Novel Diagnostic Ocular Toxoplasmosis Biomarkers

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Preface

To say I am a “dwarf standing on the shoulders of giants” is a gross understatement. My achievements both past, present and future are due entirely to the incredible support I receive from my family, professors, colleagues, and friends. Over the course of my life I have been fortunate to be immersed in a nurturing environment and tutored by incredibly caring and thoughtful individuals. The following manuscript has been put together not only by me but the countless others who have led me, taught me and fought with me to get to this point. Though it would be impossible to acknowledge everyone who has been with me over the course of my studies, I would like to take the time to mention those who had a direct impact.

Without Dr. Miguel N. Burnier Jr. this study would have never occurred. He built a laboratory for this exact type of interdisciplinary work. The environment he created brings together physicians and researchers from across McGill and from around the world. This allowed me to collect samples in Brazil and analyze them seamlessly in three different laboratories scattered around McGill. I could never have asked for a better supervisor. He allowed me to see my potential, while providing me with every opportunity to grow. Equally, I want to thank Dr. Phil Gold for simply being the mentor every student dreams of. He is the most caring, understanding and brilliant man I know. After every talk we have I have a renewed hope for the future.

Thank you to the amazing team at the Henry C. Witelson Ocular Pathology Laboratory. In particular I would like to single out Dr. Rubens N. Belfort whom without I would have lasted neither my first week nor last days in the lab without you. His guidance as a friend will not be forgotten. Christine Straccini the award winning friend and proteomics wizard who taught me what research is really about as well as how to perform the experiments, analyse the data and run the statistics. You are the true hero this study. Dr. Bruno F. Fernandes an amazing coach who always has time to listen to me and guide me especially in how to prepare and edit texts. Dr Momar Ndao, who helped immensely in the design of this study and the preparation of this thesis; his willingness to advise me in your capacity of external thesis advisor, and for providing me with unrestricted access to the CDC/McGill *T. gondii* outbreak data was a tremendous gift. Dr Manon Auger for agreeing to act as my internal thesis advisor, her professional guidance was more help than she can ever imagine. Dr. Bernard Gibbs for performing the protein identification experiments and aiding me in analysing the data generated. Dr Rubens Belfort Jr., who gave me the opportunity to share my ideas with hundreds of people and, of course, along with Dr. Rubens N. Belfort for providing the patient material and data as well as Dr. Edith Zorychta for introducing me to the field pathology, developing the program and providing me with editorial support. Finally, I want to thank Mom, Dad, Grandma, Grandpa, Gregory and Rachel for being so caring, understanding and patient with me, while providing me with the ideal environment to succeed. Also, a big thank you to Dr. Catherine Potvin, whose expertise in phytophysiology might seem out of place in pathology thesis but she showed me that I was capable of independent research and more importantly thought. To her and everyone else, I am indebted and ever grateful.

Abstract

Purpose: Ocular toxoplasmosis is the most common etiology of posterior uveitis. The high incidence of macular scarring associated with ocular toxoplasmosis is a leading cause of visual morbidity. Serum biomarkers of the disease would aid in its diagnosis. This work was designed as a pilot study to detect potential biomarkers.

Methods: Blood serum samples were collected from four groups of nine patients each; healthy, uveitic, one single-toxoplasmic event and recurrent events. Protein profiles were generated by SELDI-ToF-MS and 2-DE. Proteins were sequenced using tandem-MS. A tree-based decision classification system was developed.

Results: 50 markers of ocular toxoplasmosis and 46 markers of recurrent disease were discovered by MS; 47% were cross-validated; 14 biomarkers were selected for validation and all were visualized by SDS-PAGE. 2-DE yielded 57 differentially expressed bands, 20 of which were excised and identified.

Conclusions: This pilot study sough to elucidate blood serum biomarkers for ocular toxoplasmosis, as no biomarker for ocular toxoplasmosis exists currently. This study demonstrates the potential for SELDI-ToF-MS and well as other MS technologies to identify novel biomarkers for this disease.

Résumé

Objectif : La toxoplasmose oculaire est la cause principale de l'uvéite postérieure. Les cicatrices maculaires associées à la toxoplasmose oculaire sont à la base de la morbidité visuelle. Des marqueurs sérum sanguin de la maladie en faciliter le diagnostic.

Méthodes : Les échantillons de sérum sanguin ont été recueillis auprès de quatre groupes de neuf participants composées de patients : sains, avec uvéite, ayant subit un seul événement toxoplasmique et ayant subit des événements récurrents. Des profils protéiques ont été générés par SELDI-ToF-MS et le 2-DE. Les protéines ont été séquencées à l'aide de tandem MS. Un système de classification basé sur des arbres décisionnels a été élaboré.

Résultats : 50 marqueurs de la toxoplasmose oculaire et 46 marqueurs de récidive de la maladie ont été découverts par MS. 47% de ces marqueurs ont été validés par recoupement et 14 biomarqueurs ont été retenus pour validation. Tous ont été visualisés par SDS-PAGE. 2-DE, ce qui a abouti à 57 différentiellements exprimés en bandes dont 20 ont été excisées et identifiées.

Conclusions : Cette étude pilote visait à identifier des biomarqueurs sériques potentiels de la toxoplasmose oculaire. Ici, on a dénoté l'utilité de la technologie SELDI-ToF-MS et des autres technologies pour identifier de nouveaux biomarqueurs pour cette maladie.

Chapter 1: Introduction

1.1. Research Rational

Toxoplasmosis is caused by the obligate intracellular protozoan *Toxoplasma gondii*; one of the most common parasitic infections in the world, infecting almost one-third of all humans (Jackson and Hutchison 1989). Infection can be acquired through ingestion of food or water contaminated with oocysts excreted from the feces of infected felines; by eating undercooked or raw meat containing tissue cysts; or transmitted congenitally via tachyzoites passage across the placenta barrier (Weiss and Kim 2007) (Figure 1). Tachyzoites, the actively dividing forms of the parasite, disseminate throughout the body by means of the blood stream and lymphatic system, invading both nucleated phagocytic and nonphagocytic cells. Multiplication of tachyzoites in a cell leads to cell lysis, with the possibly of daughter tachyzoites infecting other cells or being converted to tissue cysts containing bradyzoites (Figure 2a).

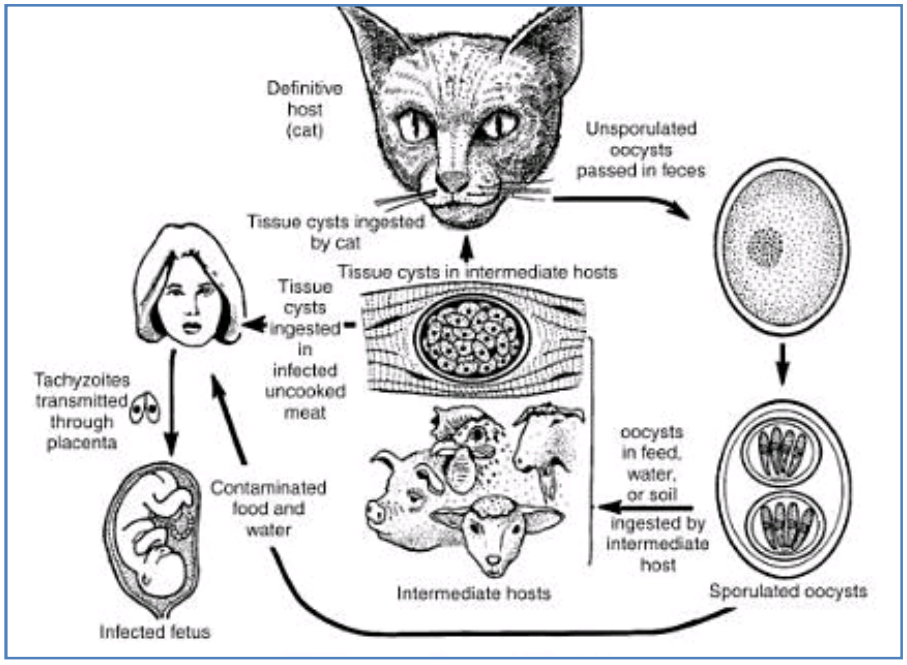

Figure 1: Life cycle of *T. gondii*. "Cats, the definitive hosts of the parasite can become infected by ingesting sporulated oocysts or, most often, through the consumption of infected animals.

The oocysts are infectious to all mammals and most birds. Toxoplasma can be transmitted to intermediate hosts through oocysts, by carnivorism, or transplacentally. Transplacental transmission is most important in humans and sheep" (Dubey 1986).

Although tissues throughout the body may be infected, in humans, cysts are found to predominate in the eye and other parts of the central nervous system. Theories explaining peripheral involvement of the CNS in toxoplasmosis include the passage of the parasite across the blood-brain-barrier and its poor clearance from the eye, though it is not known whether *T. gondii* penetrates these areas relatively easily or whether immunologically privileged sites fail to eradicate the parasite (Peterson and Remington 1997). Characteristic changes in human behaviour have been linked to *T. gondii*'s neurotrophism; impaired reaction times, leading to an increase in traffic accidents (Flegr, Klose et al. 2009) and an increase in psychiatric symptoms including schizophrenia as well as mental retardation (Zhu 2009) are but some of the examples.

Even as clinical sequelae of acute and congenital toxoplasmosis are well established, that of chronic *toxoplasma* infection remains uncertain (Holliman 1997). The majority of *T. gondii* infected patients are asymptomatic with clinical manifestation depending on the patient's immune status and the clinical setting. Once an individual is infected by *T. gondii*, "the retina is randomly undetectably 'seeded;' allowing for the possibility of local recurrences, which causes irreversible damage to the retina" (Weiss and Kim 2007) (Figure 2a). The most common pathological conditions resulting from infection are lymphadenopathy and retinochoroiditis (Montoya and Liesenfeld 2004). Non-ocular infections are not usually serious in otherwise healthy adults. In contrast, toxoplasmic retinochoroiditis is a progressive, recurring disease that can cause severe visual morbidity. The severity of the ocular lesion and its propensity to reoccur

varies among patients and may be due to the strain of *T. gondii*, chronic reinfection or the genetic characteristics of certain patient populations (Belfort-Neto, Nussenblatt et al. 2007).

It has been shown that the reactivation of ocular toxoplasmosis cannot be considered a local event (Contini, Seraceni et al. 2005) but rather a systemic one (Fortier, Aïssi et al. 1991). Once a lesion becomes active, parasite levels are detectable as *T. gondii* enters blood via choroidal circulation and/or aqueous humour drainage. Although the mechanisms of ocular toxoplasmosis reactivation are unknown, accumulating evidence suggests that senescent changes in tissue cysts with release of antigens, trauma, hormonal changes and/or fluctuation of cellular and humoural immune response could be responsible for disease recurrence (Holland 2000; Silveira, Belfort et al. 2001). Other theories include that recurrences could be induced by reinfection with a different strain, as was demonstrated in a murine model (Dao and Fortier 2001)

Ocular toxoplasmosis is the most common etiology of posterior and infectious uveitis, both in community-based practices of comprehensive ophthalmology and in tertiary referral services globally (McCannel, Holland et al. 1996). Up to 20 % of infected individuals present with retinal lesions (Vallochi, Nakamura et al. 2002) of which these infections account for a third of all infectious uveitis cases (McCannel, Holland et al. 1996). The high incidence of macular scarring associated with ocular toxoplasmosis, in absolute terms, is a leading cause of visual morbidity in the developing world causing a highly negative social and economic impact on those affected. To control ocular toxoplasmosis effectively, efforts should be directed towards developing a sensitive method for effective diagnosis. Presently there exist no diagnostic assays available that can determine which patient will develop the recurrent disease.

Currently the “gold standard” for toxoplasmosis detection is direct identification of the parasite, but this method is expensive, time-consuming and relatively insensitive (Chen, Lu et al. 2008). Pathological diagnosis of ocular toxoplasmosis can be established by: identifying the cysts in biopsies stained with hematoxylin and eosin; monoclonal or polyclonal antibodies through immunohistochemistry (Rao and Font 1977); or by polymerase chain reaction (PCR) (Brézin AP 1990). Histopathologically, ocular toxoplasmosis usually presents extensive granulomatous inflammatory infiltration of the choroid and areas of necrosis of Bruch’s membrane (Belfort, Fernandes et al. 2009) (Figure 2b).

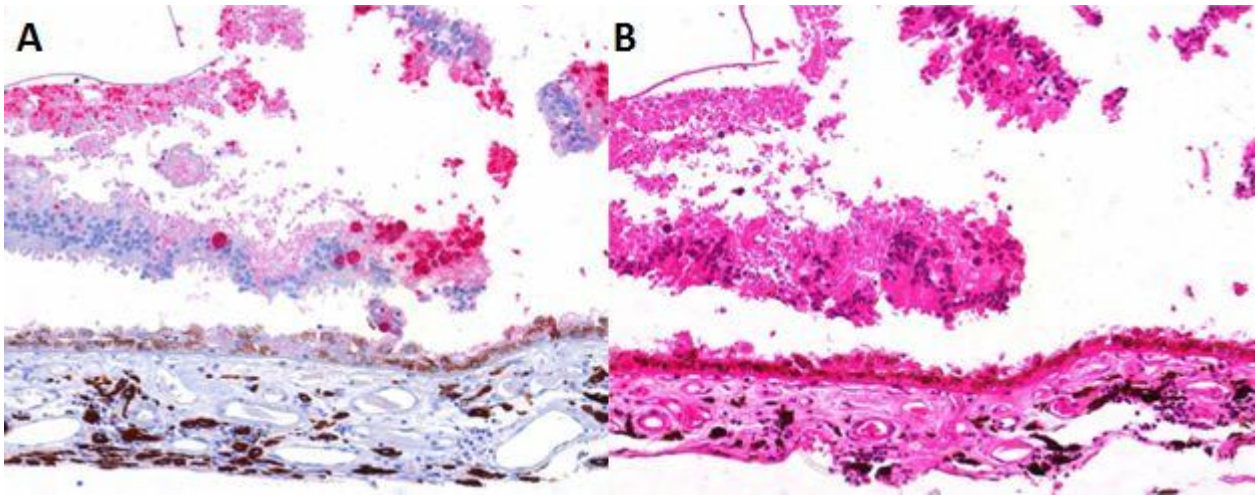

Figure 2: A) Immunostaining for small tissue cysts termed bradyzoites describes the stage of the parasite encysted in tissues. They are resistant to gastric juices whereas tachyzoites are destroyed immediately. Thus, tissue cysts are important in the life cycle of T. gondii because carnivorous hosts can become infected by ingesting infected meat. B) Hematoxylin and eosin staining diagnostic of necrotic retina due to *T. gondii*. (Courtesy of Dr. Miguel Burnier)

Typically clinical ophthalmologic findings together with positive *T. gondii* serology provide sufficient information to make a diagnosis. “Enzyme-linked immunosorbant assay (ELISA) detection of *Toxoplasma*-specific antibodies is highly sensitive and specific when compared to

the indirect immunoflurescent antibody test (IFAT) used as a reference" (van der Puije, Bosompem et al. 2000). Immunoglobulin gamma (IgG) antibody detection is widely used to diagnose *T. gondii* infection, but IgG antibodies demonstrates lifelong persistence among immunocompetent *toxoplasma*-infected individuals. Therefore, the presences of IgG antibodies cannot confirm a diagnosis of ocular infection but rather a negative result usually rules it out. Such antibodies can persist often at high titers for years after the acute infection, and there is a high prevalence of such antibodies in the general population (Ongkosuwito JV 1999). Therefore, alone this test cannot be used to discriminate between recent and more distant infection. Moreover, it may be inconclusive in patients with underlying diseases causing suppressed antibody responses or in patients receiving immunosuppressive therapy (Suzuki and Kobayashi 1987). The detection of IgM class antibodies in patients with ocular toxoplasmosis were shown to peak at two months, persisting for up to six months, though elevated levels of specific IgM have been known to be present for several years (Payne, Joynson et al. 1987). Nevertheless, the presence of IgM alone is not a reliable indication of recent infection and the detection of IgM antibodies were shown to have a low predictive value for primary *T. gondii* infection (Jenum, Stray-Pedersen et al. 1998). More recently, serum IgG avidity tests has been employed to diagnose recently acquired infections. The IgG avidity test is useful as a confirmatory test along with other conventional serological tests, where high-avidity diagnoses past infection. However, persistence of low-avidity IgG reduces the test's value as in this situation it cannot differentiate between acute and past infection (Ashburn, Joss et al. 1998) and "should therefore not be used alone as a definitive assay guiding the clinician" (Montoya, Huffman et al. 2004) (Table 1).

	Antibody Class/Test	Use in Ocular Toxoplasmosis
Indirect Detection/Serology	IgG	+ (low titres are usually seen in patients with reactivation of congenital disease; intraocular antibody production [ratio of ocular and blood antibody titres])
	IgG avidity	+ (high avidity results rule out infection in recent 3–4 months; low avidity antibodies may persist)
	IgM	+ (high titres usually in patients with acute acquired disease, negative results in patients with reactivation of congenital disease)
	IgA	-
	IgE	-
Direct Detection	PCR	+ (particulary useful in patients with atypical retinal lesions or suboptimum response to therapy [vitreous or aqueous fluid, vitreous fluid preferred])
	Histopathology (IMC)/Cell culture or mouse inoculation	- (until serological tests were developed, the diagnosis of toxoplasmosis depended on histological examination and animal inoculation)

Table 1: A major task facing researchers is the development of an assay which can serologically distinguish between congenital and recently acquired infection. This table outlines the value of various diagnostic assays (Adapted from Montoya 2004).

T. gondii DNA has been identified in ocular tissue sections of patients with presumed toxoplasmic retinochoroiditis by PCR, even when typical tissue cysts were not identified upon histopathologic examination (Brézin AP 1990; Ongkosuwito JV 1999). PCR has been shown to be of diagnostic value for detection of parasitic DNA in various biological samples, including whole blood, ocular fluids as well as retinal sections and has "gained wide acceptance because of its simplicity and sensitivity" (Klaren and Kijlstra 2002). Examination of vitreous fluids by PCR is a useful diagnostic aid establishing the differential diagnosis in patients where presentation is

atypical (Montoya, Parmley et al. 1999; Rothova A, de Boer JH et al. 2008). Nested (n)-PCR is a reliable diagnostic technique for ocular toxoplasmosis, because of the amount of specimen required, speed, cost effectiveness, and high specificity in detecting *T. gondii* DNA in intraocular fluids (Calderaro A 2006; Mahalakshmi B 2006). Although the sensitivity of n-PCR and real-time (RT)-PCR techniques are similar, RT-PCR has since replaced n-PCR as a quicker and simpler technique for quantitatively evaluating ocular samples for the presence of infectious pathogens (Lin MH 2000; Dworkin LL 2002; Rothova A, de Boer JH et al. 2008). One study found RT-PCR to have a sensitivity of 38 % for the detection of ocular toxoplamosis from aqueous humour (Fekkar A 2008). Even though sensitivity was increased to 97 % through the use of RT-PCR in conjunction with two other biological methods: Western blotting and the calculation of the Goldmann-Witmer coefficient; current laboratory diagnostics of ocular toxoplasmosis resulting from the reactivation of a latent infection on samples of aqueous humour, are inadequate because of the PCR's low sensitivity (Fekkar, Bodaghi et al. 2008).

Despite the assay's low sensitivity when sampling ocular fluids, the use of RT-PCR to detect circulating *T. gondii* from peripheral blood during a recurrent episode offers not only a reduction in the potential of product carryover between assays, but also limits patient risks associated with biopsy retrieval. A randomized controlled trial of 32 patients was able to detect less than 0.5 organisms per sample demonstrating the use of RT-PCR with the fluorescent SYBR Green I in conjunction with the 529bp gene from the whole blood in patients with *Toxoplasma* posterior uveitis, but only in two individuals (Belfort, Isenberg et al. 2010). To date, the respective contributions of both aqueous humour and peripheral blood in PCR tests for the diagnostic

confirmation of ocular toxoplasmosis is still not clear (Fardeau, Romand et al. 2002). No reliable, clinically available method demonstrating *T. gondii* in the peripheral blood of ocular toxoplasmosis patients is able to establish diagnosis in atypical cases, monitor treatment efficacy and/or to study disease mechanisms including recurrence has been described. Furthermore, no clinical trial to validate such methods in different settings of patients with toxoplasmosis to been established or evaluated.

1.2. Objectives of the research

As proteins represent the vast majority of biologically active molecules responsible for cellular function and with PCR diagnostics proving to be inconclusive, protein biomarkers could be useful in the future diagnosis of ocular toxoplasmosis. To date, no single, or group of, protein biomarkers have been described for the diagnosis of either ocular or systemic toxoplasmosis because the technology needed for the detection of non-invasive biomarkers has only recently become available. Clinical proteomics, the quantitative study of protein expression between samples to identify disease-specific proteins, has now been made possible due to the accumulation of DNA and protein sequence databases, improvements in mass spectrometry (MS), and the development of computer algorithms for database searching (Graves and Haystead 2002) as well as advances in two-dimensional protein separation.

One of the first methods developed for the analysis of complex protein extracts from cells, tissues or other biological samples is two-dimensional gel electrophoresis (2-DE). 2-DE is a multistep process that takes days to complete. Nevertheless, the concept behind the technology

is simple: the first dimension is resolved by the isoelectric focusing (IEF), proteins are separated according to their isoelectric points; following that the second-dimension is resolved through the use of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), which separates the proteins according to their molecular weight (O'Farrell, 1975). Using O'Farrell's technique the pathophysiology and phenotypes of various diseases including acute lymphoblastic leukemia and acute myeloid leukemia were able to be elucidated (Hanash et al. 1988).

Despite these successes, 2-DE has been kept from the forefront of biomarker discovery research even when combined with MS due to concerns over the quantification of protein expression differences as well as the need for protein characterization and identification (Hanash 2003). The results obtained by 2-DE are difficult to reproduce, even within a single laboratory, and more difficult between laboratories. Reasons for this limited reproducibility like the batch-to-batch variability of carrier ampholytes of the IEF, pH gradient instability over time and cathodic drift have been largely overcome by the development of immobilized pH gradients (IPGs), the method employed in this study (Frobel 2009).

These IPGs are based on the use of bifunctional immobiline reagents, ten chemically well-defined acrylamide derivatives, which form a series of buffers with different pK values between one and less than 12. They are co-polymerized with the acrylamide matrix and generate extremely stable pH gradients (Bjellqvist et al., 1982). Nevertheless, conventional 2D-PAGE analysis is not suitable for high-throughput studies because the samples have to be separated on individual gels and quantification is time-consuming and inaccurate. As such we chose to perform 2-DE in parallel with MS.

MS is an analytical technique for the determination of the elemental composition of a sample. Increasing in the past decade MS-based proteomics has become an indispensable tool for molecular and cellular biology and for the emerging field of systems biology. These include the study of protein-protein interactions via affinity-based isolations on a small and proteome-wide scale, the mapping of numerous organelles, the concurrent description of the various organismal genomes and proteomes, and the generation of quantitative protein profiles from diverse species.

It is precisely the potential for MS to yield a compressive summary of a given biological fluid or tissue's proteome, without the need to first carry out protein separations, by gels for example, which has garnered much interest (Wright Jr. and Cazares et al. 1999). Theoretically, such a method is well suited for biomarker discovery because of high-throughputs, rapid analysis, and minimal sample requirement. Indeed, surface-enhanced laser desorption/ionization time-of-flight mass spectroscopy (SELDI-ToF-MS), has been described and developed to meet these precise needs (Hutchens and Yip 1993).

The three MS techniques principally used today in biomarker discovery studies are laser desorption/ionization (LDI), matrix-assisted laser desorption/ionization (MALDI) and SELDI. All three techniques employ the same general principle of ionizating the solid-state sample by photoinduction and then detecting the ionized proteins. This is done first by presenting the sample as either crystals or as a thin film on a sample support called a probe and then using the energy of a focused laser beam to promote the creation of gaseous ions from solid-state matter (Scot R. Weinberger, Lee Lomas et al. 2007). The ionized gaseous molecules then enter the

time-of-flight mass spectrometer (ToF-MS) region of the instrument, which measures the mass-to-charge (*M/z*) of each protein, based on the time requirement for the ions to "fly" down the vacuum tube towards an oppositely charged electrode called the detector (Figure 3). Ions reach the detector at different times depending on their weight; heavier ones take longer, according to their initial amount of kinetic energy[1]. Each ion that strikes the electrode is registered as a component of the data spectrum that emerges from the analysis. The output generated from the ToF-MS analysis is a series of peaks showing the relative abundance versus the molecular weights of the detected proteins.

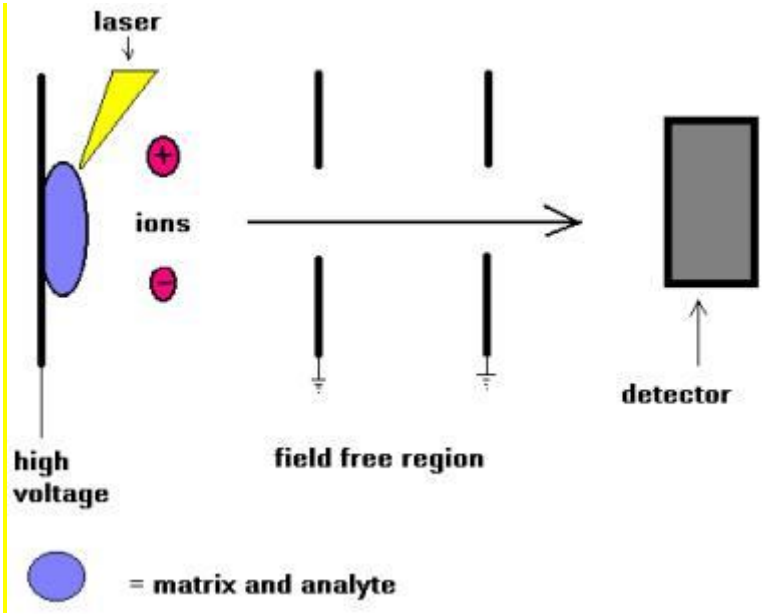

Figure 3: A simplified diagram of a mass spectrometer. Regardless of technology, when referring to MS the three most important components are the ion source (typified by the matrix and its analyte), the mass analyser (field free region) and the detector. All derivative technologies, including LDI-, MALDI- and SELDI-MS-ToF are based on these concepts (Courtesy of A.E. Ashcroft).

[1] Kinetic energy = $(0.5)mv^2$, therefore $\mathcal{V}xy=(2KE/m)^{0.5}$. In other words, as mass increases, velocity decreases by a constant $2x^{0.5}$

The main difference between the three techniques is that in both LDI and MALDI the probe simply presents the sample to the mass spectrometer for analysis, playing a passive role in the overall analytical scheme, whereas in SELDI the probe plays an active role in sample presentation. In practical terms this means that although all three techniques are suitable for the study of purified protein products; only the SELDI platform allows for the analysis of heterogeneous materials such as serum.

The study of heterogeneous material by SELDI is enabled by the implementation of two subset technologies: surface-enhanced affinity capture (SEAC) and surface-enabled neat desorption (SEND). It is SEAC that allows the probe to accept heterogeneous samples; playing an active role in the extraction, presentation, structural modification and/or amplification of the sample. This is accomplished through the use of chemical surface arrays derived from classical chromographic separation moieties. Through mechanisms including hydrophobic, electrostatic, coordinate covalent bonding and Lewis-acid/base interactions potential biomarkers bind to various chemical surface arrays such as reverse phase, ion exchange, immobilized metal affinity capture and normal phase media. These surfaces have broad binding properties allowing for their use in *de novo* biomarker discovery, where large populations of proteins are compared with the goal of elucidating and then detecting differentially expressed elements. SEAC is the SELDI technology that has shown the greatest utility thus far. In fact, SEAC is so synonymous with SELDI that it often referred to as such in the literature. SELDI further differs from LDI and MALDI due to its SEND technology, a process where the analyte are desorpted and ionized without the application of a matrix. Rather, SEND is accomplished through the addition of an

UV-absorbing compound to the probe's surface by covalent modification and physical adsorption.

SEAC, SEND and ToF-MS technologies, together with a suite of bioinformatics software, make up a SELDI-ToF-MS platform capable of identifying differences in protein expression profiles of two or more distinct samples. This is particularly useful when analyzing complex clinical samples. Once the spectra of the samples are obtained, software can convert the peaks into one-dimensional gel view or a simplified map view to more clearly display expression differences between samples. This results in a list of the MW of proteins whose relative expression differed across the groups. Proteomic pattern analysis relies on the pattern of proteins observed rather than the identification of a traceable, binary biomarker (Figure 4). In order to find out the identities of those proteins, mass and sequence information need to be elucidated by purifying the proteins followed by a tandem ToF-MS.

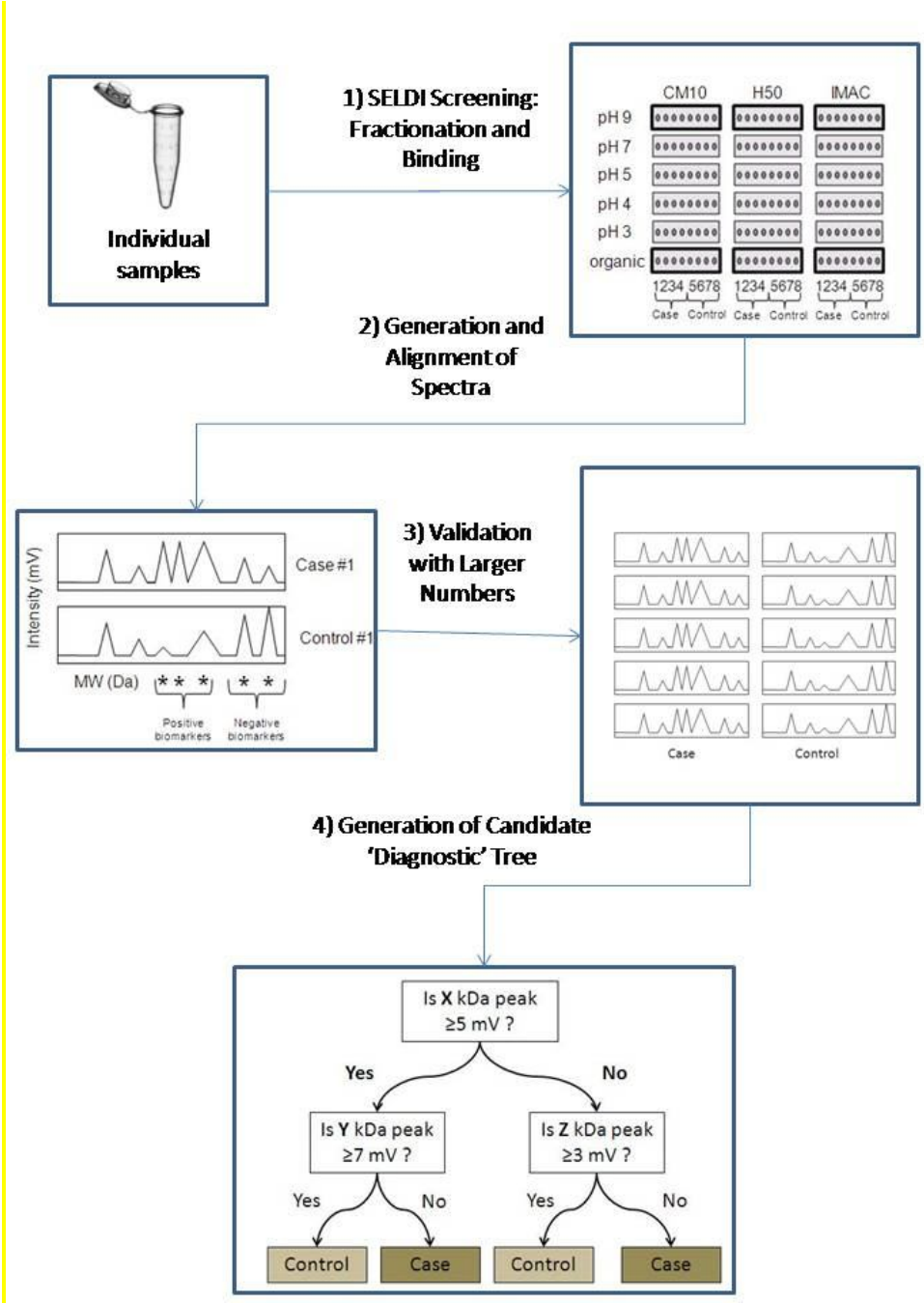

Figure 4: A schematic of SELDI-MS-ToF methodology. Individual samples are fractionated then bound and washed to a ProteinChip selected for its chemical properties. MS proceeds and spectra are generated, aligned and normalized. Results are validated in larger numbers and used to generate a diagnostic 'tree.'

Understanding that the direct measurement of protein expression can accurately indicate underlying cellular dysfunction and disease progression, SELDI-ToF-MS has been used to discover serum biomarkers for infectious diseases in the fields of virology (hepatitis B and C viruses, severe acute respiratory syndrome, HIV-1, human T-cell leukemia virus-1 and BK virus), parasitology (trypanosomiasis) and bacteriology (intra-amniotic inflammation, tuberculosis and bacterial endocarditis) (Table 2). An analysis of the 20 SELDI studies shows that the mean number of samples analyzed is 38 ± 24 cases, 33 ± 27 controls and 38 ± 25 positive controls. In terms of sample preparation, 60 % of the studies denature their serum with CHAPS/urea and use 1.25±0.55 ProteinChip types. There is no overall consensus on data analysis methodology but direct comparison and decision trees are used in the plurality of cases. Only 20 % of the studies included clinical data and only 45 % of them identified their protein peaks. Finally, independent validation and cross-validation were the preferred validatory methods.

n	Disease and/or etiological agent	Samples analyzed	Sample preparation	ProteinChip(s) used	Data analysis	Addition of clinical data	Validation	Peak ID
1	Bacterial endocarditis	Endocarditis n = 23, Healthy n = 36	Serum Q-Hyper D anion-exchange resin fractionation	CM10	Direct comparison of normalized peak intensities, Partial least squares, Logistic regresions	No	Iterative, random Sampling, Independent validation set: endocarditis n = 11; healthy n = 18	No
2	BK virus	Stable graft n = 29, BKV nephopathy n = 21, Acute allograft rejection n = 28	Urine dentured with CAPS/urea	CM10, IMAC (Cu)	Support vector machines, Random forest clasification, regression tree	No	No	No
3	Hepatitis B	Chronic hep B n = 46	Serum denatured with CHAPS/urea	CM10	Significance analysis of microarrys, Decision trees	Yes	Cross-validation	No
4	Hepatitis B	Liver cirrhosis n = 25, Liver cancer n = 20, Healthy n = 25	Serum denatured with CHAPS/urea	WCX2	Decision trees	No	Independent validation set: Liver cirrhosis n = 15; Liver cancer n = 10; Healthy n = 12	No
5	Hepatitis C	HCC n = 57, Liver cirrhosis n = 36, Non-liver cirrhosis n = 38, No liver disease n = 39	Serum denatured with CHAPS/urea	CM10, Q10, IMAC (Zn)	Decision trees	Yes	Cross-valdation, Independent validation set: HCC n = 56; Healthy n = 42	No
6	Hepatitis C	Responders n = 68, Non-responders n = 28	Serum denatured with CHAPS/urea	CM10, IMAC (Zn)	Logistic regression	Yes	Cross-valdation, Independent validation set: Responders n = 38; Non-responders n = 13	No
7	Hepatitis C	HCC n = 55, Chronic hep C	Serum denatured with CHAPS/urea	WCX2	Direct comparison of normalized peak intensities	No	Immuno assay, semi-quantitative Western blot	Yes
8	Hepatitis C	HCC n = 60, Non-HCC n = 84	Serum denatured with CHAPS/urea	IMAC (Cu)	Artificial neural nework	No	Cross-validation, Independent validation set: HCC n = 17; Non-HCC n = 21.	Yes
9	Hepatitis C	F1/F2 fibrosis n = 39, F4 fibrosis n = 44, HCC n = 34	Serum Q-Hyper D anion-exchange resin fractionation	CM10	Decision trees	Yes	Cross validation, Identity of apolipoprotein C-1 cofirmed by ELISA	Yes
10	HIV-1	HIV Seropositive n =31, Seronegative n = 10	Cellular lysates of cultured monocyte-derived macrophages in Triton X-100	WCX2	Decision trees	No	Cross-validation	No
11	HIV-1	HAD n = 11, Non-HAD n = 13, seronegative n = 9	Secreted proteins from cultured macrophages/monocytes/CHAPS/urea	WCX2	Direct comparison of normalized peak intensities	No	ELISA of lysate and supernatants for TNFa and lysozyme	Yes
12	Human T-cell leukemia virus type 1 (HTLV-1)	ATL n = 32, HAM/TSP n = 40, Healthy n= 28	Serum denatured with CHAPS/urea	IMAC (Cu)	Decision trees	No	Independent validation set: ALT n = 10; HAM/TSP n = 10; Healthy n = 10	Yes
13	Intra-amniotic inflamation	PLT+WBC+AFC n = 21, PTL+WBC-AFC n = 7, PTL-WBC+AFC n = 8, PTL-WBC-AFC n = 24, Healthy n = 17	Amniotic fluid diluted in binding buffer	H4	Direct comparison of normalized peak intensities	No	Independent validation set: n = 27 (details n/a)	Yes
14	Intra-amniotic inflamation	3 mothers with twins n = 6	Amniotic fluid applied directly to array	H4	Direct comparison of normalized peak intensities, mass restricted score of four proteins	No	No	Yes
15	SARS	SARS n = 8, Non-SARS n = 15	Serum denatured with CHAPS/urea	CM10	Direct comparison of normalized peak intensities	No	Independent validation set: SARS n = 7	No
16	SARS	Acute SARS n = 37, Non-acute SARS n = 74	Serum denatured with CHAPS/urea	WCX2	Decision trees	No	Independent validation set: Acute SARS n = 37; Non-SARS n = 993	No
17	SARS	SARS n = 28, Non-SARS n = 72, Healthy n = 10	Serum Q-Hyper D anion-exchange resin fractionation	CM10, IMAC (Cu)	Direct comparison of normalized peak intensities	No	Longitudinal follow-up tracked the correlation between SAA levels and pneumonia	Yes
18	SARS	SARS n = 39, Non-SARS n = 39	Serum denatured with CHAPS/urea	CM10	Significance analysis of microarrys, Hierarchical clustering	No	No	Yes
19	Trypanosomiasis	Typanosomiasis n = 45, Healthy n = 77	Serum denatured with CHAPS/urea	CM10	Artificial neural nework, Genetic algorithm, Decision tree	No	Cross-validation. Independent validation set: trypanosomiasis n = 40; healthy n = 69	No
20	Tuberculosis	TB n = 102, Postive controls n = 79, Healthy n = 12	Serum denatured with CHAPS/urea	CM10	Single- and multiple-layer perceptron, Tree classifiers, Support vector machines	No	Cross-validation, Independent validation set: TB n = 77; Positive controls n = 70; Healthy n = 9	No

Table 2: List of SELDI ID studies summarizing the patient/control populations investigated, sample preparation and data analysis methods used in the studies. The following are the acronyms used in the table above: *AF: Amniotic fluid; AFC: AF culture positive; ALT: Adult T-cell leukemia; CHAPS: 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate; CM10: Weak cationic exchange array; ELISA: Enzyme-linked immunosorbent assay; H4: Hydrophbic assay (see H50); HAD: HIV-associated dimentia; HAM/TSP:Human T-cell leukemia virus type 1-associated myelopathytropical paraparesis; HCC: Hepatocellular carcinoma; HTLV-1: Human T-cell leukemia virus type 1;IMAC: Immobilized metal affinity array; PTL: Pre-term labour; Q10: Strong anionic exchange array; SAA: Serum amyloid: WCX2: Weak cationic exchange (see CM10).* (Adapted from: Hodgetts 2007).

To date, there have been no mass spectroscopic studies performed for either systemic or ocular toxoplasmosis. As such, two hypotheses have been developed for this study: The first, there exists differential protein expression between individuals with toxoplasmic retinochoriditis and non-toxoplasmic uveitis; and the second there exists differential protein expression between individuals with their first episode of ocular toxoplasmosis and those who have had multiple episodes.

The purpose of this study is to determine the feasibility of hypothesis-driven proteomics based research in the elucidation of serum protein biomarkers for an accurate diagnosis of ocular toxoplasmosis, specifically to aid in determining the etiology of uveitis. Through the uses of SELDI-ToF-MS, along with 2-DE and LC-MS/MS, this pilot study intends to demonstrate an ability to differentiate individuals with ocular toxoplasmosis from those with non-*toxoplasma* uveitis. Furthermore, it will attempt to effectively group individuals with ocular toxoplasmosis into either single or multi-episodic ocular toxoplasmosis. The ability to differentiate between toxoplamosis and non-toxoplasmosis uveitis as well as the type of ocular toxoplasmosis would confer an advantage to our patients and their physicians.

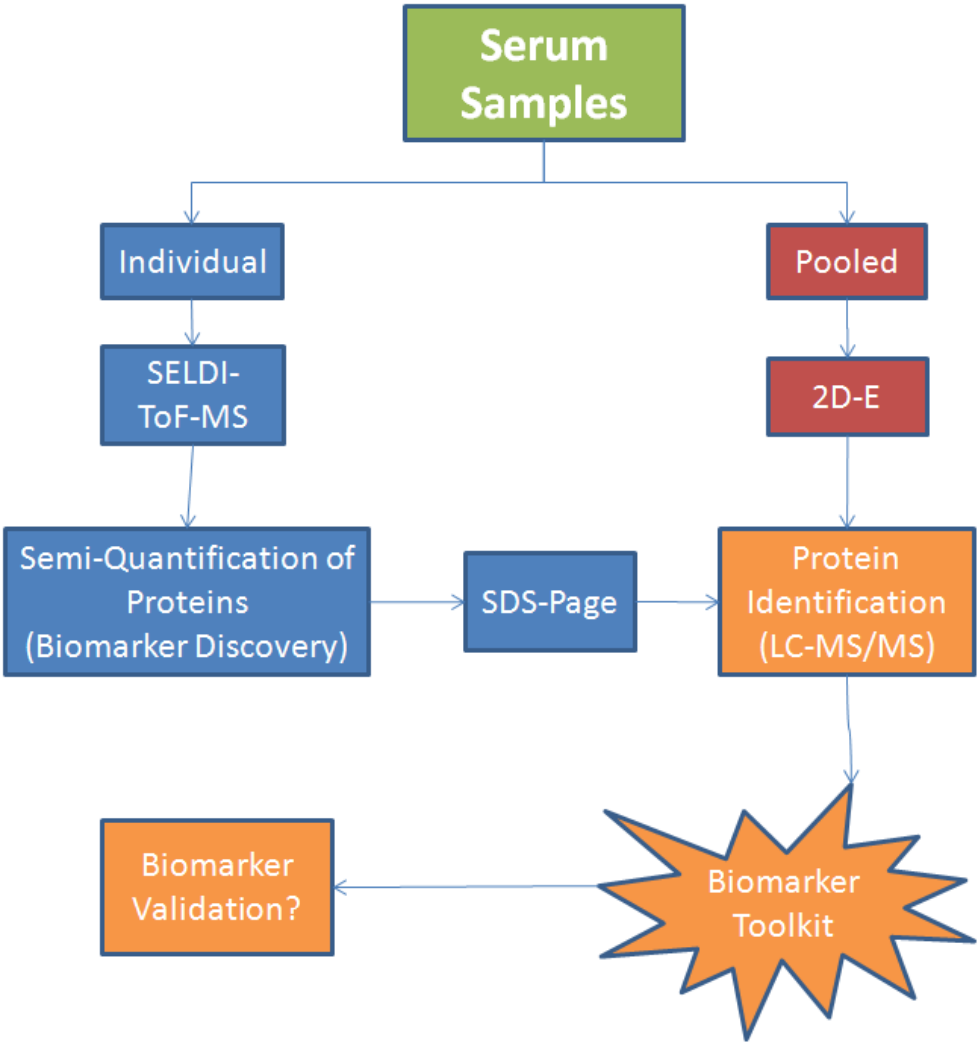

Figure 5: Study design. Ocular toxoplasmosis biomarker pilot study uses two independent technologies for biomarker discovery. To do this, serum samples were aliquoted in two; one set to be analysed individually by SELDI-ToF-MS and the other to be pooled with samples from the sample patient group and analysed with 2D-E. Proteins of interest found by the SELDI method are to be visualized by SDS-PAGE, digested with trypsin, and sequenced. A similar method will be followed for bands of interest found by 2D-E. Proteins of interest are to be studied for their clinical relevance and validated through the use of commercially available antibodies and by comparing results to previous studies (unpublished courtesy of Dr. Momar Ndao, McGill University).

Chapter 2: Review of the Literature for Ocular Toxoplasmosis

2.1. Population structure

Despite a sexual phase in the life cycle occurring in feline enterocytes, it was accepted until recently that the population structure of *T. gondii* was highly clonal; demonstrating low genetic variability. Parasitic isolates have been classified into three genetic types: I, II and III, first based on their virulence in mice then reassessed on the basis of restriction fragment length polymorphisms (RFLPs) analysis (Howe DK 1995). Less than one percent of the previously studied strains contain unique genotypes; demonstrating a high divergence in their DNA sequence, and are consequently considered 'exotic' or 'atypical' strains (Ajzenberg, Banuls et al. 2004). The low levels of genetic diversity previously reported may be due to a systematic underestimation of the total number of *T. gondii* strains; strains in the vast majority of previous studies were collected from patients and domesticated animals in North America and Europe. Ajzenberg et al. studied the genetic diversity, clonality and sexuality in *T. gondii* through the construction of genetic diversity indices through microsatellite genotypes analysis and the phylogram development. Results suggested that the global *T. gondii* population is more diverse than previously thought. This is not a characteristic of a clonal organism and in this way *T. gondii* presents a complex population structure with a mix of clonal and sexual propagation as a function of the environmental conditions (Ajzenberg 2004).

Brazil as a whole, and the southern regions in particular, has a disproportionately high incidence and severity of ocular toxoplasmosis when compared to Europe and North America (Holland

2003). *T. gondii* strains from remote and underserved areas, such as the Amazon, are still largely underrepresented in the phylogeny of the parasite. The use of Brazilian *T. gondii* isolates along with recently described markers of genetic characterization demonstrated a higher genetic variability than has been previously reported (Ajzenberg, Banuls et al. 2004; Lehmann, Graham et al. 2004). Today PCR-RFLP assays allow for the parasite to be classified as either one of the three 'classical' clonalities or as atypical genotypes (Ajzenberg, Cogne et al. 2002).

The outcome of toxoplasmosis depends on the interaction of many factors, including the functions of immune system and parasitic factors, such as the inoculum, infective parasite stage, and genotype of *T. gondii* isolate. Regardless of the debate surrounding parasite classification, the type II strain is responsible for more than 70 % of symptomatic human cases in France and the United States (Howe DK 1997; Nowakowska D 2006). Although there is no patient data characterizing the difference in strain expression in Brazil for systemic toxoplasmosis, various studies of wild and farm animals have been carried out demonstrating the high prevalence of type I, III atypical strains over type II (Dubey JP 2006; Pena, Gennari et al. 2008; Yai, Ragozo et al. 2009). As such, the type I strain seems to be responsible for the majority of ocular infections in Brazil (Vallochi AL 2005). In the southern Brazilian city of Erechim the population exhibits a 17 % prevalence of ocular toxoplasmosis. There, the type I and atypical strains predominate (Jones JL 2006), with the type I strain found in sources of potable water (De Moura L 2006) and atypical *T. gondii* genotypes isolated from porcine tongue and diaphragm obtained from local abattoirs (Belfort and Rasmussen, et al. 2008). Furthermore, a recent analysis of the genotypes of *T. gondii* isolated from ocular toxoplasmosis patients from Erechim and Sao Paulo were

highly atypical compared to previously described clonal lineages (Khan A 2006). Importantly, it is "these atypical strains that may be playing an increasingly important role in the acquired infection ocular manifestations" (Holland 2000), a suggestion that is in-line with the Ajzenberg hypothesis.

2.2. Ocular manifestations

Toxoplasmic retinochoroiditis can be seen in the setting of congenital or postnatally acquired disease as a result of acute infection or recurrence (Nussenblatt RB and Belfort Jr. R 1994; Montoya and Remington 1996). This disease typically affects the posterior pole of one eye, and the lesions can be solitary, multiple or satellite to a pigmented retinal scar (Figure 6). Nevertheless, the appearance, duration and intensity of toxoplasmic retinochoroiditis lesions vary and may be related to host, parasite, or environmental factors. The genotype of the infecting parasite, as described above, "appears to be an important determinant of disease severity in immunocompetent patients" (Holland GN 2004).

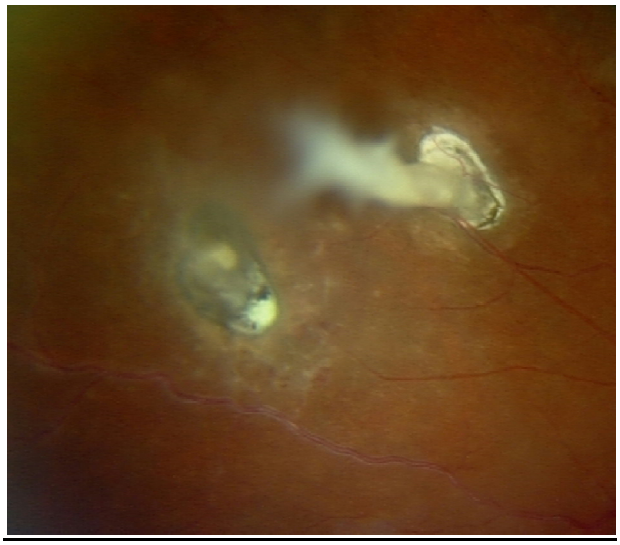

Figure 6: Ocular toxoplasmosis with vitreous strand and vasculitis. (Courtesy of the Henry C. Witelson Ocular Pathology Laboratory, McGill University)

The active lesions present as grey-white focus of retinal necrosis with adjacent choroiditis, vasculitis, hemorrhage and vitreitis (Figure 7a, b and c). Scarring occurs from the periphery towards the centre of the lesion, with variable pigmentary changes. Anterior uveitis is a common finding, with mutton-fat keratic precipitates, cells and flare, and posterior synechiae (iris-lens adhesion) (Nussenblatt RB and Belfort Jr. R 1994). Although the retina is the primary site of ocular *T. gondii* infection, the choroid, vitreous and anterior chamber are also involved; the choroid is secondarily affected as choroidal lesions cannot occur in the absence of retinal infection. Furthermore an intense secondary iridocyclitis may also be present (Nussenblatt RB and Belfort Jr. R 1994; Holland GN 2004) and the optic nerve head may also be involved (Figure 7b) (Eckert GU, Melamed J et al. 2007). Older or immunosuppressed patients may present with more aggressive, bilateral and/or multifocal disease (Figure 7d). Also, recently infected elderly individuals demonstrate a higher prevalence of ocular involvement. Other atypical presentations include: punctate outer retinal toxoplasmosis, retinal vasculitis, retinal vascular occlusions, rhegmatogenous and serous retinal detachments, unilateral pigmentary retinopathy mimicking retinitis pigmentosa, neuroretinitis as well as other forms of optic neuropathy, peripheral retinal necrosis and scleritis (Smith JR and Cunningham Jr. ET 2002; Bonfioli AA and Orefice F 2005). Ocular complications are numerous and can include: choroidal neovascularization, cataract, glaucoma, optic nerve atrophy and retinal detachment, though the latter is more frequently observed in children (Bosch-Driessen LH, Karimi S et al. 2000). It is important to note an association between ocular toxoplasmosis and Fuchs' heterochromic cyclitis has been described

(Toledo de Abreu M, Belfort R Jr et al. 1982) which was later confirmed (Schwab IR 1991; La Hey E and Baarsma GS 1993; Ganesh SK, Sharma S et al. 2004).

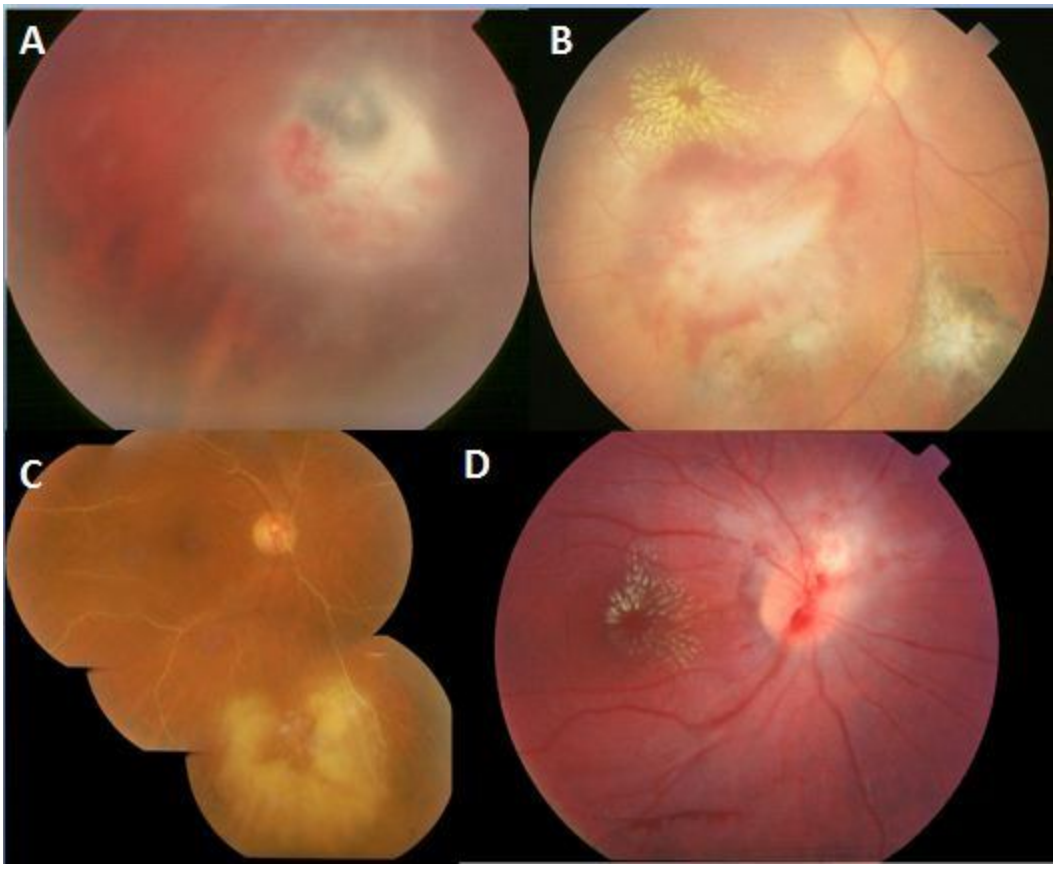

Figure 7: A) Retinochoroiditis with vitreitis B) Ocular toxoplasmosis with old pigmented scar and inferior recurrence to the macula C) Inferior area of retinochoroiditis and difuse vasculitis; D) Toxoplasmic scar in the macula and acute CMV in a patient with AIDS (Courtesy of the Henry C. Witelson Ocular Pathology Laboratory, McGill University).

Retinal vasculitis and associated inflammatory reactions may be the only ophthalmic sign during the early stages of a newly acquired *T. gondii* infection. Later development of retinitis or scars consistent with toxoplasmic retinochoroiditis in the same eye suggests that the initial, isolated inflammation may have been caused by the parasites (Silveira C 2001). Recurrent toxoplasmic retinochoroiditis is not associated with systemic symptoms and recurrence risk may be

influenced by patient age. Ocular lesions may first develop many years after *T. gondii* infection and are often asymptomatic (Nussenblatt RB and Belfort Jr. R 1994).

Transplacental transmission of *T. gondii* to the fetus during pregnancy is another important source of infection. The mother can transmit the parasite to the fetus if infected during pregnancy or a few months before conception (Montoya JG and Liesenfeld O 2004). The infection can result in visual and hearing loss, mental and psychomotor retardation, seizures, hematological abnormalities, hepatosplenomegaly or death (Montoya JG and Remington JS 2008). Retinochoroidal scars are the most characteristic ocular manifestation of a congenital or prenatal infection (Mets MB and Chhabra MS 2008) (Figure 8). Maternal infection in the first trimester of gestation has a lower chance of congenital transmission, but more severe consequences for the fetus when compared to the third (Montoya JG and Remington JS 2008). Nevertheless, congenitally infected individuals, their parents, and siblings should be aware that late-onset retinal lesions can occur many years after birth. However, "the overall ocular prognosis of congenital toxoplasmosis is satisfactory when infection is identified early and treated accordingly" (Wallon M, Kodjikian L et al. 2004).

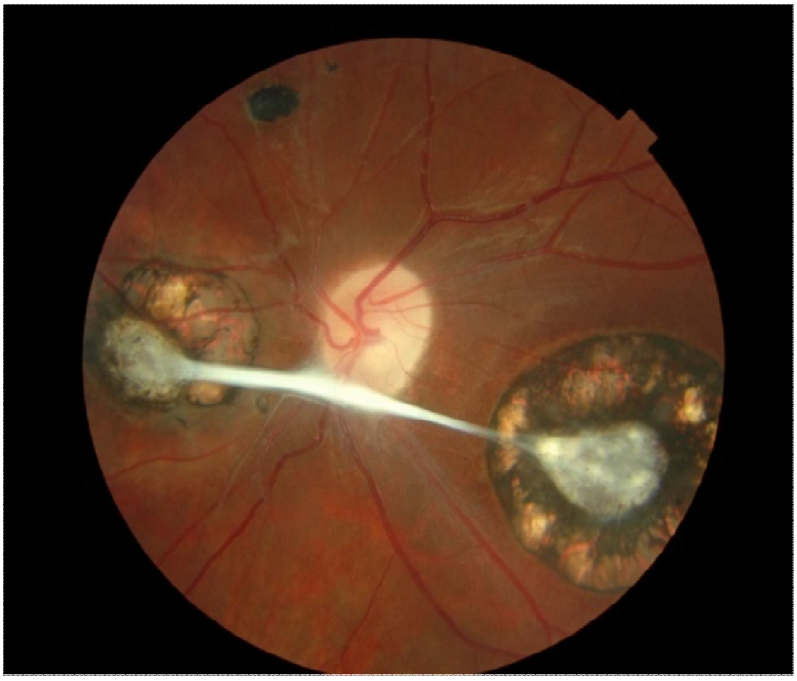

Figure 8: Retinal scars linked by vitreous strand in congenital toxoplasmosis (Courtesy of the Henry C. Witelson Ocular Pathology Laboratory, McGill University).

A British survey assessing the risk of visual impairment in 281 congenitally infected children with mean follow-up of 4.8 years demonstrated that 17 % of the children presented at least one retinal lesion. Out of 44 children, where information on visual acuity was available, 9 % suffered from severe bilateral impairment. Also, 52 % of the children with a posterior pole lesion and 17 % of those with only peripheral lesions were found to be visually impaired in the affected eye (Tan HK 2007). Many children with congenital toxoplasmosis present with substantial retinal damage at birth; yet vision may be remarkably good in the presence of large macular scars. Active lesions become quiescent with treatment and may recur at any age (Mets MB 1997).

A French study evaluating 430 children treated for congenital toxoplasmosis, found that ocular involvement was present in 30 % after a median follow-up of 12 years. The overall functional prognosis of these congenitally infected children was better than would be expected on the basis of literature findings, with only two of the 130 children suffering bilateral visual impairment (Kodjikian L 2006). Although it is classically known that only during primary infection the mother could transmit the infection to the fetus, there are a few reports supporting the possibility of chronically infected women transmitting the disease congenitally (Silveira C 2003). Reports have also suggested that *T. gondii* immune mothers are susceptible to reinfection and therefore parasite transmission to the fetus (Gavinet, Robert et al. 1997).

2.3. Treatment of ocular toxoplasmosis

Ocular toxoplasmosis therapy may include systemic antimicrobial drugs with or without corticosteroids. Some ophthalmologists treat all ocular toxoplasmosis cases while others only those with posterior pole lesions, intense vitreitis, and lesions close to the optic disk or immunosuppressed patients (Rothova A 1989). Several drugs have been proposed including pyrimethamine, sulfadiazine, spiramycin, clindamycin, and trimethoprim-sulfamethoxazole (Pleyer U 2007; Antoniazzi E, Guagliano R et al. 2008).

Results of a study comparing three drug combinations: association of pyrimethamine, sulphadiazine and corticosteroids; association of clindamycin, sulphadiazine and corticosteroids; and association of cotrimoxazole (trimethoprim and sulphamethoxazole) with corticosteroids

showed no difference in the resolution of inflammatory processes (Rothova A 1989). The same group showed a reduction in size of the retinal inflammatory lesion for 49 % of the pyrimethamine-treated patients compared to 20% of the untreated patients (Rothova A 1993). The most frequent side effects were associated with pyrimethamine and included hematologic complications such as thrombocytopenia and leucopenia, though folinic acid supplementation is believed to prevent side effects related to pyrimethamine treatment (Rothova A 1989). It should be noted however that folic acid does not prevent such complications and should not be used as a substitute for folinic acid (Belfort, Fernandes et al. 2009). The use of pyrimethamine, sulfadiazine, and corticosteroids is considered 'the classical' therapy for ocular toxoplasmosis and is the most common drug combination used (Montoya JG and Liesenfeld O 2004). Patients with active toxoplasmosis may also be treated with trimethoprim-sulfamethoxazole with or without adjunctive clindamycin and prednisone for four to six weeks. Trimethoprim-sulfamethoxazole appears to be a safe and effective substitute for sulfadiazine, pyrimethamine, and folinic acid in treating ocular toxoplasmosis (Opremcak EM 1992; Soheilian M 2005). The therapeutic benefit from the use of pyrimethamine in combination with azithromycin was similar to that of pyrimethamine and sulfadiazine. Multidrug therapy with the combination of pyrimethamine and azithromycin appears to be "an acceptable alternative treatment for sight-threatening ocular toxoplasmosis" (Bosch-Driessen LH 2002).

As previously mentioned, the causes of recurrences in ocular toxoplasmosis remain unknown. They may be related to the rupture of dormant retinal cysts (Abreu MT, Belfort Jr R et al. 1987), or circulating parasites in the peripheral blood (Silveira C, Vallochi AL et al. Manuscript

submitted). In some patients, recurrent toxoplasmic retinochoroiditis remains a major problem and can be associated with severe visual morbidity if disease extends to the macula and optic disk. Moreover, recurrent disease may cause visual morbidity resulting from inflammation or complications including retinal detachment and/or choroidal neovascularization. In patients with frequent recurrences, long-term intermittent treatment with trimethoprim combined with sulfamethoxazole was shown to reduce the rate of recurrent toxoplasmic retinochoroiditis from 23.8 % to 6.6 % (Silveira C 2002). Nevertheless, traditional short-term treatments of the active toxoplasmic retinochoroiditis lesions neither prevent subsequent recurrences nor did they have an effect on visual outcomes or future recurrence rates, with the exception of a poor visual outcome for patients who received corticosteroids without antiparasitic drugs (Bosch-Driessen LH 2002). Nevertheless, the relationship between the use of systemic corticosteroids and reactivation of ocular toxoplasmosis has yet to be elucidated (Morhun PJ, Weisz JM et al. 1996).

Intravitreal injection of clindamicyn with or without steroids may be used in patients that have contraindication of systemic therapy specific for toxoplasmosis (Aggio FB, Muccioli C et al. 2006; Sobrin L, Kump LI et al. 2007); intravitreal clindamycin injection was associated with resolution of toxoplasmic retinochoroiditis (Sobrin L, Kump LI et al. 2007). On the other hand, intravitreal injections of clindamycin and dexamethasone (Kishore K 2001) as well as subconjunctival injections of clindamycin (Colin J and Harie JC 1989) have demonstrated their potential to be employed as alternatives to the use of the ‘classical’ anti-toxoplasmic ocular therapy.

Chapter 3: Patients and Methods

3.1. Serum Samples

Blood serum samples were collected from four groups of nine patients each (n=36): healthy, uveitic (*T. gondii* IgG negative), those who have had one ocular toxoplasmic event and those who have had recurrent events (multi-episodic). Serum samples were collected in collaboration with Clinica Silveira in Erechim, Brazil and the Vision Institute at the Federal University at São Paulo (UNIFSP), São Paulo, Brazil. Patients provided informed consent while also participating in prospective clinical study of RT-PCR diagnostics in ocular toxoplasmosis (Belfort, Isenberg et al. 2010).

3.2. Serum Fractionation

Sera were fractionated using a Ciphergen Q HyperD F strong anion-exchange resin filtration plate. The filtration plate was re-equilibrated through the addition of: 200 µl rehydration buffer; 50 mM Tris–HCl at pH 9.0; and then placing it on a MicroMix 5 Orbital Vortex (Beckman Coulter), with a form of 20 and amplitude of 7 for 60 min at room temperature (RT). The rehydration buffer was then removed by vacuum and the resin was washed four times with 200 µl rehydration buffer and four times with 200 µl U1 solution containing: 1 M urea; 0.2 % 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS); and 50 mM Tris–HCl at pH 9.0. Serum samples were thawed on ice and centrifuged at 17,300 g for 5 min at RT to remove particulates. Twenty microlitres of sample were added to a v-bottom 96-well microplate (Costar Corning) with 30 µl of U9 buffer containing; 9 M urea; 2% CHAPS; 50 mM Tris–HCl at pH 9.

The microplate was sealed and placed on a MicroMix 5 orbital vortex with a form of 20 and amplitude of 5 for 20 min at RT. Fifty microlitres of sample were added to the equilibrated resin with 50 µl of U1 buffer. The filtration plate was sealed and placed on the MicroMix 5 orbital vortex with a form of 20 and amplitude of 7 for 30 min at RT. The fraction was collected by vacuum. One hundred microlitres of pH 9 buffer containing 50 mM Tris–HCl and 0.1 % octyl β-d-glucopyranoside (OGP), buffered to a pH of 9, were added to the wells of the filtration plate using the Biomek Robot Automation System (Beckman Coulter). The microplate was then placed on the MicroMix 5 orbital vortex at RT for 10 min and the fraction collected by vacuum. One hundred microlitres of the following buffers were added in two consecutive applications and collected by vacuum: pH 9 buffer, pH 7 buffer (50 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 0.1% OGP, pH 7); pH 5 buffer (100 mM sodium acetate, 0.1% OGP, pH 5); pH 4 buffer (100 mM sodium acetate, 0.1% OGP, pH 4); pH 3 buffer (50 mM sodium citrate, 0.1% OGP, pH 3); and organic wash buffer (33.3% isopropanol, 16.7% acetonitrile (ACN), 0.1% trifluoroacetic acid (TFA)). The two 100 µl eluants from each pH fraction were pooled, aliquoted and stored at −20 °C.

3.3. Binding of Fractions to ProteinChip Arrays

Two of the six pH fractions, pH 9 and the organic layer, of serum were profiled on a weak cation-exchange (CM10), immobilized metal affinity capture (IMAC) and reversed-phase hydrophobic surface (H50) ProteinChip (Invitrogen Life Science) Arrays according to the manufacturer's instructions. All steps were performed at RT. Briefly, the ProteinChip arrays were placed in a Ciphergen bioprocessor (C503-0006) and washed twice with 200 µl low-stringency binding buffer containing 0.1 M sodium acetate and 0.1% Triton X-100, buffered to a

pH of 4, and placed on a multi-tube vortexer (VWR VX-2500) at speed 1 for 5 min. Each of the fractions were bound to the chip by adding 10 µl of sample in 90 µl of binding buffer; the bioprocessor was placed on the multi-tube vortexer for 60 min. The samples were discarded, the ProteinChip arrays washed three times with 200 µl of binding buffer, placed on the multi-tube vortexer for 5 min and washed twice with 200 µl 1 mM HEPES buffered to a pH of 7.4 for 1 min. The ProteinChip arrays were air-dried prior to matrix application. Serum samples were all spotted randomly on the arrays.

3.4. Preparation and Application of Matrix

For protein analysis, a saturated sinapinic acid (SPA) solution was freshly prepared by adding 50% ACN/0.5% TFA solution. Half a microlitre of the matrix was added to each spot on the ProteinChip array and air-dried prior to adding an additional 0.5 µl of matrix.

3.5. SELDI-ToF-MS Analysis

ProteinChip arrays were read using a Ciphergen PCS4000 SELDI-TOF MS reader (Bio-Rad Laboratories). Profiles were collected in the low and high ranges: 0–10; and 10–200 kDa. The intensity and sensitivity of the instrument was adjusted for each of these ranges on each day of analysis. The instrument was calibrated for dataset collection using an all-in-one peptide standard (Bio-Rad Laboratories) when collecting data both in the 0–10 and 10–200 kDa ranges, varying the intensity and sensitivity of collection. Spectra from profiling experiments are an average of data from 110 laser shots.

3.6. Ciphergen Express Software Analysis

Spectra were normalized by total ion current intensity starting and ending at the *M/z* of the collection ranges of 0-10 and 10-200 kDa. The spectra were then aligned to a spectrum with the normalization factor nearest 1. This was only done if the percentage coefficient of variation for the given spectra was reduced after the alignment. Afterwards, any spectra identified to be greater than two standards of deviation away from the normal were rejected. First pass data analysis began when peaks from the different spectra were aligned using the Cluster Wizard function of Ciphergen Express Software (Bio-Rad Laboratories). Peak detection was completely automated within the *M/z* range of analysis, where a peak was automatically detected on the first pass when its signal-to-noise (S/N) ratio was five and it was three times the valley depth. User-detected peaks below threshold were deleted and all first-pass peaks were preserved. Clusters were created within 0.3 % of mass for each peak detected in the first pass for low mass range and 2 % for high mass range. When no peaks were detected, the peak intensity was estimated at the centre of the cluster. The peaks were manually inspected to determine if they were multi-charged entities. P-values and the receiver operation characteristic (ROC) values were calculated through the use of the P-Value Wizard which compared: healthy to uveitis (non-toxoplasma); one *toxoplasmic* event to recurrent events; uveitic to one and multiple events; and healthy to uveitic to one *toxoplasmic* event to recurrent events. In this way uveitic and *T. gondii* specific proteins were removed from the analysis, retaining only makers for one ocular toxoplasmic event, multiple events and healthy controls from which p-values below 0.05 were considered statistically significant. Second pass analysis followed a methodology similar to first pass analysis. Here, all peaks with a p-value of less than 0.001 were retained and relabelled. The peak detection software was then placed on manual mode with the following settings: detect

user labelled peaks only, increase of the mass cluster to 2 % of mass for each peak for the low mass range. Subsequently, p-value and ROC was calculated using the P-Value Wizard by comparing one *toxoplasmic* event to recurrent events.

3.7. Decision Tree Classification

Construction of the decision tree classification algorithm was performed by Ciphergen Biomarker Pattern software version 5.0 (Bio-Rad Laboratories). Classification trees split the data into two nodes using one rule at a time in the form of peak intensity. The splitting decisions in this case were based on the normalized intensity levels of peaks from SELDI protein expression profile. The process of splitting was continued until terminal nodes were created. After V-fold cross validation of 50, the accuracy of each classification tree was then challenged with the blinded test set.

3.8. Two Dimensional Gel Electrophoresis

3.8.1. Microscale Isolelectric Focusing in Solution

Nine serum samples from the uveitic, single event and recurrent groups used in the SELDI-TOF-MS analysis were thawed and made into pools of 20 µL each. The lysate is formed when 900 µL of 1.1 x IEF denaturant buffer (Invitrogen Life Sciences), containing: 7.7 M urea; 2.2 M thiourea; and 4.4% (w/v) CHAPS, 10 µL of a 100 x protease inhibitor cocktail, 20 µL of 1 M DDT reducing agent, 10 µL of both 0.5 M EDTA and 1 M tris-base were added to the pools following a half hour RT incubation on a platform rocker. 5.2 µL of 99 % N,N-dimethyacylatime was then added followed by another incubation period. To 1 mL of the now

reduced and alkylated lysate, 2.28 mL of IEF denaturant buffer, 35 µL of carrier ampholytes (pH 3−10) and 20 µL of 1 M DDT, were added for a final volume of 3.5 mL.

The ZOOM Isoelectric Focusing (IEF) Fractionator (Invitrogen Life Sciences) was assembled according to the manufacturer's instructions. 650 µL of the pooled diluted samples described above was pipetted into each of the 5 chambers of the instrument: chamber 1 pI 3−4.6; chamber 2 pI 4.6−5.4; chamber 3 pI 5.4−6.2; chamber 4 pI 6.2−7.0; chamber 5 pI 7.0−10.0. The anode chamber was filled with 17.5 mL of anode buffer containing 8.4 g of urea, 3.0 g of thiourea, and 3.3 mL of Novex IEF Anode buffer (Invitrogen Life Sciences), adjusted to pH 3, and made up to a total volume of 20 mL with deionized water. The cathode chamber was filled with 17.5 mL of cathode buffer containing: 8.4 g of urea; 3.0 g of thiourea; and 2 mL of Novex IEF Cathode buffer (Invitrogen Life Sciences) and made up to a volume of 20 mL with deionized water. IEF was then performed for three hours: 100 V for 20 min; 200 V for 80 min; and at 600 V for 80min. The current and power were limited to 2 mA and 2 W. This was repeated for each group. Samples were then removed from wells and placed into three 200 µL aliquots per chamber and kept at –20 ^{o}C until the protein gel was run.

3.8.2. Protein Gel Elctrophoresis

Following isoelectric focusing, each sample was thawed, centrifuged and desalted with 1400 µL of methanol, 200 µL of chloroform, 800 µL of deionized water, then left to air dry. Samples were then equilibrated and reduced, according to the manufacture’s recommendations, by the addition of NuPAGE LDS Sample Buffer and NuPAGE Reducing Agent (Invitrogen Life

Science), containing 500 mmol·L^{-1} DTT. Samples were then heated at 70 ^{o}C for ten minutes. The second-dimension electrophoresis was performed by aligning the ZOOM strip in the well of a NuPAGE Novex 4% to 12% Bis-Tris ZOOM Gel (Invitrogen Life Science) in a denaturing running buffer prepared with 25 mL of NuPAGE 20 x SDS Running Buffer made up to a total volume of 500 mL with deionized water. Samples were loaded into ten wells and Mark12 MW Marker (Invitrogen) was loaded into two wells, then electrophoresed at 200 V for 35 min. Following electrophoresis, samples were stained in a freshly prepared Commassie G250 dye containing 85 mL of methanol, 10 mL phosphoric acid, 42.5 g ammonium sulfate, and 0.25 g Brilliant Blue completed to a final volume of 250 mL. Gels were left to stain for 48 hours and then were photographed. Bands demonstrating differential expression, as determined by visual inspection, were excised and kept in 5 % acetone at 4 ^{o}C until protein identification proceeded.

3.9. Protein Separation (SDS-PAGE)

General protein separations are based on MW. Samples were fractionated following the method described in section 3.2, while one-dimensional gel electrophoresis followed the method presented in section 3.8.2.

3.10. Protein Identification

Trypsin digestion and liquid chromatography (LC)-MS/MS analysis were carried out on an Agilent micro LC connected to an ABI Q-STAR mass spectrometer at the Sheldon Biotechnology Centre, McGill University. The resulting tryptic peptides were matched against

both mammalian and other eukaryotic NCBI databases using MASCOT search engine (http://www.matrixscience.com/) for product ion confirmation.

3.11. Protein Function Search

Differentially expressed proteins identified by MS analysis had protein function assigned for each. Protein function assignation tools including the Bioinformatic Harvester program (http://www.harvester.embl.de/) and those at the Human Protein Resource Database (http://www.hprd.org/) were used in order to determine human protein function in ocular toxoplasmosis. Only proteins expressed by humans were selected, as no *T. gondii* proteins were detected.

3.12. *T. gondii* Outbreak

An outbreak of toxoplasmosis was reported between November 2001 and January 2002 in Santa Isabel do Ivai, Brazil, following which a matched case-control study was conducted from January 15 to February 2, 2002. Serum samples from case-patients and controls were tested for anti-*T. gondii* IgM and IgG antibodies (de Moura, Bahia-Oliveira et al. 2006) and an extensive biomarker study was at undertaken by Dr. Momar Ndao at McGill University's National Reference Laboratory for Parasitology in conjunction with the US Center for Disease Control (CDC) on 200 of those patient samples. This study followed similar protocols as those described above, though two different SELDI-TOF-MS machines (PBSIIC and PCS4000) were employed as well as all six fractions generating 10 800 spectra. The same study used th three ProteinChip arrays and three chip types (results unpublished). Data analysis saw the samples divided into six

groups: early vs. late; early vs G+M+; late vs G+M+;· early vs negative; G+M+ vs negative; late vs negative. All statistically significant biomarkers discovered in this pilot (129) study were compared to the biomarker outbreak database of 422 peaks, and then diagnosis, p-value as well as the receiver operating characteristics were noted. Biomarkers are matched to within 1 % of *m/z* but the bolded ones are matched to within 0.0001 %, while the *m/z* sensitivity of the SELDI-ToF-MS technology is within 0.1 %.

Chapter 4: Results

4.1. Biomarker Discovery

Individual serum samples (n=36) from all the training sets were fractionated, analysed and compared by SELDI-ToF-MS with CM10, H50 and IMAC ProteinChips generating a total of 432 spectra. All MS data were baseline subtracted and normalized using total ion current, and the peak clusters were generated by Biomarker Wizard software as outlined in section 3.5.

Table 3 shows the results of second pass analysis of the ocular toxoplasmosis compared to non-toxoplasmic uveitis groups; 50 protein peaks, 52 % of which were down-regulated in the ocular toxoplasmosis group, were discovered. The *m/z* of the 50 biomarkers ranged from 2,992 to 193,083 Da demonstrating a *m/z* mean of 23,174 ± 40,539 Da with a mean p value of 0.020 ± 0.015. The intensity of the samples analysed range from -0.53 to 146.23 mA. The mean difference in intensity (DI), defined as the two individual samples with the highest and lowest intensities for a given biomarker, is 12.25 ± 23.7 mA, with seven biomarkers demonstrating a difference greater than one standard of deviation. Of the biomarkers in the *m/z* range of 2.7 to 15.2 kDa, 75 % of them up-regulate; suggestive of humoral immune response. Two of the biomarkers are within 7 Da of each other and are up-regulated suggesting that they may be the same protein.

Table 4 shows the results of second pass analysis comparing patients who had either a single-event to those with multiple occurrences of ocular toxoplasmosis; 46 protein peaks, 74 % of which were down-regulated in multi-episodic patients were discovered. These 46 biomarkers have been checked against the healthy vs non-toxoplasmic uveitis cohort to make certain that none of them are of diagnostic value to the otherwise healthy individual. The *m/z* of the 46 biomarkers ranged from 2,755 to 178,373 Da demonstrating a *m/z* mean of 20, 263 Da ± 30,497 Da with a mean p value of 0.024 ± 0.015. The intensity of the samples analysed ranged from 0.22 to 67.8 mA. The mean DI was 8.47 ± 13.3 mA, with eight biomarkers demonstrating a difference greater than one standard deviation.

Figure 9 demonstrates an overlap of 15 biomarkers that can be used to differentiate between ocular toxoplamosis and non-toxoplasmic uveitis as well as a single event from multiple episodic ocular toxoplasmosis; representing 16 % of the total number of biomarkers discovered.

The cluster plots of figures 10, 11, 12 and 13 illustrate the distribution of a given biomarkers' intensity, where each dot represents one serum sample. Here, up- and down-regulated biomarkers comparing ocular toxoplamosis to non-toxoplasmic uveitis and single events to multiple episodic ocular toxoplasmosis with a differential intensity greater than one standard of deviation are shown. Cluster plots with less overlap between groups suggest a high degree of discrimination between groups, while a high level of differential expression between case and control for a given biomarker indicates a high level of protein expression (greater than one

standard deviation), facilitating downstream analysis. As such, figure 14 demonstrates the visualization of those highly and minimally expressed proteins as outlined in section 3.9

Sera from the same patients used in SELDI-ToF-MS analysis were pooled according to their group: non-toxoplasmic uveitis, single event, multi-episodic. Figures 15 and 16 demonstrate the results of two-dimensional gel electrophoresis whereby the pooled sera's proteins were separated according to pH and mass. In this way, 57 differentially expressed bands (potential biomarkers) were detected and excised for downstream protein identification according to section 3.10 with results featured in section 4.3. Analysis of the banding patterns suggest that 68 % of the bands are indicative of muli-episodic disease (red) whereas 21 % (blue) and 9 % (green) are markers of non-toxoplasmic uveitis and single episodes of disease.

Treatment (pH, Chip, Energy)	n	Biomarker (Da)	P-value	Max Intensity (mA)	Min Intensity (mA)	Differencial Expression (mA)	Regulated in OT? Up or Down
pH 9, CM10, 2200 mV	1	2885.206	0.004	6.912	0.052	6.860	Down
	2	3185.721	0.005	5.650	0.028	5.622	Down
	3	3471.874	0.003	7.869	0.094	7.775	Down
	4	3680.647	0.031	11.247	0.064	11.183	Down
	5	3934.063	0.031	21.176	0.215	20.961	Down
	6	4411.416	0.027	25.800	0.415	25.385	Down
	7	4424.719	0.013	19.002	0.523	18.480	Up
	8	4647.099	0.036	4.639	0.156	4.482	Down
	9	5993.037	0.020	1.992	0.239	1.753	Down
	10	6437.447	0.042	75.145	14.865	60.280	Up
	11	8142.427	0.048	3.554	0.304	3.250	Down
	12	8598.249	0.003	6.843	0.279	6.564	Down
	13	19719.642	0.031	0.051	0.007	0.044	Up
pH 9, CM10, 3500 mV	14	10462.903	0.031	9.891	-0.374	10.266	Down
	15	10522.998	0.005	9.760	-0.529	10.289	Down
	16	13780.185	0.045	6.814	1.725	5.090	Down
	17	18397.240	0.018	13.235	0.057	13.178	Down
pH 9, H50, 2200 mV	18	5882.200	0.009	2.125	0.222	1.903	Down
	19	20061.256	0.031	0.076	0.004	0.072	Down
pH 9, H50, 3250 mV	20	15200.146	0.049	45.917	0.644	45.273	Up
	21	16521.061	0.049	9.738	0.003	9.734	Up
	22	178373.073	0.012	0.323	0.007	0.316	Up
pH 9, IMAC, 2200 mV	23	2791.656	0.030	146.236	0.604	145.632	Up
	24	3537.109	0.026	12.270	0.107	12.162	Up
	25	3681.909	0.008	17.003	0.036	16.967	Up
	26	4791.793	0.009	6.731	0.104	6.627	Up
	27	4994.940	0.026	4.377	0.099	4.278	Up
	28	6112.504	0.040	1.991	1.991	0.000	Up
	29	8302.714	0.008	4.554	0.036	4.518	Up
	30	11355.281	0.035	1.738	0.056	1.682	Up
	31	13239.194	0.023	0.736	0.041	0.695	Up
	32	13361.203	0.020	1.057	0.043	1.014	Up
	33	22998.224	0.008	1.034	0.032	1.002	Down
	34	23430.977	0.026	1.311	0.038	1.273	UP
pH 9, IMAC, 3500 mV	35	10582.636	0.005	2.355	0.031	2.324	Down
	36	12931.827	0.018	0.593	0.022	0.570	Down
	37	17875.923	0.005	0.091	-0.001	0.092	Down
Organic, H50, 3250 mV	38	13903.893	0.002	68.727	2.725	66.002	Up
	39	28000.764	0.045	34.481	16.409	18.073	Down
	40	28853.508	0.031	11.483	5.276	6.207	Down
Organic, H50, 2200 mV	41	7442.130	0.040	0.540	0.074	0.465	Up
	42	13875.737	0.005	6.861	0.316	6.544	Up
Organic, IMAC, 3500 mV	43	11545.721	0.017	8.449	0.240	8.209	Down
	44	22940.960	0.002	2.069	0.678	1.392	Down
	45	50790.047	0.010	0.380	0.058	0.322	Down
	46	193083.094	0.005	0.042	0.003	0.038	Up
Organic, CM10, 3500 mV	47	11716.428	0.006	11.074	0.209	10.864	Down
	48	13911.390	0.022	30.902	6.076	24.827	Up
	49	100605.393	0.001	0.197	0.051	0.146	Up
	50	133299.773	0.002	2.675	0.648	2.027	Up

Table 3: 50 Biomarkers found able to differentiate non-toxoplasmic uveitis of unknown etiology from ocular toxoplasmosis.

Treatment (pH, Chip, Energy)	n	Biomarker (Da)	P-value	Max Intensity (mA)	Min Intensity (mA)	Differencial Expression (mA)	Regulated in Reccurence? Up or Down
pH 9, CM10, 2200 mV	1	2755.909	0.023	23.074	0.899	22.175	up
	2	2901.300	0.004	2.969	0.081	2.888	up
	3	3103.765	0.003	5.898	-0.202	6.100	up
	4	3471.874	0.050	2.543	0.094	2.450	down
	8	3893.921	0.030	23.865	0.854	23.011	up
	6	4578.105	0.030	15.201	0.747	14.454	down
	7	4796.257	0.013	1.110	0.196	0.915	down
	8	7852.476	0.013	1.579	0.203	1.376	down
	9	10542.539	0.039	0.371	0.002	0.369	down
	10	11653.571	0.050	1.013	0.020	0.994	down
pH 9, CM10, 3500 mV	11	11154.557	0.047	3.578	0.023	3.555	up
	12	17878.896	0.012	0.347	0.006	0.340	down
	13	18392.561	0.000	0.380	0.010	0.370	down
pH 9, H50, 2200 mV	14	3377.852	0.038	7.419	-0.216	7.635	down
pH 9, H50, 3250 mV	15	11910.753	0.021	29.684	0.119	29.565	down
	16	33429.155	0.021	0.443	0.036	0.407	down
	17	178373.073	0.021	0.323	0.007	0.316	down
pH 9, IMAC, 2200 mV	18	3471.211	0.019	11.219	1.552	9.667	down
	19	4185.428	0.031	17.132	1.758	15.374	down
	20	4575.417	0.019	54.007	1.698	52.309	down
	21	4791.793	0.015	6.731	0.514	6.217	down
	22	5707.175	0.002	1.527	0.060	1.467	down
	23	6112.504	0.015	1.756	0.530	1.227	down
	24	7953.295	0.015	1.937	0.137	1.800	down
	25	11355.281	0.005	1.738	0.106	1.632	down
	26	11537.512	0.003	1.450	0.050	1.400	down
	27	12573.719	0.002	0.541	0.040	0.501	down
	28	13361.203	0.047	1.262	0.099	1.162	down
	29	14703.493	0.031	3.051	0.105	2.946	down
	30	19927.809	0.015	0.203	0.009	0.194	down
	31	23434.902	0.038	1.311	0.091	1.220	down
pH 9, IMAC, 3500 mV	32	10582.636	0.012	0.698	0.031	0.667	down
	33	11627.882	0.031	0.435	0.007	0.428	down
	34	44058.481	0.015	0.353	0.006	0.347	up
	35	72270.173	0.003	0.066	0.001	0.065	down
Organic, H50, 3250 mV	36	11726.375	0.047	30.918	0.515	30.403	down
	37	13903.893	0.047	68.727	8.917	59.810	down
	38	83790.208	0.031	0.179	0.086	0.094	up
Organic, IMAC, 2200 mV	39	3971.839	0.028	3.400	-0.189	3.589	up
	40	4256.571	0.028	3.329	0.216	3.113	up
	41	6182.717	0.005	24.937	4.488	20.450	down
Organic, IMAC, 3500 mV	42	14063.305	0.021	35.466	24.463	11.003	up
	43	28032.434	0.049	40.736	16.682	24.054	up
	44	28871.829	0.037	12.658	5.190	7.467	up
	45	58597.340	0.021	1.285	0.863	0.422	down
	46	66423.505	0.049	14.590	9.449	5.141	down

Table 4: 46 biomarkers able to differentiate between single and multi-episodic ocular toxoplasmic events.

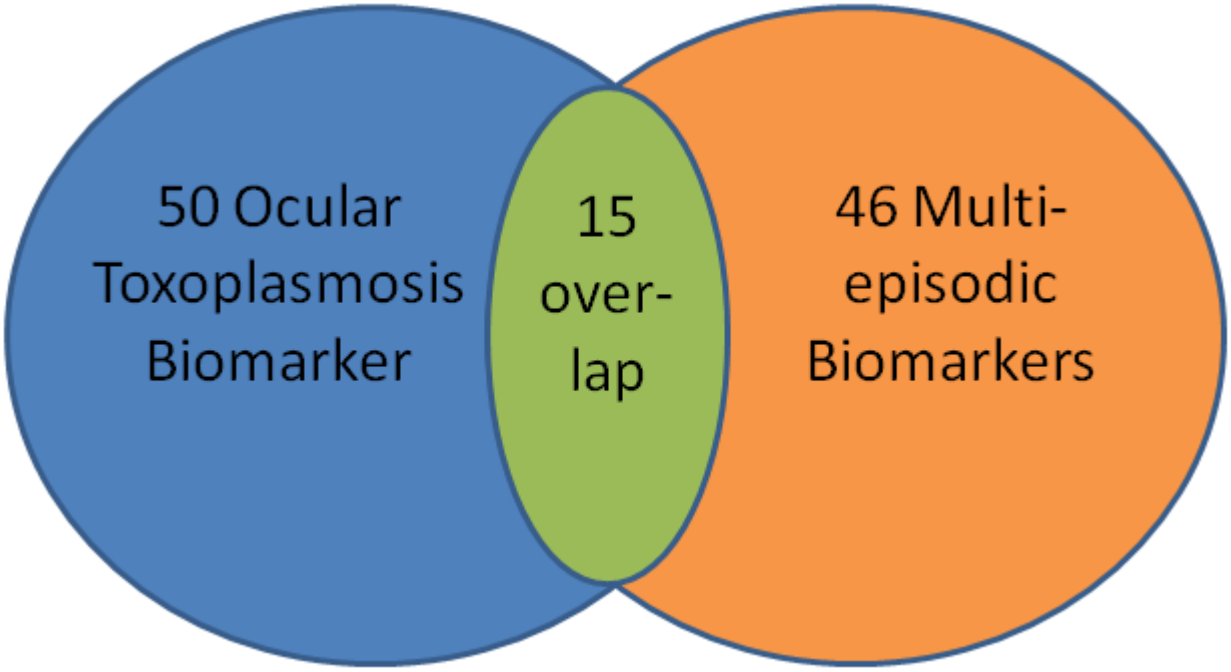

Figure 9: A Venn diagram illustrating the high degree of overlap (16 %) of biomarkers diagnostic of either ocular toxoplasmosis or multi-episodic disease.

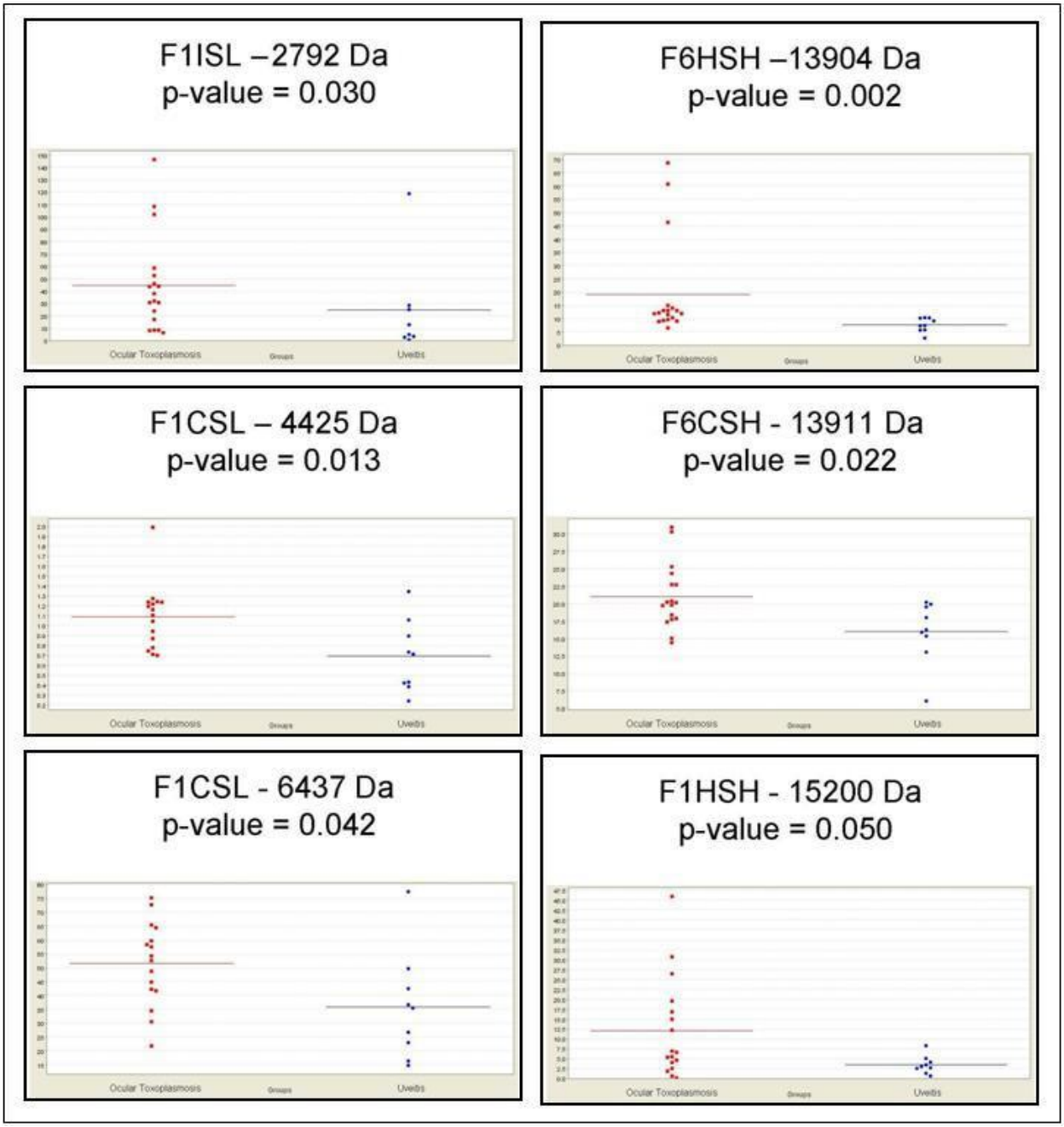

Figure 10: Cluster plot of protein biomarkers that are up-regulated in uveitis due to ocular toxoplasmosis.

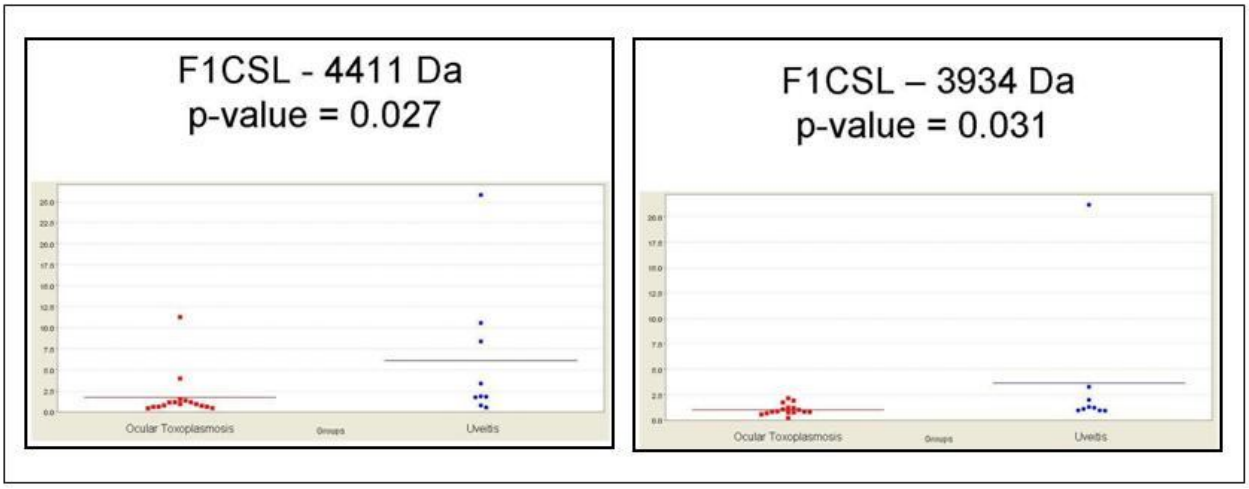

Figure 11: Cluster plot of protein biomarkers that are down-regulated in uveitis due to ocular toxoplasmosis.

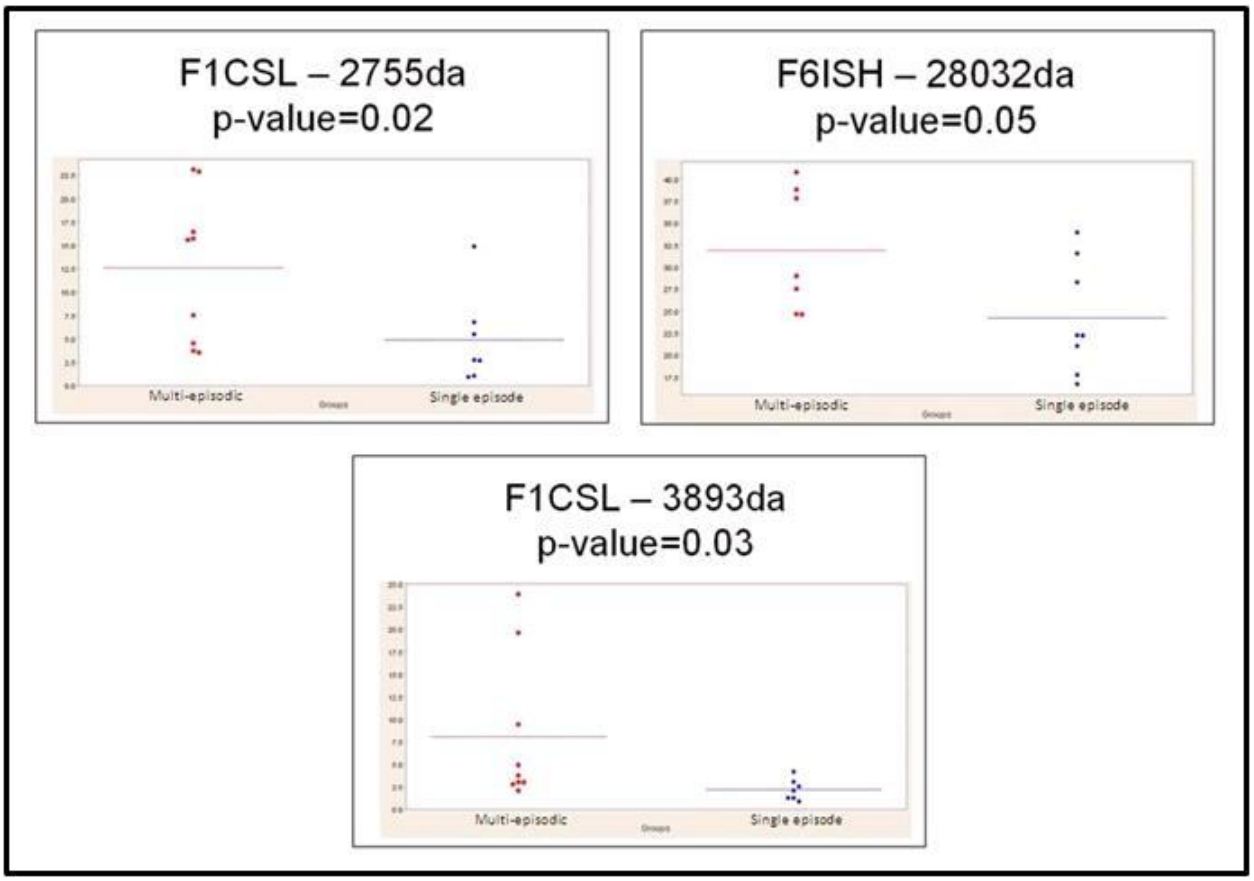

Figure 12: Cluster plot of protein biomarkers that are up-regulated in patients with recurrent uveitis due to ocular toxoplasmosis when compared to those who have only had a single event.

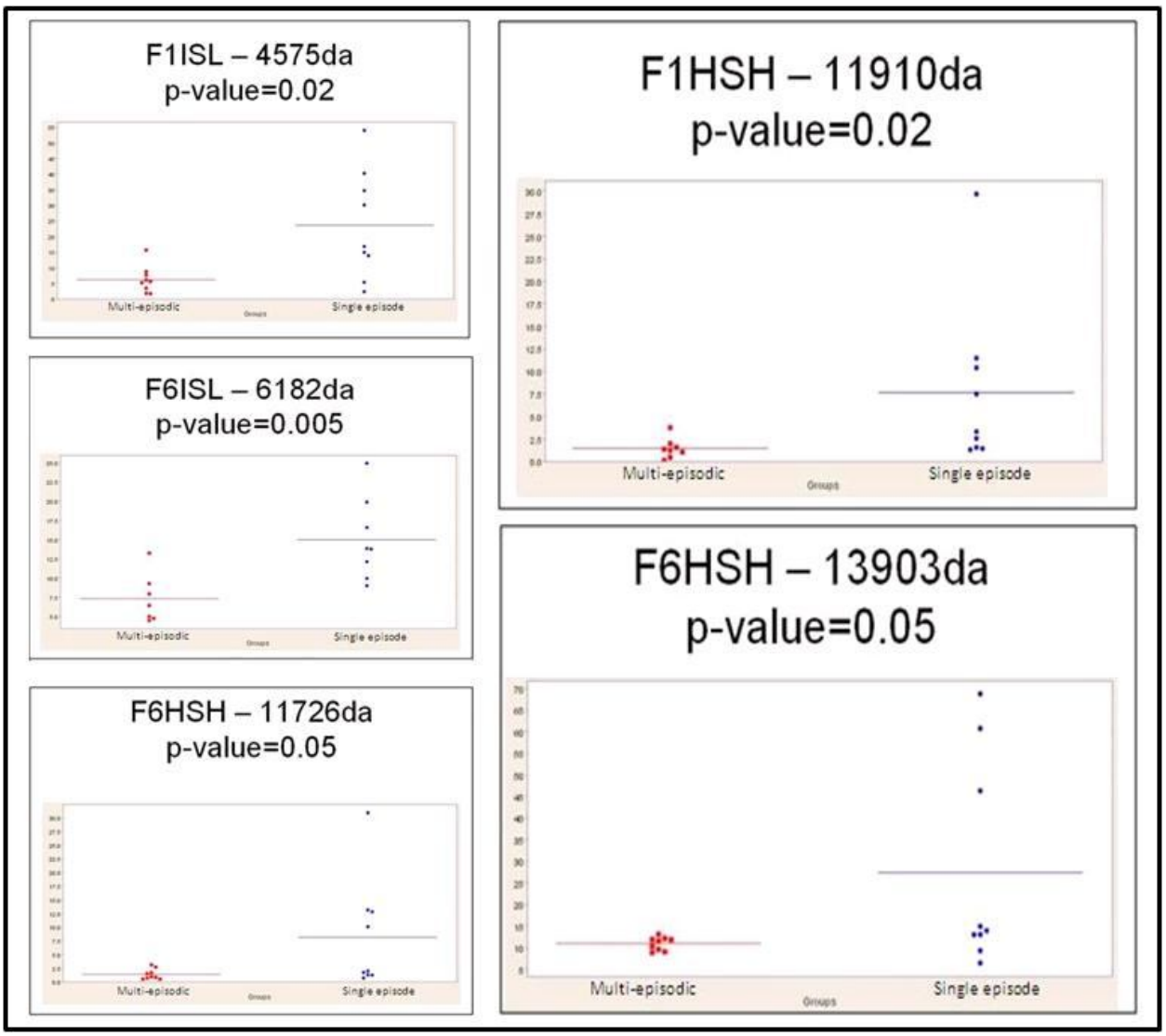

Figure 13: Cluster plot of protein biomarkers that are down-regulated in patients with recurrent uveitis due to ocular toxoplasmosis when compared to those who have only had a single event.

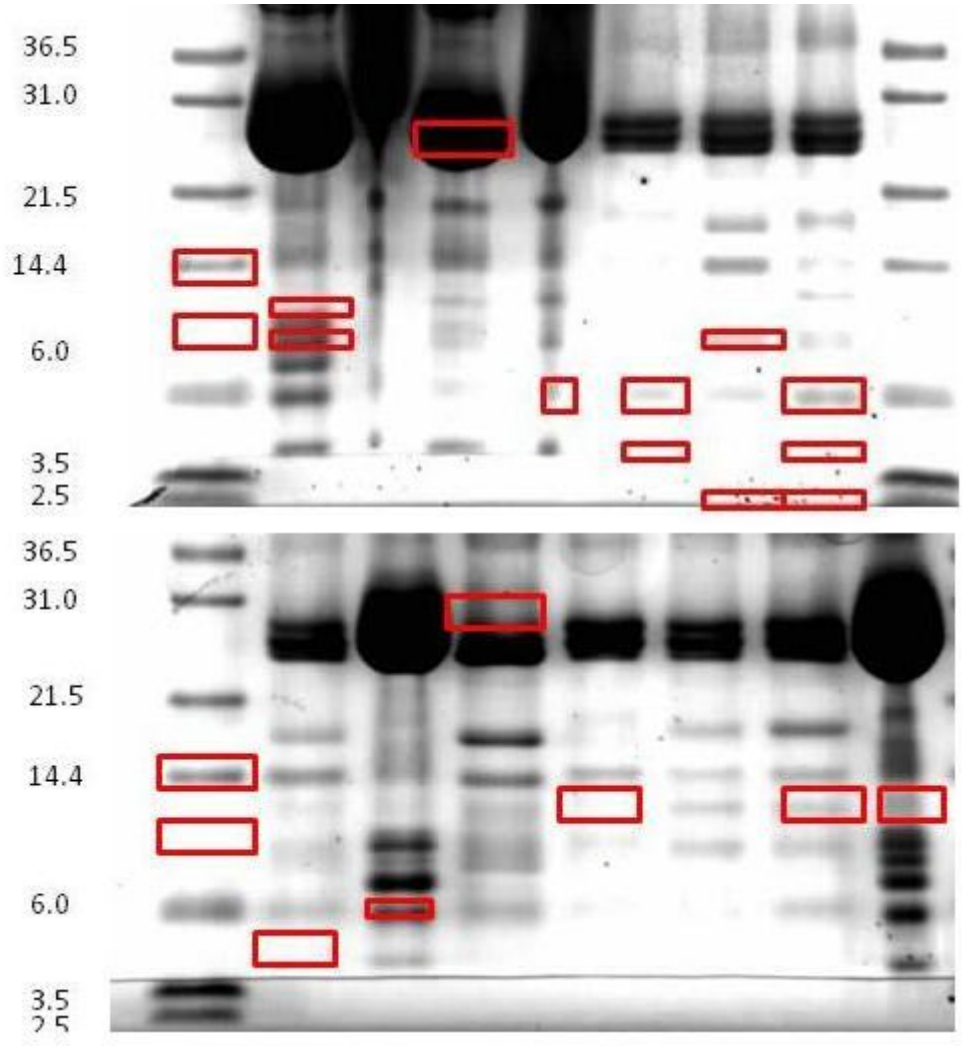

Figure 14: After SELDI-ToF-MS analysis, biomarkers which were found to have a difference in expression greater than one standard of deviation had those serum samples fractionated and proteins separated by SDS-PAGE with the goal of visualizing the biomarker. Bands in red are those that match the SELDI data and were excised for protein identification (results not shown).

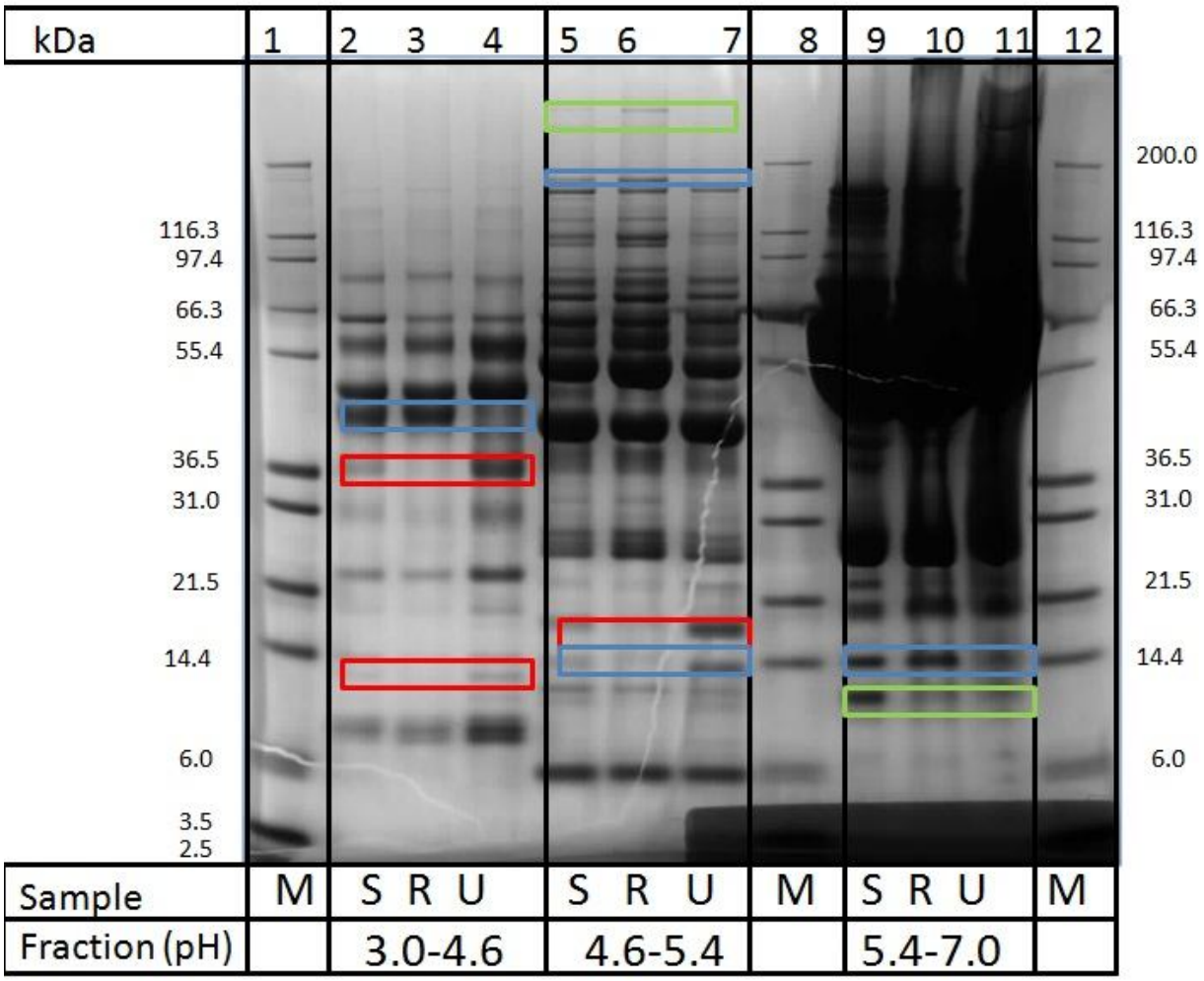

Figure 15: Two-dimensional protein gel of pooled samples ranging from a pH of 3 to 7. Here M demarks the ladder while S, R and U demark single, recurrent and non-toxoplasmic uveitis. The red (3), blue (4) and green (2) bands highlight bands that can potentially be markers of recurrent disease, non-toxoplasmic uveitis and single episode.

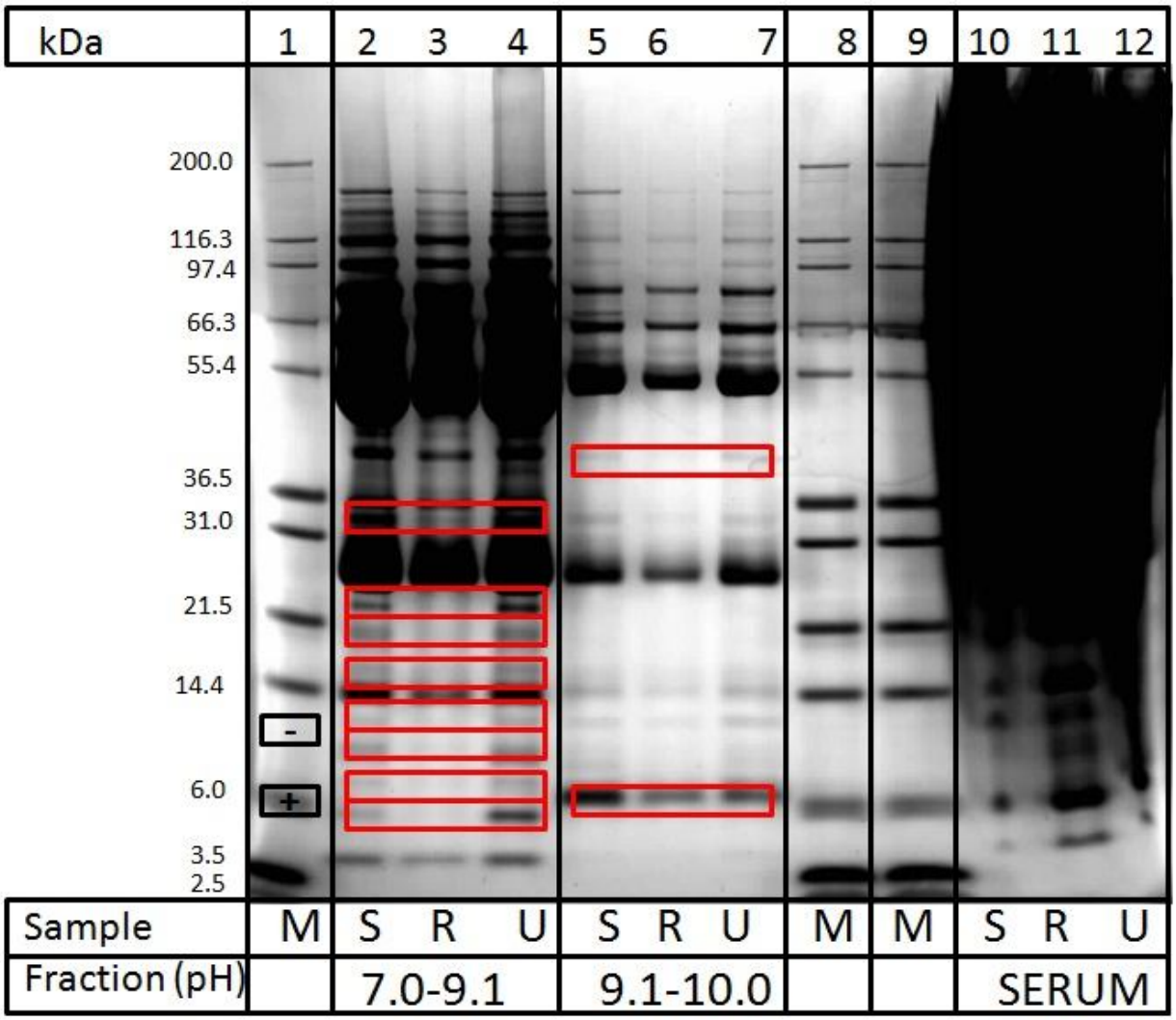

Figure 16: Two-dimensional protein gel of pooled sample ranging from a pH of 7 to 10. Here M demarks the ladder while S, R and U demark single, recurrent and non-toxoplasmic uveitis. The red bands (10) can potentially be markers of recurrent disease, non-toxoplasmic uveitis and single episode. The third lane demonstrated what happens is serum if not separated by pH and simply run according to its mass.

4.2. Biomarker Cross-Validation

Table 5 and 6 compare SELDI-ToF-MS results of ocular toxoplasmosis to the protein peaks found in the CDC/McGill toxoplasmosis outbreak study as outlined in section 3.12. Those markers that are bolded are matched within 0.0001 % while the unbolded ones are matched to within 1 %.

Figure 16 demonstrates an overlap of 9 biomarkers that can be used to differentiate between ocular toxoplasmosis and non-toxoplasmic uveitis as well as single events from multiple episodic ocular toxoplasmosis, found in both the outbreak study and the present work, repeated in section 4.1. This represents 20 % of the total number of biomarkers discovered. Interestingly, 60 % of the markers for ocular toxoplasmosis overlap, compared to only 20 % of those marking recurrent disease.

Biomarkers for comparing non-toxoplasmic uveitis of unknown etiology to ocular toxoplsmosis						
n	**OT Pilot Study**	**CDC/McGilll University *Toxoplasma* Outbreak Study**				
	Marker	Marker	Diagnosis 1	Diagnosis 2	Diagnosis 3	Diagnosis 4
1	3471.874	3476.808	Early vs Late			
2	3680.647	3682.072	G+M+ vs Neg	Late vs G+M+		
3	**3681.909**	**3683.072**	**G+M+ vs Neg**	**Late vs G+M+**		
4	4411.416	4416.557	Late vs Neg	Early vs Late	Late vs G+M+	
5	**4791.793**	**4790.725**	**Late vs G+M+**	**Late vs Neg**		
6	**5882.200**	**5883.021**	G+M+ vs Neg	Late vs. G+M+		
7	6437.447	6432.830	G+M+ vs Neg	Early vs. G+M+	Late vs Neg	Early vs Late
8	10462.903	10445.80371	Late vs. G+M+	Early vs Late	Late vs Neg	
9	11716.428	11702.68338	Late vs. G+M+			
10	13780.185	13777.669	G+M+ vs Neg			
11	13875.737	13867.677	Early vs Late	Early vs Neg		
12	**13903.893**	**13903.565**	**Late vs Neg**	**Early vs. G+M+**	**Early vs Late**	
13	13911.390	13903.565	Late vs Neg	Early vs. G+M+	Early vs Late	
14	17875.923	17911.643	Late vs. G+M+	Early vs Late		
15	28000.764	28092.08495	Late vs Neg	Early vs Late	G+M+ vs Neg	

Table 5: Comparison of results from the *T.gondii* Outbreak Study to the biomarkers discovered in section 4.1. Here, we are comparing non-toxoplasmic uveitis of unknown etiology to ocular toxoplasmosis Those markers that are bolded are matched within 0.0001 % while the unbolded ones are matched to within 1 %.

Ocular toxoplasmosis biomarkers for differentiating between single and multi-episodic events						
n	**OT Pilot Study**	**CDC/McGillI University *Toxoplasma* Outbreak Study**				
	Marker	Marker	Diagnosis 1	Diagnosis 2	Diagnosis 3	Diagnosis 4
1	3443.082	3436.513	Early vs Late			
2	3471.211	3476.808	Early vs Late			
3	3471.874	3476.808	Early vs Late			
4	**3680.647**	**3682.072**	**G+M+ vs Neg**	**Late vs G+M+**		
5	**3681.909**	**3682.072**	**G+M+ vs Neg**	**Late vs G+M+**		
6	3893.921	3883.915	Late vs Neg			
7	4411.416	4115.101	Late vs Neg			
8	4575.417	4567.464	Late vs Neg	Early vs Late		
9	4578.105	4567.464	Late vs Neg	Early vs Late		
10	**4791.793**	**4790.725**	**Late vs G+M+**	**Late vs Neg**		
11	4796.257	4790.725	Late vs G+M+	Late vs Neg		
12	**5018.141**	**5017.374**	**Early vs Late**	**Early vs. G+M+**		
13	5707.175	5711.201	Late vs G+M+	Early vs Late		
14	**5713.411**	**5711.201**	**Late vs G+M+**	**Early vs Late**		
15	**5882.200**	**5883.021**	**G+M+ vs Neg**	**Late vs. G+M+**		
16	6182.717	6191.733	Late vs G+M+	G+M+ vs Neg	Early vs Neg	
17	6437.447	6432.830	G+M+ vs Neg	Early vs. G+M+	Late vs Neg	Early vs Late
18	7852.476	7750.856	Early vs Late	Late vs Neg	Late vs G+M+	
19	7953.295	7948.186	Late vs Neg	Early vs Late	Late vs. G+M+	
20	10848.849	10841.589	Late vs Neg	Early vs Late		
21	**11726.375**	**11726.174**	**Late vs G+M+**			
22	12718.736	12712.112	Early vs Late	G+M+ vs Neg	Late vs Neg	
23	13781.995	13777.669	G+M+ vs Neg			
24	13875.737	13867.677	Early vs Late	Early vs Neg		
25	**13903.893**	**13903.565**	**Late vs Neg**	**Early vs. G+M+**	**Early vs Late**	
26	13911.390	13903.565	Late vs Neg	Early vs. G+M+	Early vs Late	
27	14703.493	14711.435	Late vs G+M+	Early vs Late	G+M+ vs Neg	Early vs Neg
28	**17312.105**	**17313.928**	**Early vs Late**	**Late vs. G+M+**	**Late vs Neg**	
29	**17910.201**	**17911.643**	**Late vs G+M+**	**Early vs Late**	**G+M+ vs Neg**	
30	33429.155	33356.781	Early vs. G+M+	Early vs Late	Early vs Neg	

Table 6: Comparison of results from the *T.gondii* Outbreak Study to the biomarkers discovered in section 4.1. Here, we are comparing single and multi-episodic events. Those markers that are bolded are matched within 0.0001 % while the unbolded ones are matched to within 1 %

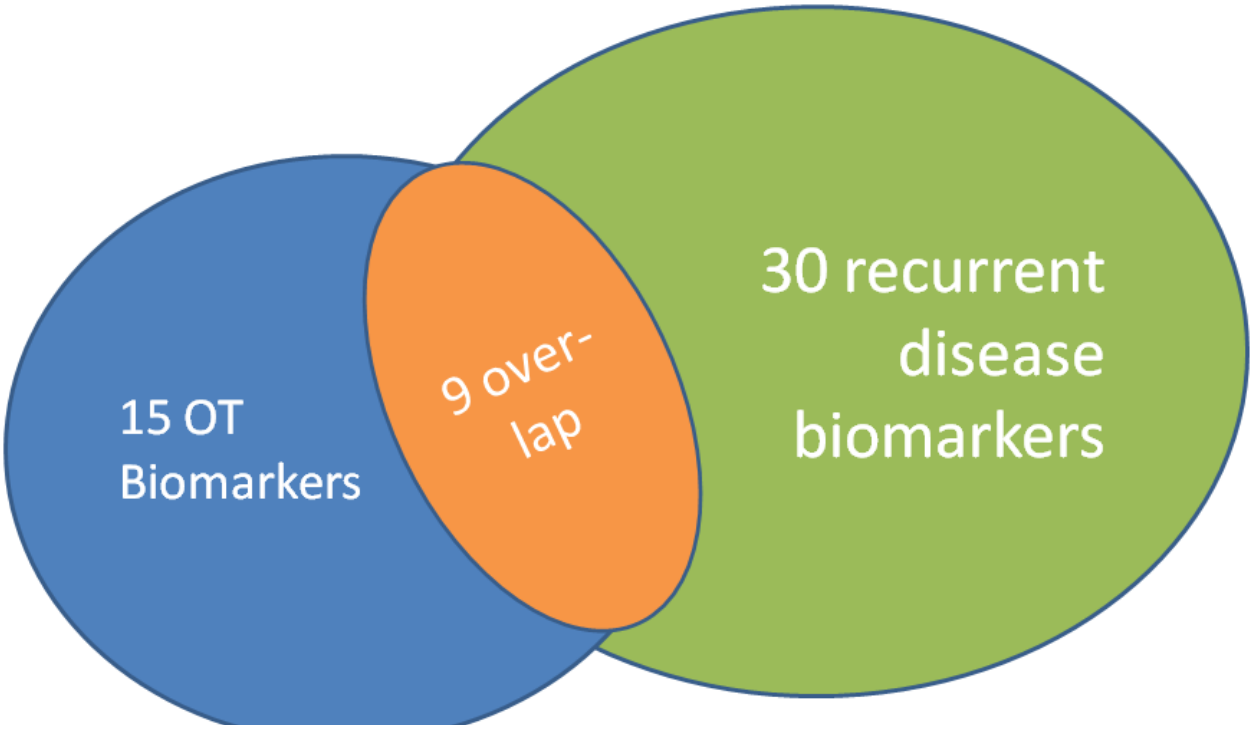

Figure 17: A Venn diagram illustrating the high degree of overlap (20 %) of biomarkers diagnostic of either ocular toxoplasmosis or multi-episodic disease found to be present in both the outbreak study of toxoplasmosis and the results of section 4.1.

4.3. Biomarker Identification

Table 7 lists all 20 biomarkers identified from 2, 3 and 4 of figure 16. Furthermore, the table describes the function of the proteins and their role in toxoplasmosis. Using the sequencing results from the 2-DE, 11 % of the markers cross referenced with the toxoplasmosis outbreak were able to be identified.

In table 8 protein peaks generated by SELDI-ToF-MS analysis were compared to the mass and sequence information from the protein identification generated from 2-DE in order to elucidate the identities of those peaks; 16 % of the SELDI generated peaks were identified.

Table 9 employed the sequence information alongside the proteins 'molecular weight search score' to develop an initial diagnostic picture using 2-DE parallel to SELDI-ToF-MS technology. Hemaglobin subunit beta, serum amyloid and complement C8 are markers of ocular toxoplasmosis. Nuclease-sensitive element-binding protein (YBOX1) is a marker of single disease. Ankyrin repeat domain-containing protein 11 (ANKRD11) and peptidyl-prolyl cis-trans isomerase A (PPIA) are markers of multi-episodic disease. Plasma retinol-binding protein 4 (RBP4), ATP-binding cassette sub-family A member 2 and bullous pemphigoid antigen 1, isoforms 6/9/10 (Trabeculin-ß) are markers of non-toxoplasmic uveitis.

Biomarker Identity and Function (all human)				
n	**Accession numbers**	**Full Name**	**Functional Classification**	**Relationship to Toxoplasmosis?**
1	ABCA2	ATP-binding cassette sub-family A member 2	ATP-binding cassette (ABC) transporters	None
2	ALBU	Albumin	Serum albumin	
3	ANR11	Ankyrin repeat domain-containing protein 11 (ANKRD11)	p53 cofactor	
4	ARI16	Probable E3 ubiquitin-protein ligase	Ubiquitin ligase	*T. gondii* proliferation
5	BPAEA	Bullous pemphigoid antigen 1, isoforms 6/9/10 (Trabeculin-ß)	Autoantigen	None
6	CO4A	Complement C4-A	Complement system	Humoral immune response to infection
7	CO8G	Complement component C8 gamma chain		
8	EF1A1	Elongation factor 1-alpha 1 (EF-1)	Translation elongation factor	None
9	HBA	Hemoglobin subunit alpha	Globin	None
10	HBB	Hemoglobin subunit beta		
11	HV101	Ig heavy chain V-I region EU	Ig Superfamily (heavy chain)	Humoral immune response to infection
12	HV106	Ig heavy chain V-I region SIE		
13	IGHG1	Ig gamma-1 chain C region		
14	IGKC	Ig kappa chain C region	Ig Superfamily (light chain)	
15	KV205	Ig kappa chain V-II region GM607 (precursor/fragment)		
16	LAC	Ig lambda chain C regions		
17	PPIA	Peptidyl-prolyl cis-trans isomerase A	Cyclophilin	Limits parasitic invasion
18	RET4	Plasma retinol-binding protein 4 (RBP4)	Lipocalin / cytosolic fatty-acid binding protein family	None
19	SAA4	Serum amyloid	Accute phase protein	
20	YBOX1	Nuclease-sensitive element-binding protein	Transcription factor, potential cancer biomarker	

Table 7: A lists of all 20 biomarkers identified from 2, 3 and 4 of figure 16 displaying protein function and involvement in toxoplasmosis. All proteins are human derived.

Identification of SELDI results		
n	Biomarker	Possible ID
1	11355.281	Ig lambda chain C regions
2	11726.375	Ig kappa chain C region
3	12573.719	Ig heavy chain V-I region EU
4	12931.827	Ig kappa chain V-II region GM607
5	13875.737	Ig heavy chain V-I region SIE
6	14703.493	Ig heavy chain V-I region SIE
7	15200.146	Hemoglobin subunit beta
8	18392.561	Peptidyl-prolyl cis-trans isomerase A
9	18397.240	
10	23430.977	Complement component C8 gamma chain
11	23434.902	
12	50790.047	Elongation factor 1-alpha 1 (EF-1)
13	58597.340	Probable E3 ubiquitin-protein ligase
14	72270.173	Albumin
15	193083.094	Complement C4-A

Table 8: Protein peaks generated by SELDI-ToF-MS analysis were compared to the mass and sequence information from the protein identification generated from 2-DE in order to elucidate the identities of those peaks

		Possible Protein Identies								
n	Protein Band	One Episode			Multi-Episodic			Non-Toxoplasmic Uveitis		
	(kDa±3)	Name	Mass	Score	Name	Mass	Score	Name	Mass	Score
1	12	HBB	16102	98	HBB	16102	243	IGKC	11773	131
		SAA4	14854	59	SAA4	14854	125	ABCA2	n/a	53
2	16	**No Results**			HBA	15305	110	HBA	15305	231
3	20	CO8G	22435	88	CO8G	22435	303	BPAEA	n/a	53
					PPIA	18229	99			
4	23	CO8G	22435	51	ANR11	n/a	51	CO8G	22435	338
								RET4	23337	47
5	33.5	YBOX1	35903	369	IGHG1	36596	233	IGHG1	36596	87

Table 9: Using protein sequence information combined with the given proteins' molecular weight search score and SELDI-ToF-MS technology we can begin to develop an initial diagnostic picture. Here, hemaglobin subunit beta, serum almyloid and complement C8 are markers of ocular toxoplasmosis. Nuclease-sensitive element-binding protein (YBOX1) is a marker of single disease. Ankyrin repeat domain-containing protein 11 (ANKRD11) and Peptidyl-prolyl cis-trans isomerase A (PPIA) are markers of multi-episodic disease. Plasma retinol-binding protein 4 (RBP4), ATP-binding cassette sub-family A member 2 and Bullous pemphigoid antigen 1, isoforms 6/9/10 (Trabeculin-ß) are markers of non-toxoplasmic uveitis.

4.4. Potential Diagnostic Panel

SELDI-ToF-MS applied tree algorithm classifications were used to evaluate ocular toxoplasmosis and multi-episodic diagnostic accuracy by classification of serum samples from patients with single and multiple episodes of uveitis due to ocular toxoplasmosis versus non-toxoplasmic uveitis (9 trees) and by comparing toxoplasmic uveitis from patients with one episode to those with who have had multiple ones (10 trees). Trees were selected for availability of protein sequence and relatively high levels of differential intensity.

Figure 18 is one such tree which can be clinically relevant in aiding in the differential diagnosis of ocular toxoplasmosis. Here ,the left and right branch node after the first layer depicts the cases equal to or less than 0.604 mA. The subsequent nodes on the left have a cut-off point for peaks 13780 Da at less than or equal to 1.178 mA and 2.483 for peak 12685 on the right.

Figure 19 is relevant for the classification of serum samples from patients with single episodes of toxoplasmic uveitis to those who have had multiple events using a decision tree algorithm. In 19.A protein peak 18397 Da was identified as peptidyl-prolyl cis-trans isomerase A (see table 8), whereas 14063 was not identified but demonstrates a high level of intensity.

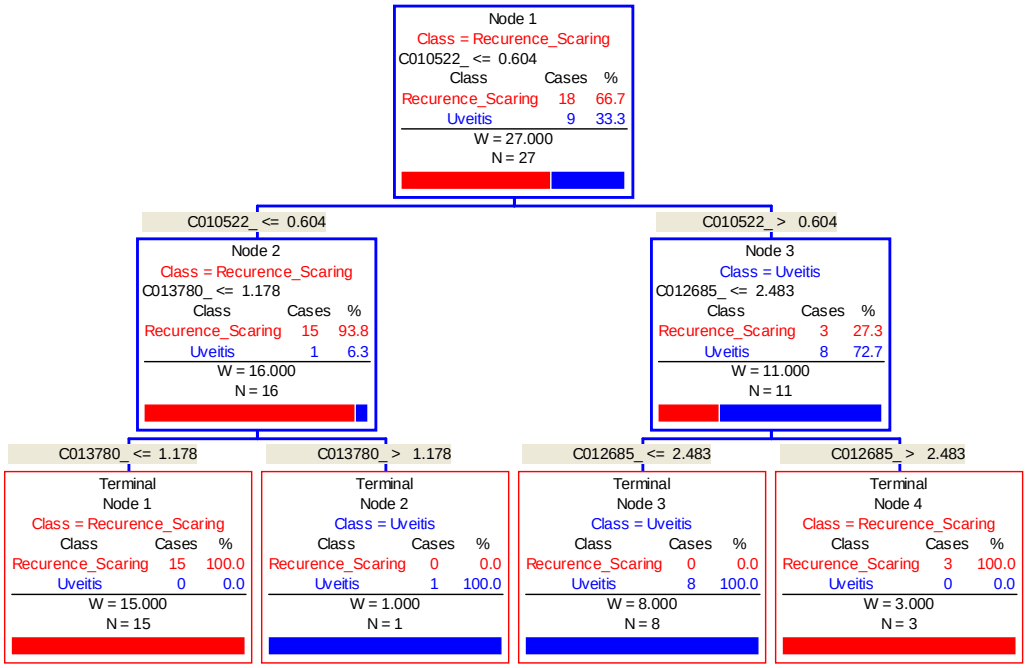

Figure 18: Classification of serum samples from patients with ocular toxoplasmosis (both single and multiple events) versus non-*toxoplasma* uveitic controls using a decision tree algorithm. This tree demonstrates a high level of discrimination between the toxoplasmic uveitis and non-toxoplasmic uveits. None of the biomarkers featured on this tree have yet to be identified.

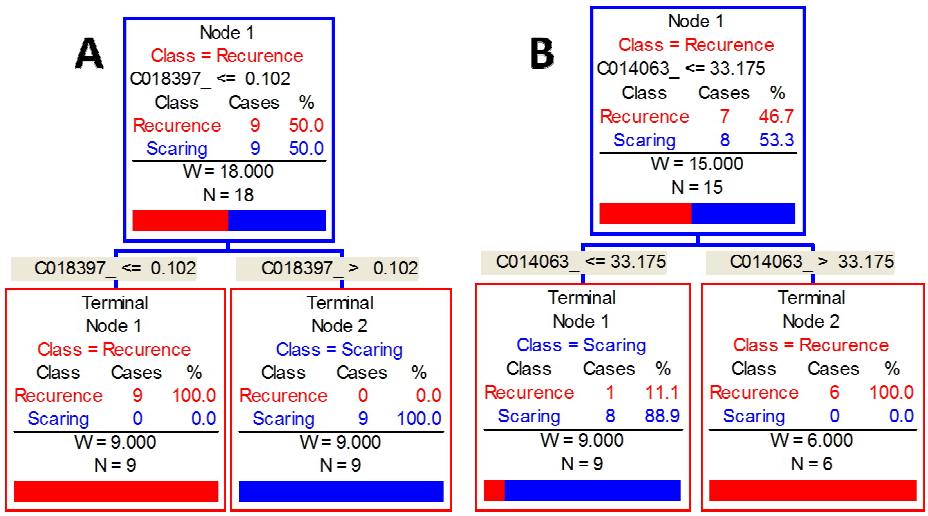

Figure 19: Classification of serum samples from patients with single episodes of toxoplasmic uveitis to those who have had multiple events using a decision tree algorithm. Protein peak 18397 Da from 19.A was identified as peptidyl-prolyl cis-trans isomerase A, whereas 14063 was not identified but demonstrates a high level of intensity.

Chapter 5: Discussion

The diagnosis of uveitis due to ocular toxoplasmosis remains a clinical challenge. To remedy this, novel non-invasive diagnostic tests, with high sensitivity and specificity, are needed. As proteins represent the vast majority of biologically active molecules responsible for cellular function and with PCR diagnostics proving to be inconclusive in disease diagnostics, protein biomarkers could be useful in the future diagnosis of the disease.

Clinical proteomics is becoming ever more accessible due to the accumulation of DNA and protein sequence databases, improvements in MS technologies, and the development of more efficient computer algorithms for database searching (Graves and Haystead 2002). SELDI-ToF-MS represents an emerging proteomic technology in biomarker discovery that allows the rapid and sensitive analysis of complex protein mixtures and yields a compressive summary of a given biological fluid or tissue's proteome, without the need to first carry out protein separations, by gels for example (Hutchens & Yip,1993; Wright Jr. and Cazares et al. 1999). Moreover, it is well suited for biomarker discovery because of its high-throughputs, rapid analysis, and minimal sample requirement. When samples are processed using SELDI-ToF-MS in parallel with the latest 2-DE methodologies, "the ability of the researcher to detect a wide range of biomarkers is sharply increased" (Boernsen 2005).

Regardless of the technology employed in the biomarker discovery process, a wide range of biological sample types can be used including: plasma, serum, urine, saliva, cerebrospinal liquid,

bronchoalveolar wash out, nipple aspirate fluid, tears, amniotic fluid and tissues or cell extracts. However, the majority of biomarker discovery studies use serum, plasma, urine, saliva and tissue extracts (De Bock 2010). When selecting the type of biological fluid to be employed in a given study the researcher must consider the many variables related to collection, storage, and conditions for sample preparations. These parameters have been commented on in literature according to the nature of the biological material. According to the Human Proteome Organization Plasma Proteome Project Specimens Committee recommendations (HUPO 2010), "plasma appears preferable to serum because it contains fewer peptides of degradation and consequently presents less variability" (Tammen 2005). However, we chose serum because proteins and peptides that "survive" the clotting procedure exhibit a stability that can be exploited in routine clinical applications. This is a major consideration because any future diagnostic assay will have to be able to perform in the tropics.

The human proteome is thought to be composed of more than half a million proteins (Banks et al., 2000). The protein content of human serum is composed of a "millieu" of proteins from most every type of cell and tissue. The serum proteome has been shown to contain information that "directly reflects pathophysiological states and represents an invaluable source of diagnostic information for a variety of different diseases" (Zhou 2004). Nevertheless, serum albumin represents approximately 60 % of whole blood protein content and its presence, along with other high abundance proteins such as immunoglobulins, complicates the detection of lower abundance proteins which could serve as useful biomarkers. The major task we faced in the biomarker discovery process is the fractionation of the entire proteome followed by processing

the massive amount of data generated into meaningful results through the use of multi-variant statistics and various bioinformatics platforms. This is due to the extraordinarily wide dynamic range of the serum proteome which spans more than 12 orders of magnitude (Anderson and Anderson 2002). Protein fractionation and profiling is performed to "increase the probability of detecting proteins with altered expression levels during disease development, progression or treatment" (Ahmed 2005). This makes it possible to combine several of these proteins into a discriminative protein pattern (Graves and Haystead 2002).

The potential of SELDI has been explored in various medical disciplines from cancer to atherosclerosis and infectious diseases (Wei 2010). To date, no single, or group of, protein biomarkers have been described for the diagnosis of either ocular or systemic toxoplasmosis, as the technology to do so has only recently come online. This pilot study demonstrates the potential of SELDI-ToF-MS combined with 2-DE to discover ocular toxoplasmosis biomarkers as well as markers of the recurrent disease from patient serum.

Results presented here demonstrate that both SELDI-ToF-MS and 2-DE can be used successfully in the search for serum biomarkers of ocular toxoplasmosis. The first goal of this study is the identification of serum biomarkers diagnostic of ocular toxoplasmic uveitis. Second pass analysis discovered 50 biomarkers of which eight were found to have a DI greater than one standard of deviation. Of the eight biomarkers one was sequenced and identified to be hemaglobin subunit beta (HBB). HBB was found to be up-regulated in toxoplasmic uveitis

when compared to the non-toxoplasmic variety. As was expected, following analysis by 2-DE, HBB was also found to be expressed in both individuals with single and recurrent disease and not in those with non-toxoplasmic uveitis. HBB is a subunit of a larger protein called hemoglobin, which is located inside red blood cells. Hemoglobin normally consists of four protein subunits: two subunits of beta-globin and two subunits of alpha-globin. Each of the four protein subunits of hemoglobin carries an iron-containing molecule called heme, which is necessary for red blood cells to bind oxygen in the lungs and deliver it to cells throughout the body. The protein has no known role in the pathobiology of the ocular toxoplasmosis. To date there is only one mention in the literature of HBB as a possible biomarker for any disease where it was found to be a colon cancer biomarker, which is mostly due to its association with hypoxic conditions within the tumour (Rho et al., 2008). However Rho and colleagues used tissue biopsies rather than serum as the biological materials for their biomarker discovery study, which may contain plasma and tissue specific proteins such as hemaglobin. Although, it is tempting to hypothesize that HBB's presence in our serum samples is due to improper removal of erythrocytes prior to serum fractionation, this is not the case as the statistics demonstrate. Its presence is most likely associated with free hemoglobin that is the hemoglobin outside of the red blood cells called serum hemaglobin (Medline Plus 2010).

Eight additional biomarkers were identified and found to be related to humoral immune response to infection. Three quarters of those markers belong to the Ig superfamily while the rest are components of the complement system. It is important to remember that the cellular immune response against *T. gondii* is critical for infection control. However, antibodies can either

enhance or block protective mechanisms and even mediate immunological damage, directly or indirectly, so the Ig superfamily proteins identified can potentially serve as disease biomarkers distinguishing either protective or pathological cellular immune events.

A 36.5 kDa band was isolated by 2-DE at a pH of between 7.0 and 9.1 and was then sequenced. Its identity was determined to be Ig gamma-1 chain C region (IGHG1), the constant region of the heavy chain associated with IgG1. IgG1 is the most abundant IgG subclass in humans at 66 %. Moreover, it demonstrates the highest affinity for the F_c receptor on phagocytic cells of any of the IgG subclasses and has a relatively high propensity to activate complement (Schroeder 2010). In toxoplasmosis, IgG1 is up-regulated by interleukin (IL)-4 and IL-13, and is therefore considered to be a biomarker of the chronic phase toxoplasmosis (Correa et al. 2007). This finding is consistent with our results, where we demonstrated IGHG1 to have an expression level three times greater in the recurrent disease when compared to non-toxoplasmic uveits, while levels of IGHG1were unremarkable in individuals with single episodes of ocular toxoplasmosis. Neither IGHG1 nor any of it fragments were detected by SELDI-ToF-MS in any of the 432 spectra, because chip type selected may not have bound either the protein or its fragments (Wright Jr. 2002). However Ig lambda chain constant region along with its two isoforms of both Ig kappa chain constant region and Ig heavy chain variable region as well as complement components C4A and C8 gamma chain were independently detected by SELDI-ToF-MS and 2-DE. They all represent potential immunological markers of ocular toxoplasmosis.

Indeed C4, a C4A precursor, is the only above mentioned immunological marker that has a connection in the literature with toxoplasmosis. The complement system helps to clear pathogens from the host by disrupting the target cell's plasma membrane and it is divided into three complimenting systems (or pathways). One of these systems is mannose-binding lectin (MBL) pathway, which allows the host's innate immunity to detect molecular patterns that specifically characterise microorganisms, including *T. gondii* (Gadjeva 2001). Among the many components and complexes that are involved in the MBL pathway are C4A and its precursor C4. In fact, the C4 component of the complement had been demonstrated to be a useful indirect marker for the prenatal diagnosis of toxoplasmosis. The use of C4 as a non-specific indicator of congenital infection demonstrated a sensitivity and specificity of 81 % and 83 % respectively and positive and negative predictive values of infection in 72 % and 89 %. These figures surpass the performance of other indirect measures of infection (Cohen-Khallas 1992). Similarly, we detected C4A to be significantly up-regulated in individuals with ocular toxoplasmosis, suggesting that the complement-precursor was activated in these individuals, which is used in its active form to potentiate opsonisation.

Aside from blood proteins and immunoglobins, we have identified the low abundance protein, peptidyl-prolyl cis-trans isomerase A (PPIase) to be a biomarker of, and have a documented connection to, ocular toxoplasmosis. The most promising of the low abundance proteins found to be a biomarker of ocular toxoplasmosis is PPIase. PPIase has four different molecular mechanisms associated with it that helps control the cis/trans peptide configuration: first, the catalysis of peptide bond cis/trans isomerisation; second, as a holding function for unfolded

polypeptide chains; third, as a presenter protein function for an unknown number of physiological ligands; and forth, a proline-directed binding function, which is specific to PPIases (Fischer 2003). In terms of toxoplasmosis, we are most interested in its folding and presenter protein function.

During invasion of host cells, *T. gondii* discharges the contents of small, apically located secretory organelles called micronemes, whose proteins are known to be necessary for both parasite motility and host cells invasion. One of the six proteins of the micronemal is TgMIC5, which possesses a peptidyl-prolyl cis-trans isomerases sequence (Brydges 2001). This PPIase sequence function may assist in the folding of other micronemal proteins arising in invasion of host cells by *T. gondii* tachyzoites. As *T. gondii* modulates pro- and anti-inflammatory responses to regulate parasite multiplication and host survival, eventually pressure from the immune response causes the conversion of tachyzoites into slowly dividing bradyzoites (Dubey 1998). This conversion is mediated by the production of nitric oxide in a CCR5-dependent manner (Ibrahim 2009). The tachyzoite produces a unique chemokine mimic that activates CCR5 via PPIase activity. As a result there is an up-regulation of downstream IL-12-dependent production of gamma interferon (IFN-γ) in dendritic cells (DCs), which allows for host survival of acute toxoplasmosis (Cai 2000). Conversely, *T. gondii*'s ability to generate such a strong protective response though the induction of IL-12 from DCs may benefit the parasite. This in turn prevents the protozoan from overwhelming its intermediate hosts thereby increasing the parasite's propensity to transmit (Aliberti 2003). Interestingly, the effect was blocked by addition of

cyclosporineA (CsA). As would be expected, our study found PPIA to be present only in multi-episodic samples isolated by 2-DE.

The second goal of this pilot study was to discover a biomarker that can differentiate between individuals with a single episode of the disease have been from those with multiple events. Thus far, 46 markers of recurrent disease discovered by MS and 39 by 2-DE, with 18 % of the markers found by the latter technique have been confirmed present by the former. Eight of these biomarkers were found to have a DI greater than one standard of deviation and one was sequenced and identified as Ig Kappa constant region. However, IgKc has no documented link to toxoplasmosis. Thirty percent of the ocular toxoplasmosis biomarkers found are also biomarkers of recurrent disease. These are lambda, kappa, two isoforms of Ig heavy chain variable regions, C8-gamma, albumin and E3, which have all been discussed with the exception of and Probable E3 ubiquitin-protein ligase (E3).

E3 ubiquitin ligase ubiquitinates histone H3 via activation of ubiquitin-like PHD and ring finger domains 1 (UHRF1) (Karagianni 2008), UHRF1 plays a central role in confirming methylation status of mother cells to daughter cells. It is precisely the E3-dependent UHRF1 activation of histone ubiquitination which is known to be required for transcriptional initiation elongation, silencing, and DNA repair (Weake 2008). Moreover UHRF1 promotes G1/S transition, which is facilitated by *T. gondii* for its proliferation and plays a role in cell cycle progression (Brunet 2008). Our results show E3 to be down-regulated in recurrent disease when compared to single

event, suggestive of correct host response to chronic infection. Accordingly, UHRF1 is over-expressed in various cancers essential for both cancer progression and *T. gondii* proliferation in infected cells. This suggests that UHRF1 targeted therapies can be effective for treating these diseases through the use of molecular targeted therapies and/or vaccination (Bronner 2007; Unoki 2009).

The data sets from this pilot study were used separately to cross-validate discoveries from the CDC/McGill *T. gondii* outbreak study (courtesy of Dr. Momar Ndao, McGill University) to detect systematic biases in data such as those because of differences in pre-analytical sample processing procedures. This differs significantly from the approaches in which data from multiple sites are pooled together and then divided through randomization into, artificially made, identically distributed training and test data sets. Considering the high cost associated with post-discovery validation, however, we believe that the conservative approach that we used is similar to the clinical environment allowing us to move more efficiently from bench to bedside given that to date there have been no published biomarker studies of either ocular or systemic toxoplasmosis. We were able to cross-validate almost half (47 %) of the biomarkers in this study. Moreover, the identification of the discovered biomarkers will allow for additional confirmation through immunoassay tests.

Chapter 6: Final conclusion and summary

T. gondii is an obligate intracellular protozoan parasite that infects up to a third of the world's population. Ocular toxoplasmosis is the most common etiology of posterior uveitis, with retinal lesions present in 20 % of those infected. The high incidence of macular scarring associated with ocular toxoplasmosis is a leading cause of visual morbidity in the developing world causing a highly negative social and economic impact for those affected. The ability to diagnose the disease from a serum sample drawn from those infected would confer an advantage to patients and their physicians.

The purpose of this study was to determine the feasibility of hypothesis-driven proteomics based research into the diagnosis of ocular toxoplasmosis and its recurrent disease, by analysis of peripheral blood. To that end, the study has reported potentially clinically relevant biomarkers diagnostic of ocular toxoplasmosis. Analysis of pooled serum by MS discovered 50 markers of ocular toxoplasmosis and 46 markers of recurrent disease. Of those biomarkers, 47% were cross-validated within 1 % of *m/z* and 31 % within 0.0001 % of *m/z*. 14 biomarkers were selected for validation and all were visualized by SDS-PAGE; protein sequencing is ongoing. Protein separation by 2-DE yielded 57 differentially expressed bands, of which 20 were excised and identified. A quarter of the identified proteins were able to be cross-validated. .

Identification and validation of all the biomarkers presented here must be completed in order to determine their clinical relevance. As such, additional studies are required to assess the broader

sensitivity and specificity of the biomarkers we have discovered and to validate them. Moreover, this study should be repeated using samples from other geographic areas as well as comparing the biomarkers found here against biomarkers of other parasitic infections. Because the biomarkers are of host origin, it will be necessary to determine what other pathological or non-pathological conditions modulate the level of these proteins in the sera. Also, simpler assays will be needed to detect the biomarkers, such as ELISA, in the future if this knowledge is to be applied to diagnosis in low resource settings. If validated in more comprehensive studies, this discovery will have a major impact on future disease diagnosis and treatment monitoring and the quality of life of the patients.

An important future goal therefore is confirmation of sensitivity and specificity for the prospective detection of ocular toxoplasmosis. Designing a trial to assess the efficacy of the approach as a stand-alone or combined with current screening options will be important (appendix 8.1). Once biomarkers of interest have been successfully validated, they should be grouped together to comprise a diagnostic panel. This panel would represent the first stage in development of an assay which would enable diagnosis the disease from a serum sample drawn from those thought to present with atypical uveitis, with the goal of confirming or ruling out ocular toxoplasmosis.

At the moment for hypothesis-driven research to be successful there needs to be careful selection of the specific features of a proteome to provide information relevant for the particular

biomedical question. This is important largely given that the bottleneck is likely to lie not in identifying the proteins but in their downstream characterisation. Today, proteomics is beginning to benefit integration of genomics initiatives, such as the sequencing of the human genome, coupled with the development of national and international proteomic funding schemes. Now more than ever proteomics-based approaches offer the researcher the potential to move beyond laboratory and into the clinic. Whether the identification of diagnostic markers for various diseases including ocular toxoplasmosis results in the development of new treatment modalities remains to be seen, but it is safe to say that the world is watching.

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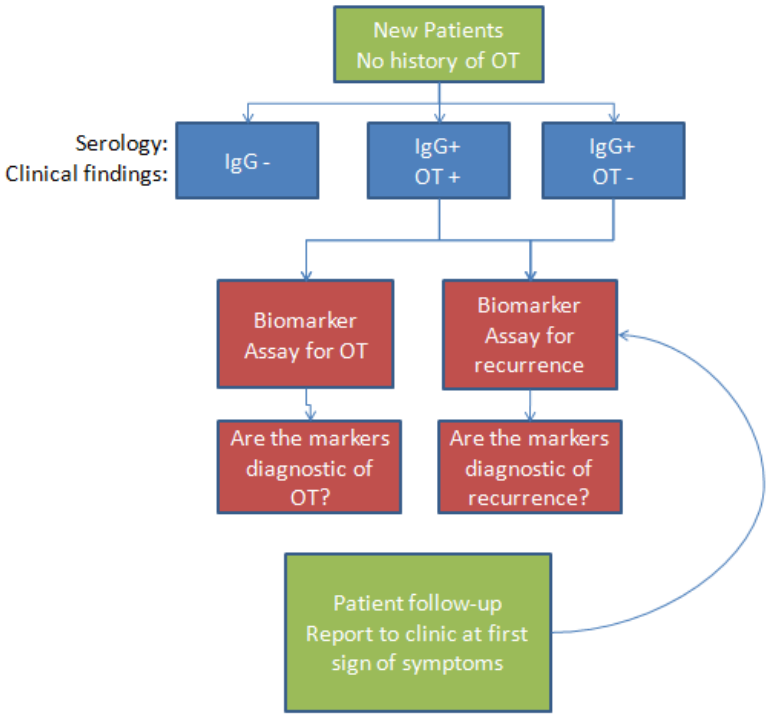
Appendix



The following is a proposal for a prospective randomized blind clinical validation of the biomarkers developed over the course of this pilot project. The goal of this study is to determine whether the biomarkers presented in this thesis are diagnostic of ocular toxoplasmosis and/or the recurrent disease. The study begins by recruiting patients who go to the clinic presenting with new symptoms of ocular toxoplasmosis. They will then undergo a full clinical work-up performed and serology taken. Those individuals who have no previous history of ocular toxoplasmosis, confirmed by IgG positive serology, will be included in the study. Excluded are those who have clinical signs of ocular toxoplasmosis but negative serology, or who have positive serology but atypical manifestations and clinical diagnosis. Aside from the routine serology and clinical assessment, serum obtained will also be used to evaluate the individuals' protein profile against a biomarker panel. Patient follow-up will be triggered only if the individual exhibits symptoms of recurrent diseases, which will consist of clinical evaluation, serology and a reassessment of the panel of biomarkers. Investigational bias will be avoided by blinding the participants of the study including the ophthalmologist performing the clinical assessment, the technician performing the serology and the researcher assigned to the biomarker panel who will not be sharing information.