Immuno-Epidemiology of *Helicobacter pylori*-Associated Autoimmune Conditions in Zimbabwean Patients

By

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DECLARATION

I Mazvita Chakawa declare that this thesis is my original work that I compiled. This thesis contains no material that has been submitted previously, in whole or in part, for the reward of any other academic degree or diploma by any other person. Except where otherwise indicated, this thesis is my own work. The work described in this thesis was done at the Asthma, Allergy and Immune Dysfunction Clinic and at the University of Zimbabwe. External Sources and facilities used during the period of the project have been acknowledged in the document.

Student: Mazvita Chakawa

Signature _____ Date _____

I am satisfied that this is the original work of the author in whose name it is being presented.

I confirm that the work has been completed satisfactorily for presentation in the examination.

Principal supervisor:

Signature_____ Date_____

TABLE OF CONTENTS

ABSTRACT

Background; The bacterium *Helicobacter pylori* (*H. pylori*) colonizes the human gastric mucosa and is a recognized causative agent for peptic ulcer disease. The prevalence of *H. pylori* infection in Zimbabwe is unknown. Treatment requires combination therapies which may predispose to antibiotic resistance. The pathogen is also implicated in development of autoimmune endocrinopathies such as autoimmune thyroiditis, Systemic lupus erythematosus and rheumatoid arthritis. While the prevalence of autoimmune diseases has been increasing in Zimbabwe, there are no studies investigating whether any association exists between *H. pylori* infection and any autoimmune disease. Effective treatment regimens to prevent the development of autoimmunity on *H. pylori* infection are unavailable. It is important to understand the immuno-epidemiology of autoimmunity and *H. pylori* so as to understand the immune system dynamics that occur during the disease. The aim of the present study was to determine the burden of *H. pylori*-associated autoimmune conditions and to characterize the cytokines in the affected patients.

Methods: Clinical and laboratory records of 1500 patients were reviewed and an analytical cross-sectional study on 385 samples was done. Archived samples of patients that warranted an *H. pylori* and/or autoimmunity test were used for detection of autoantibodies using immunoblot assays. Detection of IgG anti-*H. pylori* antibodies was done using rapid kits and IgA using ELISA. Flow cytometry was used for detection of cytokines. Ten samples were used in a prospective study where whole blood was stimulated with *E. coli* antigen, *H. pylori* antigen and pokeweed mitogen. Cytokines expressed were determined using ELISA.

Results& Discussion: The prevalence of co-existing autoimmune autoantibodies and *H. pylori* infection was 18%. The association of *H. pylori* infection and expression of autoantibodies was greater among systemic sclerosis patients, SLE and myositis, respectively. The auto antibodies associated with *H. pylori* infection are anti-RP155, anti-PM-Scl-100, anti-Th/To, anti- Ku, anti-dsDNA and anti-PCNA. Of these anti-Th/To and anti-Ku are only found in women. High levels of pro-inflammatory cytokine TNF-α and low INF-γ are associated with *H. pylori* inflammation while IL-17A was associated with autoimmune inflammation in the patients studied. The immune response to chronic *H. pylori* infection in the gastric mucosa possibly drives towards autoimmune inflammation. Infection by *H. pylori* potentially suppresses specific immune cell populations.

Conclusion: We conclude that there is an association between *H. pylori* infection and the expression of auto antibodies in Zimbabwean patients. Immune responses to *H. pylori* infection in the gastrointestinal tract may potentially drive toward autoimmune inflammation. We recommend investigation of molecular mimicry and B cell compartment studies to further elucidate this association.

Key words: *H. pylori*, infection, autoimmunity, Zimbabwe

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LIST OF ABREVIATIONS

ANA	Antinuclear antigens
APC	Antigen presenting cell
CagA	Cytotoxin-associated gene A
Vac A	Vacuolating cytotoxin A
CD	Cluster of differentiation
E. coli	Escherichia coli
ELISA	Enzyme linked immunosorbent assay
H. pylori	*Helicobacter pylori*
IL	Interleukin
INF-γ	Interferon gamma
PWM	Pokeweed mitogen
RPMI	Roswell Park Memorial Institution
Scl	Sclerosis
SLE	Systemic lupus erythematosus
SPSS	Statistical package for social sciences
STATA	Statistics and data (syllabic abbreviation)
Th1	T helper 1
Th2	T helper 2
TNF-α	Tumor necrosis factor alpha

LIST OF FIGURES

LIST OF TABLES

1.1 INTRODUCTION

1.1.1 *Helicobacter pylori*

The bacterium *Helicobacter pylori* is the main species of the genus *Helicobacter*. It is a universal species that is gram-negative, spiral, and microaerophilic which inhabits the gastric mucosa of the human stomach. The pathogen has been associated with common gastric diseases, especially with chronic and active gastritis and peptic ulcer disease (Marshall and Warren, 1984). In some cases, *H. pylori* has been associated with gastric cancer. The bacterium was first described by Marshall and Warren (1984) and its public health significance was recognized in 2005.The pathogen *H. pylori* colonizes the gastric mucosa by adhering to mucosal cells and the mucus layer lining the gastric epithelium. More than 50% of the world's population is chronically infected by the pathogen (Astl and Sterz,2015).

H. pylori is one of the most genetically diverse bacterial species, and is a major cause of at least 90%of duodenal ulcers (DUs), 70% of gastric ulcers (GUs), non-ulcer dyspepsia (NUD) and gastro-esophageal reflux disease (GORD) (Henriksen, 2001). In many societies, *H. pylori* plays a role in, mucosa associated lymphoid tissue lymphoma (MALT) and primary gastric non-Hodgkin's lymphoma (Salama *et al.,* 2013).

The bacterium's ability to adhere, invade and evade host defenses also causing tissue damage is predominantly due to its production of virulence factors (Tanih *et al.*,2010). The most important virulence factors related with the gastric pathological process are Cytotoxin-associated gene A (CagA) protein and the Vacuolating cytotoxin A (VacA) (Salama *et al.* 2013). Genes encoding the CagA virulence factors of *H. pylori* are grouped in the Cag Pathogenicity Island (CagPAI) region and some of them encode a type IV bacterial secretion protein. This protein translocates CagA into host cells in gastric mucosa. The CagA protein stimulates cell signaling through the interaction with several host proteins. This interaction leads to an increased release of cytokines, especially interleukin 1, interleukin 6, interleukin 10 and tumor necrosis factor α and regulatory molecule production (Parsonnet *et al.,* 1994). VacA is also an important virulence factor as it causes vacuolation. There are differences among *H. pylori* strains in the structure of VacA. Two types of

signal regions are known- s1 and s2. There are also two types of mid-regions known – m1 and m2. Strains bearing

the VacA s1m1 gene showed higher degrees of gastric colonization (Parsonnet *et al.,* 1991, Radosz-Komoniewska *et al.,* 2005).

Survival of *H. pylori* bacteria in the hostile environment of the stomach is facilitated by many factors. These include firm adherence of *H. pylori* to the gastric epithelium, production of urease, which converts acidic urea into ammonia and carbon dioxide so that it overcomes the acidic gastric environment in the stomach (Suerbaum and Michetti, 2002). The pathogen also evades the host immune-mediated responsiveness. Survival is also enhanced by the slow biological activity of *H. pylori's* lipopolysaccharide (LPS). There is appreciable evidence to support the idea that structural modifications of the *H. pylori* LPS influences the biological activity of the whole *H. pylori* molecule (Jirillo *et al.,* 1999).

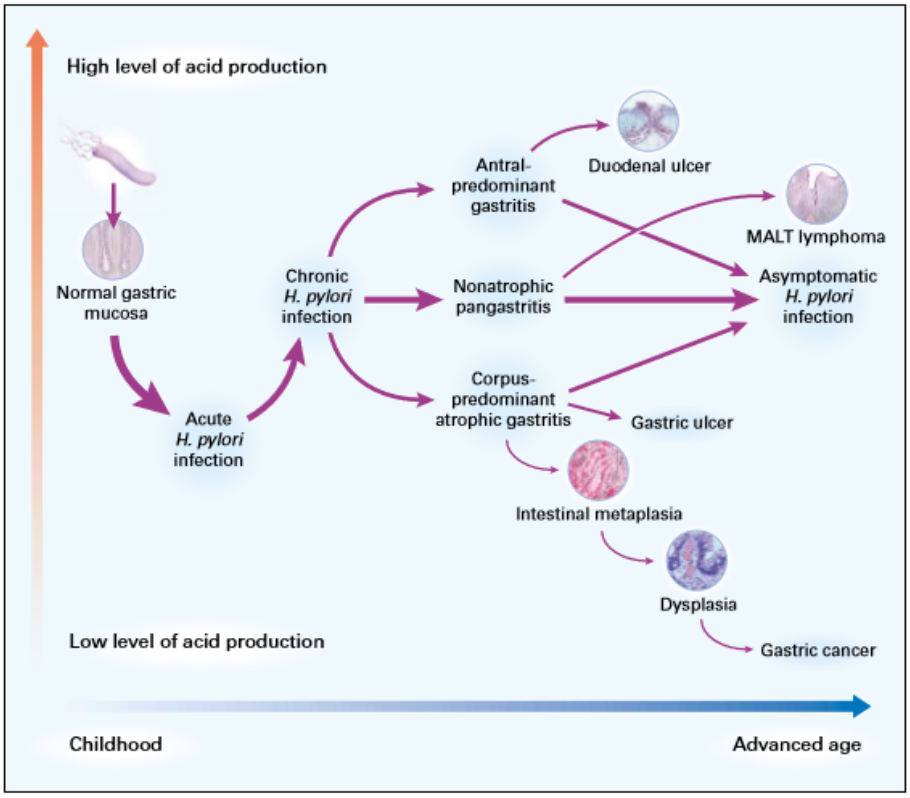

Figure 1: Development of disease from *H. pylori* infection from childhood to adulthood (Suerbaum and Michetti, 2002).

As shown in Figure 1, initial infection occurs during childhood after oral ingestion and the pathogen persists for the life of the host unless treated (Blaser, 1997; Everhart, 2000). Acute *H. pylori* infection may cause short-lived hypo-chlorhydria which is seldom diagnosed. In persistently infected individuals, chronic gastritis develops although 80% will be asymptomatic (Suerbaum and Michetti, 2002). The advanced clinical course is variable and depends on both bacterial and host immunity factors. Patients with a generally higher acid production are potentially likely to develop antral predominant gastritis, which may predispose them to duodenal ulcers. Those who have lower acid production are more likely to develop gastritis in the stomach, which may predispose them to gastric ulcer. In rare instances, this may lead to development of gastric carcinoma. The *H. pylori* infection may cause development of mucosa-associated lymphoid tissue (MALT) in the gut. Another rare complication of *H. pylori* infection as a result of MALT is malignant lymphoma (Suerbaum and Michetti, 2002).

Spontaneous remission in childhood is relatively common, but it is usually associated with the use of antibiotics to treat unrelated illnesses (Tindberg *et al.*, 1999). Spontaneous eradication of *H. pylori* rarely occurs (Xia and Talley, 1997). The most likely mode of transmission is person to person transmission via contact with infected secretions. recent studies suggest that contaminated water and poor hygiene may be another source of transmission in low income countries (Hansi *et al.*,2011). Humans can also become infected with *Helicobacter heilmannii*, a spiral bacterium found in dogs, cats, pigs, and nonhuman primates (Solnick and Schauer, 2001).

1.1.2 Autoimmunity

An autoimmune disease arises from an abnormal immune response to a normal body organ. Autoimmune diseases are a vast array of organ-specific as well as systemic diseases, whose pathogenesis derives from a sustained and persistent immune response against self-components. The pathogenesis in autoimmunity stems from the activation of bursa-derived (B) and thymus-derived (T) lymphocytes reacting against antigens of the body's own tissues. In healthy individuals this onslaught is prevented by self-tolerance mechanisms. 'Tolerance' is the acquired ability of the immune system to avoid reactivity to self-antigens. Tolerance is achieved by the conjunctive efforts of central and peripheral mechanisms. These allow rapid and efficient removal of pathogens such as protozoa, bacteria or viruses in the absence of self-recognition. Once in a while, autoreactive cells may be activated, probably because of molecular mimicry between structures of

the invading pathogen and the self-antigens. However, these autoreactive immune responses are rapidly controlled by several immune-regulatory mechanisms.

Central tolerance happens during lymphocyte development in the thymus and bone marrow. During this process, T and B lymphocytes that recognize self-constituents are deleted before they develop into mature immunocompetent cells, preventing autoimmunity. This process is virtually active in fetal life, but continues throughout life as immature lymphocytes are produced. Positive selection occurs first when naive T-cells are exposed to antigens in the thymus. T-cells which have receptors with sufficient affinity for self-MHC molecules are selected. Other cells that do not show sufficient affinity to self-antigens will undergo a deletion process known as death by neglect which involves apoptosis of the cells. The positive selection is a definitive example of the importance of some degree of auto responsiveness.

Central-tolerance mechanisms are efficient. However, they cannot eradicate all self-reactive lymphocytes, partly because not all self-antigens are expressed in the thymus during primary lymphocyte development. Not all genes are expressed in the thymus so developing T cells cannot be exposed to all self-antigens. An additional mechanism for eradication of mature T lymphocytes is required. Such a mechanism is called peripheral-tolerance. Peripheral tolerance is crucial to control tolerance of lymphocytes that first encounter their related self-antigens outside of the thymus for example such as in the case of antigens presented during chronic infection, food allergens and developmental antigens. Mechanisms of peripheral B cell self-tolerance are also essential because after stimulation with an antigen B cells expand and undergo somatic mutation, begetting a population of B cells with new antigen specificities. Some of these cells may be responsive to self-antigens.

In some cases, T cells are not deleted but become specifically unresponsive to antigen stimulation therefore do not proliferate. This phenomenon is called clonal anergy. After the antigen is removed, these anergic cells recover their responsiveness over time. One of the molecular mechanisms responsible for inducing anergy is signaling via CTLA4, a molecule expressed by activated T cells.

Breakdown of self- tolerance results in the pathogenesis of autoimmunity. Several mechanisms for the tolerance breakdown have been proposed. These include genetic inability to delete all autoreactive T- and B-cell clones during ontogenesis, molecular mimicry where an immune

response initially targeted against a foreign antigen will also attack self -antigens due to sequence or structural similarities between epitopes. These similarities may occur randomly or due to phylogenetic preservations during evolution (Goodnow, 2007).

The cause for the breakdown of self-tolerance which then leads to autoimmunity is not yet clearly understood however genetic and environmental factors may influence susceptibility to autoimmunity. Much of the research documenting genetic risk factors associated with autoimmune disorders have focused on the MHC genes, commonly known as human leukocyte antigen (HLA) genes. Different HLA gene subtypes have been shown to be associated with an increased risk for several autoimmune disorders. Most autoimmune disorders are related to a particular class I or II HLA molecule (Klein, 2000).

The interaction of genes can also influence the risk for autoimmune disorders. Researchers have shown that people who have the HLA genetic marker called DQ2 in conjunction with a gene for a certain subtype of immunoglobulin have a much-increased risk for autoimmune-mediated myositis. Myositis is a muscle destroying condition with many causes (Miller, 1995). It has also been shown that HLA DR2 is positively associated with Systemic Lupus Erythematosus (SLE), multiple sclerosis and narcolepsy, while it is inversely related to diabetes mellitustype 1. The involvement of genetic factors is strengthened by the affirmation that, nonbiological siblings such as adopted siblings, do not have a greater risk of developing an autoimmune disease compared to genetically related siblings such as identical twins. In monozygotic twins, inheritability is accounted for by about 30 % genes. Generally, women are more susceptible than men. This is because sex hormones and genes linked to the X and Y chromosomes come into play.

In most cases, autoimmune diseases are multigenic. The abnormal phenotype is produced by many susceptibility genes working together. Generally, polymorphisms occur in normal individuals but exist harmoniously with the normal immune function. Autoimmunity arises when the polymorphisms occur with other susceptibility genes (Encinas and Kucgroo, 2000). Some of the susceptible genes confer a greater risk of autoimmunity than others. Examples of such genes are those for the major histocompatibility complex (MHC), which contribute to disease susceptibility.

Genetic predisposition for autoimmune diseases can, in most of the cases, only explain a part of the disease risk, supporting the view that the increasing prevalence triggered in addition by environmental factors. Studies done by the Federation MSI in 2008 have shown a higher

prevalence of multiple sclerosis especially in developed countries (Fearnhead, 2004). Several hypotheses been proffered to justify these observations. The main source of Vitamin D is exposure to sunlight. Vitamin D generation is decreased in regions of lower sunlight exposure such as Northern Europe such that patients with multiple sclerosis were found to have lower blood levels of vitamin D (Ascherio *et al.,* 2014). In other autoimmune diseases, vitamin D deficiency may be a risk factor. The effect of vitamin D in autoimmunity suggests an immunomodulatory capacity with anti-inflammatory action (Schleithoff *et al.,* 2006).

Moreover, the hygiene hypothesis states that people who have never been exposed to certain infections early in life but growing up in high hygiene standards may develop a hyperactive immune system which in turn may favour the occurrence of autoimmune diseases (Fleming, 2007). According to the hygiene hypothesis, some pathogens may be protective in developing autoimmunity. However, others may increase the risk. The Epstein–Barr virus (EBV) is associated with multiple sclerosis especially when infection takes place during late adolescence. A potential explanation for this observation might be the resemblance of the EBV nuclear antigen (EBNA)-1 to the myelin surface protein in the brain. Commensal gut bacteria among other physiological functions, such as providing fermentation products for food digestion, compete with potential infectious agents (Louis and O'Byrne, 2010).

Microbiota is essential for effective functioning of the human immune system. Changes in the gut microflora have been observed in diseases such as intestinal bowl disease allergies (Thorburn *et al.,*2015). These changes may be induced by factors such as medication such as corticosteroids, diet or stress. This can result into a dysbiosis. The usual consequence of a dysbiosis is the altercation of the mucosal immune system leading to gut inflammation and alterations of the intestinal immunity. A study done by Cani *et al.,*2008) showed that a dysbiotic microbiome could result in T regulatory lymphocyte (Treg) deficiency and a stimulation of Th17 proinflammatory cells which in turn produce pro-inflammatory cytokines and cross reactivity of adaptive immune cells.

Hormones have a possible role in the evolution of autoimmunity. Most autoimmune diseases are more prevalent in women than men. It is estimated that nearly 70% of individuals with autoimmune diseases are women except diabetes mellitus, ankylosing spondylitis and inflammatory heart disease which frequently occur in men. The source of external hormones can

be drugs such as birth control pills, food such as soy or skin products. Sex hormones both natural and synthetic may directly be associated with autoimmunity (Jorg *et al.*,2016). Figure 2 below represents the interplay of genes and environmental factors in the development of autoimmunity.

Figure 2: The contribution of genes and environmental factors in the progression from autoimmunity to autoimmune disease adapted from DeLisa Fairweather and Rose, 2007.

Autoimmune responses are generated in the process of mounting an immune response to pathogens, but autoimmune disease results if the autoimmunity response persists and is poorly regulated (DeLisa Fairweather and Rose, 2007). Autoimmune diseases can be organ specific or systemic. Some of the most common organ specific autoimmune diseases are diabetes mellitus and Graves's diseases where the thyroid is affected and is characterised by hyperthyroidism. Other organ specific diseases are Hashimoto's thyroiditis, Myasthenia gravis among many others. Common systemic autoimmune diseases are Rheumatoid arthritis, where joints are affected, Systemic lupus erythematosus where DNA, erythrocytes, nuclear proteins, cytoplasmic proteins and platelet membranes are affected. Multiple sclerosis and systemic sclerosis are some of the common systemic autoimmune diseases.

1.1.2.1 Organ specific autoimmune diseases

Hashimoto's thyroiditis

In Hashimoto's thyroiditis, antibodies elaborated by the humoral response target the thyroid gland causing autoimmune damage which results in decreased thyroid function and hypothyroidism. It is characterized by the presence of circulating antibodies against thyroid peroxidase, thyroglobulin microsomal proteins and other constituents of thyroid cells. The condition is also characterised by

a mononuclear lymphocyte infiltrate within the thyroid gland. The aetiology of this disease is generally unknown, triggers may include initial damage to the organ from trauma or a viral infection. Associated to susceptibility to the disease are HLA linked genes.

Grave's disease

This autoimmune thyroid condition is characterized by hyperthyroidism. Auto-antibodies produced target the receptor for Thyroid Stimulating Hormone (TSH) which is expressed on thyroid follicular cells. The antibodies stimulate the chronic overproduction of thyroid hormones. The auto-antibodies act as agonist for their target molecules thus stimulating TSH activity. Grave's disease is usually treated by surgery which aims to reduce thyroid function or use of radioactive iodine.

1.1.2.2 Systemic autoimmune diseases

Systemic sclerosis

Systemic sclerosis also known as scleroderma is a connective-tissue disease of unknown pathogenesis that affects 30- to 50-year-old women four times as often as it affects men (Foy *et al.*, 1996). This type of sclerosis is characterized by overproduction of collagen, which leads to fibrosis of visceral organs. The overproduction of collagen results from an autoimmune dysfunction, in which the immune system attacks the kinetochore of the chromosomes. This leads to genetic malformation of nearby genes. Organs affected by these changes are predominantly the skin, however internal organs such as the respiratory and gastrointestinal tracts can be involved in scleroderma. Many patients with systemic sclerosis do not have significant symptoms despite abnormalities of internal organ function. Thinning of the lips and reduced oral apparatus are frequent. Furthermore, tightening of the perioral skin with restricted ability to open the mouth. Atrophy of the mucous membrane and tongue papilla with impaired taste perception has been reported and may contribute to malnutrition. The esophagus is the most common affected gastrointestinal site.

Rheumatoid arthritis

Rheumatoid arthritis is an autoimmune disease of unknown pathogenesis that affects 1% of the population. Three women in 4 people of ages between 20 and 50 are usually affected (Beratsky *et*

al., 2006). The classic clinical presentation is chronic symmetric polyarthritis. In rheumatoid arthritis, autoantibodies are directed against the Fc fragment of IgG antibodies among other host constituents such as nuclear proteins. The anti-IgG autoantibodies are called rheumatoid factor, and are mostly IgM. The resultant immune complexes are deposited in various sites within the vasculature and joints. In these locations they can cause tissue damage through the fixation of complement and the attraction of poly mononuclear cells which result in synovitis and vasculitis which are characteristic of the disease. In rheumatoid arthritis, the oesophagus may show decreased distal peristalsis, hiatal hernia and inflammatory synovitis. Gastrointestinal manifestations are common, Peptic ulcer disease in RA is probably the result of pharmacologic therapy with non-steroidal anti-inflammatory drugs (Cojocaru *et al.,* 2011).

Polymyositis

Polymyositis and dermatomyositis are a group of systemic autoimmune diseases characterized by inflammation of striated, skeletal and smooth muscle. Polymyositis patients initially present with progressive weakness of proximal striated muscles. Polymyositis is also characterized by chronic muscle inflammation and weakness involving skeletal muscles. Women are affected more often than men during childhood and middle adulthood although most cases are observed in adults between 35 and 60 years. A characteristic skin rash accompanies dermatomyositis. The gastrointestinal tract may be affected throughout its entire length, but the proximal oesophagus is mostly involved. Patients may have difficulty in swallowing and nasal regurgitation, reduced gastrointestinal motility, abnormal gastrointestinal peristalsis and bloating. Diagnosis of polymyositis can be made using magnetic resonance imaging and often requires a muscle biopsy.

Systemic Lupus erythematosus

Systemic lupus erythematosus (SLE) is an autoimmune disease of unknown cause, characterized by the presence of autoantibodies to a wide variety of self-antigens such as ribonucleic acid (RNA), histones, de-oxyribonucleic acid (DNA), ribonucleoproteins, proliferating cell nuclear antigens (PCNA) and other cellular elements. These auto-antibodies damage a wide variety of tissues and organs including skin, causing lesions known as erythema. The auto-antibodies also damage vocal cords which result in a characteristic hoarseness. The presentation of the disease is usually systemic and includes weight loss, fever, fatigue and malaise. The disease preponderantly affects women aged between 25 and 55 years than men in the ratio 9:1 respectively. Gastrointestinal

manifestations and symptoms are common in patients with SLE and can be due to primary gastrointestinal conditions, complications of therapy or the disease itself alone.

1.1.3 Relationship between *H. pylori* and autoimmunity

A number of pathogens have been implicated as possible environmental agents contributing to the development of autoimmune disease in genetically susceptible individuals. The pathogen *H. pylori* is one such microorganism that has been implicated in the development of autoimmunity. Possible mechanisms involved in the relation of *H. pylori* and autoimmunity are epitope spreading, polyclonal lymphocyte activation which may lead to generation of high levels of pro-inflammatory cytokines, molecular mimicry, bystander activation, and activation by super-antigens. In a review by (Getts and Miller, 2010), the role of infectious agents in autoimmunity suggested that autoimmune disease is triggered by these mechanisms working 'simultaneously and/or sequentially. 'There is evidence for the role of infectious agents in diseases such as rheumatic fever and Guillain-Barre syndrome is convincing (Bach, 2005).

The bacterium *H. pylori* may alter cellular immunity leading to loss of self-tolerance and autoimmune destruction of melanocytes. This autoimmune reaction is as a result of elevated levels of C-reactive protein, high levels of tumor necrosis factor- α, production of auto-antibodies, chronic inflammatory condition and antigenic stimulation (Gasbarrini *et al.*, 1997). Local inflammation of gastric mucosa due to *H. pylori* infection results in the release of inflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL) 17A, IL-8, IL-23 and IL-1β that produce systemic inflammation. This inflammation subsequently affects skin microenvironment with loss of functional melanocytes (Moss and Calm, 1994).

Although *H. pylori* infects the stomach, a variety of systemic inflammatory effects have been observed. These among others include a higher number of leucocytes, lymphocytes and basophils (Kattunen *et al.,* 1996). Various cardiovascular, skin, blood, endocrine and rheumatic disorders have been reported to be linked with *H. pylori* infection. However, in most of these reports, the information has been inconsistent (Leontiadis *et al.*, 1999). In a study done by Showji *et al.*, in 1996, the association between *H. pylori* and SLE was not proven. African-American patients with SLE were less likely to react against *H. pylori* antigens compared to controls. The study indicated

a significantly lower prevalence of *H. pylori* infection in African-Americans with SLE and no association observed in European-Americans or Hispanics. However, subgroup analysis unveiled that the association was present in African-American women. No difference was noted between the African-American male patients with SLE and their age-matched controls (Showji *et al.*, 1996).

The pathogen *H. pylori* has been implicated in development of autoimmune endocrinopathies such as autoimmune thyroiditis (AT). Induction of immune-suppression activates gastric and extra-gastric diseases such as gastric ulcers, gastritis and some gastric malignancies. The pathogen causes persistent lifelong infection despite local and systemic immune response (Mendall *et al.*, 1996). Chronic inflammation due to infection by *H. pylori* can lead to gastric autoimmune reactivity by a mechanism called molecular mimicry. This involves cross-reactivity between antigens expressed both on *H. pylori* and on gastric parietal cells in proton pump, H+, K+-ATPase Besides gastric disorders, the *H. pylori* etiology is implicated in connection with the development of different extra gastric diseases such as vascular, skin and autoimmune diseases such as autoimmune thyroiditis (Bergman *et al.*, 2005).

In a study done by Yanaoka *et al.*, 2009 patients with autoimmune thyropathies, mainly with autoimmune thyroiditis, Graves' thyrotoxicosis and Hashimoto's thyroiditis, had an increased prevalence of *H. pylori* infection. In support of the observation, serological tests revealed elevated levels of anti-*H. pylori* IgG antibodies. Patients suffering from autoimmune thyroiditis and *H. pylori* infection also had abnormalities in the secreting function of the stomach where high levels of pepsinogen I and II and gastrin were found.

The present study investigates possible association of *Helicobacter pylori* and autoimmunity in Zimbabwean patients. The possible association has not been documented and epidemiological studies have not been conducted. The project aims to interrogate the immune-epidemiology of *H. pylori* associated autoimmunity in Zimbabwean patients.

1.2 LITERATURE REVIEW

1.2.1 The epidemiology of *H. pylori* infection

1.2.1.2 Global overview of *H. pylori* infection

The bacterium *Helicobacter pylori* (*H. pylori*) is a gram-negative microaerophilic pathogen that infects the gastric mucosa and epithelial lining in the stomach. Infection by *H. pylori* was once debilitating but now can be dependably cured with antibiotics. However, there are intensifying concerns due to increasing antibiotic resistance. In most countries, the prevalence of *H. pylori* infection has decreased in relation with improved hygiene and general standards of living. On the contrary, the prevalence of *H. pylori* remains ubiquitous (Graham, 2014). The bacterium is the main cause of peptic ulcer disease, chronic gastritis and the chief known etiological agent for gastric cancer. In most cases, the major mechanism of transmission is intra-familial. The infection is acquired by oral ingestion of the bacterium and is mainly transmitted within families in early childhood before developing to a chronic disease in adulthood. The continuous rise in the prevalence of *H. pylori* with age is largely due to a cohort effect, reflecting intense transmission during the time when members of earlier birth cohorts were children. Although very rare, spontaneous eradication of the bacterium may occur in childhood. The prevalence of *H. pylori* remains high in many low-income countries and is generally associated with the socio-economic status and levels of hygiene in these countries or regions within them. Global and regional prevalence of *H. pylori* had not been systematically reported until 2015 (Yokota *et al.,* 2015).

Although infection by *H. pylori* occurs worldwide, the prevalence varies immensely among countries and among regions within the same country. Prevalence also varies within population groups within the same region in a country. The prevalence among middle-aged adults is above 75 percent in most low-income countries, as compared with 30-55 percent in industrialized countries. It has been proposed that in industrialized countries, *H. pylori* is predominantly directly transmitted from person to person by feces, vomitus and saliva. In low income countries, other transmission routes include consumption of contaminated water (Feldman, 2001). Currently there is no documented evidence for zoonotic transmission, although the bacterium can be found in primates and occasionally in other animals (Handt *et al.*,1994).

Infection by *H. pylori* continues to be a public health issue of concern worldwide. A global systematic review done by Hooi *et al.,* revealed that in 2015, about 4.3 billion people in the world were approximated to be positive for *H. pylori* infection. This review was established on population-based studies on the prevalence of *H. pylori* infection in different countries around the world and at different time periods ranging from January 1970 to January 2016. So far, this study is the most conclusive and broad up-to-date systematic review of the worldwide prevalence of *H. pylori* infection. A broad fluctuation in the prevalence of infection between countries and regions within a country was affirmed. Africa was found to have the highest prevalence of *H. pylori* infection which is 80%. Second and third highest prevalence was found to be Latin America (63.4%), and Asia (55%) respectively. The lowest prevalence of *H. pylori* infection is in Northern America (37.2%) and in Oceania with 24.2% (Hooi *et al.*,2017).

It has been noted that there can be significant differences in the prevalence of *H. pylori* within the same country. Different racial communities in the United States have varying *H. pylori* prevalence. It was shown that the prevalence of infection among non-Hispanic whites ranges from 18% to 24.2% while that in non-whites ranges from 36% to 65% (Everhat *et al.*,2000). In industrialized nations such as Denmark, Australia, Norway and Switzerland, the rate of acquisition of *H. pylori* has decreased considerably over the most recent decades. A possible explanation of this could be the improvement in hygiene and administration of effective antibiotics.

Worldwide, recent epidemiological studies suggest that the different prevalence of *H. pylori* is as a result of various levels of sanitization, socio-economic status, access to clean and safe water as well as high level of urbanization. This differential gap in prevalence of infection has significant implications on the prevalence of diseases associated with *H. pylori* such as gastritis, gastric cancer and peptic ulcer disease. As shown in Figure 3, which represents the global prevalence of *H. pylori*, the highest prevalence was found in certain regions in Russia, Asia and Africa as well as in Latin America. Most parts of Arica have unknown prevalences as few studies have been conducted. Low prevalence was reported in North America and Australasia.

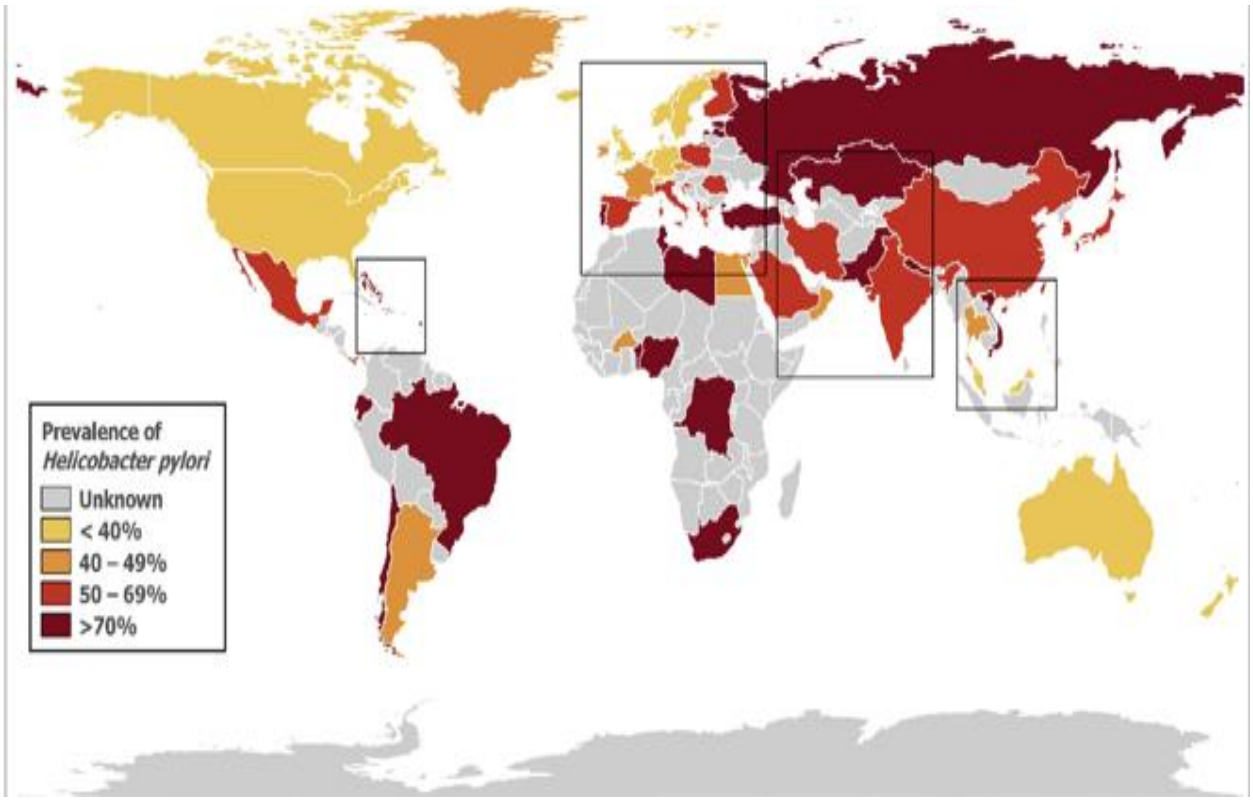

Figure 3: Global overview of the prevalence of *H. pylori* infection (https://people.ucalgary.ca/wggkaplan/HP2016.html.)

1.2.1.2 *H. pylori* infection in Africa

As alluded in the previous section, the prevalence of *H. pylori* infection increases with increase in age and is higher in lower income countries or among low socio-economic populations within a country. Although the socio-demographic prevalence of *H. pylori* infection varies, the incidence of morbidity caused by the infection remains high. Infection by *H. pylori* is the major cause of about 90% of duodenal ulcers and 70% of gastric ulcers in Africa. Cameroon and Ghanaian patients with gastritis showed a similar pattern of *H. pylori* involvement (Louw *et al.,* 1993). As expected in most lower income countries, *H. pylori* infection appears to be common in South Africa. Studies that have described the prevalence of the pathogen in the continent have adopted a sero-prevalence approach, which inadequately provides the prevalence picture as it only provides information on the presence of antibodies, some of which may be poor indicators of active infection. In a study done by Tanih *et al.,* in 2000, the prevalence of *H. pylori* in non-ulcer

dyspepsia (NUD) in Cape Town was a 63%. These results were based on histology reports. Based on serology testing, a prevalence of 81% in of *H. pylori* infection in patients with NUD was reported.

Serological studies done in various regions of Africa have shown that the majority of participants (60-95%) are infected with *H. pylori*. Fifty percent of these were children below the age of ten in the Ivory Coast. In northern Nigeria and Gambia, 55% of children under the age of 5 are infected (Asrat *et al.,* 2004). Studies in South African populations have reported acquisition of infection during early childhood. Infants from Bloemfontein have been reported to have a seropositivity rate of 43% although this might have been an overvaluation as maternal antibodies were not taken into account (Henriksen, 2001). A cohort study in Soweto revealed that approximately 48% of children at 1 year are infected with *H. pylori* and 100% of children at 12 years are infected (Ally and Segal, 1998).

1.2.1.3 *Helicobacter pylori* infection in Zimbabwe

The burden of *H. pylori* infection in Zimbabwe is unknown as there have not been any recent concrete epidemiological studies to address the prevalence question. However, it has been observed that there seems to be increasing incidences of peptic ulcer disease, gastric ulcer disease as well as some intestinal bowl diseases that are related to *H. pylori*. Moreover, a significant number of H. *pylori* seropositive patients have been observed to be positive for some auto-antibodies when an antinuclear antibody (ANA) screen is performed. This rise in *H. pylori* infection and its possible association with autoimmunity motivated the researcher to determine the prevalence of *H. pylori* and its immuno-epidemiology.

1.2.1.4 Immune responses to *H. pylori* infection

The bacterium *H. pylori* is a noninvasive pathogen occupying principally in the extracellular mucus layer. However, studies have evidenced the ability of the pathogen to invade gastric epithelial cells of humans and primates both *in vivo* and *ex-vivo*. Recent studies have also shown that the pathogen can bind onto red blood cells in the micro vessels of the lamina propria (Semino-mora *et al.,* 2003). Macrophages, B and T lymphocytes play a major role in the immune responses to *H. pylori* infection. The bacterium *H pylori* induces both cell-mediated and humoral immune

responses. The B lymphocyte triggered antibody responses include IgG, IgA and IgM isotypes. Studies conducted in mice showed that immunization with *H pylori* antigens could generate protective immunity. The pathogen causes an inflammatory reaction with polymorphonuclear (PMN) and mononuclear cells. Patients with *H. pylori* infection have elevated levels of pro-inflammatory cytokines such as IL-8, IL-1β, IL-6 and tumor necrosis factor alpha (TNF-α) (Yamaoka *et al.,* 1999).

Chronic infection by *H. pylori* elicits an inflammatory response in the gastric mucosa. This stimulation results in elevated generation of interleukin (IL)-12, which in turn leads to a T helper type 1 (Th1)-polarized response and high levels of Th1 cytokines. The products of the local inflammatory reactions may affect extra-gastric locations, resulting in an association between H. *pylori* infection and the pathophysiology of various extra-gastric diseases, including some autoimmune disorders (Valle *et al.,* 1996).

Macrophages

Macrophages are the chief innate responders to the pathogen in the gastric mucosa. They also receive signals from epithelial cells in contact with the pathogen. In *H. pylori* infection, monocytes, dendritic cells and macrophages are activators of adaptive immunity by producing IL-12 which stimulates Th1 cells (Mayor *et al.,* 2003). Studies have shown that neutrophil-activating protein (NAP) of *H pylori* plays a role in stimulation of Th1 cells by stimulating IL-12 and IL-23 secretion from neutrophils and monocytes (Amedei *et al.,*2006). In the CagA strain of *H. pylori,* IL-12 generation in the lining of the gastric mucosa is related to the development of peptic ulcers largely due to the stimulation of the Th1 response. Macrophages produce pro-inflammatory cytokines such as IL-1 and tumor necrosis α which contribute to the amplification of the inflammatory response. Indeed, the host inflammatory response can be enhanced by macrophage activation. However, when the activation results in apoptosis the consequences become greater as dying cells may release more cytokines which could heighten the inflammation.

B lymphocytes

The IgG and IgA responses to *H pylori* provide protective immunity. However, the IgA response may confer negative effects (Akhiani *et al.,* 2005). It has been shown that naïve mouse splenocytes that would have been exposed to *H pylori* are protected from spontaneous apoptosis hence they

undergo proliferation low infection (Bussiere *et al.*,2006). The constant B cell activation or the surviving phenotype may play a role in development of mucosa-associated lymphoid tissue lymphoid tissue (MALT). Apart from production of antigen-specific antibodies against *H. pylori*, B cells have been demonstrated to generate autoreactive antibodies that may be pathogenic and may lead to development of autoimmunity (Yamanishi *et al.*,2006). The urease enzyme secreted by *H. pylori* has been found to stimulate B-1cells due to the presence of urease-specific IgA producing B-1 cells. The B-1 autoantibodies secreted include IgM-type rheumatoid factor, anti-phosphatidyl choline antibodies and anti-single stranded DNA antibodies. These autoantibodies have been implicated in autoimmune diseases such as rheumatoid arthritis, Idiopathic thrombocytopenic purpura (ITP) and MALT. This study investigates possible associations with systemic sclerosis, systemic lupus erythematosus and dermatomyositis.

T lymphocytes

A characteristic of *H. pylori* infection is an accumulation of mononuclear lymphocytes which sometimes aggregate to form MALT. Amongst these are T lymphocytes. Patients infected with *H. pylori* have an elevated level of IFN-γ-producing T cells, consistent with a Th1 cytokine response, (Karttunen *et al.*,1995). The specific T-lymphocyte clone in the gastric mucosa against H *pylori* has a Th1-profile. This is observed in individuals suffering from peptic ulcer disease. (Bamford et a.,1998). The mucosal T lymphocytes produce excessive levels of Th1 cytokines IFN-γ, TNF-β and IL-2, and lower levels of Th2 cytokines IL-4 and IL-6. The production of IL-12 from innate immune cells contributes to the activation of a CD4+ response. Gastric epithelial cells may also act as antigen presenting cells which may activate CD4+ cells (Hida *et al.*,1999).

1.2.1.5 Diagnosis and Treatment of H. pylori infection

Diagnosis of *H. pylori* infection

Infection by *H. pylori* can be diagnosed by noninvasive methods or by invasive methods. Noninvasive methods include the urea breath test and serological evaluation for both IgA and IgG antibodies, invasive testing includes endoscopic biopsy of the gastric mucosa. The selection of the method of diagnosis relies on the clinical presentation and extent of disease. Noninvasive methods of diagnosis are serological tests such as rapid kit tests and ELISA assays, urea breath tests, and

stool antigen tests. The urea breath test depends on the profuse urease action from *H. pylori* infection in the stomach. The test qualitatively detects active infection and has a sensitivity of more than 95%. The test can be used for initial diagnosis of *H. pylori* infection and for determining the effectiveness of eradication therapy. In follow up testing, the urea breath test should be done after a four-week period has elapsed so as to avoid false negative results. The urea breath test can be used reliably in children above the age of six (Howden and Hunt, 1998).

Serologic testing in *H. pylori* detection is relatively inexpensive. Rapid kits are widely used. These may detect the presence of IgM and IgG antibodies in serum. Enzyme linked Immunosorbent assays (ELISA) are more specific and sensitive serological tests.

The stool antigen tests are an alternative test to the urea breath test and serological tests. These have a sensitivity and specificity of 98%. Stool tests can be used as follow-up tests in 8-week intervals and can be used in both children and adults.

Patients with suggestive symptoms such as gastrointestinal bleeding, or weight loss, and those above 55 years of age should undergo endoscopy for the diagnosis infection and the exclusion of ulcers or malignancy. A urease test is performed on the biopsy specimen. This is an inexpensive and quick way of determining urease activity in the antral-biopsy sample. The test has 80- 100% sensitivity (Graham and Qureshi, 2001).

For initial *H. pylori* infection diagnosis, culture of *H. pylori* with antibiotic-sensitivity testing is not usually done. This is usually recommended after the failure of second-line therapy (Bazzoli F, 2001).

Treatment of *H. pylori* infection

Treatment of *H. pylori* infection is done by antibiotics. However, the luminal acidity determines the effectiveness of some antibiotics that target *H. pylori*. A triple combination of antibiotics and proton-pump inhibitors or with ranitidine bismuth citrate, the pylobact kit is the standard of care. There are triple therapy combinations of two antibiotics and one anti-secretory agent which can be administered for use for one or two weeks. These have been evaluated and found to be effective. The use of two or more antimicrobial agents in combination increases cure rates while reducing

the chances of antimicrobial resistance. The major first line antimicrobial agents are metronidazole, bismuth, clarithromycin and/or amoxicillin. Resistance to amoxicillin or tetracycline is rare. However, resistance to clarithromycin is about 10% (Lind *et al.*,1996). If resistance occurs after administration of the first line antimicrobials, second line therapy is administered. This is usually a quadruple therapy involving, a proton-pump inhibitor or an H2-receptor antagonist given in addition to a bismuth-based triple regimen with high metronidazole dosage. Eradication of the bacteria using this strategy is about 75% (Hojo *et al.,* 2001).

1.2.2 The epidemiology of Autoimmunity

1.2.2.1 Global overview of autoimmunity

Autoimmune diseases account for the second largest cause of chronic sickness that also causes morbidity in most women in the United States. Approximately 24 million people have autoimmune diseases in America (Smale *et al.,* 2001). Autoimmune diseases are included in the top 10 causes of death in America. It has been observed that autoimmune diseases are on the rise not only in the United States, but around the world hence making autoimmune diseases a significant public health issue which is as important as cancer and heart disease. Scientists in the United States have juxtaposed the increased frequency of autoimmune diseases with a drop in infectious diseases. This is partly explained by the hygiene hypothesis which postulates that a drop in the exposure to certain microbes may increase the odds of developing an autoimmune disease (Walsh, 2000).

It is difficult to accurately develop a prevalence and cost for the burden of all autoimmune diseases which are over a hundred due to unavailable epidemiological studies of many of the diseases. However, according to The National Institutes of Health it is estimated that about 24 million Americans have an autoimmune disease (National Institutes of Health Fiscal, 2006).

The prevalence of multiple sclerosis in Italy has risen from a million cases to 4 million over the past decade (Lerner *et al.,* 2015). The prevalence of diabetes in children below the age of 10 has also rise from 7 million to over 20 million in Norway and Sweden while type 1 diabetes has doubled In Finland. Worldwide, celiac disease and intestinal bowel diseases (IBD) have been observed to be on the rise.

Generally, the frequency of autoimmunity diseases has increased in the last 35 years more so in the Northern and Western countries, compared to Southern and Eastern countries. (Agmon-Levin *et al.,* 2011). There has been an outbreak of autoimmune diseases in low income countries. This has brought interest in the factors contributing to this increased prevalence. Since the genetics aspect seems constant, attention has focused on environmental factors, and the western lifestyle (Lerner and Matthias, 2015).

1.2.2.2 Autoimmunity in Africa

The most common autoimmune diseases in Africa seem to be those of the thyroid particularly Graves' disease. In Tunisia the overall prevalence of Autoimmune thyroid disease is 10%. This was in conjunction with 6.5% of other autoimmune disease such as multiple sclerosis (Dowman *et al.,* 2012). In Ethiopia, the prevalence of autoimmune thyroid disease is reported to be 1.5% while reports from Libya show a prevalence of 3.9%. The main feature of Grave's disease is thyrotoxicosis in Africa. This scenario may be due to financial challenges in seeking treatment and late presentation. Also, by miss diagnosis by general practitioners. (Elhamel *et al.,* 1988).

Systemic auto immune diseases such as rheumatoid arthritis, systemic lupus erythematosus, systemic sclerosis and inflammatory myopathies had previously been rarely reported among black Africans although they are common among black Americans. Different hypotheses to this have been proffered. However, studies from some African countries have shown that these conditions may not be rare after all. Population studies from South Africa and some reports from Zimbabwe indicate that the prevalence of rheumatoid arthritis may be as equally high as among Caucasians and that there are no differences in the incidences between the rural and urban populations (Segal and Mitchel, 2001).

1.2.2.3 Autoimmunity in Zimbabwe

Unlike most western countries, the burden and epidemiology of autoimmunity in Zimbabwe has not been studied and documented. It is therefore difficult to calculate the overall cost burden that comes with autoimmunity. However, it has been observed that the incidence of autoimmune conditions may be on the rise in Zimbabwe. These observations have been made as a result of the increasing frequency of patients suspected to have an autoimmune condition that are referred to

the Asthma, Allergy and Immune Dysfunction clinic in Harare which is the major clinic for immune dysfunction referrals in Zimbabwe. It has also been observed that the autoimmunity patients present symptoms and clinical history of peptic ulcer disease and other gastric disturbances suggesting the possible role of *H. pylori* infection in the pathology of autoimmunity in Zimbabwean patients. Coupled with the observation of increasing *H. pylori* incidences at the same clinic, the present study interrogates the possible association of autoimmunity and *H. pylori* infection, factors associated with the association and the immune-epidemiology of the *H. pylori* associated autoimmunity in Zimbabwean patients.

1.1.2.4 Diagnosis and Treatment of autoimmunity

Diagnosis

Autoimmune diseases can be diagnosed based on the clinical features supported by the identification of present auto-antibodies and also by identification of clinical symptoms that are associated with disease. Immunoassays are the golden standard in diagnosis of autoimmunity. The chief principle for all autoantibody immunoassays is the capture of auto-antibodies from serum using immobilized autoantigens. The various techniques used to generate specific tests for detecting autoantibodies include immunofluorescence, immunodiffusion, enzyme linked immunoassays and immunoblotting techniques.

In the diagnosis of systemic autoimmune diseases, different autoantibodies are highly specific for certain diseases. Examples are anti-ribosomal P autoantibodies anti-dsDNA in systemic lupus erythematosus (SLE), and anti-topoisomerase I (Scl-70) in scleroderma, anti-Jo-1 in polymyositis or dermatomyositis. Generally, in diagnosis of systemic autoimmune diseases, detection of anti-RNP, Jo-1, SS-A/Ro, Scl-70, SS-B, and Sm is clinically useful. The Sm and RNP antigens are heterogeneous molecules which consist of peptides that with small ribonucleic acids. The SS-A/Ro antigen is a ribonucleoprotein of molecular weight 62kDa.The SS-B/La antigen is a 45 kDa phosphoprotein related to a different small RNAs. The Jo-1 antigen resides in the cytoplasm and is similar to histidyl-tRNA synthetase and is present in the cytoplasm. The Scl-70 antigen has elevated topoisomerase enzyme activity of 70 kDa molecular weight (Hayashi *et al.*,2001).

In diagnosis of organ-specific diseases, detection of specific autoantibodies against a particular organ is done. In thyroiditis, autoantibodies targeting the thyroglobulin (TGA) and thyroid peroxidase enzyme (TPO) can be detected. In type I diabetes mellitus, anti-insulin and anti-glutamic acid decarboxylase (GAD) autoantibodies can be detected. The autoantibodies either show disease activity or may give insight on a pathogenic condition in the near or far future. The PM-Scl antigen is a complex of 10-17 proteins of varying molecular weights (Cook, 1998).

Indirect immunofluorescence

The indirect immunofluorescence (IIF) technique, uses the human tumor cell line (HEp-2) as an antigenic source. In this technique, autoantibodies from the patient's serum recognize undefined antigens and produce patterns that are specific to a particular autoimmune disease. The tissue section has different antigenic targets hence results in a satisfactory overall evaluation. Antinuclear antibodies with undefined specificities are detectable in serum of patients with several different autoimmune diseases as well as infectious diseases. The HEp-2 cells however do not have dependable ability for detection of auto-antibodies against SS-A/Ro-52 and Jo-1 (Wiik, 2005). The limitations of the IIF method of diagnosis are substrate variations and poor specificity which may result in misleading interpretation. The method is labor intensive and requires highly skilled personnel (Bossuyt *et al.*,2004).

Enzyme linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) can be used in diagnosis of autoimmune diseases. Autoantibodies in patient sera are detected using the specific antigen-antibody binding principle. Auto-antigens are embedded into a microplate and serum with autoantibodies against the antigen is added. A second antibody conjugate attached to an enzyme whose substrate is then added is introduced. A chromogenic product whose absorbance is then determined. This is proportional to quantity of auto-antibodies present in the serum. The ELISA technique can be done on antigens prepared from nuclear extracts of the human tumor cell line (HEp-2) or from recombinant antigens. The disadvantage of using antigens from human tissue is that these are not pure and are of poor reproducibility. Proteins are present in limited amounts and other potentially antigenic targets may need to be removed in the purification process. The specificity of ELISAs

depend on the purity of isolated antigens. If recombinant antigens are used, it is important that an antigen should have the exact conformation and post-translational modifications as the human antigen (Haass and Lehmann,2001).

Line blot immunoassay

The line-blot immunoassay is a multiplexed assay in which various types of autoantibodies can be detected all at once. In this assay, recombinant antigens are immobilized in straight lines on a nylon strip. The test strip is then incubated with patient serum and the autoantibodies present in the sample recognize the autoantigens on the strip. A chromogen detection system which depends on the activity of alkaline phosphatase is used to visualize the bound auto-antibodies. The color intensities of the antigens on the strip are compared to those on the cut off lines.

Flow cytometry

Flow cytometry can be used as an alternative to ELISA and immunoblotting. In this technique, immunoassays are done with micro-beads. These detect and quantify auto-antibodies in patient sera. The potential low cost and time saving may be a reason for the routine use of these assays in the research and clinical laboratories (In Fritzler,2006). This technique, polystyrene microspheres which are labeled with various ratios of two different fluorochromes are used. Each fluorochrome has different degrees of fluorescence intensity, therefore creating a broad spectrally addressed bead interface. Antigens that correspond to auto-antibodies are bound to the microspheres. Each microbead bears a specific antigen immobilized onto it and the microbeads can be differentiated by their fluorescence intensities. A green laser excites fluorescent light so as to quantify the specific reaction associated with each autoantibody (Gonzales-Buitrago, 2006).

Treatment

Autoimmune diseases are incurable but can be controlled by managing the symptoms keeping the autoimmune process in check and maintaining good immune function. Corticosteroids and immunosuppressant drugs are the key drugs administered. These may not work in some patients and may have hostile side effects as a result of immunosuppression. In organ specific diseases, remedial medication may be administered to address the poor functioning of the organs. For

instance, patients with Addison's disease, may require therapy to replace lost generation of steroid hormones.

Immunosuppressive drugs include cyclophosphamide, methotrexate and cyclosporine. In some autoimmune diseases like systemic sclerosis and myositis, during the first phase of inflammation, convectional anti-inflammatory drugs such as ibuprofen or corticosteroids such as prednisone can be administered.

In some autoimmune diseases like SLE, patients may be given antimalarial drugs. These block the action of intracellular toll-like receptors. Hydroxychloroquine and chloroquine can be used in treatment of SLE. Anti-malarial are associated with less disease flares, maintaining autoimmune disease in remission and also reduce damage associated with disease. Antimalarial drugs also have good efficacy against arthritis (Ruiz-Irastorza and Khamashta, 2008).

1.2.3 The mechanisms of association between Helicobacter pylori and autoimmunity

Several autoimmune diseases have been associated with *H. pylori* infection. These include, Immune thrombocytopenic purpura (ITP), rheumatoid arthritis and systemic sclerosis and Sjogren's syndrome. In rheumatoid arthritis, (Yamanishi *et al.*, 2006) reported that B cells chronically sensitized with urease generated by *H. pylori* had the ability to produce IgM rheumatoid type autoantibodies. There is clinical correlation between *H. pylori* infection and rheumatoid arthritis and patients that have rheumatoid arthritis are at high risk of developing peptic ulcer disease. The extensive use of non-steroidal anti-inflammatory drugs (NSAIDS) in rheumatoid arthritis patients may contribute to a significant risk for peptic ulcer disease. (Tanaka *et al.,* 2005). Several studies have shown some clinical improvement in rheumatoid inflammation after successful eradication of *H. pylori* bacteria although the C-reactive protein which is characteristic of rheumatoid inflammation, did not seem to decrease, which still poises an unclear relationship between *H. pylori* and rheumatoid arthritis (Seriolo *et al.,* 2001).

Studies have also suggested a convincing contribution of *H. pylori* in the development of ITP. The Cag protein of the *H. pylori* pathogen initiates an autoimmune response by providing an antigenic stimulus for the generation of antiplatelet antibodies (Pordeus *et al.,* 2008). Moreover, several studies have indicated improvement in platelet counts after treatment of *H. pylori* infection (Emilia

et al., 2001). These findings help to affirm the relationship between the pathogen and autoimmunity in ITP.

In SLE, unlike the other infectious agents, a rather uncommon relationship exists between *H. pylori* and the disease. Similar to results obtained in studying the relationship between rheumatoid arthritis and *H. pylori*, it was found that urease is capable of inducing SLE-related anti-ss-DNA autoantibodies in mice. (Yamanishi *et al.,* 2006). However, in a different study by Showji *et al.,* it was demonstrated that SLE patients have lower anti-*H. pylori* antibody titres in comparison to other autoimmune diseases and also that the anti-*H. pylori* antibody levels seen in SLE patients were similar to those observed in controls (Showji *et al.,* 1996). Despite the evidence that demonstrates that *H. pylori*-related proteins may induce anti-ssDNA antibodies in rodents, it appears that infection with *H. pylori* may actually confer a protective effect on the development of SLE.

1.2.3.1 The role of pro-inflammatory cytokines in *H. pylori* -associated autoimmunity

Cytokines have a significant role in the pathogenesis of autoimmune diseases. Studies done in experimental models of human autoimmune diseases as well as observations in patients have shown that pro-inflammatory cytokines impart the initiation or propagation of autoimmune inflammation. Anti-inflammatory cytokines help regress autoimmune inflammation and recovery during the early stages of the disease. Inflammation of gastric mucosa as a result of *H. pylori* infection leads to secretion of pro-inflammatory cytokines such as TNF-α, interleukin (IL)-8, IL-IL-1β and IL-6. These produce systemic inflammation which then affects skin microenvironment characterized with loss of functional melanocytes especially in sclerosis (Moss and Calm,1994).

Previously, the pathogenesis of autoimmune diseases was analyzed predominately in the context of the T helper 1 (Th1)/Th2 cytokine imbalance, where the two T cell subsets reciprocally cross-regulate each other (Coffman,2006). In autoimmune diseases, if the nature of the main immune reactions is cell-mediated, the autoimmune diseases could be categorized as predominantly Th1-driven or largely Th2-driven if the main immune mediators are antibodies.

As shown in Figure 4 below, there are various subsets of effector and regulatory T cells, whose balance in their activity is important for an effective immune response that is symmetrical to the inciting stimulus. Excessive, decreased, or aberrant cytokine responses contribute significantly to autoimmune inflammation that underlies several autoimmune diseases.

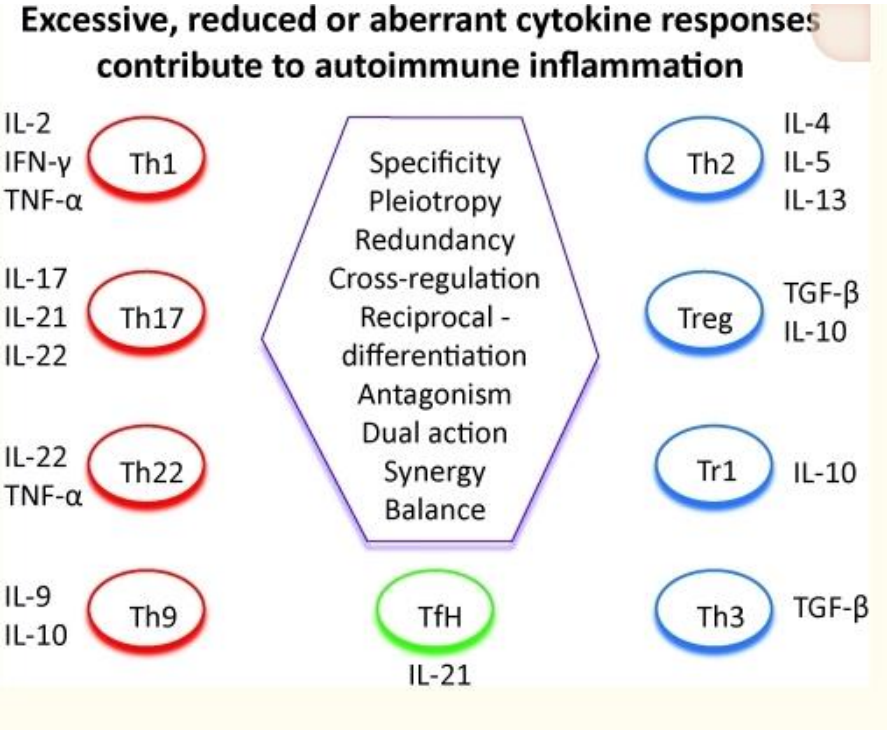

Figure 4: The role of various T cell subsets and their secreted cytokines in the pathogenesis of autoimmune disorders (Moudgil and Choubey, 2011).

In most autoimmune diseases such as multiple sclerosis and rheumatoid arthritis, IL-1 is involved in the pathogenesis of disease. The cytokine has many pro-inflammatory properties of TNF-α. The inflammatory process of rheumatoid arthritis is caused largely by TNF-α. However, IL-1 largely contributes to the pathogenesis of articular cartilage degradation. Interleukin-1 receptor antagonist (IL-1Ra) clinical trial was found to improve the condition of rheumatoid arthritis patients. However, treatment with anti-TNF-α therapy was more effective. Clinical trials using combined anti-TNF-α and IL-1Ra therapy are being developed (Bresnihan *et al.,* 1998).

In a study done by McInnes *et al.,*2011, it was reported that the production of TNF-α is elevated by direct T cell to macrophage contact through a process that depends on IL-15 which results in

activation of T lymphocytes. In this study, synovial fluids and membranes of rheumatoid arthritis patients were shown to have high levels of IL-15 protein. This observation was made together with high levels of IL-17 suggesting that IL-15 may trigger inert synovial fluid T lymphocytes to generate IL-17 In addition, IL-17A has been observed to be elevated in individuals with myositis and scleroderma (Ziolkowska *et al.,* 2000).

Interleukin 23 is a cytokine that shares a common p40 subunit with cytokine IL-12. Inhibition of the p40 protein has proved to be effective in the treatment of inflammatory bowel disease. Therapies that modulate or regulate cytokine production and action are powerful immune-suppressants (Kastelein *et al.,* 2007).

In the present study, the role of cytokines in development of *H. pylori* associated autoimmune diseases is investigated. It is hypothesised that inflammatory cytokines from *H. pylori* infection potentially drive autoimmune inflammation. It is also important to enumerate the lymphocytes involved in *H. pylori* infection and those in autoimmunity.

The effects on cytokine production can be analysed by detection of the relative concentrations of various cytokines in the circulation after experimental treatment or stimulation of peripheral blood mononuclear cells (PBMCs) or diluted whole blood. Cytokine production has been analysed successfully in stimulated whole blood as the stimulation assays mimic the natural environment *in vivo* (Liu *et al.,*2009). Mitogens, also known as polyclonal lymphocyte activators, are capable of inducing mitotic proliferation in cells. They do so in non-specific reaction influencing various lymphocyte subpopulations. The most commonly used mitogens are phytohemagglutinin (PHA), concanavalin A (Con A) and pokeweed mitogen (PWM). The main target of their action is the plasma membrane, where they bind to carbohydrate moieties of membrane glycoproteins. Pokeweed mitogen and PHA bind to N-acetyl- D-glucosamine while Con A binds to a-D-mannose. The binding of mitogens enables membrane receptors to activate adenylate cyclase where synthesis of cyclic adenosine monophosphate which result in signal transduction from membrane to the nucleus of lymphocytes (Wimer BM ,1996) The *In vitro* stimulation of lymphocytes by mitogens makes the cells produce appropriate cytokines to elicit an immune response. Compared to other stimulation methods, the whole blood assay requires minimal sample manipulation to

evaluate cytokine production as no labour-intensive separation and culturing of specific cell sub-populations is required (Wattrang, 2012)**.**

1.2.3.2 The role of molecular mimicry between antigens in *H. pylori* -associated autoimmunity

Molecular mimicry is an antigenic and operative similarity between microbial structures and host molecules which may lead to generation of self-reactive antibodies contributing to the onset of autoimmune conditions. This is a common strategy used by pathogens to adapt to the host organism and avoid host immune response mechanisms. Combined with the ability of T lymphocytes to circumvent the mechanisms of tolerance, molecular mimicry has been hypothesized as a potential mechanism implicated in the pathogenesis of various autoimmune diseases, including systemic sclerosis, diabetes mellitus, inflammatory bowel disease and spondylo-arthropathies. There are similarities within or between linear amino acid sequences as well as spatial structures that have been identified (Quaratino *et al.,* 1995).

The pathogens *Klebsiella pneumoniae* and *Campylobacter jejuni* stimulate the generation of cross-reactive antibodies that target the human leukocyte antigen (HLA)-B27 or the gangliosides. Certain viruses like the Epstein-Barr virus and hepatitis B virus have similar peptide sequences with some proteins in the central nervous system (CNS) (Sfriso *et al.*,2010).

Molecular mimicry between *H. pylori* surface antigens and some host antigens has been suggested as a mechanism for the relationship between infection and autoimmunity. Efforts have been made to identify homologous sequences between *H. pylori* and host polypeptides. These include similarities between the VacA, and urease beta chain in comparison with the gastric H+K+-ATPase, the P-type adenosine triphosphate (ATP)-ases, CopA and CopP that are involved in heavy metal iron transport, the pathogen heat shock protein B (HspB) in comparison with the host 60-kDa Hsp as well as the pathogen hemagglutinin/protease (hap) in comparison with carbonic anhydrase (Guarneri *et al.,* 2005).

In pernicious anemia, an autoimmune condition characterized by two phenomena, that is atrophy in the fundus of the stomach and autoantibody generation against parietal cells (PC) as well as their secretory component called an intrinsic factor (IF). CD4+ T cells recognize parietal cell

H+/K+ ATPase and have been demonstrated to be involved in the development of autoimmune gastritis. The enzyme H+/K+ ATPase is secreted by the parietal cells during normal cell turnover before it is captured and processed by antigen - presenting cells (APCs) (Claeyset *et al.,* 1998).

Infection by *H. pylori* may initiate the onset of autoimmune gastritis and pernicious anemia by molecular mimicry between gastric H+/K+ ATPase and *H. pylori* at the T cell level where there is activation of autoreactive T cells that target H+/K+ ATPase. Auto antibodies against gastric H+/K+ ATPase and their secretory classes are produced by B cells in cooperation with CD4+ antigen-specific T cells (Amedei *et al.*,2003).

In *H. pylori* infected patients, the presence of antibodies that react against the gastric mucosa suggests that the pathogen stimulates production of autoantibodies which may play a role in the *H. pylori* - associated inflammatory response. It has been suggested that these auto antibodies could be stimulated by molecular mimicry between a variety of Lewis (Le) antigens such as Le^x, Le^y and $Le^{x/y}$ present in the lipopolysaccharides (LPS) of *H. pylori* strains and those on human cells including polymorphonuclear white blood cells, endothelial cells and epithelial cells of the gastric mucosa. Studies revealed that the lipopolysaccharide O-specific chain of the *H. pylori* reference strain NCTC (National Collection of Type Cultures) 11637 possesses certain determinants that are similar to the human Le^x blood group antigens, while that of MO19 strain has determinants similar to human Le^y. There are other blood group antigens such as the H type 1, Le^a, sialyl Le^x, and blood group A but not H type 2 that have been detected in various *H. pylori* isolates (Heneghan *et al.,* 2000).

1.2.3.3 The role of the B-cell compartment in *H. pylori*-associated autoimmunity

As alluded before, infection by *Helicobacter pylori* may be associated with autoimmune conditions like rheumatoid arthritis (RA) or idiopathic thrombocytopenic purpura. Such autoimmune diseases are frequently associated with autoreactive antibodies generated by B-1 lymphocytes, which are a sub-population of B cells. The B-1 lymphocytes are located in the pleural cavity or mucosal compartment. It is hypothesized that the presence of $CD5^+$B-1 cells that produce anti-*H. pylori* urease-specific immunoglobulin A (IgA) in the gastric mucosa as well as their specific IgM in the sera of acutely infected patients suggests a possibility that urease stimulates mucosal innate immune responses. A study done by Yamanishi *et al.,* in 2006 demonstrated that purified *H. pylori*

urease greatly stimulates murine B-1 lymphocyte population rather than B-2 cells. In their study, the addition of polymyxin B did not affect the stimulation of B-1 cells shows that the effect of purified *H. pylori* urease was not due to contamination by bacterial LPS. The autoantibodies that were produced by B-1 cells sensitized to urease were anti-phosphatidyl choline, IgM-type rheumatoid factor and anti-single-stranded DNA antibody. These results suggest that *H. pylori* urease is an environmental trigger that may initiate various autoimmune disorders by the production of autoreactive antibodies via activation of B-1 lymphocytes.

Two distinct types of murine B-cell lineages exist. The first lineage comprises of conventional B cells commonly known as B-2cells. These are found predominantly in the spleen and lymph nodes forming systemic acquired immunity. The other comprises of B-1 cells which are found mainly in the mucosal compartment. Several studies provide evidence which suggests that the B-1 cells produce low-affinity and less-mutated antibodies. The repertoire of these antibodies is reactive with T-cell-independent (TI) antigens such as phosphatidyl choline. The B-1 cells produce IgM and IgG3 antibodies with few somatic mutations. The B-1 cells do not create long term memory unlike the B-2 cells (Martin and Kearney, 2000).

1.3 Rationale

Infection by *H. pylori* is increasing among the Zimbabwean population. The reasons for this have not been elucidated, there are suspicions that the general decline in hygiene standards in the country may be contributing factors. Autoimmune diseases are also on the rise. There has been a noted association between *H. pylori* and autoimmunity in patients referred to the Asthma, Allergy and Immune Dysfunction clinic. Reasons for this association are still unclear although various hypothesis have been proffered. The association of *H. pylori* and autoimmunity in Zimbabwean patients has not been studied and documented. Moreover, the epidemiology and clinical manifestations of *H. pylori* infection has been fluctuating, especially in developed countries. For example, peptic ulcer prevalence has declined in Western Europe, the United States, and Japan. Global eradication schemes require updated information regarding *H. pylori* incidence and disease burden (Hooi *et al.*, 2017).

The immuno-epidemiology of *H. pylori* associated autoimmunity in Zimbabwe is still not known. As studies on *H. pylori*-associated autoimmunity have not been documented in Zimbabwe,

effective treatment regimens to curb or prevent the development of autoimmunity after or during *H. pylori* infection are therefore unavailable. It is important to understand the immuno-epidemiology of autoimmunity and *H. pylori* so as to understand the immune system dynamics that occur during disease. This would reveal the most vulnerable groups of people, gender and therefore inform decisions on effective diagnosis and treatment plans.

The researcher performed a serological evaluation of archived laboratory analysis of remnant samples and correlated the results with the archived clinical data with the aim of reporting *H. pylori* and *H. pylori* associated autoimmunity prevalence in Zimbabwe over a period of 2 years. This data would provide essential updates about *H. pylori* associated autoimmune disease burden and the information can be used to plan appropriate strategies for allocating health care resources.

Elevated, aberrant or unregulated pro-inflammatory cytokines and unregulated T lymphocyte activation have been hypothesised as possible causes for the association of *H. pylori* and autoimmunity thus resulting in the high prevalence of *H. pylori*-associated autoimmunity. Again, information on the particular cytokines that play a role in *H. pylori*-associated autoimmune disease among Zimbabwean patients is not known and has not been documented. It is important to study cytokine profiles of patients with various autoimmunity-*H. pylori* statuses in Zimbabwe not only to contribute to scientific knowledge regarding this phenomenon. Identification of active pro-inflammatory cytokines and knowledge of cytokine-mediated immune mechanisms in autoimmunity will give insights in designing effective immunodiagnostic tools and treatment regimens.

It is in this light that the aim of the study was to determine the immuno-epidemiology of *H. pylori* associated autoimmunity as well as the role of cytokines in *H. pylori*- associated autoimmunity.

1.3.1 Research question

What is the burden of *H. pylori*-associated autoimmunity in Zimbabwe and what is the role of cytokines in development of disease?

1.3.2 Main objective

To determine the burden of *H. pylori*- associated autoimmunity and the role of cytokines in disease development in Zimbabwean patients.

1.3.2.1 Specific objectives

i. To determine the proportion of people presenting with different autoimmune conditions that are reactive to the *H. pylori* antigen
ii. To determine if the proportion of *H. pylori* reactive patients is significantly different between different autoimmunity conditions and gender
iii. To compare antinuclear (ANA) reactivity profiles of autoimmunity and *H. pylori* positive patients with autoimmunity positive and *H. pylori* negative patients
iv. To characterize cytokines in groups of patients with different autoimmunity-*H. pylori* conditions

2.1: MATERIALS AND METHODS

2.1.1 Ethics

Ethical approval to conduct this study was sought from the Medical Research Council of Zimbabwe (MRCZ/B/1479). The participants gave full consent to participate in the study. Consent forms were availed to participants for signing in English and in Shona. Participants were assured of confidentiality and no patient identifiers such as patient names were used. Unique laboratory reference numbers were used to identify the participants during the study, for laboratory analysis, data capture and in reporting work done. Samples used in the study were disposed by incineration after use.

2.1.2 Study setting

The study was carried out using serum and whole blood samples of people suspected to have autoimmunity conditions and or *H. pylori* infection, who were referred to the Asthma, Allergy and Immune Dysfunction clinic (AAIC) in Harare, Zimbabwe. There was no gender, ethnic, racial or age restriction in the study.

2.1.3 Study design

The research is an analytical cross-sectional study with a retrospective arm involving available laboratory and clinical records of patients that presented symptoms that warrant an *H. pylori* and autoimmunity test. The study also has a prospective arm where walk in patient serum was used for laboratory analysis. The participants in the study gave consent to have their leftover serum samples used for research.

2.1.4 Study population

The study population comprises Zimbabwean patients who presented symptoms to warrant an *H. pylori* laboratory test for diagnosis or those who presented symptoms suggestive of autoimmune disorders and were tested for these at the Asthma, Allergy and Immune Dysfunction clinic in Harare from January 2016 to February 2018. The participants included those referred to the laboratory by other physicians as well as those that were the clinic's regular patients.

2.2 Sampling

2.2.1 Sample size calculation

The sample size for this study was calculated using the following formula:

The total number of participants required n to determine the prevalence of autoimmunity and *H. pylori* infection at 95% confidence level is given by:

$$n = \frac{z^2 p(1-p)}{\Delta^2} = 385$$

Where n -sample size

Z-1.96 standard score value from the normal distribution at 95% confidence interval

p-0.5 standard value used for an unknown prevalence of *H. pylori* infection and autoimmunity burden

Δ-0.05 precision of the estimated prevalence

$n = (1.96/0.05)^2\ 0.5(1-0.5)$

= 385samples.

2.2.2 Sampling frame

Random sampling was done on 1500 serum samples of autoimmunity and *H. pylori* patients referred to the Asthma Allergy and Immune Dysfunction clinic from January 2016- February 2018.

2.2.3 Sampling unit

Each sampling unit was a serum sample intended for autoimmunity testing or a serum sample requested for *H. pylori* testing by the clinician.

Summary of procedures

Figure 5 below summarizes the procedures done in the study

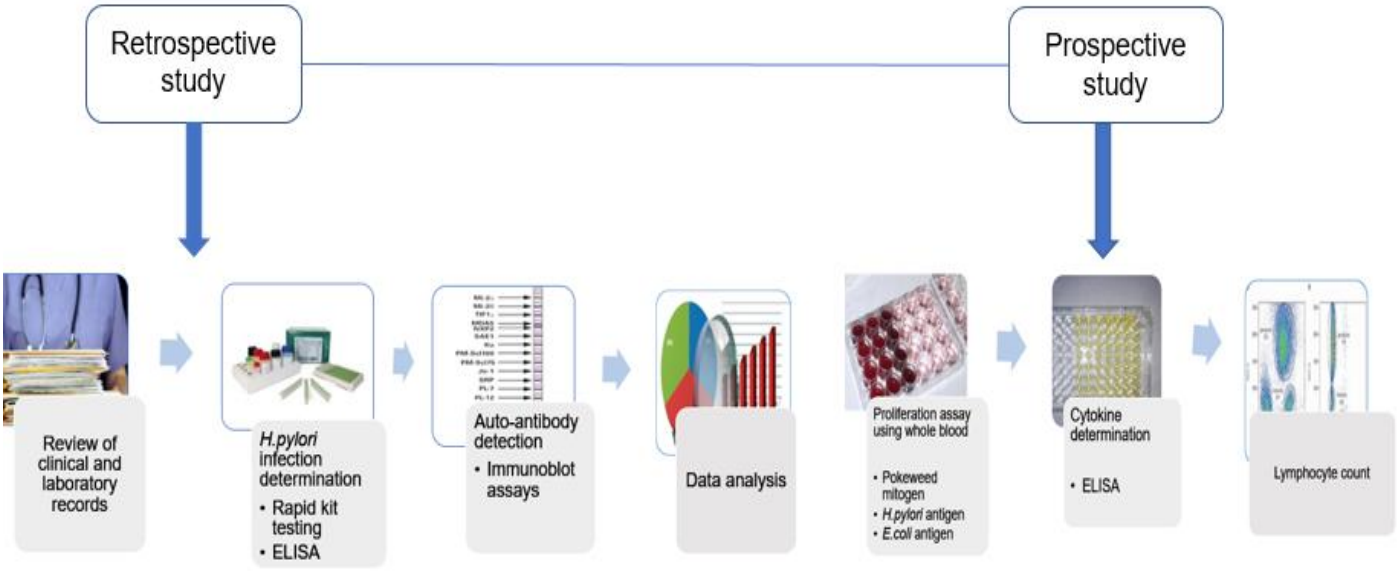

Figure 5: Summary of all the procedures done throughout the study.

The steps included the review of archived records, determination of *H. pylori* infection and autoimmunity in remnant samples and data analysis in the retrospective study. In the prospective study steps included stimulation of whole blood with a mitogen, *H. pylori* and E. coli antigens as well as enumeration of white blood cells before and after stimulation.

2.3 Review of archived records

In the retrospective element of the study, archived clinical and laboratory records of patients with remnant samples were reviewed. This data was for patients positive or negative for autoimmune conditions. A total of 1500 patient files were reviewed and samples retrieved. However, for laboratory processing of these samples, 385 samples were selected based on adequacy of sample volumes for proposed repeat assays. The variables that were reviewed were laboratory reference numbers, age, gender and symptoms presented during examination by clinician, presence or absence of autoantibodies and *H. pylori* status as shown in
Table 1 overleaf.

Table 1: The study participant variables that were reviewed from 2016-2018.

Variable Number	Variable	
1	Laboratory reference number	
2	Gender	Female
		Male
3	Age at diagnosis in years	≤ 12
		13- 19
		20- 45
		46- 60
		≥60
4	Auto antibody status	Present
		Absent
5	*H. pylori* infection status	Positive
		Negative
6	Clinical symptoms	Musculoskeletal
		Gastrointestinal
		Ocular
		Respiratory
		Cardiac
		Swelling
		Dermatological
		Other

2.3.1 Autoimmune antibody detection of remnant samples

Immunoblot assay

An immunoblot assay was done to detect human auto-antibodies of the IgG class to different nuclear antigens in serum. The assay is a multi-parameter assay containing test strips coated with parallel lines of highly purified antigens. The test has two steps. In the first step, diluted patient serum or plasma samples are incubated with the immunoblot strips. In the case of positive samples,

the antibodies in the serum bind to the antigens on the strips. In the second reaction, an incubation with an enzyme-labelled anti-human conjugate is done where the bound antibodies are detected by a color reaction.

Preparation of reagents

All reagents were brought to room temperature that is 18-25 ºC for 30 minutes before use. The positive control is a 100X concentrate which was diluted with sample buffer in the ratio 1:101 respectively. The enzyme conjugate is a 10X concentrate which was diluted with sample buffer in the ratio 1:10 respectively. Similarly, the wash buffer was diluted with distilled water in the ratio 1:10 respectively. The patient serum samples were diluted with sample buffer in the ratio 1: 101 and mixed well by vertexing.

Pre-treatment

Test strips with labelled corresponding patient reference numbers were placed in separate channels on the incubation tray. A volume of 1.5ml sample buffer was added to each test strip and incubated for 5minutes at room temperature on a rocking shaker. After the 5 minutes, all liquid was aspirated off.

Incubation assays

Each channel with test strip was filled with 1.5ml of diluted serum samples and incubated for 30 minutes at room temperature (25 ºC) on a rocking shaker. All liquid was then aspirated off from each channel and washed 3 times, each wash for 5 minutes with 1.5ml of working phosphate buffer on a rocking shaker. A volume of 1.5ml diluted alkaline-phosphatase-labelled anti human IgG conjugate was pipetted into each channel before an incubation of 30 minutes at room temperature. The liquid was aspirated and washed as described above. A volume of 1.5ml nitrobluetetrazoliumchloride (NBT)substrate solution was added and incubated for 10 minutes. All liquid was aspirated off and strips were washed 3 times with distilled water for a minute. Test strips were then placed on the evaluation protocol, air dried and evaluated.

Table 2 represents the general ANA screen antigens tested and their arrangement on the strip from the top of the strip to the bottom.

Table 2: ANA screen antigens

Position	Antigen code	Antigen origin
1	nRNP/Sm	Native calf U1-nRNP
2	Sm	Native Sm from bovine spleen
3	SS-A	Native SS-A from bovine thymus
4	Ro-52	Recombinant Ro-52 from baculoviral in insect cells
5	SS-B	Native SS-B from calf thymus
6	Scl-70	DNA topoisomerase from bovine thymus
7	PM-Scl	Recombinant PM-Scl cDNA expressed in baculovirus system in insects
8	Jo-1	Native histidyl-tRNA synthetase from calf thymus
9	CENP B	Recombinant centromere protein B expressed in baculovirus system
10	PCNA	Recombinant PCNA expressed in baculovirus system
11	dsDNA	Native from salmon testes
12	Nucleosomes	Native from calf thymus
13	Histones	Mixed types from calf thymus
13	Rib.P protein	Native from rabbit thymus
14	AMA M2	Native pyruvate-dehydrogenase complex

The antinuclear antigen (ANA) test done is a general autoimmunity diagnostic test that is initially done to verify presence of any auto antibodies, screen, rule out or point towards a more specific autoimmune disease. Presence of certain antibodies suggested a further interrogation of disease specific auto antibodies such as myositis and systemic sclerosis. These followed the same procedure and interpretation of results.

Table 3 overleaf represents the antigens tested for diagnosis of myositis and their arrangement on the strip from the top of the strip to the bottom

Table 3: antigens detected in the diagnosis of myositis

Position	Antigen code	Antigen origin
1	Mi-2α	Recombinant protein expressed in baculovirus system in insect cells
2	Mi-2β	Recombinant protein expressed in baculovirus system in insect cells
3	TIF1γ	Recombinant protein expressed in *E. coli*
4	MDA5	Purified MDA protein
5	NXP2	Recombinant NXP2 protein expressed in *E. coli*
6	SAE1	Recombinant SAE1 protein expressed in *E. coli*
7	Ku	Recombinant Ku protein expressed in baculovirus insect cells
8	PM-Scl175	Recombinant Pm-Scl protein expressed in baculovirus insect cells
9	Jo1	Native Jo-1 histidyl- tRNA synthetase from calf thymus
10	SRP	Recombinant SRP protein expressed in baculovirus insect cells
11	PL-7	Recombinant theoyl-tRNA synthetase expressed in baculovirus insect cells
12	PL-12	Recombinant alanyl-tRNA synthetase expressed in baculovirus insect cells
13	EJ	Recombinant glycyl-tRNA synthetase expressed in *E. coli*
14	OJ	Recombinant isoleucyl-tRNA synthetase expressed in *E. coli*
15	Ro-52	Recombinant Ro-52 expressed in baculovirus insect cells

Table 4 below represents the antigens tested for diagnosis of systemic sclerosis and their arrangement on the strip from the top of the strip to the bottom

Table 4: antigens detected in the diagnosis of systemic sclerosis

Position	Antigen code	Antigen origin
1	Scl-70	Native Scl-70 DNA topoisomerase 1 from calf thymus

2	CENP A	Recombinant centromere protein A expressed in baculovirus system in insect cells
3	CENP B	Recombinant protein B expressed in baculovirus system in insect cells
4	RP11	Recombinant subunit of POLR3K of human RNA Polymerase III expressed in *E. coli*
5	RP155	Recombinant subunit of POLR3A of human RNA Polymerase III expressed in *E. coli*
6	Fibrillarin	Recombinant fibrillarin protein expressed in *E. coli*
7	NOR90	Recombinant NOR90 protein expressed in E. coli
8	Th/To	Recombinant Th/To expressed in *E. coli*
9	PM-Scl 100	Recombinant PM-Scl protein (100kDa) expressed in baculovirus insect cells
10	PM-Scl 75	Recombinant PM-Scl protein (75kDa) expressed in baculovirus insect cells
11	Ku	Recombinant Ku protein expressed in baculovirus insect cells
12	PDGFR	Recombinant PDGRF receptor expressed in mammalian cells
13	Ro-52	Recombinant Ro-52 expressed in baculovirus insect cells

2.3.2 Detection of *H. pylori* infection

Detection of IgG antibodies using rapid kits

To detect the presence of antibodies against *H. pylori* in both autoimmunity positive and negative samples, the Combo Rapid test (CTK Biotech, catalogue number R0191C) was used. The test is a lateral flow chromatographic assay which detects IgG, IgM or IgA antibodies against *H. pylori* in human serum. A reagent is ready to use as supplied. The regents were brought to room temperature before use. The rapid testing device was labelled with the corresponding specimen number and placed on a clean surface. Thirty microliters of serum were dispensed into the sample well using a dropper included with the kit. One drop of sample diluent was added to the sample and liquid was allowed to migrate the sample along the nitrocellulose membrane for 15 minutes. After 15 minutes, the results were read. Positive results appeared as a red line on the test line of the device as well

as on the positive control line. The rapid kit was a screening method to aid in identifying *H. pylori* positive samples from the autoimmune positive and negatives. To differentiate between active (current) or historical *H. pylori* infection, an IgA ELISA assay was performed. This detects the presence of active *H. pylori* infection, IgA antibodies whose half-life is approximately 6 days more accurately confirm active, current infection and is an indication for therapy.

Detection of IgA antibodies using ELISA

All reagents were brought to room temperature before use for an hour. To each of the required wells except those for the controls, 25 µl of IgA sorbent (ref. S001) was added. Five microliters of sample followed by 75 µl of the serum diluent was added to each well. The control wells were prepared by adding 95 µl of the serum diluent to each well followed by 5 µl of the positive control. A volume of 5 µl of the cut off control and 5 µl of the negative control was added to the corresponding wells in duplicate. The ELISA plate was placed in a plate shaker for 3 minutes in order to homogenously mix the reagents. The plate was covered with parafilm and incubated at 37ºC for 30 minutes. After incubation the seal was removed and liquid aspirated from all wells before the plate was washed 4 times with 300 µl of washing solution per well. Excess liquid was drained off before 100 µl of conjugate solution was added immediately to each well. The plate was covered again and incubated at 37ºC for 30 min. A second washing step was done as described earlier and excess liquid drained. Hundred microliters of substrate solution were added into each well and incubated at room temperature for 20 minutes in a dark cupboard. Color development was stopped by adding 50 µl of stopping solution into the wells. Absorbances were read using a spectrophotometer at 450nm. Sample were considered positive if their absorbance were 20% above the cut off absorbance.

After detection of historical and present *H. pylori* infection was done using the IgG and IgA antibodies respectively, samples were grouped according to four different autoimmunity-*H. pylori* conditions as illustrated in Figure 6 overleaf.

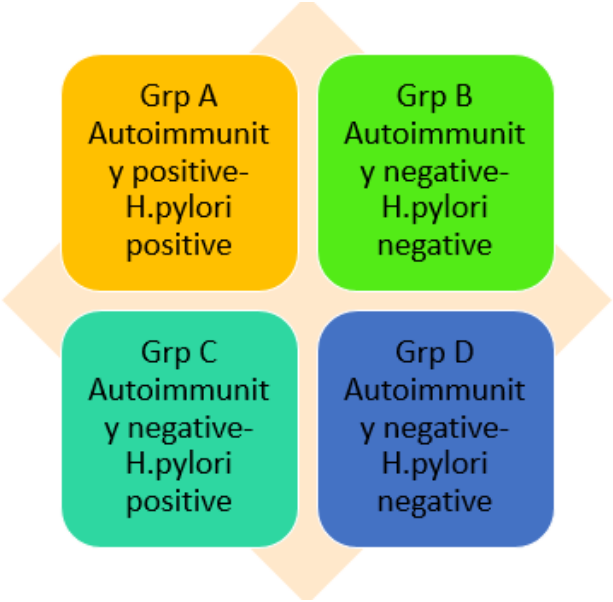

Figure 6: Groups of different autoimmunity-*H. pylori* conditions from autoimmunity and *H. pylori* diagnosis.

2.3.3 Cytokine analysis using flow cytometry

Flow cytometry was used to detect the presence of Th1 and Th2 cytokines from available selected samples from the four groups A-D. Simultaneous analysis of IL-2, TNF-α, INF-γ (Th1), IL-4, IL-6, IL-10 (Th2) and IL-17A (Th17) cytokines was done in a single sample. A total of 20 samples were analyzed using the BD™ Cytometric Bead Array (CBA). The flow cytometer employs a series of particles with discrete fluorescence intensities to simultaneously detect multiple soluble analytes as illustrated in Figure 7 overleaf.

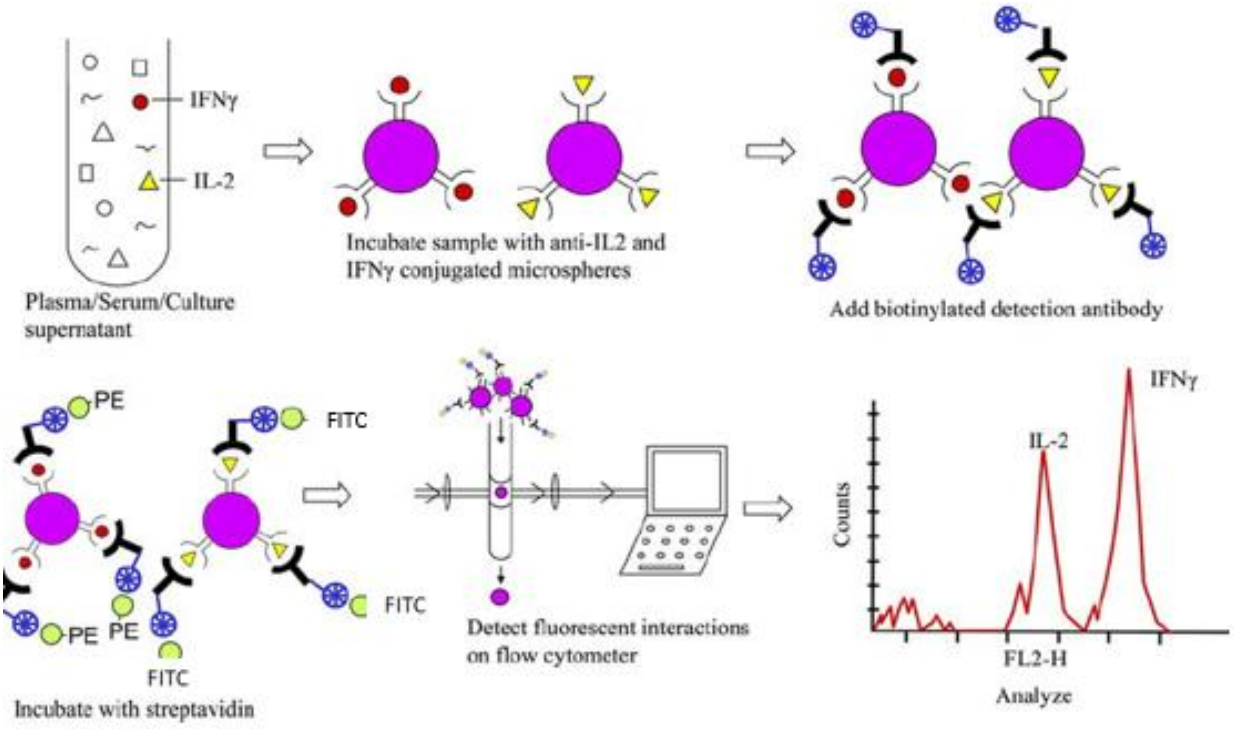

Figure 7: Sequence of events in flow cytometric detection of cytokines in serum adapted from Salamunic, 2010.

Preparation of standards

The lyophilized human Th1/Th2 Cytokine Standards were reconstituted with 2.0ml of assay diluent and serially diluted before mixing with the capture beads and the PE detection reagent. The reconstituted standards were allowed to equilibrate for 10 minutes before dilutions were made. Reconstituted protein was mixed by pipette only. Tubes were labelled and arranged in the following order: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256 for serial dilutions. A volume of 300 µl of assay diluent was pipetted to each of the tubes and serial dilutions were made, mixing the tubes before the next dilution. One tube containing assay diluent alone was used as the 0 pg./ml negative control.

Preparation of cytokine capture beads

Preparation of capture beads was done to reduce the chances of false-positive results due to the effects of serum or plasma proteins. Mixed capture beads were centrifuged at 200 × g for 5 minutes and the supernatant discarded. The mixed capture bead pellet was re-suspended in Serum

Enhancement Buffer and vortexed thoroughly. The mixed capture beads were incubated for 30 minutes at room temperature and kept in a dark cupboard to protect them from direct exposure to light.

Cytokine analysis assay

Prepared standards, test samples and capture beads were transferred to the appropriate assay tubes for incubation and analysis. After vertexing, 50 µl of the mixed Capture Beads were added to assay tubes before an equal volume of the Human Th1/Th2 PE detection reagent was added to the assay tubes. Fifty microliters of the Human Th1/Th2 Cytokine Standard dilutions were added to the control assay tubes. To each of the assay tubes, 50 µl of test samples were added and incubated for 3 hours at room temperature in the dark. During this incubation the Cytometer Setup procedure was done. After incubation, 1.0 ml of wash buffer was added to each assay tube and centrifuged at 200 × g for 5 minutes. The supernatant was carefully aspirated and discarded. The pellet was re-suspended in 300 µl of wash buffer, vortexed for 5 seconds then analyzed on the BD5 Calibur flow cytometer.

The results were retrieved from the output computer and analyzed.

2.4 Prospective study sample analysis

A total of 10 patients of different *H. pylori* infection statuses was determined using ELISA analysis as described above were used for the prospective cytokine study. Five milliliters of patient whole blood were collected into EDTA tubes. A full blood count was done using the BC 3000 from Mindray. and the total white blood and lymphocyte percentage for each sample was documented. The $CD3^+$, $CD4^+$, $CD8^+$ and $CD45^+$ lymphocytes were enumerated using flow cytometry. Cytokine detection was done following whole blood stimulation with *H. pylori* antigen (case), E. coli antigen (case control), and Pokeweed mitogen (positive control).

2.3.1 Lymphocyte enumeration using flow cytometry

Anticoagulated blood collected in EDTA tubes and stored at room temperature before analysis. Sample tubes were labelled with sample identification number. A volume of 20 µl of BD multi-test CD3/CD8/CD45/CD4 stain into the bottom of the 12 × 75mm tubes. Fifty microliters of well

mixed, anticoagulated whole blood were pipetted into the bottom of the tubes. The tubes were capped and vortexed gently to mix before a 15-minute incubation step was done in the dark at room temperature. Erythrocytes were lysed following staining using 450µl of 1X BD FACS lysing solution. The tubes were capped, vortexed and incubated in the dark for 15 minutes before they were analyzed on the BD FACS flow cytometer. Results were recorded and analyzed using Statistical Package for Social Sciences (SPSS) software.

2.3.2 Whole blood stimulation

Whole blood stimulation was done using E. coli bacteria, *H. pylori* positive antigen, and pokeweed mitogen as positive control.

Antigen preparations

Five milligrams of Pokeweed mitogen (PWM) were reconstituted in 0.9% NaCl to give a stock concentration of 1mg/ml. Hundred microliters of PWM stock solution were added to 10ml of RPMI media to give a working concentration of 10µg/ml. The bacterial E. coli was cultured in 100ml 2X Luria Bertani broth for 18hours at 37° C before it was serially diluted to 10^{-5} dilution. Four hundred microliters of E. coli suspension were added to 10ml of RPMI media. Four hundred microliters, concentration at 10µg/ml of *H. pylori* antigen was taken from the positive control of the ELISA Human anti *H. pylori* IgA kit.

Stimulation assay

One milliliter of RPMI media with antigen was pipetted into each well in a 6-column culture plate. Row A was the negative control in which 1ml of antigen free RPMI media was pipetted. Rows B, C and D had 1ml of E. coli (case control), *H. pylori* (case) and PWM (positive control) media respectively. Columns 1-6 had samples 1-6 respectively where 250µl of well mixed whole blood was pipetted into. Samples 7-10 were loaded in a similar manner on a different plate. Each sample was cultured with 3 different antigens and a negative control without any antigen as illustrated in Figure 8.

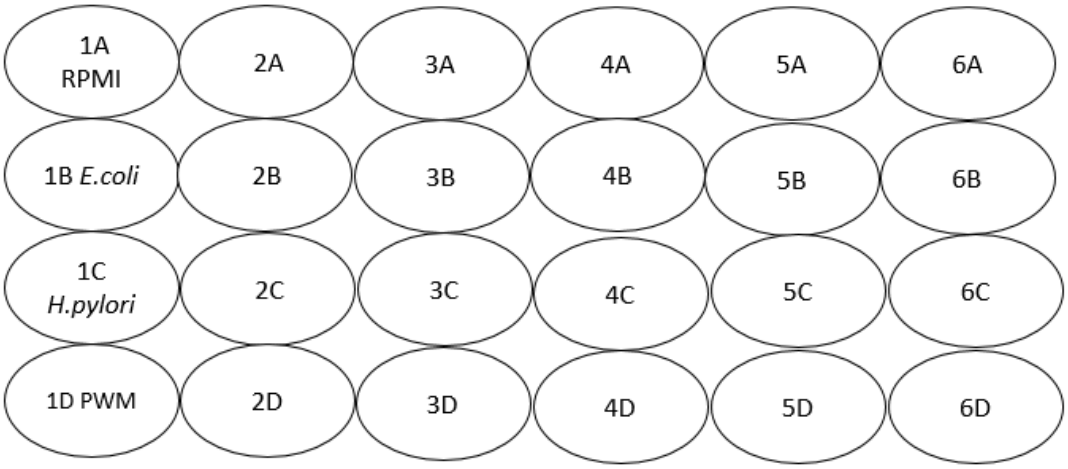

Figure 8: Whole blood stimulation assay with RPMI negative control, *E. coli* antigen, *H. pylori* antigen and Pokeweed mitogen.

Each culture plate was covered with parafilm and placed in a culturing jar with CO_2 bags. The culture jar was tightly closed and the plates were incubated for 48 hours at 37° C. After incubation, whole blood was aspirated from the wells and put into correspondingly labelled centrifuge tubes before they were centrifuged at 200 g for 3 minutes. The supernatant for cytokine detection was aspirated and stored at -20 ºC. The pellet was used for lymphocyte count after the stimulation using flow cytometry as described above.

2.3.3 Cytokine detection using ELISA

The cytokines IL-2, IL-4, TNF-αand INF-γ were detected from culture supernatant using sandwich Mabtech ELISA (Mabtech Sweden, product code 3510-1H-20) development kits purchased from Sweden.

Buffers and Reagents

Phosphate saline buffer (PBS, 1X) was made by mixing 8 g Sodium Chloride (137 mM), 0.2 g Potassium chloride (2.7 mM), 2.9 g di-Sodium Hydrogen orthophosphate anhydrous (10 mM) and 0.2 g Potassium di-Hydrogen Phosphate (10 mM) to 1 litre of distilled water and the pH was adjusted to 7.4 with an automatic pH meter. Tris buffered saline (TBS, 1X) was made by mixing 6.05 g of Tris (50 mM) and 8.76 g of sodium chloride (150 mM) mixed to 1 litre of distilled water and the pH was adjusted to 9.6 by an automatic pH meter. Phosphate citrate buffer (1X) was made by mixing 7.19 g di-Sodium Hydrogen orthophosphate anhydrous and 5.19 g citric acid to 1 litre

distilled water and the pH was adjusted to 5.0 by an automatic pH meter. The wash buffer was made by adding 1 litre TBS in a reagent bottles and mixing with 400 µL Tween-20 (0.04%). The blocking buffer was made by adding 0.5% bovine serum albumin (BSA) and 1 litre of TBS. The incubation buffer was made by mixing 0.5% BSA with 1 litre of TBS and 0.04% Tween-20. The substrate used was 0.4 mg/ml ortho-phenyldiaminedihydrochloride (OPD) which was added to phosphate citrate buffer and 30% hydrogen peroxide (H_2O_2) at concentration of 1 µg/L.

Preparation of standards

Cytokine standards were reconstituted in 1ml TBS buffer with 1% BSA to a stock solution concentration of 1µg/ml. These were left at room temperature for 15 minutes and vortexed. Ten microliters of stock solution were added to 990µl of TBS buffer and 200 µl serially diluted into an 800µl volume of TBS. Two hundred microliters were then serially diluted into 500µl of buffer. The 7 serial dilutions that followed were made by aliquoting 200µl into 500µl volumes of TBS buffer. A standard curve was plotted at the end of the assay and concentrations of analyte samples were extrapolated from the curve.

ELISA assay

A 96 well microtiter plate (Nunc MaxiSorb, Denmark) was coated with 100 µL in each well of specific cytokine monoclonal antibody (capture mAb) diluted 2 µg/mL in PBS and incubated overnight at 4ºC. After 24 hours the plate was washed twice with TBS (pH 7.4) and the wells were blocked by adding 200 µL of blocking buffer to block any nonspecific binding sites. The plate was left to incubate for 1 hour at room temperature on shaker at 200 rpm, then was washed 5X with TBS containing 0.05% Tween-20 (wash buffer) and gently tapped to remove excess blocking buffer. One hundred microliters of the different samples and standards were added in the wells in distinctive known columns and rows. The plasma in the plate were left to incubate overnight at 4 ºC to allow maximum binding of the cytokine to the capture antibody. The plasma was washed 5X with wash buffer to remove any unbound cytokines and tapped gently on a multi wipe to remove any excess wash buffer. Incubation buffer was mixed with biotinylated monoclonal antibody (detecting mAb) at 1 µg/mL and 100 µL of the mixture was added to each well to bind to the cytokine bound to the captures antibody and incubated for 1 hour. Streptavidin with horse-radish peroxide attached (conjugate mAb, enzyme-linked antibody) was mixed with incubation buffer at

the ratio of 1:1000. One hundred microliters of the conjugate antibody used to quantitate the amount of bound detecting antibody was added to each well and left for incubation for 1 hour. After an hour the plate was washed 5X with the wash buffer and the and gently tapped to remove any excess wash buffer. Freshly prepared substrate was (100µL) was added to each well and the plates were left to incubate in the dark for 30 minutes. Successful binding of the enzyme linked antibody to the antibody allowed catalysis of the substrate by enzyme resulting in a yellow color development. The absorbances of the samples were read at 450 nm using an ELISA plate analyzer from the Biomolecular Interactions Laboratory in the Department of Biochemistry. Concentrations of cytokines were extrapolated from the standard curve.

Data Analysis

All data obtained in the study was analyzed using the SPSS, STATA and Graph pad software. Prevalence was calculated at 95% confidence level. Comparisons of quantitative data were performed using nonparametric Chi-square and Fisher's exact tests. P values < 0.05 were considered significant.

3.1: RESULTS

3.2 Retrospective study

Clinical and laboratory data was collected from patients who attended the Asthma, Allergy and Immune Dysfunction clinic from 2016 to 2018. There is one autoimmunity specialist clinic in Zimbabwe, Harare and hence data reported is representative of Zimbabwean statistics. The patients attending this clinic had been offered clinical consultation by the autoimmune disease specialist and laboratory diagnosis of autoimmunity and *H. pylori* infection done. For the purpose of this study, patients who were sensitized to various allergens were excluded to avoid confounding effects.

Detection of autoantibodies

Detection of autoimmunity or presence of anti-nuclear auto antibodies was done using the immunoblotting technique. The assay is a multi-parameter assay containing test strips coated with parallel lines of highly purified antigens. In the case of positive samples, the antibodies in the serum bind to the antigens on the strips and are seen as dark lines on the strip. A general ANA screen was first done to determine presence of any autoantibodies. A more specific systemic sclerosis and myositis panel was used to detect presence of disease-specific autoantibodies. Results were interpreted on the EuroLine scan. After the reaction was stopped using deionized water, the test strips were placed onto adhesive foil paper and pressed hard using filter paper and then left to air dry. This ensured that the strips stuck to the adhesive foil. The dry test strips were then scanned using a flatbed scanner from EUROIMMUN. After scanning, the scanned data was uploaded into a EUROLineScan software. This software follows a number of commands and produces a soft copy result. Positive results appeared as a dark band on a particular antigen embedded on the test strip. The darker the band the larger the quantity of that particular auto-antibody in the serum. Evaluation of band intensities was based on signal intensity. Table 5 overleaf represents the diagnostic band intensity grading.

Table 5: Diagnostic band intensity grading for auto-antigens

Signal Visual evaluation	Signal intensity EUROLineScan	Result	
No signal	0-5	0	Negative
Very weak band	6-10	(+)	Borderline
Medium	11-25	+	Positive
Strong band	26-50	++	Positive
Very strong band (comparable to control band)	≥ 50	+++	Strong positive

Figure 9 represents an ANA screen positive results for systemic sclerosis, myositis and SLE.

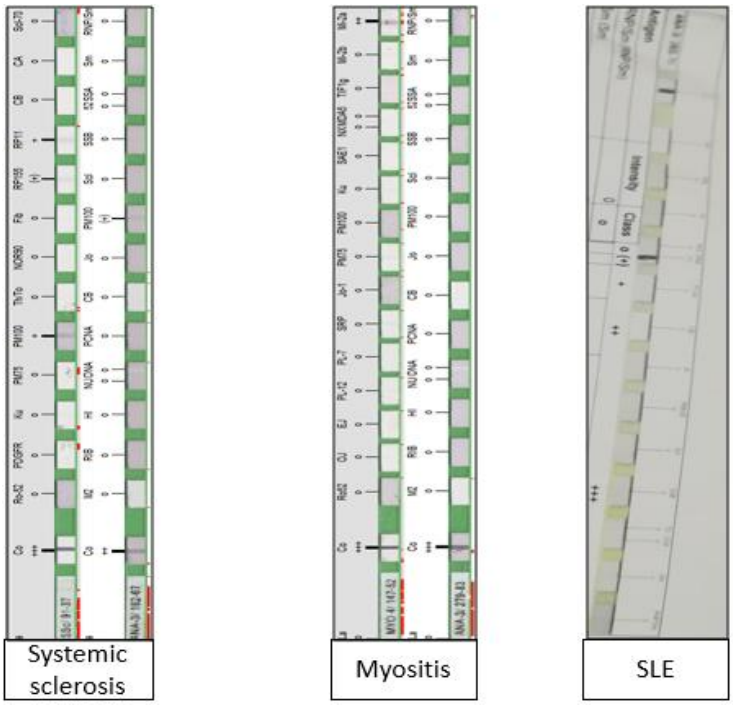

Figure 9: Immunoblot detection of antinuclear autoantibodies in systemic sclerosis, myositis and SLE patient sera. The dark bands are representative of antibody and antigen interaction between IgG and various auto antigens.

From the 385 samples tested for auto-antibodies, 193 were positive and 192 were negative. This data suggests an autoreactivity prevalence of 50% in this specialist center. Table 6 summarizes the prevalence of autoantibody reactivity.

Table 6: Prevalence of autoimmunity

Autoantibody expression	**Cases**
Autoantibody positive	n = 193 (50%)
Autoantibody negative	n =192 (50%)

Determination of anti-*H. pylori* IgG and IgA antibodies

Historical *H. pylori* infection was determined by the presence of anti-*H. pylori* IgG antibodies using rapid kits. Positive results were seen as a pink line on the control and positive line on the rapid strip as shown in Figure 10a below. Patients either positive or negative to autoimmunity were tested for *H. pylori* infection. Current infection was determined by quantification of anti-*H. pylori* IgA antibodies in IgG positive samples using ELISA as shown in Figure 10b below. Absorbances above the cut-off point absorbance were considered positive.

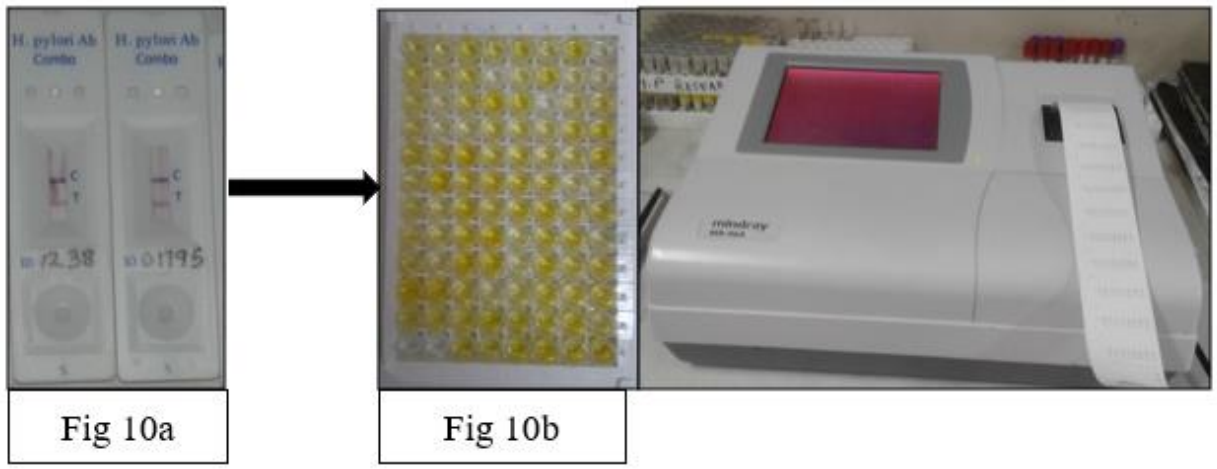

Figure 10: Anti-*H. pylori* IgG detection and anti-*H. pylori* IgA detection respectively. Color intensity in 10b was proportional to absorbance and IgA concentration.

Table 7 represents the prevalence of *H. pylori* infection

Table 7: Prevalence of *H. pylori* infection

Variable		Cases
H. pylori infection status	*H. pylori* positive	n = 126 (33%)
	H. pylori negative	n = 259 (67%)
Gender of infected patients	Female	n = 69 (55%)
	Male	n = 57 (45%)
Antibody isotypes	IgG	n = 76 (60%)
	IgA	n = 50 (40%)

Four autoimmunity-*H. pylori* categories were obtained from autoantibody detection and *H. pylori* infection determination as shown in Table 8 overleaf.

Table 8: Autoimmunity-*H. pylori* categories obtained from *H. pylori* infection determination and autoantibody detection.

	***H. pylori* positive**	***H. pylori* negative**
Auto-antibody positive	Grp A: n=68 (18%)	Grp B: n= 126 (33%)
Auto-antibody negative	Grp C: n= 59 (15%)	Grp D: n=132 (34%)

As shown in Table 8, the prevalence of people expressing autoantibodies and *H. pylori* infection (Group A) is 18%. Patients who were positive for auto antibodies but negative for *H. pylori* infection (Group B) were 33%. Patients who were positive for *H. pylori* infection only (Group C) were 15% while those negative for both *H. pylori* infection and auto antibodies (Group D) were 34%.

3.2.1 Immuno-Epidemiology analysis

The epidemiology, prevalence and gender distribution of *H. pylori*-associated autoimmunity was studied in the 4 groups. The prevalence of people with different autoimmune conditions and were reactive to *H. pylori* categorized in group A was 18%. The prevalent autoimmune diseases that were associated with *H. pylori* infection in Zimbabwe were systemic sclerosis (50%), SLE (39%) and myositis (11%) respectively as shown in Figure 11 overleaf:

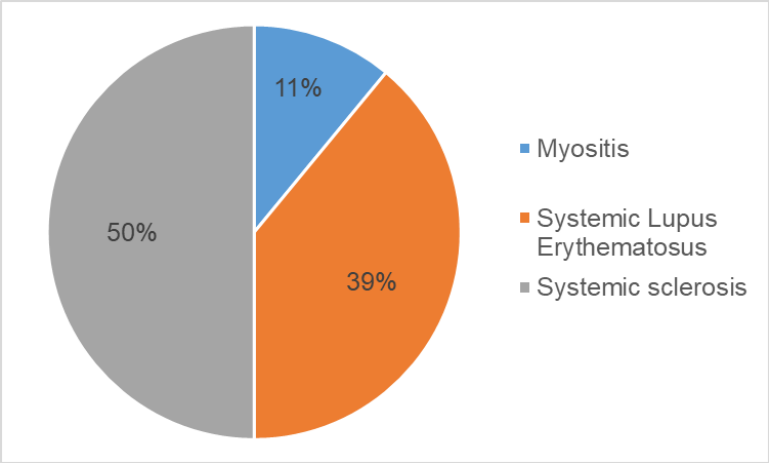

Figure 11: Autoimmune diseases associated with *H. pylori* infection in Zimbabwe (p= 0.046)

As shown in Figure 11, systemic sclerosis was observed to be the autoimmune condition that has the most association with *H. pylori* infection followed by SLE and myositis. A chi-square test performed in the 95% confidence interval was done to determine association between these conditions and *H. pylori* infection.

The gender distribution among the 3 conditions associated with *H. pylori* infection was studied and represented in Figure 12 below:

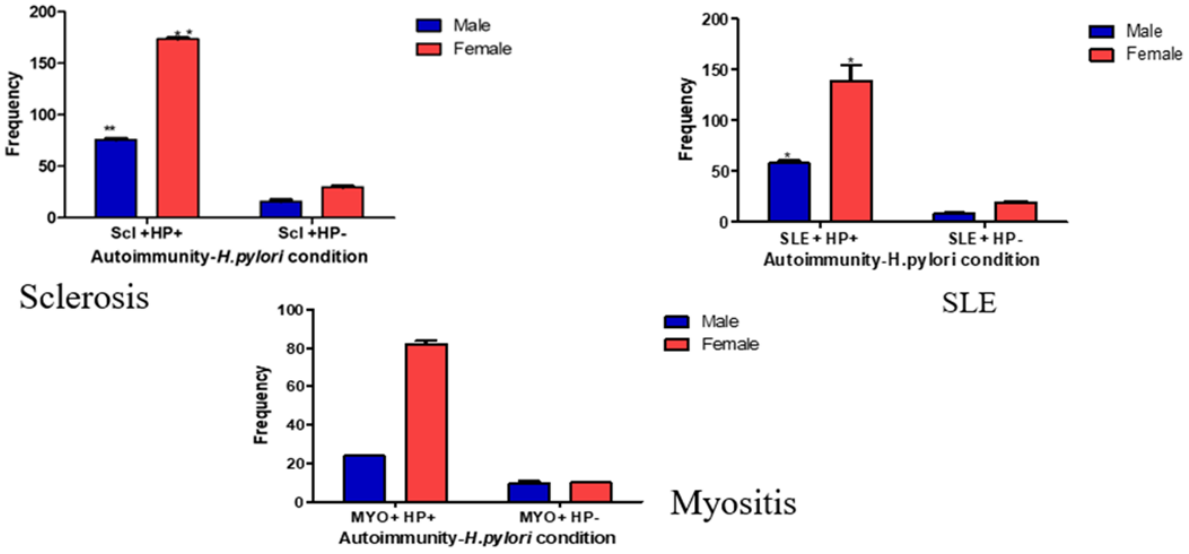

Figure 12: Gender distribution among prevalent *H. pylori*-associated autoimmune conditions. Amongst *H. pylori* infected patients and in the presence of any autoimmune condition (Systemic sclerosis, SLE or myositis) there was a consistent preponderance of females

As shown in Figure 12, there was a higher prevalence of co-existing disease in all three autoimmune conditions compared to existence of autoimmune conditions alone without *H. pylori* infection. There was a significant prevalence difference between co-existing disease and autoimmunity alone particularly in the case of systemic sclerosis (p=0.038) and in the case of SLE (p= 0.048). With respect to myositis, there was no significant difference in the prevalence of co-existing myositis and *H. pylori* infection (p>0.05). Generally, there was a higher incidence of co-existing disease in female patients compared to male patients. The general prevalence of both co-existing disease and autoimmunity disease alone was high in sclerosis followed by SLE and myositis.

Age distribution of patients with co-existing disease was studied and represented in Figure 13. The ages were categorized according to the standard criteria used in central hospitals for patient assessment, that is children (0-12 years), adolescents (13-19 years), adults (20-45 years), middle aged (46-60) and the aged (above 60).

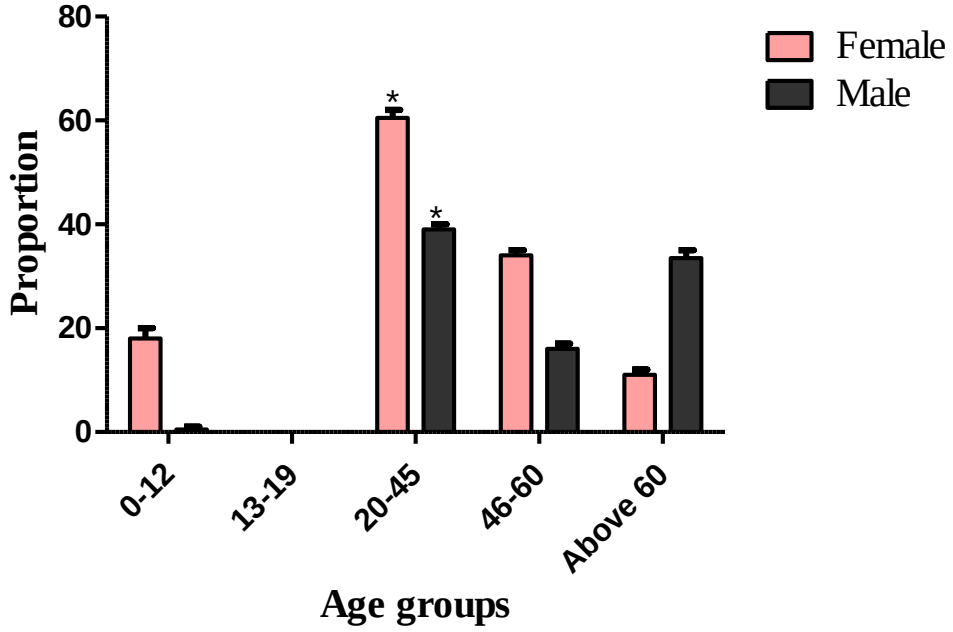

Figure 13: Graph of various age groups in patients with *H. pylori* infection and autoimmunity conditions.

As shown in Figure 13, the highest prevalence of co-existing disease was observed in the 20-45 years age group particularly in women. Followed by the 46-60 and above 60 ages respectively.

There was significant relationship between age and autoimmunity-*H. pylori* condition in the 20-45 age group and (p=0.048) after chi-square testing. Men above 60 had a higher co-existing disease incidence compared to women. No association of autoimmunity and *H. pylori* infection was observed with the adolescent group (13-19 years) and very few males were observed to have had co-existing disease in children aged between 0 and 12.

The general age distribution pattern for systemic sclerosis, SLE and myositis is shown in Figure 14 overleaf.

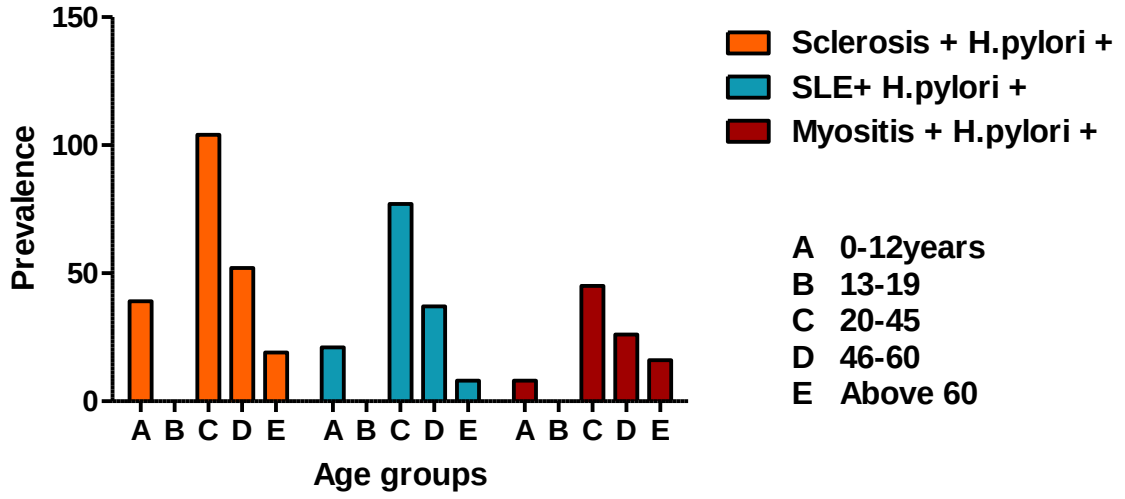

Figure 14: General co-existing disease age distribution patterns.

A higher prevalence of co-existing disease was observed with the adult group of ages between 20 and 45 for all 3 autoimmune diseases. No association between autoimmune disease and *H. pylori* infection was observed for the 13-19 age group for all 3 autoimmune conditions.

The ANA profiles of patients with co-existing disease and those with autoimmune conditions alone were studied and compared. The ANA reactivity profile for people with co-existing disease is represented in Figure 15 overleaf.

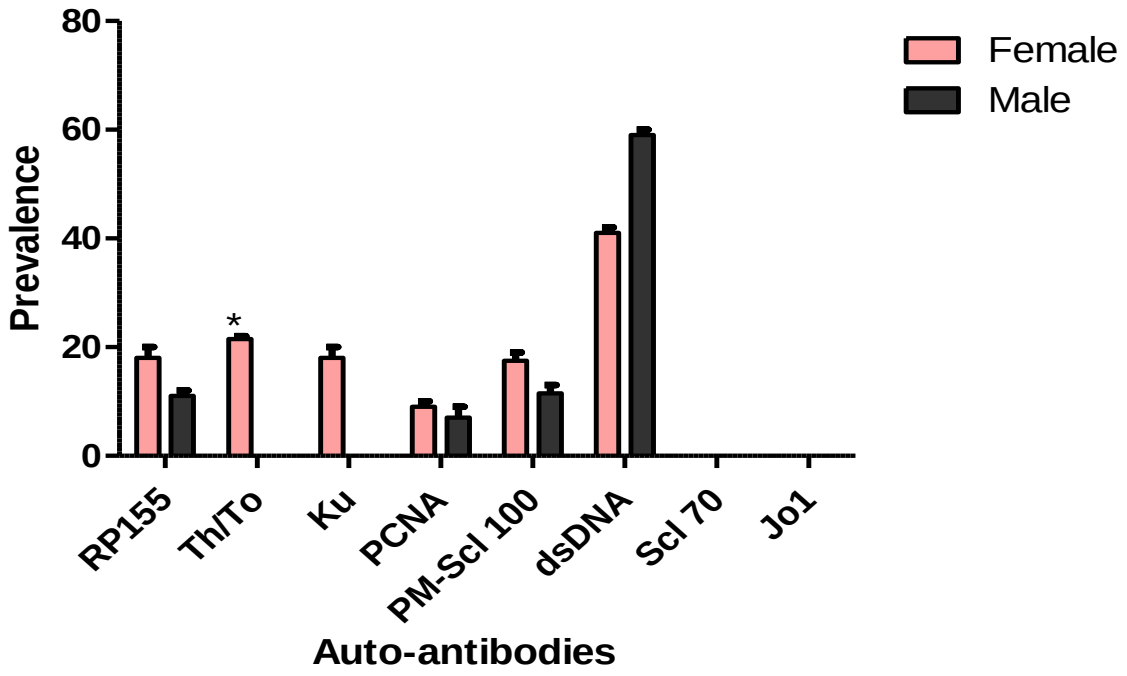

Figure 15: Auto-antibodies in patients with *H. pylori* infection and autoimmune conditions.

The auto antibodies that were prevalent in people with co-existing disease were mostly systemic sclerosis specific auto antibodies RP155, PM-Scl 100, Th/To and Ku. The auto antibody dsDNA was also prevalent in this group although it is specific for SLE. Systemic sclerosis specific Scl 70 and Jo1 auto antibodies were not expressed in co-existence with *H. pylori* infection. The auto antibodies Th/To and Ku were only expressed in female patients with co-existing disease. There was significant gender bias in the expression of Th/To ($p=0.023$) after a Fisher's exact test was performed.

Figure 16 represents prevalent auto antibodies expressed in patients with autoimmune conditions alone.

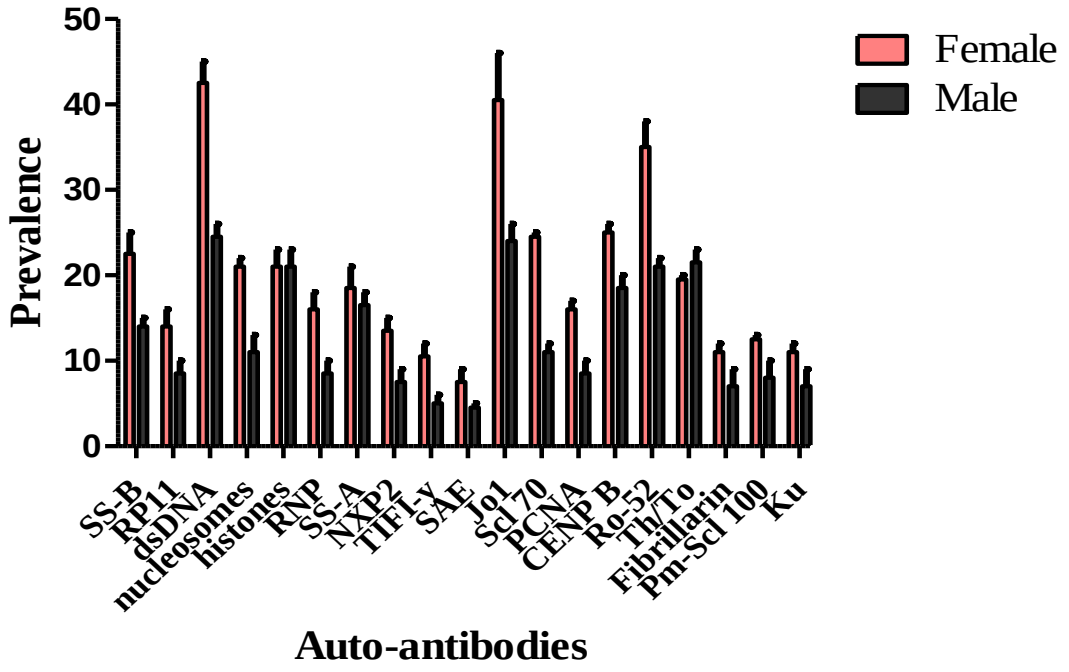

Figure 16: Auto-antibodies in patients with autoimmune conditions and no *H. pylori* infection.

The most prevalent auto antibodies expressed in patients with various autoimmune conditions without *H. pylori* infection were dsDNA, SS-B, Jo1, anti-histones, NXP2, Scl 70, and nucleosomes. There was expression of a wider range of autoantibodies in this group of patients and there is no sufficient evidence to support a bias in the expression of autoantibodies towards any particular autoimmune disease. Generally, there was high expression of auto antibodies that may be used in the diagnosis of systemic sclerosis and SLE such as SS-B compared to those that can be used in specific diagnosis of myositis such as TIFI-γ. In comparison to Figure 15, where no expression of Jo1 and Scl 70 was observed in patients with co-existing disease, there was expression of these auto antibodies in patients with autoimmune conditions alone.

3.2.2 Cytokine analysis

Cytokine analysis was done using flow cytometry. The cytokine profiles of autoimmune positive patients with different *H. pylori* conditions categorised in groups A and B are represented in Figure 17 below:

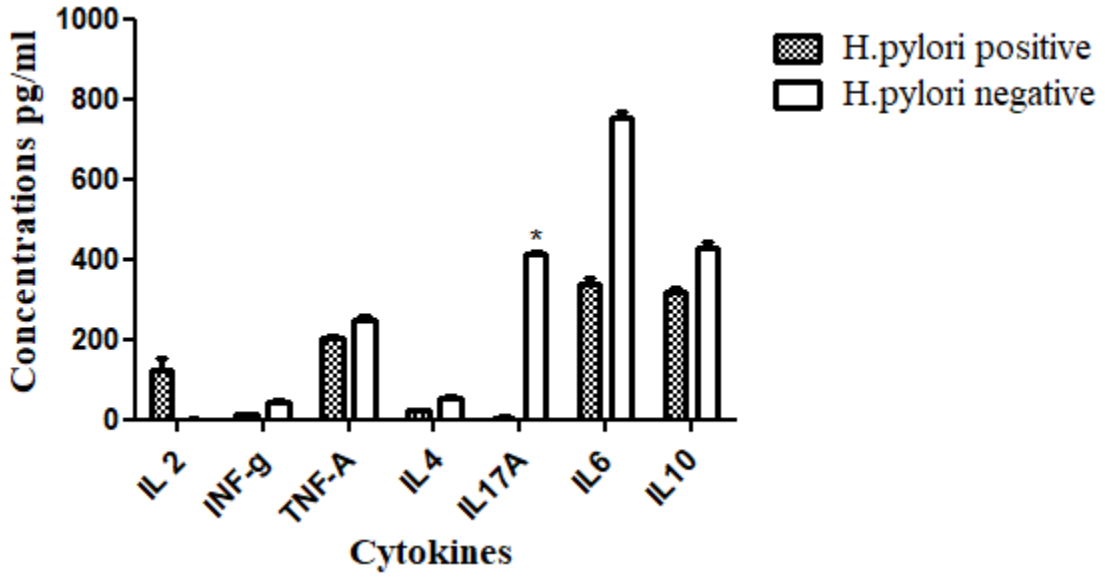

Figure 17: Cytokine profile of autoantibody positive patients that were positive (group A) and negative (group B) to *H. pylori* infection.

As shown in Figure 17, there were high concentrations of IL6, IL10 and TNF for both groups. The cytokine IL17 was expressed mostly in autoimmune patients without *H. pylori* infection (Chi-square test, p= 0.242). The cytokine IL4 and INF-γ were generally low in both groups.

The cytokine profiles of autoimmune negative patients with different *H. pylori* conditions categorised in groups C and D are represented in Figure 18 overleaf.

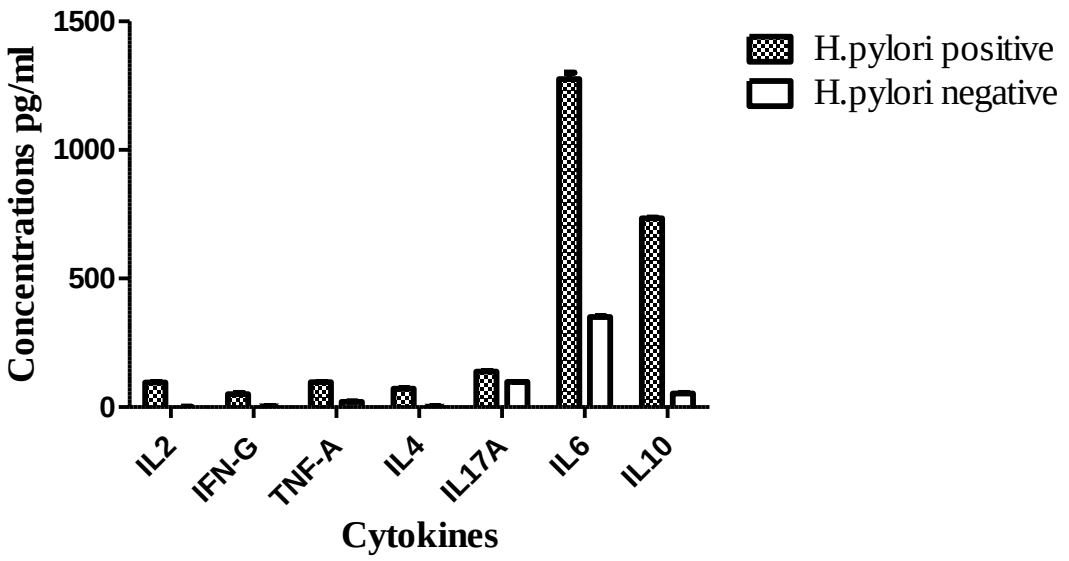

Figure 18: Cytokine profile of autoantibody negative patients that were positive (group C) and negative (group D) to *H. pylori* infection.

As can be observed in Figure 18, all cytokines were generally higher in patients that are positive to *H. pylori* infection compared to those that were negative to *H. pylori*.

3.3 Prospective study

3.3.1 *H. pylori* detection

From the 10 whole blood samples collected for use in this part of the study, 6 were positive for *H. pylori* infection after an anti-*H. pylori* IgA ELISA was done. Four were negative for *H. pylori* infection.

3.3.2 Lymphocyte enumeration

A total lymphocyte count before in-vitro stimulation of cells by different antigens was done on the 10 whole blood samples using flow cytometry. The CD4+, CD8+, CD3+ and CD45+ populations were stained by APC, PE, FITC and PerCP respectively. The results were reported as the percentage of positive cells per microliter of blood (absolute count). The BD Multiset clinical software was used to determine the absolute counts and cell populations were represented in dot plots as shown in Figure 19.

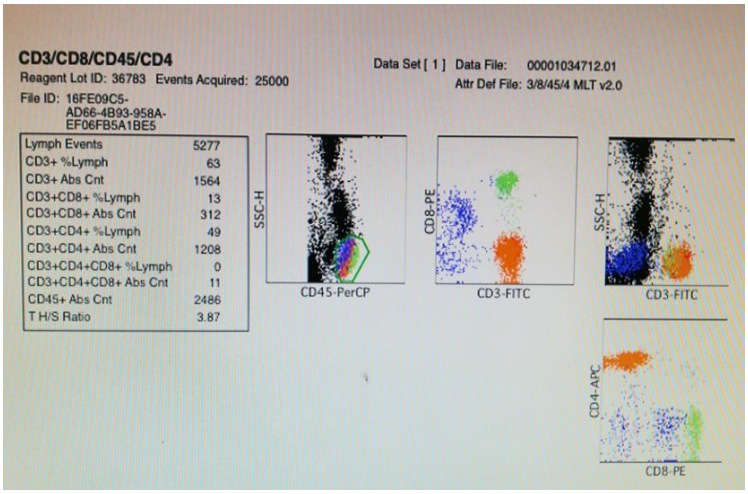

Figure 19: Dot plots of cell populations obtained from flow cytometry using the BD Multi-test flow cytometer.

Figure 20 below represents the general lymphocyte population of *H. pylori* positive and negative patients before in vitro stimulation by different antigens

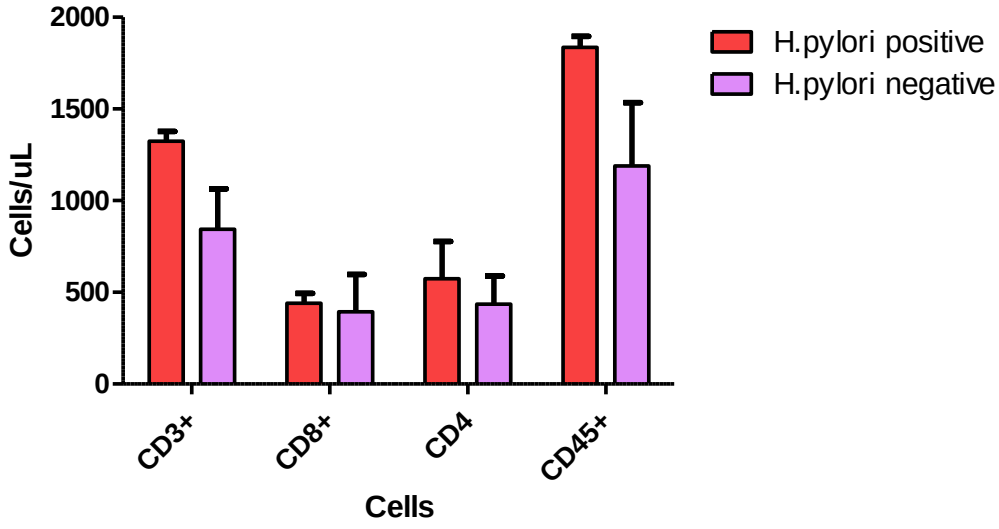

Figure 20: Lymphocyte population of *H. pylori* positive and negative patients before in vitro stimulation by different antigens.

As can be seen in Figure 20, cells populations were generally high in *H. pylori* positive patients compared to *H. pylori* negative patients. The CD45+ cell population was generally higher in all patients while the CD8+ population was lowest.

3.3.3 Whole blood stimulation assays

B and T lymphocyte (CD45+) proliferation in response to the *H. pylori* antigen was conducted. The *E. coli* bacterial lysate was used as a positive bacterial case control. Antigen free samples were incorporated as negative controls. PWM, which preferentially or selectively, stimulates CD19+ B lymphocytes was used as a positive control. The results of proliferation of the total lymphocyte populations, (CD45+) when unstimulated (Control) or stimulated with mitogen (PWM), *E. coli* or *H. pylori* are shown in Figure 21 below.

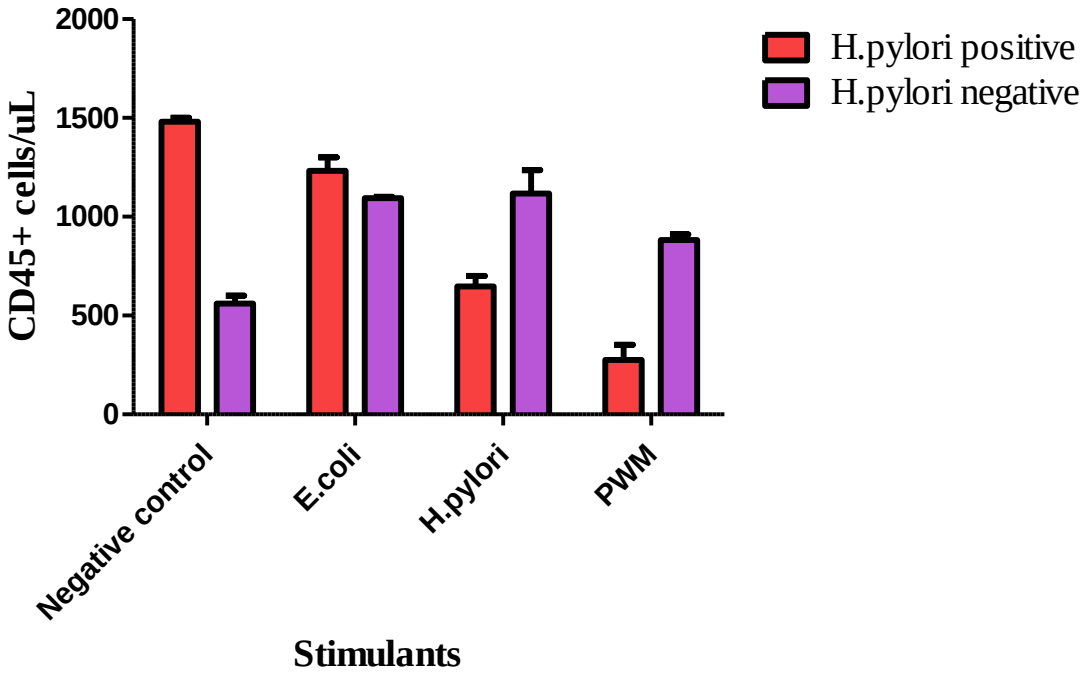

Figure 21: Total lymphocyte count of *H. pylori* positive and negative cells stimulated by E. coli, *H. pylori* and pokeweed mitogen. No stimulant was added in the negative control.

As shown in Figure 21, In the absence of antigen or mitogen (negative control) there was three-fold (1500cells/µl) higher (CD45+) proliferation in *H. pylori* infected compared to uninfected patients (500cells/µl). Co-incubation of the same whole blood samples with *E. coli* lysates showed comparable proliferation between *H. pylori* infected and uninfected patients. Co-incubation of *H.*

pylori negative whole blood samples with *H. pylori* antigens yielded lymphocyte proliferation values that were comparable to those obtained upon incubation with the *E. coli* extracts (1100cells/µl). By contrast the coincubation of *H. pylori* positive whole blood samples with the *H. pylori* extract resulted in a significant (p= 0.046) reduction in CD45+ T lymphocyte proliferation.

Stimulation of the whole blood samples with PWM and measurement of CD45+ lymphocytes showed a reduction in proliferation in *H. pylori* positive compared to *H. pylori* negative patients.

Figure 22 represents the various cell population quantities obtained after stimulation.

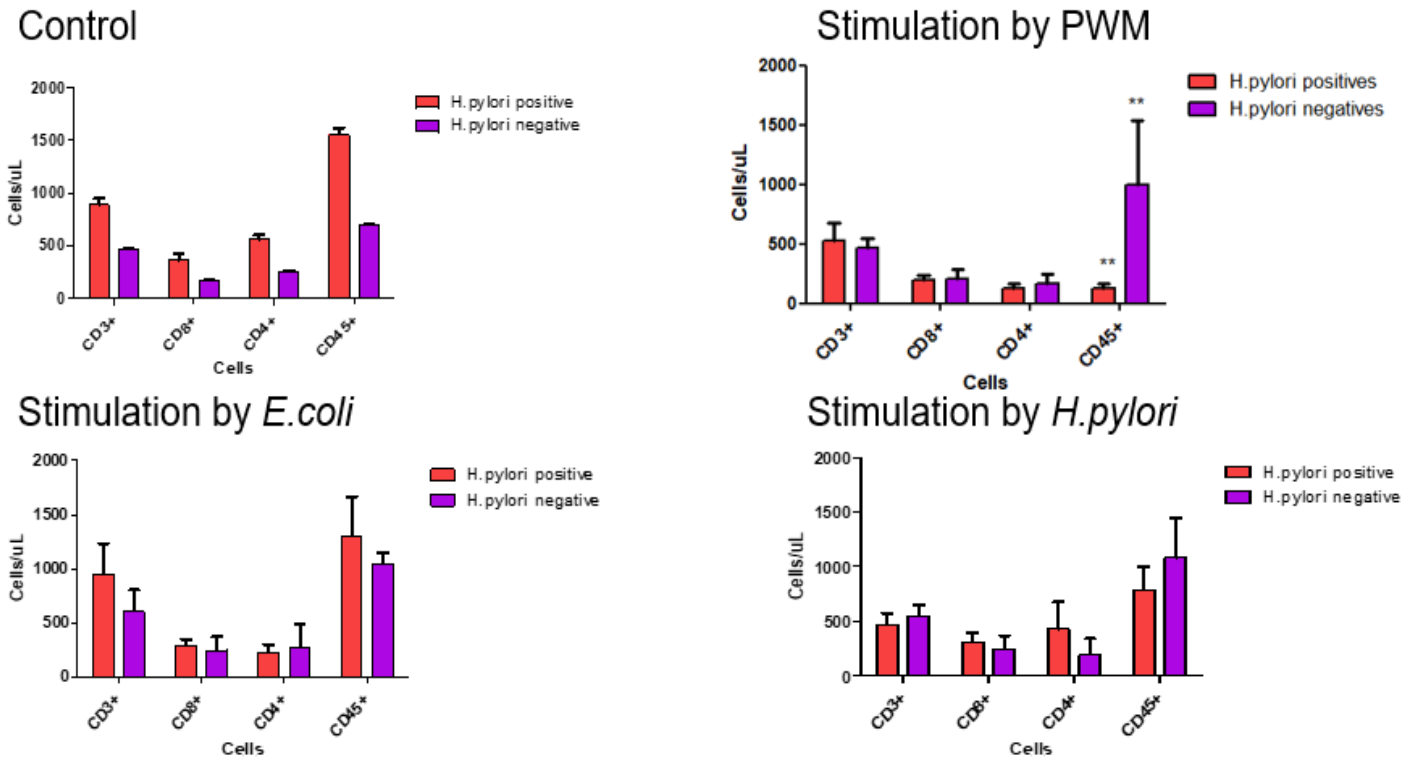

Figure 22: Lymphocyte enumeration post in-vitro stimulation of whole blood by *E. coli*, *H. pylori* and pokeweed mitogen (PWM). No stimulant was added in the control samples.

As shown in Figure 22, The proliferation of CD45+ lymphocytes in the presence of PWM shows a substantial difference between *H. pylori* positive versus *H. pylori* negative patients. Likewise, proliferation of CD45+ lymphocytes stimulated with *H. pylori* is lower in *H. pylori* positive compared to *H. pylori* negative patients. This pattern differs from that observed when the same cells are stimulated with *E. coli* antigens, where a much higher CD45+ count is observed,

suggesting a specific effect of *H. pylori* infection on the proliferation of certain lymphocyte subpopulations.

The CD8+ cells were higher in *H. pylori* positive samples when stimulated by both E. coli and *H. pylori* compared to the *H. pylori* negative samples. The *H. pylori* positive patient samples had a slightly higher CD4+ count when stimulated with *H. pylori* compared to when stimulated by E. coli. Estimated CD19+ cells were calculated as the difference between the CD+45 and CD3+ cells. Generally, the CD19+ estimates were lowest in *H. pylori* positive samples stimulated with *H. pylori* antigen and when the same samples were stimulated with PWM.

3.3.4 In-vitro cytokine determination-ELISA

Cytokine determination after in-vitro stimulation was done using ELISA. Standards were run and a standard curve was plotted from where cytokine concentrations were extrapolated from. The standard curve is shown in Figure 23 below

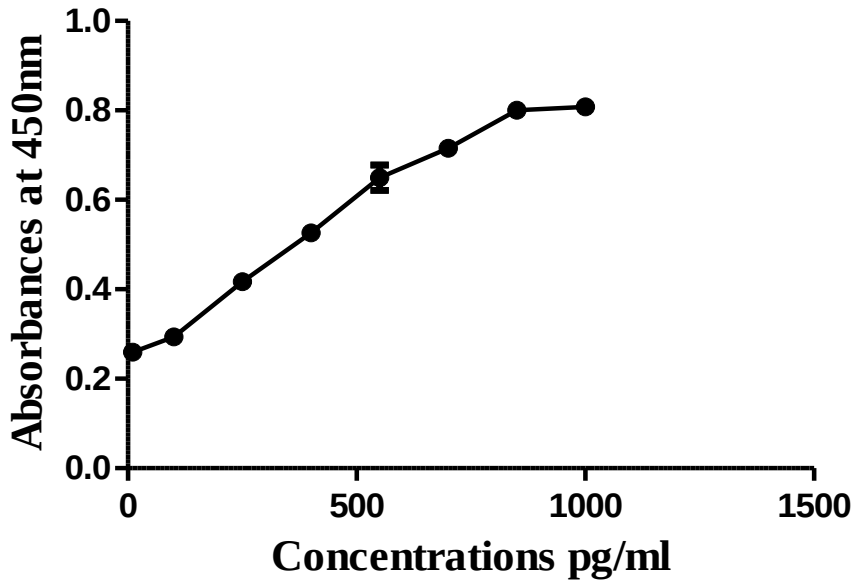

Figure 23: Standard curve of cytokine concentrations against absorbances read at 450nm wavelength.

Absorbance values were directly proportional to cytokine concentrations.

Figure 24 represents the cytokine quantifications done post stimulation by different antigens.

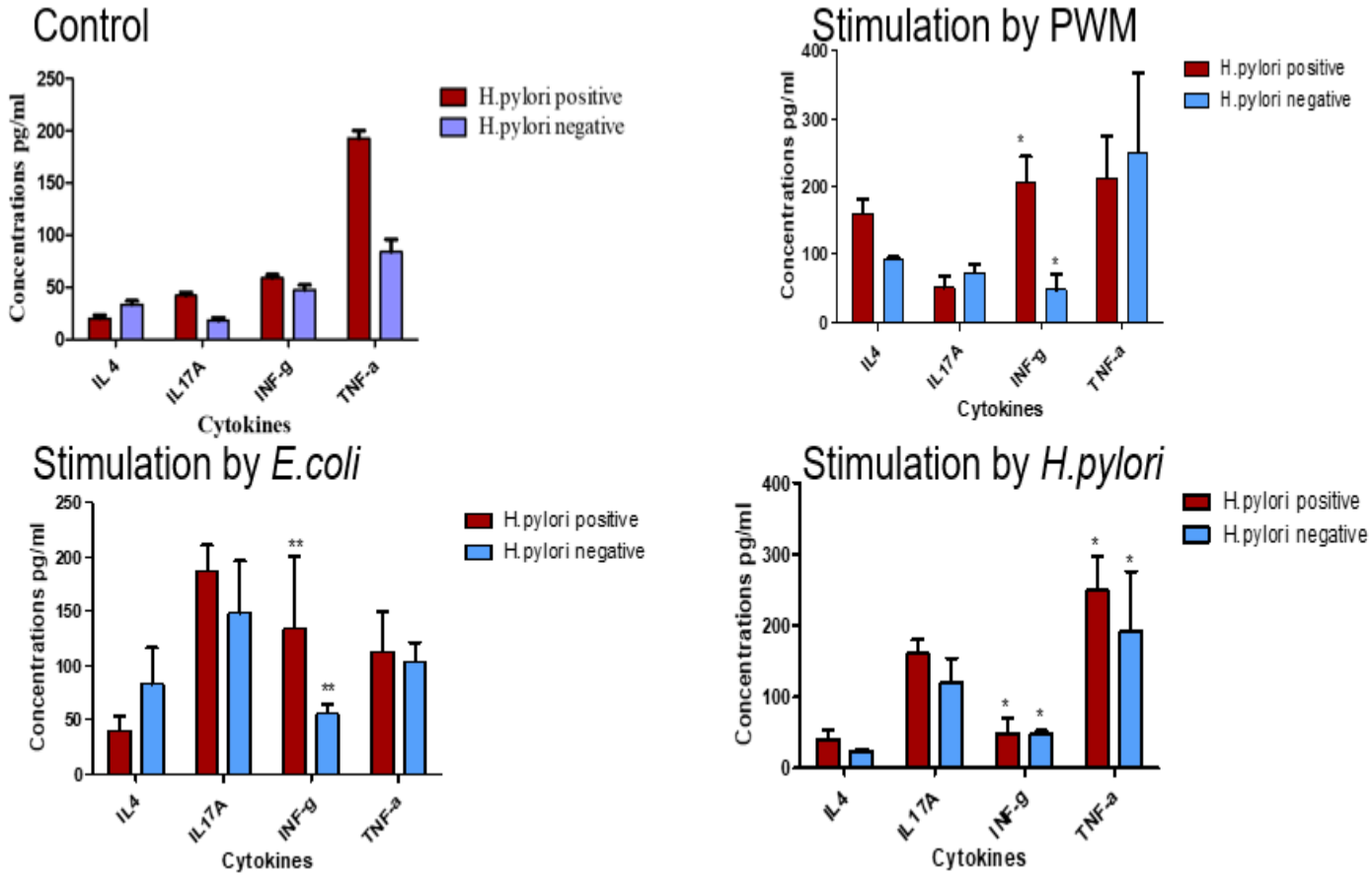

Figure 24: Cytokine level determination after stimulation with E. coli, *H. pylori* and PWM. No antigen was added in the control.

As shown in the above Figure, cytokine levels in the control samples were generally lower than those obtained after stimulation by antigens. The cytokine TNF-α was however high in *H. pylori* positive patients in the control samples.

Stimulation with PWM

When stimulated with PWM, higher TNF-α was produced especially in *H. pylori* positive samples. The levels of INF-γ (205pg/ml) were four-fold higher in *H. pylori* positive samples compared to the negative samples (50pg/ml). There was a significant difference in expression of IFN-γ between the *H. pylori* positive and negative samples, (p=0.034). Low levels of IL17A and IL4 were observed although IL4 levels were slightly higher in *H. pylori* positive samples.

Stimulation the E. coli lysate

When stimulated with *E. coli*, high IL17A (170pg/ml). Stimulation by E. coli resulted in the highest expression of IL17A in both negative and positive samples to *H. pylori*. High INF-γ levels (130pg/ml) were observed. After a Fisher's exact test was perfume, there was a significant difference in the expression of INF-γ between *H. pylori* positives and negatives (p=0.047). The cytokine IL4 was the lowest although slightly higher in *H. pylori* negatives. The expression of TNF-α was generally the same in *H. pylori* positives and negatives (100pg/ml).

Stimulation with H. pylori

Stimulation of whole blood samples with the *H. pylori* antigen resulted in expression of high levels of IL17A (180pg/ml) and TNF-α (260pg/ml). These cytokines were mostly expressed in large quantities by *H. pylori* positive samples. Stimulation by *H. pylori* did not upregulate or produce high levels of IL4 (43pg/ml) and INF-γ (50pg/ml). Production of INF-γ was the same for both *H. pylori* negative and positive samples. After a Fisher's exact test was done, significantly lower levels of INF-γ expressed in both *H. pylori* positives and negatives were observed as compared to the TNF-α expression (p= 0.047).

Cytokines expressed after stimulation by *H. pylori* antigen only are shown in Figure 25 below.

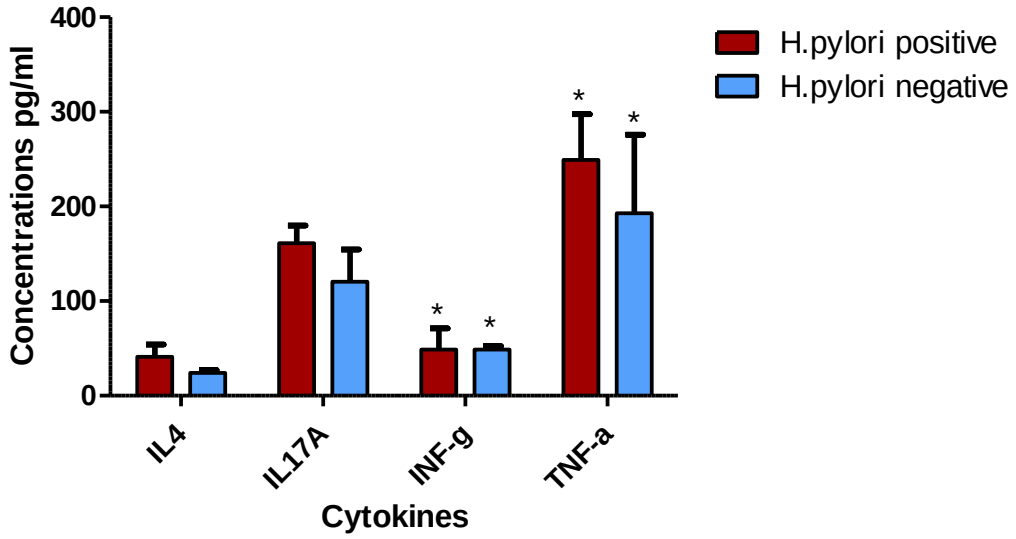

Figure 25: Graph of cytokine levels after in-vitro stimulation of whole blood cells of *H. pylori* negative and positive samples by *H. pylori* antigen

4.1: DISCUSSION

4.1.1 Retrospective study

The retrospective study on archived remnant samples of patients diagnosed with autoimmunity and or *H. pylori* was done so as to give an insight of the prevalence and immune-epidemiology of autoimmunity, *H. pylori* and *H. pylori*-associated autoimmunity in Zimbabwe. Samples used in the retrospective study were from January 2016 to January 2018 so as to give a most recent picture of the prevalence and epidemiology of the mentioned conditions as no studies regarding *H. pylori*-associated autoimmunity have been done leading to unknown prevalence of disease until this study was done. This part of the study gave a pre-amble to the prospective study where samples from walk in patients were used to determine if the immunological responses of disease in the archived samples were the same in a much recent era so as to effectively conclude on the prevalence and immune-epidemiology of *H. pylori* and *H. pylori*-associated autoimmunity in Zimbabwe. The clinical records and laboratory review also gave direction and highlighted he main focal points for the prospective study and the prospective study gave insight to avenues for further research on the study topic.

The retrospective study section summaries the results of an audit review of the profile of 1500 patients who attended the AAIC, a specialist referral center in Harare, Zimbabwe. There is one autoimmunity specialist clinic in Zimbabwe therefore an assumption was made that the patients attending the clinic were representative of the whole population. The epidemiology patterns are therefore a reasonable representation of the population and should provide a credible panorama of the epidemiology patterns in Zimbabwe. These patients were clinically evaluated, met the obtaining international clinical and laboratory guidelines for the diagnosis of the respective conditions. Medical practitioners in private practice primarily referred all the patients to the AAIC. The need for referral suggests that the symptoms could have been more severe than can be managed at the level of general practitioners and other physicians.

Detection of autoantibodies in the samples analyzed revealed a prevalence of 50% patients that have autoimmune conditions regardless of their *H. pylori* condition as shown in Table 6. Detection of *H. pylori* infection revealed a prevalence of 33% infected people regardless of their autoimmunity condition as shown in Table 8. This prevalence was slightly lower than that found

in a cohort study done by Mitchel and Segal in 2000, where the *H. pylori* prevalence in Soweto was 48%. The *H. pylori* incidence was not significantly different between men and women suggesting that anyone can be infected by the pathogen and that there is no preferred gender. Forty percent of the population had current (IgA positive) infection. This is a significant statistical figure which may be associated to poor hygienic standards in the country especially in the capital CBD. Another factor contributing to the high prevalence is antibiotic resistance as treatment of the infection require antibiotic combination therapies. Moreover, the genome of *H. pylori* changes continuously during chronic infection of the gut by recruiting small pieces of foreign DNA from other *H. pylori* strains during transient mixed infections (Falush *et al.*,2001).

The prevalence of *H. pylori*-associated autoimmunity was 18%. This may be statistically low but has biological significance. Considering that most patients included in the study were referral patients, there is a possibility that some patients could have had symptoms of disease but had no access to the private clinic as it is the only autoimmunity health care facility in Zimbabwe. The prevalence of co-existing *H. pylori* infection and autoimmune disease could have been higher than reported in this study.

4.1.2 Immuno-epidemiology analysis

As shown in Figure 11, systemic sclerosis is the autoimmune condition that is mostly associated with *H. pylori* infection in this study. It is not clear whether *H. pylori* infection causes autoimmunity or whether autoimmunity predisposes one to *H. pylori* infection. The association between *H. pylori* infection and systemic sclerosis could be due to the bacterium's spiral nature that prefers to bore through thickened tissues of the gastric mucosa as a result of the mucosal changes that are a feature of gastrointestinal manifestations of systemic sclerosis. The high prevalence of *H. pylori* infection in systemic sclerosis patients could also be as a result of defective peristalsis which is one of the gastrointestinal manifestations of the disease. This was presumption is supported by the observation of an association between gastrointestinal dysfunction and the frequent presence of anti-*H. pylori* IgG antibodies in the same patients. In contrast to our findings, a study done by Danese *et al.*, 2000 did not report any significant difference in the prevalence of the *H. pylori* infection in systemic sclerosis patients as compared to controls although they reported 85% presence of *H. pylori* CagA strain in scleroderma patients compared to the VacA strain found in the controls (Danese *et al.*,2000).

Thirty-nine percent of autoimmunity patients with *H. pylori* infection had SLE. A study done by Yamanishi *et al.,* revealed that urease, secreted by *H. pylori*, was able to induce SLE-related autoantibodies in mice, such as anti-ssDNA (Yamanishi *et al.,* 2006). In our study, we found that the autoantibody mostly expressed in SLE patients positive to *H. pylori* was dsDNA. This could suggest that the *H. pylori* strain in Zimbabwe induces expression of dsDNA. Contrary to our findings however is a study done by Sawalha *et al.,* 2004 which found that seropositivity to *H. pylori* was less likely in SLE patients compared to controls. From their study it appears that infection by *H. pylori* may indirectly have a protective effect on the development of SLE although the exact mechanism in this observation remains elusive (Sawalha *et al.,* 2004).

Myositis was least associated with *H. pylori* infection. Generally, myositis prevalence is low in Zimbabwe according to our findings in this study. However, its association with *H. pylori* infection could be due to simultaneous usage of antibiotics with omeprazole in treatment of *H. pylori* where omeprazole has been observed to potentially render toxic effects such as myopathy. A study done by Visruthan in 2012 revealed a pediatric Asian patient who was suspected to have developed myositis as a result of combination therapy that included omeprazole. The exact mechanism for omeprazole-induced myositis is not understood. However, the cytochrome CYP2C19 metabolizes omeprazole where mutations in this cytochrome may lead to inefficient metabolism of omeprazole. The high omeprazole levels in the patient system would cause toxicity implicated in the pathogenesis of muscle disorders (Visruthan *et al.,*2012).

Figure 12 represents the gender distribution of co-existing disease against presence of autoimmunity disease alone. Generally, women had a higher incidence of co-existing disease than men. This trend was also observed in the general prevalence of autoimmunity regardless of *H. pylori* infection. This could be due to an interplay of hormones. Estrogen upregulates secretion of auto antibodies while testosterone suppresses cytokine BAFF which is elevated in *H. pylori* infection. Cytokine BAFF stimulates B-1 cells to secrete IgM and IgG type auto antibodies that are implicated in the pathogenesis of autoimmunity. In women to avoid double expression of genes on the X-chromosome, one of the two X chromosomes is inactivated. No particular order in as far as which X chromosome is inactivated is used for this inactivation and this process happens by chance. Usually there should be 50:50 distribution of the 2 cell types (Quintana-Murci *et al.,* 2001). The inactivation can be skewed where there is substantial deviation from a 50:50 distribution and this skewed X-chromosome inactivation predisposes women to autoimmunity if the active

chromosome has X-linked autoantibody genes. These genes are not sufficiently presented in the thymus during tolerance induction but can be expressed in the peripheral organs enough to trigger the onset of auto antibody production.

In females, a Th2 response to infections that is mediated by IL4 protects them from severe inflammation by the inhibition of IFN-γ production and by increasing anti-inflammatory Treg cell populations. In males, an increased proinflammatory Th1 response to infections induces severe acute inflammation putting men at risk of early death such that males that are susceptible to develop chronic autoimmune diseases may not survive long enough to develop disease. This generally explains why the prevalence of co-existing disease is higher in women than in males.

Another observation that we made from the results in Figure 12 was that co-existing *H. pylori* infection and autoimmune disease seems to be more prevalent than autoimmunity alone for all the three *H. pylori* associated diseases that we investigated. This suggests that chronic inflammation brought about by the response to *H. pylori* infection in the gut possibly drives a trigger for autoimmunity. Persistence of *H. pylori* infection increases secretion of urease which triggers B-1 cells to produce auto-antibodies as alluded before.

Figure 13 and 14 represent the age distribution of patients with co-existing disease. The most prevalent age group being the adult's group (20-45years) particularly females. Higher prevalence was found to be in people above 20. Generally, the risk for developing an autoimmune disease increases as the number of autoantibodies increases, and the number of autoantibodies increases with age, regardless of gender (Fairweather and Rose, 2007). This aligns with our findings where expression of auto antibodies was in people 20 years and above. Considering that initial *H. pylori* infection occurs during childhood and in early life, it is possible that inflammation from chronic infection by *H. pylori* during in the earlier years drove towards expression of autoantibodies and development of autoimmune disease later in life.

There was no relationship between autoimmunity and *H. pylori* in adolescent patients aged between 13 and 19 as shown in Figure 13. Disease may have existed alone and not in co-existence. This could have been due to the fact that this period is associated with the onset of puberty and production of sex hormones. Sex hormones such as estrogen indirectly upregulate the production of auto antibodies. As production of the sex hormones only begins during this period, the development of auto antibodies may not be rapid and would only occur during one's middle life

and in advanced age. From literature, autoimmunity alone is generally known to be prevalent in middle-aged women and in children (Eaton *et al.*, 2007). We observed a relationship between *H. pylori* infection and autoimmunity in female children as shown in Figure 13. Considering that *H. pylori* initially colonizes the gut during childhood and autoimmunity is naturally prevalent in children, this relationship was expected.

The prevalent auto antibodies that are associated with *H. pylori* infection are shown in Figure 15. These were mostly those specific for systemic sclerosis. The most associated systemic sclerosis-specific auto antibodies were RP155, PM-Scl 100, PCNA, Th/To, and Ku. The SLE specific autoantibody dsDNA was also prevalent. This provides more evidence that *H. pylori* infection is mostly associated with systemic sclerosis followed by SLE as alluded earlier. Interestingly Jo1 and Scl 70 were not associated with *H. pylori* infection at all although PM-Scl 100 and Scl 70 together make the exosome complex. This suggests that there could have been strict molecular mimicry between *H. pylori* nuclear or surface antigens and nuclear antigens used in scleroderma diagnosis. Studies done by Heneghan *et al.*, 2000 showed that the lipopolysaccharide O-specific chain of the *H. pylori* reference strain NCTC (National Collection of Type Cultures) 11637 has some antigenic determinants that are similar to human Le^x blood group antigens, while that of MO19 strain has determinants similar to human Le^y. Other blood group antigens such as the H type 1, Le^a and sialyl Le^x have been detected in various *H. pylori* isolates.

Expression of Th/To and Ku *H. pylori*-related autoantibodies was gender specific. These were only expressed by females. After performance of a Fisher's exact test, there was significant gender bias towards the expression of Th/To ($p= 0.023$). A possible explanation for this gender specificity in the expression of these 2 auto antibodies is that they could be found predominantly on the male X chromosome where after skewed inactivation, these are predominantly expressed. If there are certain epitopes on the bacterial surface that mimic Th/To and Ku during infection, anti-Th/To and Ku autoantibodies could be mounted against host tissue leading to the *H. pylori*-associated autoimmune conditions in women.

In comparison to patients with co-existing disease, we studied the auto antibodies expressed by patients with autoimmune conditions only. Figure 16 represents an array of the predominant autoantibodies. We found that the prevalent auto antibodies were those that can be used for diagnosis of either scleroderma or SLE. The autoantibody SS-B is usually a rare autoantibody

associated with severe disease. Others were sclerosis specific including Jo1 and Scl 70 which were absent in patients with co-existing disease. Other autoantibodies were specific for SLE such as dsDNA and histones, while others were specific for myositis although in lower frequencies. This further suggests that there is possible molecular mimicry of antigens in the case of co-existing *H. pylori* infection and autoimmune disease. Another reason for expression of Scleroderma autoantibodies in *H. pylori* infection is irregular activation of B cells by *H. pylori* that stimulates antibody secretion that is polarized to sclerosis specific autoantibodies.

After a review of the symptoms presented by patients with co-existing disease, and correlation of autoantibodies detected and symptoms presented, we found that the most predominant symptoms were joint pain, thickness of skin, white dusty skin and urticaria. These co-relate largely with scleroderma suggesting a strong link between *H. pylori* and sclerosis.

Cytokine analysis done on the remnant samples using flow cytometry were carried out in order to determine the cytokine profile in *H. pylori* associated autoimmunity. To effectively analyze this analysis was done in autoimmune positive patients that were either positive or negative to *H. pylori* shown in Figure 17. These patients were compared to patients negative to autoimmunity but were either positive or negative to *H. pylori* infection shown in Figure 18.

As shown in Figure 17, where all patients represented here had autoimmune conditions, there were high levels of IL-6, IL-10 and TNF-α in both *H. pylori* positive and negative patients. Considerably significant levels of IL2 were observed in *H. pylori* positive patients while IL17A was high in *H. pylori* negative patients. As expected in *H. pylori* positive patients, TNF-α and IL2 were high as the response to infection in the gut is Th1 mediated. However, the INF-γ levels were unexpectedly lower. This was in contrast to a review done by Elios *et al.,* 2004 where IFN-γ levels were higher in gastric ulcer patients (Elios *et al.,*2004). This could be due to antigenic variations of the pathogen strain that preferentially stimulates production of TNF-α more than INF-γ as *H. pylori* may incorporate foreign DNA pieces in mixed strain infection.

The cytokine IL 10 was high in both groups and this was expected as it is an immunoregulator of inflammation brought about by both diseases. The T regulatory cells (Treg) are thought to suppress immune responses. A study done in mice infected with Cag A *H. pylori* strain showed that the T regulatory (T-regs) lymphocytes suppressed inflammation and encouraged persisting colonization. In the infected mice, IL10 levels were higher (Mohammadi *et al.,* 1996). This Treg response may

contribute to the prolonged harmonious co-existence with the human host. Infection with *H. pylori* is associated with reduced proliferation of CD45+ lymphocytes. Further studies are required in order to establish which subsets are preferentially inhibited and to determine the possible mechanistic association between the reduced proliferation and the persistence of *H. pylori* infection. The cytokine IL17A was only high in *H. pylori* negatives suggesting that it is elevated in autoimmune inflammation. Cytokine IL6 can act as a pro or anti-inflammatory cytokine. In both cases it was elevated. In the case of *H. pylori* infection, its high levels could have been to pro-inflammatory action as it can be secreted by macrophages in response to *H. pylori* pathogen-associated molecular patterns (PAMPs) which bind to Toll-like receptors (TLRs). These are found the cell surface components and induce intracellular signaling cascades that results in inflammatory cytokine production. In the case of autoimmunity alone, IL6 could have been an anti-inflammatory cytokine to regulate autoimmune inflammation especially in SLE patients as it is a marker of inflammation in these patients. The Treg cytokines in *H. pylori* positive patients with autoimmunity were reduced compared to the *H. pylori* negative patients. This suggests that the disturbance of T regs is permissive to infection by *H. pylori*.

Figure 18 represents all patients who had no autoimmune condition but were either positive or negative to *H. pylori* infection. As can be seen, all patients had relatively low cytokine levels particularly the group that was neither positive to *H. pylori* nor autoimmunity. In the *H. pylori* positive patients however, slightly higher IL6, IL 10 and TNF-α were seen. This is expected due to the nature of immune response to *H. pylori* infection that has been discussed above. Generally pro-inflammatory cytokines were higher in co-existing disease more than in either disease.

To verify if pro-inflammatory cytokines induced by *H. pylori* infection would trigger autoimmune inflammation, a prospective study was done where in-vitro stimulation of whole blood cells of *H. pylori* patients was done and cytokines produced determined. Autoimmune inflammation specific cytokines such as IL17A were measured in the *H. pylori* positive patients to determine if *H. pylori* infection could possibly drive towards autoimmune inflammation.

4.1.3 Prospective study

In the prospective determination of cytokines by ELISA as shown in Figure 24, stimulation of cells by *H. pylori* antigen resulted in high TNF-α and IL17A cytokines. These were particularly higher

in *H. pylori* positive samples. The cytokine INF-γ was again in low quantities and were generally the same for both positive and negative patients to *H. pylori*. The high TNF-α and low INF-γ seen in the remnant samples was observed again in the in-vitro stimulation study. This suggests that the *H. pylori* strain in Zimbabwe could possibly have specific antigens that preferentially stimulate TNF-α-mediated response. This information may imply that the lipopolysaccharide or nuclear antigens could be the immunodominant antigens on *H. pylori* which has preferential presentation on specific Th1 cell population which is polarized towards more of TNF-α production.

High levels of IL17A were observed to be associated with high TNF-α levels when cells were stimulated with *H. pylori* antigen as shown in Figure 25. This suggests that *H. pylori* infection indirectly upregulates production of IL17A which is elevated in autoimmune inflammation suggesting that untreated, resistant or chronic *H. pylori* induced inflammation could drive autoimmune inflammation. The IL4 levels were generally low in both *H. pylori* positives and negatives suggesting limited involvement of Th2 cells in the response to *H. pylori*. Several studies have however suggested that the polarized Th1 response in *H. pylori* infection is related to severity of gastric ulcer disease where activation of a Th2 response and reduction of the Th1 response attenuated dyspeptic symptoms in these patients (Elios *et al.*,2004).

When cells were stimulated with *E. coli,* there was high INF-γ production in contrast to the *H. pylori* stimulation. High TNF-α and elevated IL17A were however observed. Low IL4 levels were also observed as was the case in stimulation by *H. pylori*. We suggest that in this cohort, there is no preferential Th1 cell activation by *E. coli* as was proposed to be the case with *H. pylori* infection.

The idea of using a mitogen as a positive control was due to the fact that it is a non-specific proliferator of cells with no expected antigens to polarize towards production of cytokines from a specific subset of lymphocytes. We used PWM which is a B lymphocyte stimulator. In the study, as expected in *H. pylori* positive patients, there were high levels of TNF-α and even INF-γ which was unexpectedly lower when stimulation by *H. pylori* was done. This indicates that even the cells that otherwise not stimulated by *H. pylori* to produce INF-γ were stimulated by the non-specific mitogen enough to produce high INF-γ. This assured us that there were no reagent or procedural incompetence that could have resulted in detection of low INF-γ levels in cells stimulated by *H. pylori* antigen.

In-vitro proliferation assays have been used successfully in many studies, to determine cytokine production. However, there are several limitations with these methods. Firstly, cytokines act mainly at a localized level where after exerting their functions are quickly cleared from circulation. Cytokines are also active at low concentrations where they are only expressed after cellular activation which makes their determination rather difficult in-vitro. The culture procedures done prior to quantification of cytokines may wash out essential immune molecules that are important for molecular networks between immune cells (Foster, 2011).

4.1.4 Lymphocyte enumeration

Lymphocytes were counted using flow cytometry. Infection by HIV was excluded in all samples by antibody testing using rapid kit testing. HIV infection has confounding effect on the lymphocyte populations. As shown in Figure 20, CD3+, CD4+, CD8+ and CD45+ lymphocyte populations were higher in *H. pylori* positive patients than in *H. pylori* negative patients. The pathogen elicits an antigen -specific cell mediated and humoral response as shown by the high CD45+ cells, which include both B and T lymphocytes.

Elevated T cells in the mucosa supports this observation. Infection by *H. pylori* leads to the production of gastric lymphoid follicles which decline in number after eradication of infection. Lymphoid follicles are B cell rich. This suggests that specific stimulation by *H. pylori* antigen is a pre-requisite. The CD4+ T cells play an important role in antigen-specific immune responses therefore infection is related to an increase in gastric CD4+ T cells (Hatz *et al.*, 1996). A study done by Romi *et al.*,2011 revealed that live *H. pylori* bacteria activate CD3+ cells and also upregulate CD69 which is an early activator marker antigen of lymphocytes. Activated CD3+ cells are responsible for secretion of TNF-α and INF-γ seen in *H. pylori* infection. The CD4+/ CD8+ ratio was generally low in both *H. pylori* positive and negative patients. This suppression of cells could be as a result of possible autoimmunity inflammation.

4.1.5 Lymphocyte enumeration after stimulation with antigens

Figure 21 represents the total lymphocytes obtained after stimulation was done by *E. coli*, *H. pylori*, PWM and what was observed in the negative control with no stimulant. The highest CD45+ count was in *H. pylori* positive control samples and also in both *H. pylori* positive and negative samples stimulated with *E. coli*. The least CD45+ was observed in the positive controls, stimulated

with PWM, especially in *H. pylori* positive samples. This low stimulation was attributed to by the fact that PWM is a mitogen that mainly stimulates B-cells more than T-cells hence there could have been preferential stimulation of B-cell population. This means that the stimulated cells in the CD45+ population was not representative of the entire lymphocyte population. Stimulation of CD45+ cells by *H. pylori* antigen was lower than in the case of *E. coli* particularly in *H. pylori* positive samples. Infection with *H. pylori* is associated with reduced proliferation of CD45+ lymphocytes, which include all T lymphocyte subsets as well as all B lymphocyte subsets. This may imply that presence of H. *pylori* infection may suppress the immune cells particularly the lymphocytes rendering patients considerably immune-deficient.

It is important to establish which subsets are preferentially inhibited and to determine possible mechanistic association between the reduced proliferation and the persistence of *H. pylori* infection. This will require proliferation assays using sorted lymphocyte subsets and a differentiation of this effect on B1 and B2 lymphocytes. A correlation should be made between these lymphocytes and TLR to establish whether impaired innate TLR recognition has a role to play in the persistence of *H. pylori* infection.

The stimulation profile of specific cell populations by various antigen were studied and represented in Figure 22. When the negative control stimulation profile was compared to the positive controls, all cell populations were higher in the negative control where no stimulant was used. This was due to the B-cell polarized stimulation that occurred when mitogen was used and non-specific stimulation that occurred in the negative control.

Comparing stimulation by *E. coli* to that observed by *H. pylori*, the CD45+ and CD3+ lymphocytes were generally higher in stimulation by *E. coli* than in *H. pylori*. When stimulated by *H. pylori*, these cells were particularly lower in *H. pylori* positive samples. Impaired innate TLR recognition may have a role in persisting *H. pylori* infection. This may predispose to the development of autoimmunity on a background of chronic *H. pylori* colonization. The B-cell population recognized by the CD19+ count, was not measured in the study however it can be estimated by the difference between the CD45+ and CD3+ counts. In the case of *E. coli* stimulation, the estimated B-cell population in both *H. pylori* positives and negatives was higher than the estimate in *H. pylori* stimulation. Moreover, as mentioned earlier that PWM particularly stimulates B- cells, the observation that the CD19+ estimate was significantly suppressed in *H. pylori* positives indicates

that the B- cell population is critically suppressed in *H. pylori* infection. This alteration in the B-cell compartment may have various reparations such as low production of antibodies against the pathogen which may indirectly contribute to persisting infection.

The CD8+ and CD4+ cell populations were generally lower in both cases although slightly higher CD4+ cell count was observed in *H. pylori* positive samples stimulated by *H. pylori* antigen. The slight increase in CD4+ cells may indicate slight activation of a Th2 response that may occur during prolonged colonization by *H. pylori* in addition to the initial Th1 response. This slight involvement of Th2 response may initiate systemic autoimmune inflammation. Contrary to our findings, a study by Hayata *et al.*,2004 reported higher CD8+ in *H. pylori* positive samples stimulated by *H. pylori* antigen. Their study also revealed a high CD3+ count and the explanation for this response was that the gut entraps these cell subsets as a typical host immune response to *H. pylori* infection.

4.2: CONCLUSION AND RECOMMENDATIONS

4.2.1 Conclusion

The study aimed at investigating the immune-epidemiology of *H. pylori*-associated autoimmunity in Zimbabwean patients. The study revealed an autoimmunity prevalence of 50% and an *H. pylori* infection prevalence of 33% in Zimbabwe. The prevalence of autoimmunity and *H. pylori* infection is higher among women. The prevalence of co-existing autoimmune autoantibodies/disease and *H. pylori* infection is 18%. The association of *H. pylori* infection and expression of autoantibodies is greater among systemic sclerosis patients, SLE and myositis respectively. Adult women of ages between 20 and 45 are the most vulnerable to *H. pylori*-associated autoimmunity in Zimbabwe. The auto antibodies associated with *H. pylori* infection are anti-RP155, anti-PM-Scl-100, anti-Th/To, anti- Ku, anti-dsDNA and anti-PCNA where anti-Th/To and anti-Ku are only found in women. There could be possible molecular mimicry between the antigens on the lipopolysaccharide or nuclear compartment of *H. pylori* bacteria and nuclear antigens found on the human host to which the associated auto-antibodies react with.

Elevated levels of pro-inflammatory cytokine TNF-α and low INF-γ are characteristic of *H. pylori* inflammation in Zimbabwean patients. Interleukin 17A is characteristic of autoimmune inflammation in Zimbabwean patients. The immune response to chronic *H. pylori* infection in the gastrointestinal tract possibly drives towards autoimmune inflammation. The Treg cytokines are possibly permissive to *H. pylori* infection. Infection by *H. pylori* potentially suppresses the immune cells particularly the B-lymphocytes. The slight involvement of the Th2 cells in immune response to *H. pylori* infection may drive towards systemic autoimmune inflammation.

We conclude that there is an association between *H. pylori* infection and the expression of auto antibodies in Zimbabwean patients.

4.2.2 Recommendations

Now that we have established the prevalence, immune-epidemiology and vulnerable groups of people affected by *H. pylori*-associated autoimmunity, the next step is to work towards effective immune-diagnostic tools in diagnosis and management of patients. We recommend that patients infected with *H. pylori* who have history of autoimmunity in their families be tested for autoimmunity as well. This diagnosis may include determination of pro-inflammatory cytokine levels. If these patients have elevated levels, immune modulators can be administered as part of the therapy prescribed for treatment of *H. pylori* infection. This may lower the chances of a patient developing autoimmunity as a result of chronic inflammation from *H. pylori* infection.

As we suspect that molecular mimicry plays a role in the association of *H. pylori* infection and autoimmunity, we recommend further studies to be done to investigate the role of molecular mimicry. Bioinformatics tools may be used to determine any similarities in the structures of the antigenic epitopes involved in *H. pylori* bacteria and autoimmunity. We also recommend further studies on the B-cell compartment in *H. pylori* and autoimmunity as we suspect that the B-cells have a role to play in autoimmunity and may be suppressed in *H. pylori* infection.

As *H. pylori* infection is associated with poor hygienic and sanitation standards, we recommend that health and sanitation standards be improved for all citizens in the country including access to clean and safe water for all. Little is known about autoimmunity in Zimbabwe and there is only one specialist in the field. We recommend that more work and funding be directed to

autoimmunity-related research in order to have a better understanding of the disease dynamics and management of patients in Zimbabwe.

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APPENDICES

Appendix 1: Cytokine Standard curve. The standard curve was plotted using duplicate measurements of the standards. A mean of the two values was use =d to plot the reference curve (Figure 23)

Standard concentration (pg/ml)	**A_{450}**	**A_{450}**
10	0.263	0.257
100	0300	0.288
250	0.413	0.422
400	0.520	0.532
550	0.621	0.678
700	0.714	0.716
850	0.800	0.804
1000	0.811	0.812