

Your Title

Master Degree Project in Systems Biology

45 ECTS

1st version

Written Assignment

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# Background

## *Glaucoma*

Glaucoma categorizes a set of eye diseases which collectively represent the leading cause of irreversible blindness globally, affecting nearly 70 million individuals worldwide (Zhang et al., 2021). This category is primarily divided into open-angle and angle-closure subtypes, which are then further divided into primary or secondary disease types, where primary refers to variants which present with no discernible pathological cause resulting from other physiological conditions such as inflammation, trauma, etc. (Davis et al., 2016). To date, the only identified modifiable risk factor for glaucoma is measurement of intraocular pressure (IOP), which is believed to cause damage to the optic nerve. However this is convoluted by the fact that not all cases of high IOP result in damage to the optic nerve (Kass et al., 2002). While reduction of IOP is shown to be effective at slowing the progression of damage to retinal ganglion, the study by Kass et al. (2002) supports the hypothesis that some yet-undiscovered factor contributes to the progression of retinal nerve damage. Increasing evidence points to autoimmunity as a major contributor to this pathogenesis (Gramlich et al., 2013).

## *Autoimmunity*

Autoimmunity describes any immune response which reacts with a self-antigen, i.e., molecules produced as a normal component of the organism in which the response occurs (Delves, 1998). While this is a normal and essential regulatory component of the immune system, abnormalities in this regulatory system contribute to the pathogenesis of about 80 known diseases (Hayter & Cook, 2012). Several pathways have been proposed which may contribute to autoimmune dysfunction, including but not limited to self-reactive adaptive immune cell escape, inhibitory molecule deficiencies across adaptive cells, expression of sensors reactive to self nucleic-acids across innate cells, and disruptions to the microbiota ecosystem (Theofilopoulos et al., 2017).

The widely accepted clonal selection theory of acquired immunity informs that ordinarily, B and T lymphocytes which express antigen receptors specific to self are clonally deleted (Burnet, 1959). In certain circumstances, such as that seen in the syndrome ‘autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy’ (APECED), the impairment of the central deletion of autoreactive T cells can lead to the escape of adaptive immune components which subsequently causes systemic inflammation (Mathis & Benoist, 2009). However, numerous experiments have demonstrated that the mechanism by which self-reactive lymphocytes are deleted does not play an absolute role in preventing self-reactivity (Yu et al., 2015). For instance, Yu et al. (2015) demonstrated that CD8+ T cells are widely abundant in the blood of healthy adults, and other studies have found similar results for T cells reactive to endogenous antigens in mice (Bouneaud et al., 2000, Zehn & Bevan, 2006). Perhaps unsurprisingly, this is made possible in part by inhibitory molecules expressed on the surface of lymphocytes which help control against over-reactivity. A key example of this is the Cytotoxic T lymphocyte antigen-4 (CTLA-4) receptor, which was demonstrated in CTLA-4-/- mice to regulate peripheral T cell tolerance, with the knockout mice developing fatal autoimmune pathology within the first month of life (Paterson & Sharpe, 2010b).

While the adaptive system gives us a picture of a complex balancing act between components, the innate system provides perhaps an even more confounding myriad of interactions. These interactions are principally governed by a wide array of pattern-recognition receptors (PRRs), most notably toll-like receptors (TLRs), which respond to a variety of molecular stimuli (Blasius & Beutler, 2010). Receptors along the surface of cells such as TLR1, TLR2, TLR4, etc., are able to detect various pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPS) such as lipopeptides, peptidoglycan, etc., from various pathogens and self, while receptors expressed on the endosome detect nucleic acids shed by both self and invaders (Kumar et al., 2009), (Roh & Sohn, 2018). These receptors when activated induce production of pro-inflammatory cytokines and interferons, in turn activating the wider immune inflammatory response. Theofilopoulos et al (2017) suggest an autoimmune pathogenesis wherein self nucleic acids which are contained in neutrophil extracellular traps and microparticles from dead cells may gain access to the endolysosomal compartments of dendritic cells (DCs) and/or B cells, engaging TLRs and upregulating expression of MHC and the engagement of autoreactive T cells. This would lead to a feedback loop where autoantibodies are interacting with the DC receptor FcR then amplify and sustain the response. This activation by the innate immune system through an array of PRRs which may be erroneously activated by self DNA or RNA is thought to promote systemic autoimmunity as seen in many autoimmune diseases (e.g. Rheumatoid Arthritis, Sjögren’s syndrome, psoriasis, etc.) (Theofilopoulos et al., 2011).

Additionally, growing research points to the microbiota living on and within the body as having a critical regulatory role for the immune system (Honda & Littman, 2016). For example, experiments in mice and rats with strains of Clostridia have shown that the bacteria strongly induce Treg cells in the colon, preferentially enhancing the accumulation of those which express the RORγt receptor, and facilitate the expression of IL-10 and CTLA-4 (Atarashi et al., 2011). Introduction of 17 strands of these Clostridia also reduced the severity of graft-versus-host disease in mouse models (Mathewson et al., 2016). These sorts of regulatory processes demonstrate that the biota of healthy individuals maintains a homeostasis with the immune system, and so it follows that a bacterial dysbiosis may have a role in the pathogenesis of certain autoimmune diseases (Ruff & Kriegel, 2015).

Glaucoma shares several characteristics with the autoimmune disease rheumatoid arthritis (RA), and patients with glaucoma often also suffer from RA (Geyer & Levo, 2020). In particular, heat shock proteins (HSPs) have previously been demonstrated to be an apparent link between the microbiota regulation and the autoimmune pathology of RA (van Eden et al., 2019). Antibodies to HSP have been seen in both glaucoma patients and animal models (Joachim et al., 2007), and it has been observed that following transient elevation of IOP, expression of HSP is induced within retinal ganglion, which subsequently initiates HSP-specific T-cell infiltration into the retina and leads to aggressive glaucomatous neurodegeneration (Chen et al., 2018). It is unclear whether all or most types are due to an autoimmune pathology, but there is considerable evidence that at least a subtype of the disease may be autoimmune in nature (Wakefield & Wildner, 2020).

## *Biomarker Discovery*

By developing a profile of which proteins are implicated in the pathogenesis of a given autoimmune condition, a biomarker model may be constructed to enable detection of the condition and monitor response to treatments (Prince, 2005). Furthermore, understanding the targets of this renegade autoimmunity in certain diseases has allowed for the development of complex biologic therapies which can mediate response, often through the mimicry of human antibodies which target B- and T- cells within the immune system, shortening their lifespan or inhibiting function (Rosman et al., 2013).

In the case of glaucoma, in previous studies, several serum autoantibodies have been identified to be differentially regulated among glaucoma patients as compared to healthy counterparts. Notably, both upregulation of several antibodies such as against heat shock proteins (HSP27, HSP70), neuron specific enolase, glial fibrillary acidic protein (GFAP), etc, and selective downregulation (anti-GFAP, anti-14-3-3) have been observed in glaucoma patients (Gramlich et al., 2013). This offers compelling evidence that a biomarker profile may be developed for this condition to facilitate early detection and treatment.

A previous study utilizing a microarray method to profile 30 serum samples taken from Scandinavian glaucoma patients found five biomarkers (IRAK4, FUT2, PFKFB1, VAV2, GPATCH8) which may also be of particular interest in developing a methodology for early detection of glaucoma onset (Khan, 2019). In particular, Khan (2009) principally suggests that the IRAK4 molecule serves a proinflammatory function and the observed absence of autoantibodies in diseased patients and not in controls may be an effective identifier of the autoimmune dysfunction contributing to this condition. This observation is corroborated by the observation that IRAK4, among other members of the IL-1 receptor-associated kinase family, is upregulated in other autoimmune conditions (D. Y. Chen et al., 2013). Khan also presents several hypotheses of function for the other mentioned antibodies, which were found almost exclusively in the cohort of diseased patients.

Expanding upon the work done by Khan, this study will expand upon these findings through the analysis of 90 serum samples taken from 30 patients with glaucoma and 30 anonymous blood donors, with some samples randomly duplicated.

## *Machine learning*

The search for useful biomarkers and modelling of biological systems is one of many areas where the utility of employing machine learning techniques has been increasingly demonstrated, providing an often-improved approach to interpretation, classification, and validation of biological data (Larrañaga et al., 2006). Of particular interest, artificial neural networks, which create a multilayered approach to the classification of data, have shown remarkable predictive capability in a myriad of different applications related to biological data (Tang et al., 2019). Based on the success compared to state-of-the-art statistical models of neural networks like DeepChrome, designed by Singh et al. (2016), which uses a convolutional neural network (CNN) not unlike those used in image-recognition software in order to classify gene expression data based on histone modification data. These types of tools will likely be foundational in the development of more complex biomarker modelling.

Deep learning algorithms employ a multi-layered abstraction where all input nodes are passed to intermediate layers which take as input weighted values from the preceding layer and output a single value, passed on to subsequent layers (Graves et al., 2020). These intermediate layers discover non-linear relationships between the inputs and outputs and pass along these higher-level features to subsequent nodes. A characteristic trait of such models is that typically they demand a large quantity of input data, however some success has been shown in smaller-scale studies using relatively simple neural networks to analyze serum biomarkers to predict cancers (Flores et al., 2013), (Li et al., 2017). Additionally, a 2019 study attempted to classify cases of primary open angle glaucoma using artificial neural networks against serological proteome analysis data (Beutgen et al., 2019). In this study, Beutgen et al. (2019) discovered CALD1, PGAM1, and VDAC2 as new biomarker candidates and were able to classify subjects with a sensitivity of 81% and specificity of 93% using these three new biomarkers in conjunction with previously identified biomarkers VIM and HSPD1.

# Aim

The aim of this study is to identify proteins which are differentially expressed in glaucoma patients compared with the healthy cohort in order to identify possible diagnostic biomarkers. Secondarily, the study will examine the viability and predictive power of employing deep learning as compared with more traditional statistical methods for classification and validation on protein expression datasets.

# Methods

This study is based on an autoimmunity profiling test conducted by SciLifeLab. This test conducted analysis on 258 antigens and 4 controls using a suspension bead array with COOH-NH2 chemistry. Quality control steps were also conducted by SciLifeLab using R.

Using the data from this trial, analysis will primarily be conducted using R version 4.1.2. Differentially expressed antibodies between healthy and diseased cohorts will be selected based on two-sample t-test for means and corroborated using Fisher’s test due to the medium to small sample size. To minimize false-discovery, correction with Benjamini-Hochberg procedure will be used.

Following this basic data preparation, gene set enrichment analysis and visualization with Cytoscape will be conducted in order to explore the pathways implicated by the differentially expressed genes. Multiple supervised classification methods, including linear discriminant analysis, logistic regression, and support-vector machine will be separately tested and validated using leave-one-out cross-validation.

Finally, a novel neural network will be constructed using Python version 3.1 in order to classify the dataset for the purpose of comparing predictive accuracy against the above methods.

# Ethical aspects

Data used in this study was supplied and processed absent of personally identifying information. The burden that blindness from glaucoma puts on the individual, family, and society at large demonstrates clear necessity for methods which can slow or halt the progression of this illness. The development of biomarkers which can reliably classify those at risk of developing glaucoma is essential to early diagnosis, or even prevention. What’s more, further construction of advanced models of immune function, particularly autoimmune function as it pertains to disorders like this, may be paramount to the eventual relief of countless autoimmune disorders and diseases. Use of artificial neural networks for these models often enables detection of patterns at much higher levels of abstraction than conventional statistical methods, which will be helpful and perhaps necessary in reliably modelling such complex systems as immune function.

# Time plan

|  |  |  |  |
| --- | --- | --- | --- |
| **Task/activity** | **Start date** | **End date** | **Duration (weeks)** |
| **Written assignment** | **22 Nov 21** | **06 Jan 22** | **6** |
| Subtask 1: Literature studies | | | |
| Subtask 2: Planning of experiments | | | |
| Subtask 3: Writing the written assignment | | | |
| ***Date for submission of written assignment to examiner: 06 January 2022*** | | | |
| **Laboratory assignment** | **07 Jan 22** | **20 Apr 22** | **16** |
| Subtask 1: T-Test, Fisher’s test, plotting, BH correction | | | |
| Subtask 2: Gene set enrichment analysis, cytoscape analysis | | | |
| Subtask 3: Classification methods (LDA,LR), validation | | | |
| ***Date for half-time seminar: 13 February 2022*** | | | |
| Subtask 4: Subtask 4: Construct, train, test artificial neural network | | | |
| **Essay** | **01 May 22** | **30 May 22** | **4** |
| Subtask 1: Writing of report | | | |
| Subtask 2: Correcting comments from the supervisor | | | |
| ***Date for submission of thesis report to the examiner: 30 May 2022*** | | | |
| **Presentation** |  |  | **1** |
| Subtask 1: Preparation of presentation and poster | | | |
| Subtask 2: Preparation of opposition | | | |
| ***1Preliminary date for oral presentation/opposition: 10 June 2022*** | | | |

1As thesis presentations are only scheduled a few times each semester you can only give a preliminary date based on when you are planning to be ready for oral presentation.

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