(译)	AEGERA Therapeutics公司.		
当前申请(专利权)人(译)	AEGERA Therapeutics公司.		
[标]发明人	BHAN VIRENDER GILLARD JOHN W HEBB ANDREA HOLCIK MARTIN KORNELUK ROBERT G ROBERTSON GEORGE		
发明人	BHAN, VIRENDER GILLARD, JOHN, W. HEBB, ANDREA HOLCIK, MARTIN KORNELUK, ROBERT, G. ROBERTSON, GEORGE		
IPC分类号	C12Q1/68 C12N15/12 C12P19/34 C12Q1/00 G01N33/53 C07K16/18 C07K14/81		
CPC分类号	C12Q1/6883 C12Q2600/112 C12Q2600/158 G01N33/564 G01N2800/285		
优先权	60/765734 2006-02-07 US		
其他公开文献	EP1991693A4		
外部链接	Espacenet		

摘要(译)

通过分析凋亡蛋白抑制剂 (IAP) 家族成员的基因或蛋白质表达来区分多发性硬化亚型的方法: X连锁凋亡蛋白抑制剂 (XIAP) , 人凋亡蛋白-1抑制剂 (HIAP-I) , 人凋亡抑制蛋白-2 (HIAP-2) , 生存素和神经细胞凋亡抑制蛋白 (NAIP) 。该方法包括a) 通过qRT-PCR或蛋白质印迹确定血液样品, 外周血单核细胞样品或静息T淋巴细胞样品中表达的IAP基因或蛋白质的量; b) 将IAP基因或蛋白质表达水平的数量与Relap sing-Remitting (RR) , Secondary-Progressive (SP) , Primary-Progressive (PP) , Relap sing-Remitting Quiescent (RRQ) 和/的关联相关联。或复发 - 缓解活动 (RRA) 多发性硬化亚型。