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A study examining chronic stress and the immune system, measuring cortisol and salivary IgA

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ABSTRACT

This study aims to assess the effect of Long-term stress {chronic stress} on the immune system. It involved the measurement and statistical analysis of Hormone cortisol concentrations and Immunoglobulin A (IgA), in saliva samples.

These samples were obtained from healthy undergraduate students who consented to giving their saliva samples. These subjects were studied over a period of six months. Health questionnaire and stress questionnaire were used to monitor their response in the six-month period. On a monthly basis, blood pressure readings, heart rate and samples of whole saliva were collected, in addition to the health and stress questionnaire response. These samples were analysed using ELISA method to determine IgA concentrations, the Cortisol Kit was used to determine cortisol level.

The results were processed by statistical analysis; it showed a decrease in Mean IgA levels (0.27µg/ml) in relation with high Perceived mean stress rating (26.0) by Pearson's correlation ($r = -0.33$, $p = 0.03$). There was also a negative correlation for IgA and cortisol concentrations, Spearman's ($r = -0.73$, $p = 0.04$),

Kendall's_tau correlation ($r = -0.88$, $p = 0.02$). There was a positive correlation for Stress and Cortisol but the p values were not significant.

DEDICATION

This work is dedicated to the Almighty God for his Grace to successfully complete this project. To my Parents Mr and Mrs Momodu, and to all aspiring Biomedical scientist.

Hypotheses

Hypothesis one

H0: There is no significant decrease in salivary IgA concentrations as stress increases over time. (Chronic stress).

H1: There is a significant decrease in Salivary IgA concentrations as stress increases over time. (Chronic stress)

Hypothesis two

H0: There is no significant increase in salivary cortisol concentrations as stress increases over time. (Chronic stress).

H1: There is a significant increase in salivary Cortisol concentrations as stress increases over time.

Hypothesis three

H0: There is no significant increase in salivary cortisol concentrations as IgA concentrations decreases during chronic stress

H1: There is a significant increase in salivary cortisol concentrations as IgA concentrations decreases during chronic stress

Hypothesis four:

H0: There is no change in Health status for individuals who suffer continuous stressed situations over time.

H1: There is a change in health status for individuals who suffer continuous stressed situations over time.

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CHAPTER 1: Introduction

1.1 Definition of stress

On a daily basis we as humans encounter one form of stress or the other, which is actually very vital for survival, these includes response by the body e.g. the fight/flight response in threatening situations, immune response to infections. Academic examinations and assessments are common encounters for any student and are the key contributing factors to assessing the general outcomes of a student. Research has shown that this activity has been found to be quite stressful for most students... (Vivian et al, 2003).

Stress is a strong stimulus both Physiological and Psychological that can cause a physiological response. The physiological response was termed "general adaptation syndrome". … (Hans's selye…). The modern man comes in contact with less physical threatening situations that don't require physical response but psychological response (examples are: Exam conditions and other mentally stimulating task). The duration and course over which we as individuals encounter these stressful events can then define the stress as acute (short lived) or chronic (long-lived). (Suzanne.C…et al, 2004).

1.1.1 Effect of Stress and Perception

Psychological stress is known to affect immune function and predict infectious disease susceptibility…. (Sheldon…et al, 2001). Moderate or extreme stress of Academic assessments and exams can affect student performance… (Vivian Ng *et al*., 2003), these assessments are necessary to achieve their qualification over the stated

period of study. The way each student would perceive the assessment would determine his or her response to it, as been moderately or extremely stressful.

A stressful lifestyle can be deteriorating to the individual's physiological function leading to an increased level of cortisol. This can be damaging to the brain and other tissue…. (Mary Ann & Even star…. 2005). Perception of stress is usually based on what an individual would classify as a stressor. There are some experiences that are stress predictors and the frequency of encountering these experiences does have a great effect on stressing the individual, examples are chronic illness, depression, anxiety disorder, sleeping difficulties e.t.c…. (Dusselier et al…2005).

1.2 The Immune system and stress: Immunoglobulin A

Immunoglobulin A or IgA is an Antibody. This antibody reflects our capacity to fight disease. This is due to the function, which is effective in stopping germs and viruses when we breathe or eat…. (nceph.anu.edu.au/health_for_life..2004).

Immunoglobin A is very functional in oral health; it is the main immune mechanism that fights pathogen invasion in the oral cavity. Its production is regulated by neuroendocrine control. There are factors that can alter the normal functioning of this neuroendocrine control and one of those factors is stress….(Teeuw W, et al…2004).

According to. …(Armelle et al…2004) analysing the components of the immune system can help assess the effect of long-term stress, which is said to cause immunosupression. The sensitivity of the immune system to stress has been shown to be the major reason for alterations in the immune system during stress periods, particularly chronic stress….(Lourdes et al….2001).

It has been shown that an increased stress level does affect student performance. … (Vivian Ng…. 2003). There are other classes of Antibodies which includes; IgM, IgE, IgD, IgG. The function of these antibodies is intertwined with the unique structures that enables them function properly.

1.3 Hormones, cortisol and Stress

Cortisol hormone reflects how the body responds to stress and help us adapt on a daily basis to challenges. This is produced by the HPA axis activity where the secretion of corticotrophin releasing hormone (CRH) activates other secretion that produces cortisol. … (Carmine et al…2003). The main hormones produced by the body during stress periods are catecholamines by the adrenal medulla and the glucocorticords by the cortex, which results from HPA activity.

The production of glucocorticoids (cortisol hormone is in this group), is increased by increased activity of the HPA in long-term stress… (Armelle…et al, 2004). The function of the hormones produced by the activity of HPA axis varies depending on the situations. Certain studies have acknowledged the effect of the glucocorticoids in Phobias… (Soravia et al…2006). The hormone of interest to this study is the glucocorticoid hormone Cortisol, this hormone is the primary hormone produced during stressful situations. Cortisol has been said to affect the initiation of some chronic diseases like Rheumatoid Arthritis and idiopathic Arthritis…. (Straub et al…2005).

The release of cortisol in the body follows a pattern referred to as the circadian cycle or pattern. In this pattern cortisol levels is highest in the mornings soon after awakening, then peaks and starts to fall. The levels of cortisol are usually lowest in

the night. Repeated stress activation of cortisol can alter the circadian pattern… (Angela Clow…2004). The varied function of cortisol helps facilitate survival of stressful events by mechanisms such as; Suppressing Inflammation, Stimulating Cardiac output, breaking down muscle protein and fats and a host of other mechanisms all to aid survival in stressful conditions.

1.4 Previous Studies on IgA and Stress

This was a study of 30 healthy day-active Young adults, which involve using their awakening cortisol response to measure IgA and Cortisol, it was observed that the levels of IgA measured in saliva samples of healthy individuals was down regulated during periods of Chronic stress. This study had results as following:

- Marked elevation of cortisol from awakening level over succeeding 30mins
- IgA showed the opposite response with a marked fall from the highest first awakening concentration in the same samples over same period.
- This lead to a conclusion that there is a possibility for vulnerability to infection, since IgA is a major mucosal immunity.

(Hucklebride.F.et al…1998).

Another study conducted to review the effect of Psychological stress and Antibody response stated that there is supporting evidence to conclude that psychological stress alters immune status in human. Although these evidence were based on mostly data from in vitro studies. These studies involved the removal of immune cells or tissues, and assessing their functional capabilities. This established a proof that stress-induced immune changes do occur and this has clinical implications on altering response to immunization. (Sheldon Cohen …et al…2000).

1.5 Previous Studies on cortisol and stress

Health professionals and public sectors, have taken the consequences of Psychological stress seriously due to the effect on health of individuals .An estimate of one in every five employees are suffering from high level of stress. … (Angela Clow 2004).

In this study, salivary cortisol response was measured by using a Standardized stressor (the trier social stress test). It was stated that Cortisol hormone was identified as the primary biomarker of stress, which is due to the activity of the HPA axis following the perception of a stressor. Cortisol has been found to have diverse set of actions, which includes balance of the immune system and effect on blood pressure. It was also noted that clinical depression, which is a stressor has been associated with hyper secretion of cortisol and flattened diurnal cortisol profiles.
(Angela Clow… Cortisol as a biomarker of stress, 2004).

In another study of hormones, brain and stress, it was observed that there are two pathways to the stress system that is called the CRH1-immediate response mode, and the CRH2- the slow mode, these two have receptors, which are co-localised in the limbic neural circuitry.
Balance in both systems is essential for cell homeostasis, mental performance and health. Imbalance can be induced by chronic stressors, which would change specific neural signalling pathways. These pathways are underlying psychic domains of cognition, emotion, anxiety and aggression, which lead to cortisol-induced stress-related disorders e.g. severe depression. (de Kloet ER……Hormones, brain and stress…2003)

In a related study on chronic stress and Depression it was stated that Chronic psychosocial stress has been associated with the origin and development of depression, where increased levels of cortisol has also been observed in chronic psychosocial stress and also in depression.

(Tafet.G.E et al….2004)

1.6 Aim of study

This study like similar ones measured cortisol levels in relation to stress as perceived by the individual in half a year (six months). Assessments made monthly showed when these students had encountered stressful events, when their health statuses were different from the norm. Cases where some students had recurrent infections, insomnia, and increased consumptions of alcohol and increased smoking rates were noticeable and this was documented in their stress and health questionnaires.

The assessment made in this project work gives the basic facts about the response of healthy individuals from a student population. This population was not restricted to any ethnic minority; the individuals represented the vast population expected in a university setting with students from different ethnicity. This study is to emphasis the fact that an increased stress level does not only affect student performance but also the immune system, and this occurs over time. The experimental design made variables of correlations between cortisol, IgA and resulting assessment, which showed how much effect stress had on the immune system over the six-month period of time.

CHAPTER 2: Literature Review

2.1 Introduction

This chapter focuses on giving an in depth information on the literatures, articles, journals and other citations in the introduction. It also gives a better understanding of the variables used in this study like cortisol, immunoglobulin A, blood pressure and heart rate readings. The correlations between these variables and chronic stress would also be stated. Clarity of issue of study would also be emphasised in the various subset headings.

2.1.1 Types of Stress

According to earlier study by Hans's selye in 1935, there was a concept of stress, which he defined, that has been put in use ever since then. Stress was defined as an external strong stimulus both physiological and psychological that caused a physiological response from our body. This response according to Hans Selye was termed '*General adaptation syndrome'*. The Classification of stress as been positive (eustress) or negative (distress) was also part of his work.

In more recent times there have been various modifications to the earlier studies carried out and stress is now a more prevailing term which is used in day to day life. In a recent study by V.Vasudevan stress was defined as a conflict between the two control centres of the brain, this makes emphasis on the definition coined by Lazarus Richard a renowned stress Researcher. (V.Vasudevan…2003).

The main types of stress have been classified, as been positive type or the negative type, which still confirms Hans's selye classification of Eustress and Distress. Most researchers have different viewpoints of classifying stress. The main classification has been put forward as:

- Physiological or systemic stress: The disturbance of tissue and system.
- Psychological stress: Stress with cognative factors leading to threat.
- Social stress: stress with the disruption of a social unit or system.

(Richard L…stress and coping an anthology 1977).

In this study the main emphasis is on Physiological and Psychological effect of stress. Stress is also defined based on the duration of encounter and how the body's mechanism copes. The two types of stress based on period are; acute (short term) stress, Chronic (Long term) stress…. (Andrew, B…stress and disease processes, 1992).

2.1.2 Major Chronic Stressors

A stressor is any factor that can contribute towards an individual been stressed, these factors or challenges were described as catalyst for stress to occur (Patricia et al, 2000). This project study will make main emphasis on factors considered to be chronic stressors. There are lists of factors that contribute to individuals becoming stress over a long-term period, which includes: Traumas, persisting loneliness, Long-term financial problems and emotional distress. Other factors like Academic examinations have been reported to cause psychological stress for most students. (Vivian et al…2003). The main factors to be considered in more detail are sleep disturbances and chronic pain.

Sleep disturbances and Insomnia*:* One of the major contributors to stress has been found to be sleep disturbances or Insomnia. In a study of young adults coping with sleep disturbances, it was reported that emotional response to stress for students was highly associated with their quality of sleep…(Chien-Ming-Yang…et al 2003).

It has also been found that disturbances of sleep and arousal are common among people experiencing stressful life events. Sleep and arousal patterns are useful to help identify individuals' ability to cope with the vagaries of life.

Chronic Pain: Active disease processes, tissue damage, and other insults to our body cause chronic pain. Rheumatoid arthritis, cancer, musculoskeletal problems, cardiac disease, and headache are but a few of the conditions that can lead to chronic pain. (Angela j...et al understanding chronic pain, 2002).

Chronic wide spread pain has been associated with altered hypothalamic-pituitary-adrenal (HPA) axis function. Altered HPA axis function is known to occur in stress periods when cortisol production is prolonged. This was found in clinic studies of fibromyalgia, a syndrome characterised by chronic wide spread body pain… (John et al…. 2004).

2.1.3 Chronic stress, blood pressure and heart rate

Stress has been reported to be associated with some diseases particularly cardiovascular diseases. Increased HPA activity can cause increased glucose in blood, increased heart rate and increased blood pressure, from the effect of altered cortisol secretion from the HPA axis. Chronic stress is a major cause of this altered secretion that can occur as hypo or hyper secretions. (Karen et al…2003). It was stated that chronic real-life stress in humans appears to be associated with increased arterial pressures and impaired autonomic regulation of cardiovascular function.

(Lucini et al…2005). It has been reported that Emotional distress that is physiological, if prolonged does have effect on the cardiovascular, respiratory and immune system.

2.1.4 Methods of stress analysis

Subjects involved in stress researches have always been put through various tests to be able to analyse their stress levels. In acute stress studies, written test, arithmetic or mental stimulating procedures have been used to cause the subjects to be stressed. In a recent study by Vivian Ng, written term test were used, then subjects were asked to describe their feelings of stress in questionnaire response. (Vivian Ng... et al, 2003).

In another study of stress a standardized stressor called the trier social stress test was used to analyse stress response. There are hormones produced by the body when in stress situation such as adrenaline (acute stress) and cortisol (Chronic stress), these are referred to as biomarkers and measurement of these hormones can also measure level of stress (Angela Clow... 2004). This project study measures one of those biomarkers, which is cortisol and also uses questionnaires response to asses stress response over a period of time.

2.2 HORMONE CORTISOL

Cortisol is a hormone produced by the cortex in response to stress on a long-term basis. The group of hormones produced by the cortex and the adrenal medulla are the glucocorticoids and the catecholamines. Cortisol is a glucocorticoid hormone that is predominantly produced in primates during the activation of the HPA axis.
(Armelle… et al 2004).

2.2.1 Body Stress mechanism

The body always has a way of handling most challenges that it faces and one of such challenges is stress. The body stress mechanism combines the body and brain responses to the environment; this leads to an immediate response. The first response is by the hypothalamus, this produces corticotrophin-releasing hormone (CRH) via

CRH-1 receptors. The second slow mode response is by the urocortins (AVP Argininne Vasopresin) acting via CRH-2 receptors. The type one receptor is referred to as (Mineralocorticoid-MR) high affinity receptor and type two is the (Glucocoticord receptor-GR) lower affinity type two. Balance between these two receptors is very important to keep homeostasis in cells, mental performance and health. It was reported that chronic stress could alter or induce an imbalance in these system, leading to certain disorders e.g. severe depression. (de Kloet ER... 2003).

2.2.2 The Hypothalamus-Pituitary-Adrenal-Axis (HPA):

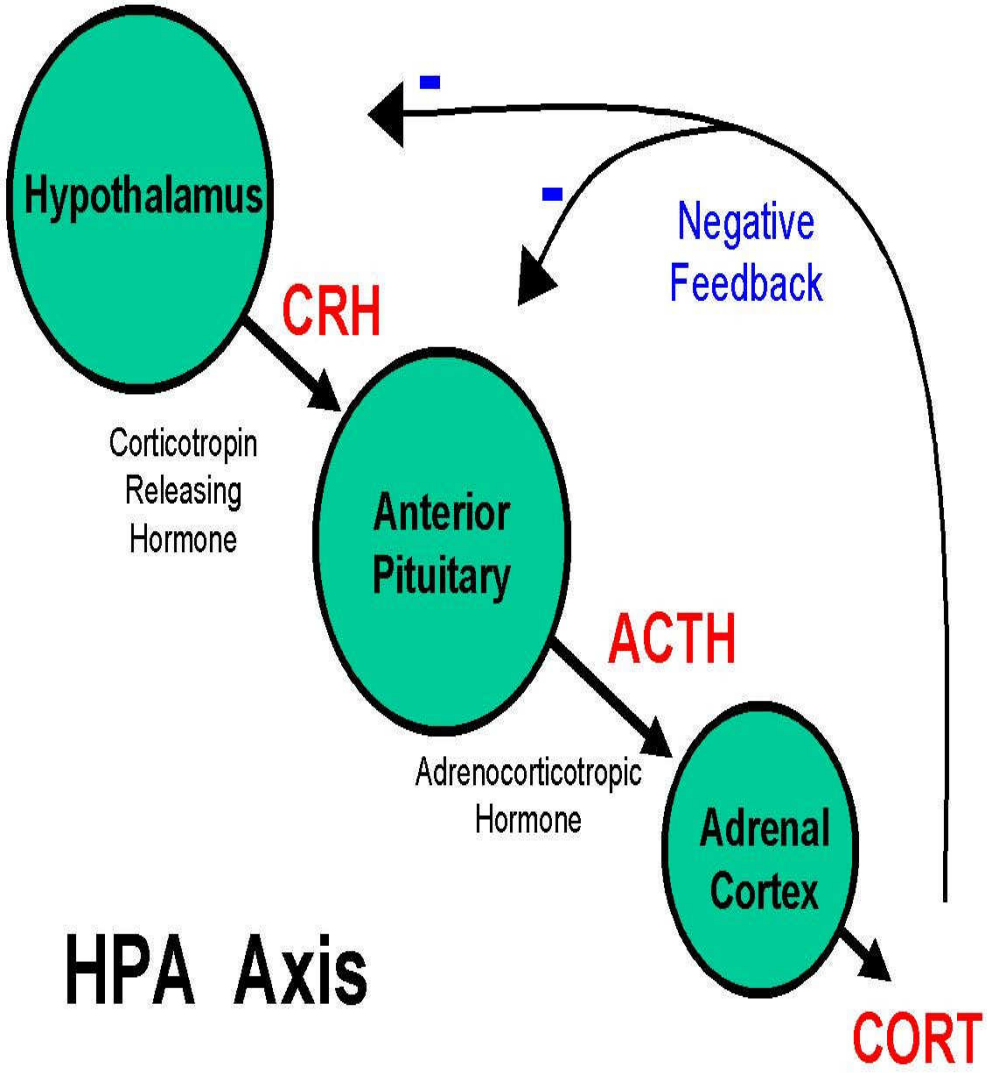
The system that responds to stress applied to the body is called the Hypothalamus-Pituitary-Adrenal (HPA) Axis. This is a major part of the neurological system and is involved in the body's reaction to stress. This is achieved by the system creating a balance with the hormones released… (Carmine et al… 2003).

The anatomy of the HPA Axis comprises part of the Hypothalamus, the anterior lobe of the Pituitary gland, the Adrenal cortices, which consist of a feedback loop that respond to cortisol levels in the body from adrenal gland and back to Hypothalamus.

The HPA axis has varied function due to the fact that it produces other hormones apart from cortisol. A stressor activates the HPA; which causes the secretion of CRH and AVP stimulates the pituitary gland to produce ACTH (Adrenocorticotropin hormone). The ACTH goes to the Adrenal cortex and causes the release of cortisol. (Femina et al…2001).

Cortisol increase is regulated by the body's endocrine and autonomic responses. The situation is different when there are elevated cortisol levels for extended periods. This leads to altered physiological function. Some of the functions that could become difficult to maintain are blood sugar levels, balance of other hormones, mucosal integrity, recovery and repair. (Functional adrenal stress profile….2005, biodia.com).

Fig 1: Diagram of the HPA axis in response to stress and cortisol production



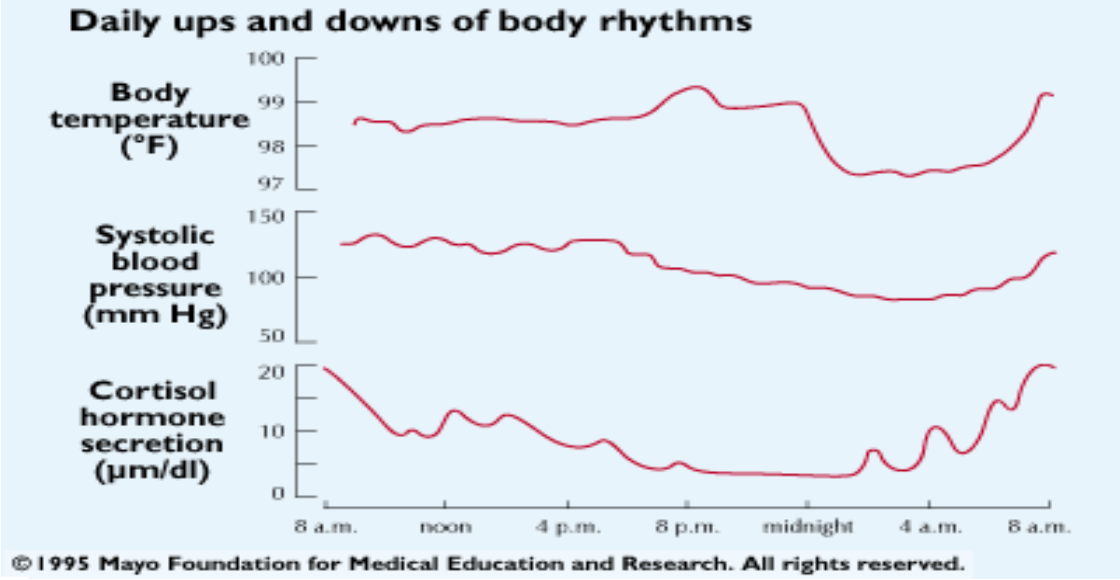
www.biology.ucr.edu/.../ Garland/HPA_axis.jpg

2.2.3 Circadian Pattern of Cortisol

The Circadian pattern of cortisol secretion is the pattern in which cortisol is secreted and regulated. This is usually seen as a steep increase in the early morning, followed

by a gradual tapering off till late evenings… (Adrenal stress test…2005). The production of cortisol been highest in the morning's help the body prepare for the challenges of the day. This pattern of cortisol is what is found in a normal adult. Variations to the pattern of cortisol secretion have been seen in cases of severe or prolonged stress situations. This is due to the fact that cortisol level stays increased. The effects of an altered pattern could suggest the presence of an abnormality. It was found that hyper secretion of cortisol was associated with flattened diurnal cortisol profiles… (Angela Clow…. 2004).

Fig 2: Diagram showing cortisol pattern, blood pressure and body temperature at different times of the day



2.2.4 Methods of Cortisol analysis

The hormone cortisol can be found in blood, urine, and saliva. The process involved in the analysis of cortisol can be very distressing if the wrong method of obtaining

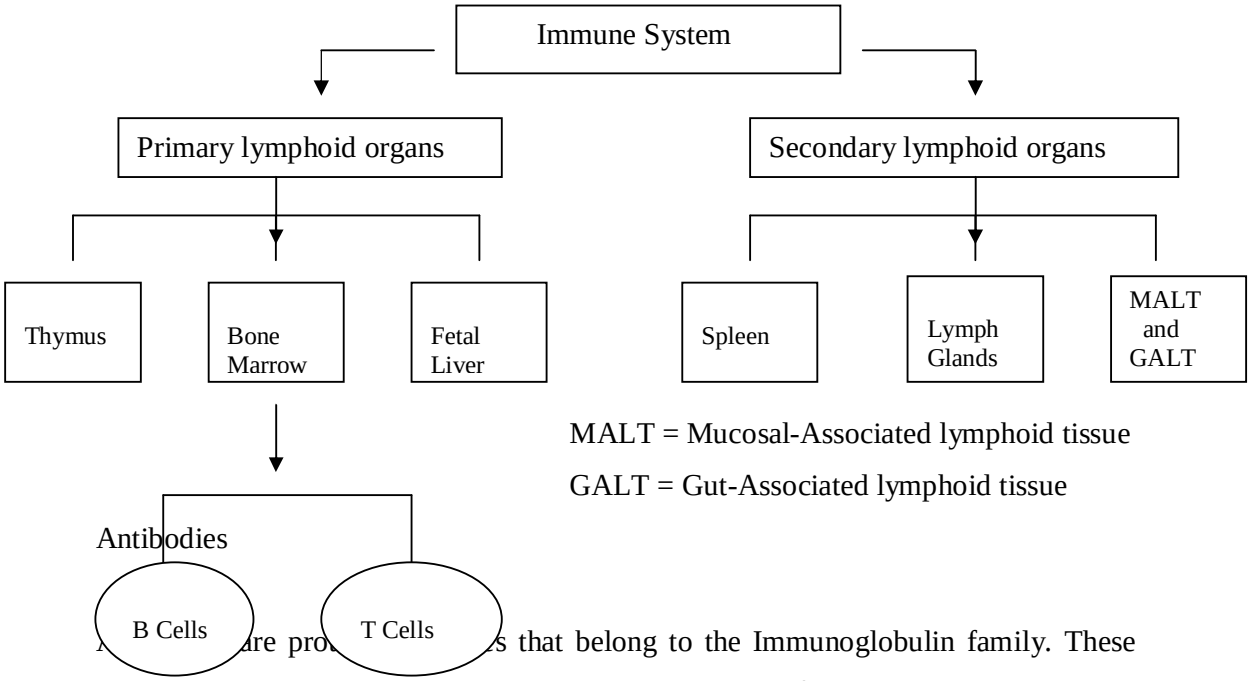
any of these samples is used. The major factor to be considered is that the method of sample collection should be less invasive as possible.

Recent research has shown that success rates have been better with the use of salivary cortisol, which shows reduced invasion to the subjects involved. In comparism to blood sampling involving vein puncture and other distressing mechanisms, these methods in themselves poise as stressful situations to the body… (Armelle et al…2004).

2.3 Immunoglobulins (Antibodies)

The immune system is made up of different components. These are the primary and secondary lymphoid organs. Antibodies are produced by the primary lymphoid organs and are known as B-lymphocytes or B cells… (Goldsby et al…Kuby Immunology…2000).

Fig 3: Flow chart showing the Basic organs of the immune system.



Antibodies are proteins that belong to the Immunoglobulin family. These molecules bind to invading micro organisms and prevent further invasion. Antibodies respond to antigens (protein molecules, micro organisms, virus and other pathogens), but in a specified manner. This mechanism is referred to as specific antigen response.

There is the primary response and the secondary response of antibodies… (Sheldon Cohen et al…2001).

2.3.2 Immune System Response

The immune system is the vital system for protection and fighting against pathogens. It has specific mechanisms of responding to challenges or situations of invasion by pathogens and other disease causing organisms. An immune response usually comprises of two parts. The first part is recognition of pathogen, the next part is to set up a reaction to remove or eliminate the pathogen. The response mounted by the immune comes in two different forms.

Innate (non-adaptive) immune response: This is known as the natural immunity, this function by a number of defensive barriers that prevent the entry of pathogens. This type of immune response is usually non specific, these can occur as:

1. Anatomic barriers: Skin surfaces of mucous membranes, very effective as a barrier to the entry of organisms. If this barrier is broken it can result in infections e.g. mosquitoes spread malaria.
2. Inflammatory response: This is the response of the body in reaction to tissue damage or pathogen invasion. This response causes vasodilatation, an increase in capillary permeability and influx of phagocytes.
3. Physiological barriers: These include soluble factors such as lysosomes, complements and interferon. Other factors like body temperature, Acidic pH e.g. in stomach, and fever response inhibits bacterial growth.
4. Phagocytic and Endocytic barriers: This involves the ingestion of part or whole microorganisms and destroying them. Some immune cells have specialised function to ingest pathogens. E.g. macrophages, dendritic cells.

Acquired (adaptive) immune response: This is a more specific type of immune response that gets better with each encounter of the organism. This response has a characteristic referred to as memory where the immune cells encounter a specific antigen and when that antigen is re encountered the immune cells remembers and is able to deal with the antigen. This type of immune response is not natural from birth it has to be acquired either by encounter or by immunization… (Goldsby et al…Kuby Immunology text…2000).

In recent studies it has been reported, "Chronic stress seems to impair the immune system's capacity to respond to glucocorticoids hormones that are normally responsible for terminating an inflammatory response following infection or injury."…. (Gregory E…Chronic stress can interfere with Immune system, 2002).

2.3.3 Structure and Function of Immunoglobulins (Antibodies)

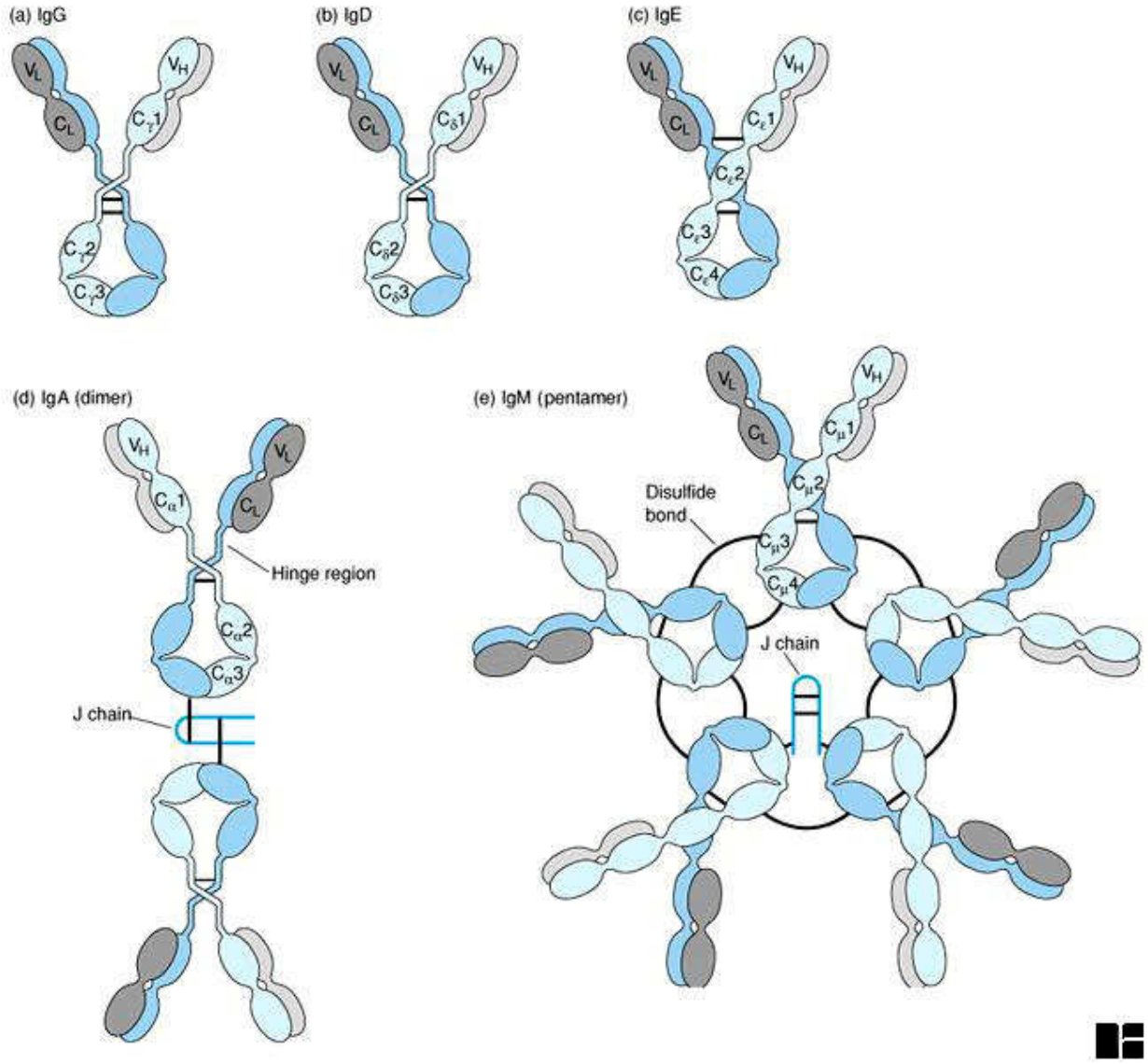
There are various classes of Immunoglobulins. These classes vary in function and structure. The main known classes are five, and these are IgA, IgD, IgM, IgE and IgG. The structure of each immunoglobulin class is made up of a heavy and light chain section, disulphide bonds. The major classes are further divided into subclasses as would be discussed.

Table 1: A table showing immunoglobulin types and their function.

Antibody class	No of subtypes	Function	Place to find it
IgA	IgA1 and 2	1. Fights pathogens that are ingested, inhaled or in contact with body surface.	Mucosal tract Intestine, stomach

			Tears, saliva, maternal milk.
IgD	No subclass	Functions as antigen receptor.	Plasma proteins membrane And Serum.
IgM	IgM1-5	Complement Activation	Serum
IgE	No subclass	Defence against parasitic worms Respond to hypersensitivity.	Saliva and other outer secretions.
IgG	IgM1-4	Protection to Foetus. Bind pathogens by activating complement.	Blood, tissue liquids, can cross placenta.

Fig 4: Five Major Classes of Immunoglobulins



General structure of the **five major classes** of secreted antibody. Light chains are shown in shades of gray and heavy chains in shades of blue; thick black lines indicate disulfide bonds. Note that the **IgG**, **IgA**, and **IgD** heavy chains contain four domains but no hinge region. The polymeric forms of **IgM** and **IgA** contain a polypeptide, known as the **J chain**, which is linked by two disulfide bonds to the Fc region in tow different monomers. Serum **IgM** is always a pentamer; most serum **IgA** exists as a monomer, although some dimmers, trimmer, and even tetramers sometimes are present. Not shown in these figures are intrachain disulfide bonds and disulfide bonds linking light and heavy chains.

W. H. Freeman & Co. and Sumanas, Inc.

Immunology, January, 1997 last updated June 25, 1997

2.3.4 Immunoglobulin A and Stress

Antibody IgA is produced by the immune system within a few weeks of antigen exposure. In the case of immunization the production is continuous for years. S-IgA in saliva is produced by the plasma cells in the salivary glands and gingival… (Sheldon Cohen et al….2001). Chronic stress has been associated with low levels of secretory IgA, which gave vulnerability for increased risk of infection… (Hucklebridge et al….1998). It was reported that mucosal immunity as reflected by salivary immunoglobulin A (IgA) levels is influenced by psychological stress. Chronic stress suppresses the production of immunoglobulin… (Vivian Ng…2003). Chronic stress has also been found to impair the immune system's ability to respond to its own anti-inflammatory signals. (Gregory E….2002).

2.3.5 Analysis of Secretory IgA in Saliva

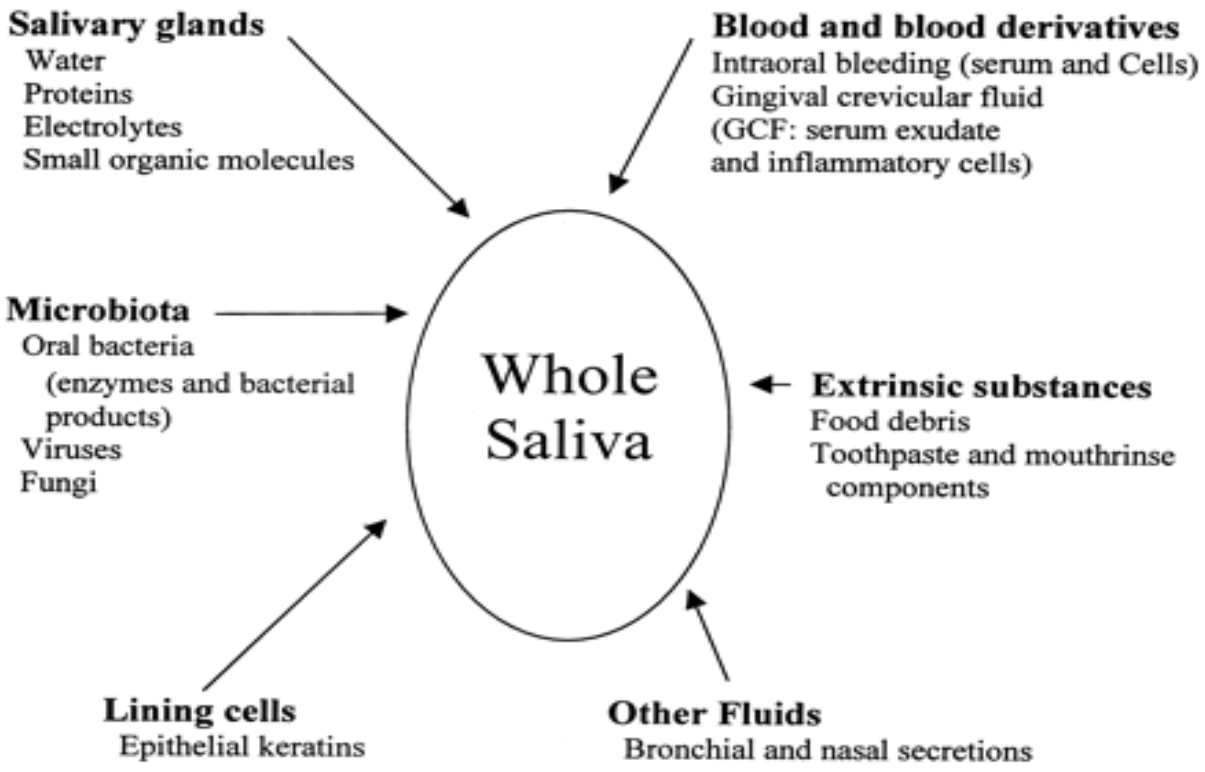
The analysis of salivary IgA would have to involve obtaining whole saliva samples from the subjects to be studied. In most research where IgA is measured in saliva, the mode of saliva collection is an important criterion to ensure that the samples are not altered. In a study of Dental carries salivary IgA in children with Down syndrome, saliva samples were collected by spitting into a tube for 5mins and IgA analysed using ELISA…

(S R lee et al….2004). In another study of undergraduate dental studies saliva samples were obtained without been stimulated for 5 mins and IgA analysed by Salimetrics HS-IgA Kit…(Vivian Ng…et al …2003). In this project study similar procedures were used to collect saliva samples and IgA was analysed using ELISA.

2.4 Contents of Saliva

Saliva samples are easy to obtain, its quick, simple and non invasive. It also contains secretory IgA and IgG, which is part of the immune system. Cortisol is another hormone found in saliva. Saliva is secreted by salivary glands; there are three main types of salivary glands paratoid glands, submaxillary and sublingual glands. The clusters of cells on the secretory glands are known as acini. These cells secrete fluids that contain mucus, electrolytes, enzyme and water. All these are collect outward and flow into the collecting duct to the mouth. One of the varied functions is in Oral hygiene where it constantly flushes the mouth cavity, taking away debris of food and other substances that can harbour germs.

Fig 5: Components of Whole Saliva



(Kaufman et al….The Diagnostic applications of Saliva…2004).

2.5 Summary

There has been a critical review of various literatures and major facts have been backed up. The various results of most of the researches analysed shows how much work has been done on the issue of stress especially long-term stress. This project study would be able to buttress these facts by the end of the study and prove all the hypotheses real.

CHAPTER 3: Methods and Materials

3.1 Introduction

This chapter gives details about the experimental design and vivid descriptions of all the procedures carried out in the study. The materials used were also documented and a step by step protocol for the monthly sample collections. The materials required completing ELISA protocols and those required for stress analysis were all documented in this chapter.

3.2 Materials

These consist of the buffers and the reagents used for ELISA protocols. Some of them were already in the laboratory and others were purchased from various companies. All the antibodies, hydrogen peroxidase, SDS (Sodium dodecyl sulphate) and ABTS (2, 2-azino-bis 3-ehtylbenzthiazoline-6-sulfonic acid) were purchased from sigma chemical Co (Poole, UK). Unless other wise stated product catalogue numbers are included in brackets.

3.2.1 Materials for Salivary IgA analysis

Reagents used in the analysis of Salivary IgA in ELISA were:

- Anti human IgA (alpha specific) peroxidase conjugate A-4165 sigma, developed in goat sigma.
- Anti human IgA (alpha chain specific) 1-0084 sigma, developed in rabbit-sigma.
- Human IgA colostrums (1-2363 sigma).
- ABTS substrate (2,2-azino-bis- 3-ethylbenthiszoline-6-sulfonic acid), (A-9941, sigma).
- Casein-Marvel milk powder

- Citric acid
- De-ionised/distilled water
- Hydrochloric acid (concentrated HCL)
- Hydrogen peroxide (30% w/w H_2O_2)
- Potassium chloride (KCL)
- Potassium dihydrogen phosphate (KH_2PO_4)
- Di-sodium hydrogen phosphate (Na_2HPO_4)
- Diz- sodium carbonate (Na_2CO_3)
- Sodium chloride (NaCl)
- Sodium hydrogen carbonate ($NaHCO_3$)
- Sodium hydroxide (1M NaOH)
- Trisodium citrate

The following tables show a list of buffers needed to complete the ELISA technique

Coating Buffer (pH9.6) ***table 2***

Vol. of distilled H_2O (ml)	Na_2CO_3 (g)	$NaHCO_3$ (g)
200	0.32	0.58
500	0.8	1.45
1000	1.6	2.9

Phosphate Buffer Saline (PBS) (pH 7.2) ***table 3***

Vol. of distilled H_2O (ml)	NaCl (g)	KCl (g)	KH_2PO_4 (g)	Na_2HPO_4 (g)
1000	8	0.2	0.2	2.9
2000	16	0.4	0.4	5.8
4000	32	0.8	0.8	11.6

Glycine Buffer (pH 10.4) ***table 4***

Vol. of distilled H_2O (ml)	Glycine (g)	$MgCl_2$ (mg)	$ZnCl_2$ (mg)
1000	7.51	0.203	0.136
2000	15.02	0.406	0.272

Citrate Buffer (pH 4.0) ***table 5***

Vol. of distilled H_2O (ml)	Citrate acid (g)	Tri-sodium citrate (g)
200	2.48	2.41
500	6.20	6.025
1000	12.4	12.05

3.2.2 Materials for cortisol analysis

Materials used for the analysis of cortisol were all in the ELISA cortisol Assay kit purchased.

The following reagents were provided in the kit:

- Microtitre plate: 8 wells snap-off strips, 12 strips coated with (mouse) anti-Cortisol antiserum.
- Reference standard set (standard 0-6), 7 vials, 1ml each,
- 0,2,5,10,20,40,80 ng/ml, ready to use.
- Enzyme conjugate, 26ml (Cortisol conjugated to horseradish peroxide, ready to use).
- Substrate solution- TMB, 25ml, ready to use.
- Stop solution, 30ml: Preparation involves the addition of deionised water to make up 1200ml.

3.3 Equipments

The equipments listed are the ones used in both ELISA technique and cortisol analysis.

- ELISA micro-titre plates
- Labsystems Multiwasher ELISA plate washer
- Automatic Finn pipettes (0.5-10µl, 5-50µl, 50-250µl, 200-1000µl, 1-10ml)
- Eppendorf tubes (1.5ml)
- pH meter
- Pipette tips

- Universal tubes (20ml)
- Vortex (mixer)
- Incubator (37°C)
- Labsystem multiscan (multisoft) plate reader (414nm)
- Multichannel Finn pipette (100-500µl)
- Glass bottles (1000ml)
- Beakers and conical flask (100-500ml)
- Cortisol kit

The only equipment used on subjects was: Blood pressure monitor.

3.4 Subjects

This study involved 9 subjects, 7 out of the nine subjects stayed consistent on the procedures for the six-month study. The 9 subjects were volunteers and were drawn from different populations: Caucasians, Asians and Africans. The subjects were all year 2 undergraduate students in Biomedical Sciences at the University of Wolverhampton.

There were no particular protocols to selection of these populations. These subjects willingly volunteered to be part of the study and had general interest in the field of immunology. There were no particular circumstances in which the volunteers were selected. The subjects were mixed males and females.

3.5 Principles of ELISA for Cortisol and IgA analysis

This is an explanation of how ELISA (Enzyme Linked Immunoabsorbant Assay), helps to determine unknown cortisol concentration and unknown IgA concentrations in the subjects samples.

3.5.1 Principle of DRG Salivary Cortisol ELISA Kit

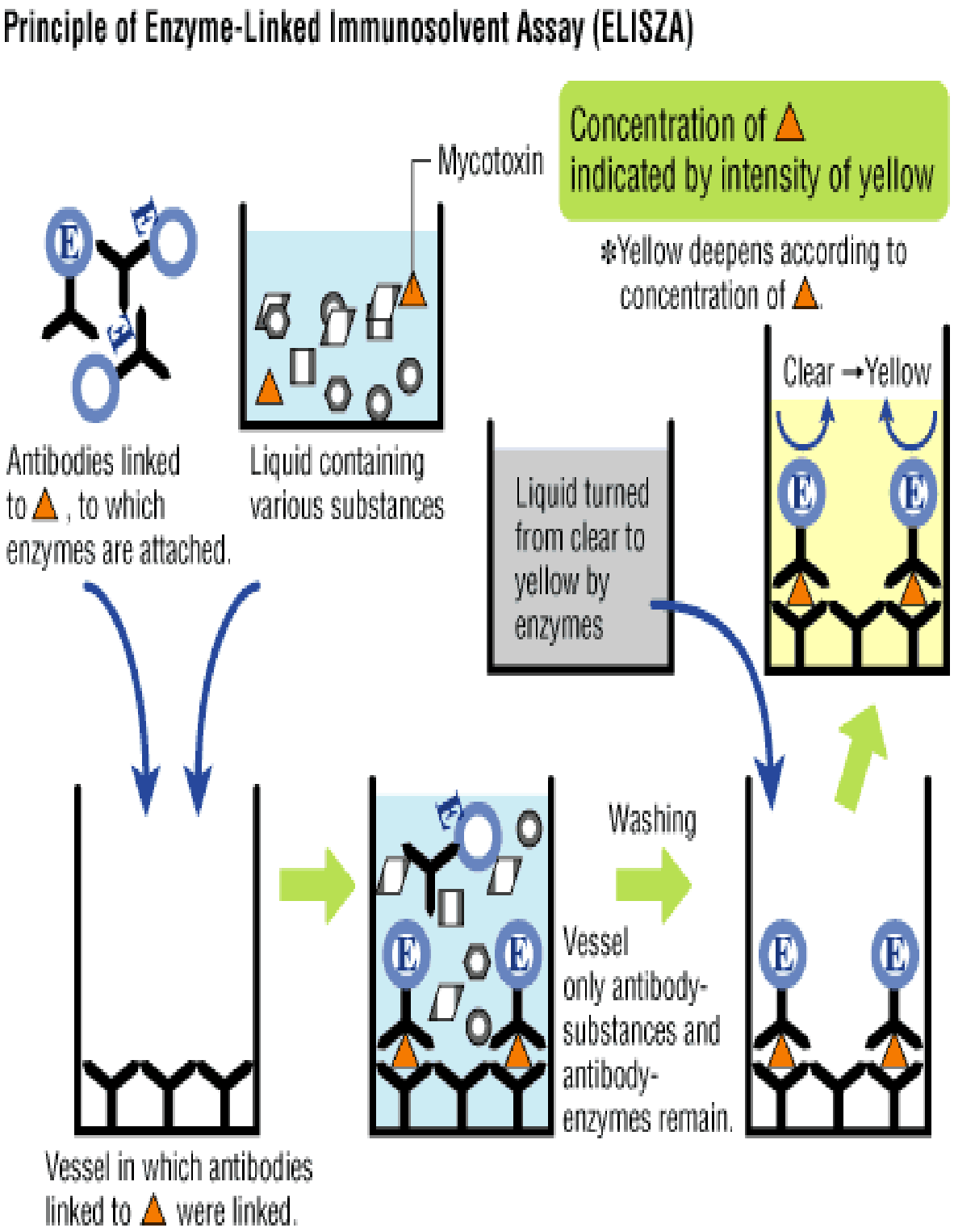
The principle is based on a competition principle and the micro plate separation. The wells of the microtitre plates are coated with mouse monoclonal Cortisol anti-serum; this has binding sites for cortisol. There is the known amount of cortisol in the standard that is conjugated with horseradish peroxide. There is also the unknown amount in the subject samples, both cortisols compete for the binding sites on the mouse monoclonal Cortisol anti-serum. The microtitre plate is washed after 1hr to stop competitive reaction. The substrate is then added which alters the cortisol concentration to be inversely proportional to the optical density measured.

3.5.2 Principle of ELISA for Salivary IgA

The technique ELISA is a method frequently used in Clinical Immunology. It is based on an antibody-antibody binding complex formation, where antibody binds to a specific antigen (e.g. proteins, hormones, peptides, drugs e.t.c.) and an enzyme-coupled antibody. The complex is incubated and produces a substrate that can be assayed and used to determine the amount of unknown substrate in samples.

It is also used to screen, detect and quantify the amount or concentration of antigens, antibodies, hormones, and peptides in samples (e.g. urine, saliva, blood e.t.c). The method of ELISA used in this study for IgA is the sandwich ELISA method. There are different ELISA techniques that work on the principle stated.

Fig 6


Principle of Enzyme-Linked Immunosolvent Assay (ELISZA)
Concentration of ▲ indicated by intensity of yellow
*Yellow deepens according to concentration of ▲.
Mycotoxin
Antibodies linked to ▲, to which enzymes are attached.
Liquid containing various substances
Liquid turned from clear to yellow by enzymes
Clear →Yellow
Washing
Vessel only antibody-substances and antibody-enzymes remain.
Vessel in which antibodies linked to ▲ were linked.

3.6 Experimental Design

This study examines Chronic Stress and the Immune system. Saliva samples were obtained from 9 subjects on a monthly basis. The major emphasis lies on the analysis and measurements of the following variables: Salivary IgA and Cortisol in the saliva samples.

Salivary IgA is one of the variables analysed, antibody IgA is the body's first line of defence and also the mucosal defence mechanism. Therefore measuring the levels of IgA is an indicator of the immune status of the subjects. The effect of lowered levels or increased levels of IgA can help assess the health status of the subjects.

Another variable considered is the hormone Cortisol, this hormone is the major hormone produced by the HPA axis during chronic stress. The measurements of this hormone can show if the subject is stressed or not.

There were other variables measured to also help asses the stress levels of the subjects. These subjects as said earlier are healthy subjects. Their blood pressure and heart rates were also monitored over a six-month period. The readings of the mean blood pressure and heart rates different from the normal range could also indicate level of stress since they are healthy individuals.

The experimental design also focused on assessing the perceived level of stress of the subjects over a six-month period. This is because chronic stress is stress over a prolonged period from weeks to even years in some cases. The subjects were normal healthy individuals but because stress comes from different sources like academic assessments, financial issues, relationship issues and a list of other issues due to varied lifestyles and personality, they are subjected to one form of stress or the other

on a daily basis depending on individual. This accumulation of little issues stressing them builds up over the months. This is the reason for the distribution of a stress questionnaire monthly to asses how stressed they have been during the month.

The subjects were not given to a stressor because that would induce the body's response to acute (short lived) stress and the body's response would differ from when encountering a long-term stress.

The key point of the design is how well the controls work. The controls used in this study were strictly in relation to the analysis of the main variables i.e. cortisol and salivary IgA. The measurement of salivary IgA by ELISA technique had various control parameters, which is discussed in section **3.7.2.** The protocol used by ELISA involved the use of key reagents: Anti-human IgA, Casein, and Standard, Conjugate peroxide and ABTS. These entire reagents were necessary to validate the presence of salivary IgA in the saliva samples. Five sets of controls were used with each set missing one of the reagents.

3.7 Procedure

This is a detailed section of how the study was carried out, which includes the experimental procedures in the laboratory and the monthly assessments of the subjects.

3.7.1 Procedure for the subjects

The subjects involved in this study were assessed over a six-month period.

- Each subject was given a copy of a consent form, which they signed. This form indicated that they were freely and willingly consenting to their saliva samples to be used and to under go any procedures that the study would entail for the six-month period.

- They were each contacted and given a date, time and venue for the meeting for the first month, this was via class email. The time was between 1-2pm on the last school day of the month.

- One the first meeting, at 1pm the subjects were all seated, and a copy of the stress questionnaire, health questionnaire and how to obtain saliva samples were distributed. The subjects were given volunteer numbers for the purpose of confidentiality. They were also given 1.5ml eppendorf tube to collect their saliva. Each tube had to be labelled with their volunteer number.

- The stress questionnaire help asses their level of perceived stress over the month. The questionnaire was designed to obtain response to certain feelings i.e. anxiety, increased alcohol consumption, fatigue e.t.c that were indicators of stress and the frequency in which the subjects had encountered these feelings over the month.

- The health questionnaires assess their health status indicating issues like infections.

- After the subjects had finished with both questionnaires, a demonstration of how to obtain their saliva sample was given.

- First subjects were advised not to chew or eat anything before the collection of the saliva samples to avoid stimulating saliva production. The saliva obtained is to be obtained by free flow.

- The eppendorf tube was placed under the tongue.

- Saliva was allowed to drip freely into the tube over a period of 3mins

- The tubes with their saliva samples were collected.

- This was placed in a box containing ice, and taken to the laboratory

- The samples were put in the freezer and stored at -20°C until ready to be assayed.

- The stress and health questionnaires were retrieved and kept.

- These procedures were repeatedly carried out every month for six months.

3.7.2 Procedure for ELISA technique

- Micro titre plates containing 96 wells were used for this procedure.

Ca=control A Cd= control D

Cb= control B Ce=control E

Cc= control C blank/control= Empty wells

Layout of a micro titre plate containing 96 wells ***table 6***

Blank	Control	Ca	Ca	Cb	Cb	Cc	Cc	Cd	Cd	Ce	Ce
Std											
Std											
Std											
Saliva											
Saliva											
Saliva											
Saliva											

The procedures carried out were similar to the normal methods used in Sandwich ELISA.

1. A dilution of anti-human IgA (a-chain specific) 1:1000 in coating buffer was prepared and 100µl of the solution was added to each well of the micro titre plate except to control well A. Duplicates of control was made.

2. This was incubated for 1hr, and then wells were washed with PBS five times using the micro titre plate washer. The plate was blotted on tissue paper, once it had been through the washer to remove any bubbles.

3. A dilution of 0.2g 'marvel' in 10mls PBS (2% Casein) was made and 100µl of the solution was put in each well except control B.

4. It was incubated for 1hr at 37°C. The micro titre plate should be covered while incubating to reduce containmination. The wells were washed with PBS and blotted as in step 2.
5. A dilution of Human IgA Standard was made in a ratio 1: 2 in PBS

Table 7 **A table showing dilution of Human IgA standard in PBS**

Dilution of Standard(mg/ml)	Volume of Human IgA standard and PBS buffer (ml)
2	10µl of Human IgA and 8990µl of PBS
1	1ml of 2 and 1ml PBS
0.5	1ml of 1 and 1ml PBS
0.25	1ml of 0.5 and 1ml PBS
0.125	1ml of 0.25 and 1ml PBS
0.0625	1ml of 0.125 and 1ml PBS
0.03125	1ml of 0.0625 and 1ml PBS
0.01525	1ml of 0.3125 and 1ml PBS
7.81×10^{-3}	1ml of 0.01525 and 1ml of PBS
3.91×10^{-3}	1ml of 7.81×10^{-3} and 1ml of PBS
1.95×10^{-3}	1ml of 3.91×10^{-3} and 1ml of PBS
9.77×10^{-4}	1ml of 1.95×10^{-3} and 1ml of PBS
4.88×10^{-4}	1ml of 9.77×10^{-4} and 1ml of PBS
2.44×10^{-4}	1ml of 4.88×10^{-4} and 1ml of PBS
1.22×10^{-4}	1ml of 2.44×10^{-4} and 1ml of PBS

6.10 x 10^{-5}	1ml of 1.22 x 10^{-4} and 1ml of PBS
3.05 x 10^{-5}	1ml of 6.10 x 10^{-5} and 1ml of PBS
1.53 x 10^{-5}	1ml of 3.05 x 10^{-5} and 1ml of PBS
7.61 x 10^{-6}	1ml of 1.53 x 10^{-5} and 1ml of PBS
3.81 x 10^{-6}	1ml of 7.61 x 10^{-6} and 1ml of PBS
1.91 x 10^{-6}	1ml of 3.81 x 10^{-6} and 1ml of PBS
9.54 x 10^{-7}	1ml of 1.91 x 10^{-6} and 1ml of PBS
4.77 x 10^{-7}	1ml of 9.54 x 10^{-7} and 1ml of PBS
2.38 x 10^{-7}	1ml of 4.77 x 10^{-7} and 1ml of PBS
1.19 x 10^{-7}	1ml of 2.38 x 10^{-7} and 1ml of PBS
5.96 x 10^{-8}	1ml of 1.19 x 10^{-7} and 1ml of PBS
2.98 x 10^{-8}	1ml of 5.96 x 10^{-8} and 1ml of PBS
1.49 x 10^{-8}	1ml of 2.98 x 10^{-8} and 1ml of PBS

5. The dilutions made above were put in the wells except for control C.

6. Whole saliva samples were centrifuged at 8990rms for 10minutes. These samples were made into aliquots of 1:200 in ice PBS.100µl of these aliquots for each subject was also added to the wells. The aliquots were made in duplicates for each subject, for each month. (I.e. subject 1, two aliquots for each month 1-6).

7. The wells were incubated for 1hr. at same temperature as before.

8. The wells were washed and blotted as in step 2.

9. A dilution of Anti-human IgA Peroxide conjugate was made in a ratio 1: 250 in PBS. 100µl of the dilution was added to all the wells except control D.

10. This was incubated for 1hr at 37°C

11. The plate was washed and blotted as in step 2.

12. 10mg ABTS tablet was dissolved in 20ml of citrate buffer; this was mixed using a vortex mixer and to it was added 5µl of 30% w/w hydrogen peroxide. 100µl of the solution was added into each well except well E at timed intervals of approximately 15 secs.

13. The plate was incubated at 37°C until the green colour developed in the wells.

14. The absorbance was read using a plate scanner at 414nm filter.

15. These procedures were one for all the saliva samples collected for the six-month period.

Controls are necessary to test the validity of an experiment.

The table below gives a summary of the controls used in the experiment.

Table 8

Control	**Missing Reagent**
A	No anti-human IgA
B	No Casein (block)

C	No Standard or Saliva
D	No Conjugate
E	No ABTS

3.7.3 Procedure for Salivary Cortisol ELISA using the DRG kit

1. The first protocol was to secure the number of coated strips in the frame holder of the microtitre plates.

2. 100µl of each Cortisol Standard was put into appropriate wells.

3. The saliva samples of the subjects for month 1-6 were put into selected wells. These samples had previously been centrifuged for 10mins at 2000rpm. A duplicate of each sample was made.

4. 200µl of the conjugate was added into each sample and standard well. This was mixed thoroughly for 10 seconds.

5. The microtitre plate was then incubated for 60 minutes at room temperature. The wells were covered during incubation.

6. After incubation, the contents of the wells were shaken out and the wells were rinsed 3 times using the diluted wash solution.

7. The wells were inverted and blotted on paper towel to remove residual droplets.

8. 200µl of Substrate was added to each well

9. The plate was incubated for 30 minutes at room temperature.

10. After incubation adding 100µl of the stop solution to each well stopped the reaction.

11. The absorbance of each well was determined at 450nm.

3.7.4 Analysis of how the correlations for the results were done

The results reported in the next chapter were correlations between IgA, Cortisol and Stress score ratings. As stated the aim of the project is to find out if there is any significant correlation between chronic stress, IgA and cortisol, over time 1-6 months.

The first correlations were:

- The mean stress scores of each month against the mean IgA concentrations of for each month. This involves finding the mean for each month for one variable and correlating it with the mean for each month of the other variable. N=6
- The mean stress scores of each month against the mean cortisol concentrations of for each month. This involves finding the mean for each month for one variable and correlating it with the mean for each month of the other variable. N=6
- The mean cortisol concentrations of each month against the mean IgA concentrations of for each month. This involves finding the mean for each month for one variable and correlating it with the mean for each month of the other variable. N=6

The second correlations were:

- The stress scores for all the months 1-6, N=42, were correlated with the IgA concentrations for all the months
- The IgA concentrations for all the months were correlated with the cortisol concentrations for all the month. 1-6, N=42. Seven subjects for six months
- The Stress scores for all the months correlated with the cortisol concentrations for all the months.

CHAPTER 4: Results and Statistical analysis

4.0 Introduction

This chapter is where all the results gotten from the various experimental procedures would be analysed. The data would be able to give a better clarity of the hypothesis.

4.1 Results for ELISA assay of Salivary IgA concentration for each subject for the six-month of study.

Table showing Actual concentration of salivary IgA (μg/ml) from Samples and Absorbance value (nm) for the six-month of study.

Table 9 for Month 1-3

Subjects	*Month 1*		*Month 2*		*Month 3*	
	Absorbance (nm)	*IgA Concentration* (μg/ml)	*Absorbance (nm)*	*IgA Concentration* ((μg/ml)	*Absorbance (nm)*	*IgA Concentration* (μg/ml)
4	0.855	0.04	0.717	0.12	0.619	0.38
5	0.631	0.30	0.529	0.38	0.622	0.38
7	0.651	0.24	0.662	0.20	0.177	20.0
10	0.555	0.78	0.835	0.06	0.199	6.32
14	0.659	0.20	0.485	0.05	0.358	2.00
19	0.579	0.30	0.485	0.05	0.530	0.04
26	0.685	0.06	0.427	1.26	0.690	0.06
Mean		0.27**(μg/ml)**		0.43**(μg/ml)**		4.17**(μg/ml)**

Mean IgA concentrations with Standard deviation

Month 1 = 0.27μg/ml (SD = 0.246)

Month 2 = 0.43 μg/ml (SD = 0.40)

Month 3 = 4.17 μg/ml (SD = 7.33)

Table 10 for Month 4-6

Subjects	*Month 4*		*Month 5*		*Month 6*	
	Absorbance (nm)	*IgA Concentration* (µg/ml)	*Absorbance (nm)*	*IgA Concentration* (µg/ml)	*Absorbance (nm)*	*IgA Concentration* (µg/ml)
4	0.668	0.08	0.464	0.50	0.464	0.50
5	0.575	0.30	0.916	2.00	0.652	0.38
7	0.419	2.52	0.219	7.96	0.146	20.0
10	0.141	10.02	0.210	20.00	0.302	6.32
14	0.421	2.00	0.299	2.00	0.301	2.00
19	0.507	0.50	0.472	0.50	0.495	0.04
26	0.558	0.78	0.493	0.50	0.256	0.06
Mean		**2.31(µg/ml)**		**4.78(µg/ml)**		**4.19 (µg/ml)**

Mean IgA concentrations with Standard deviations

Month 4 = 2.31µg/ml (SD = 3.52)

Month 5 = 4.78 µg/ml (SD = 7.21)

Month 6 = 4.19 µg/ml (SD = 7.32)

Table 11 showing Mean Absorbance value of the controls

Controls	Absorbance (nm)	Meaning of the Control well.
Blank	0.00	This well was left blank.
Ca	0.097	This is the well without Anti-human IgA.
Cb	0.418	This is the well without the blocking agent (casein).
Cc	0.120	This is the well without Standard IgA
Cd	0.088	This is the well without the Conjugate.
Ce	0.022	This is the well without ABTS.

The various controls used are needed to validate this experiment and to show that the data obtained did not occur by chance.

Table 12

Control	**Missing Reagent**	**Outcome**
A	No anti-human IgA	Salivary/Standard IgA cannot Bind, as the block reagents would cover the base of the wells (low absorbance).
B	No Casein (block)	Conjugate might have adhered to plate giving high absorbance value.
C	No Standard or Saliva	Conjugate has nothing to bind to if IgA is not present. (Low absorbance)
D	No Conjugate	Unbound IgA would not be detected. Conjugate detects the presence of bound IgA.
E	No ABTS	ABTS is a substrate that produces a product when bound to the conjugate and this result in colour development, in control E no colour resulting in low absorbance.

4.2 Tables showing mean Values of Concentration of Salivary Cortisol and Absorbance of Subjects saliva samples for the six-month of study.

Table 18 for Month 1-3

Subjects	Month 1		Month 2		Month 3	
	Absorbance (nm)	Cortisol Concentration (ng/ml)	Absorbance (nm)	Cortisol Concentration (ng/ml)	Absorbance (nm)	Cortisol Concentration (ng/ml)
4	0.995	2.0	1.091	0.9	0.928	2.9
5	1.142	0.5	0.943	3.0	1.056	1.2
7	0.829	6.0	1.052	1.2	1.016	1.5
10	0.997	2.0	0.930	2.5	0.922	3.0
14	1.043	1.5	0.881	3.9	1.049	1.4
19	0.852	5.0	1.033	1.4	1.096	0.9
26	1.138	0.4	1.053	1.3	0.996	2.0
Mean cortisol value		**2.49**		**2.03**		**1.84**

Mean Cortisol concentrations with Standard deviations

Month 1 = 2.49ng/ml (SD = 2.18)

Month 2 = 2.03 ng/ml (SD = 1.12)

Month 3 = 1.84 ng/ml (SD = 0.83)

Table 19 for Month 4-6

Subjects	Month 4		Month 5		Month 6	
	Absorbance (nm)	Cortisol Concentration (ng/ml)	Absorbance (nm)	Cortisol Concentration (ng/ml)	Absorbance (nm)	Cortisol Concentration (ng/ml)
4	1.066	1.0	1.106	0.8	1.077	0.9
5	0.983	2.5	0.928	2.9	1.142	0.5
7	0.869	4.9	1.039	1.5	1.041	1.5
10	1.210	0.3	1.199	0.3	1.046	1.5
14	1.058	1.1	0.930	2.5	1.405	0.1
19	0.846	5.2	1.021	1.5	1.106	0.6
26	1.060	1.0	1.075	0.9	0.998	1.9
Mean Cortisol value		**2.29**		**1.49**		**1.00**

Mean Cortisol concentrations with Standard deviations

Month 4 = 2.29ng/ml (SD = 2.00)

Month 5 = 1.49 ng/ml (SD = 0.94)

Month 6 = 1.00 ng/ml (SD = 0.65)

4.3 Results and analysis of Stress questionnaires for the Six-month Study.

The stress questionnaire used in this study was designed to assess subject response to stressed feelings like: anxiety, restlessness, and lack of concentration and other stressed feelings. The total numbers of stressed feelings used were ***21***. There is a copy of the stress questionnaire in the index section.

A subject is scored based on the following ratings:

Never: Meaning the subject has not had any of those feelings throughout the month and the score is 0.

Rarely: Has had some of the feelings but not often. Score is1

Sometimes: Has felt those signs more frequently. Score is 2

Often: Has had these feelings lots of times. Score is 3

Normal rating 1-20points

Stressed rating 21-41points

Very stressed rating 42-63points

Table 20 Showing Stress scores rating for the six –month of study

Subjects	Month1	Month2	Month3	Month4	Month5	Month6
4	28	11	7	4	4	4
5	28	23	25	23	23	23
7	27	11	10	11	10	10
10	27	20	14	12	16	15
14	37	31	25	19	23	23
19	17	14	14	24	17	17
26	18	26	24	29	29	29
Mean scores	26.00	19.43	17.00	17.43	17.43	17.29

Mean Stress scores with Standard deviations

Month 1 = 26.00 (SD = 6.78) Month 4 = 17.43 (SD = 8.77)

Month 2 = 19.43 (SD = 7.76) Month 5 = 17.43 (SD = 8.50)

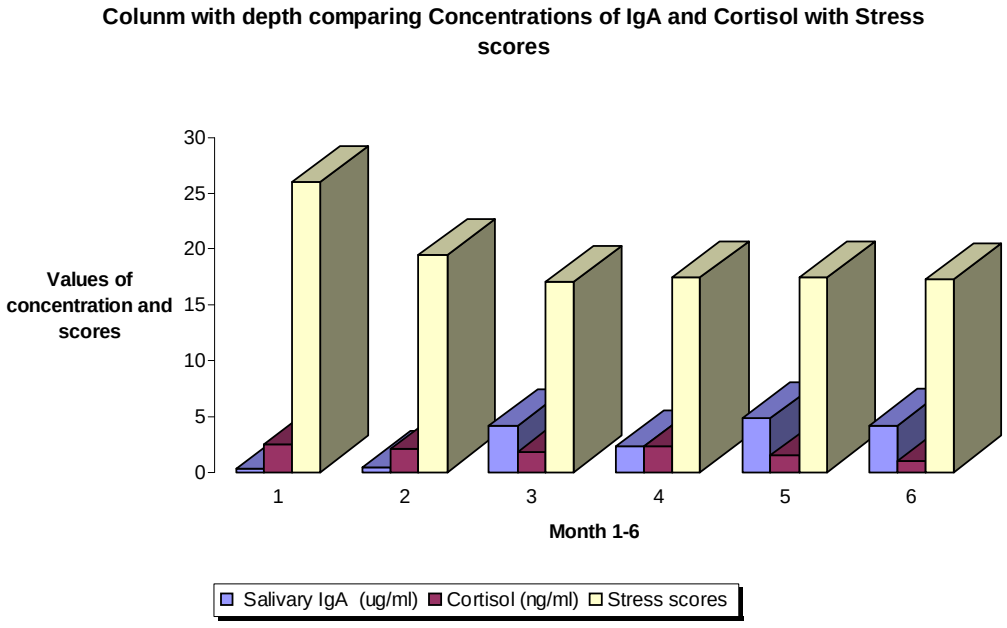
Month 3 = 17.00 (SD = 7.57) Month 6 = 17.29 (SD = 8.53)

4.4 Analysis of Result presented for IgA concentrations, Cortisol concentrations and Stress Scores for Month 1-6 table 21

Mean values Monthly	Month1	Month2	Month3	Month4	Month5	Month6
Cortisol concentrations (ng/ml)	2.49 (Sd= 2.17)	2.03 (Sd= 1.12)	1.84 (Sd= 0.83)	2.29 (Sd= 2.00)	1.49 (Sd= 0.94)	1.00 (Sd= 0.65)
IgA Concentrations (μg/ml)	0.27 (Sd= 0.25)	0.43 (Sd= 0.40)	4.17 (Sd= 7.33)	2.31 (Sd= 3.52)	4.78 (Sd= 7.21)	4.19 (Sd= 7.32)
Stress Scores	26.00 (Sd= 6.78)	19.43 (Sd= 7.76)	17.00 (Sd= 7.75)	17.43 (Sd= 8.77)	17.43 (Sd= 8.50)	17.29 (Sd= 8.54)

The data presented shows mean monthly cortisol, IgA and Stress Scores. It is quite evident that Month 1 and Month 2 show some differences from other months for all the data. Cortisol concentrations were highest in Month 1 and 2. IgA concentrations were lowest in those same months and stress scores were highest in those months. Month 1 and 2 were the months for assessments and exams in the first semester, The subjects were having lots of tests, and other academic assessment.

Fig 7



4.5 Statistical Correlations for IgA concentrations, Cortisol concentrations and Stress Scores for the 7 subjects for the six-month of study.

Correlations for Month 1-6 of Mean IgA concentrations, Mean Cortisol concentrations and mean stress scores:

N= 6 total number correlated

P= significant values of correlation

R= coefficient of correlation.

P is significant when < 0.05, the signicant correlations have been flagged *p

	Parametric Correlations	**Non-Parametric Correlations**	
Variables	**Pearson's Correlation**	**Spearman's Correlation**	**Kendall's tau_b Correlations**
Mean Stress scores	**N = 6**	**N = 6**	**N = 6**
Vs.	**R= -0.75**	**R= -0.55**	**R= -0.69**
Mean IgA Concentrations	**P= 0.08**	**P= 0.13**	**P= 0.13**
Mean IgA Concentrations	**N = 6**	**N = 6**	**N = 6**
Vs.	**R= -0.76**	**R= -0.73**	**R= -0.88**
Mean Cortisol concentrations	**P= 0.08**	***P= 0.04**	***P= 0.02**
Mean Stress scores	**N = 6**	**N = 6**	**N = 6**
Vs.	**R= 0.62**	**R= 0.55**	**R= 0.69**
Mean Cortisol Concentrations	**P= 0.19**	**P= 0.13**	**P= 0.13**

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	Mean Stress scores	19.0967	6	3.49236	1.42575
	Mean IgA concentration	2.6917	6	1.99584	.81480
Pair 2	Mean IgA concentration	2.6917	6	1.99584	.81480
	Mean Cortisol concentration	1.8567	6	.54544	.22268
Pair 3	Mean Stress scores	19.0967	6	3.49236	1.42575
	Mean Cortisol concentration	1.8567	6	.54544	.22268

Correlations for all the variables for all the months:

N= 42 total number correlated

P= significant values of correlation

R= coefficient of correlation.

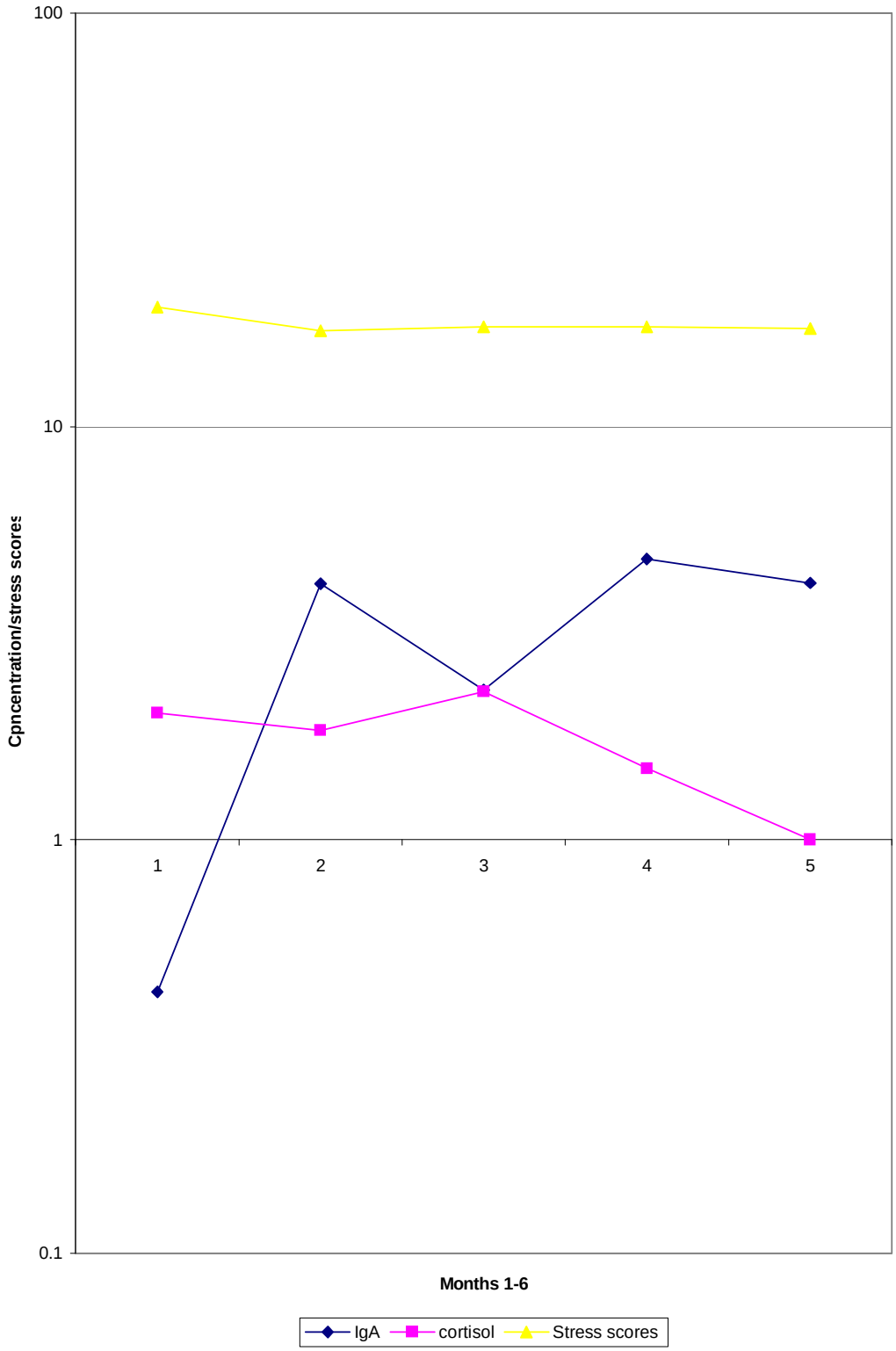
P is significant when < 0.05, the signicant correlations have been flagged *p

Variables	**Parametric Correlations**	**Non-Parametric Correlations**	
	Pearson's Correlation	**Spearman's Correlation**	**Kendall's tau_b Correlations**
Stress scores	**N = 42**	**N = 42**	**N = 42**
Vs.	**R= -0.33**	**R= -0.20**	**R= -0.26**
IgA Concentrations	***P= 0.03**	**P= 0.07**	**P= 0.09**
Stress scores	**N = 42**	**N = 42**	**N = 42**
Vs.	**R= -0.17**	**R= -0.01**	**R= -0.03**
Cortisol concentrations	**P= 0.28**	**P= 0.95**	**P= 0.83**
IgA Concentrations	**N = 42**	**N = 42**	**N = 42**
Vs.	**R= 0.17**	**R= 0.11**	**R= 0.18**
Cortisol concentrations	**P= 0.29**	**P= 0.32**	**P= 0.26**

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	Stress scores	19.0952	42	8.17733	1.26179
	IgA concentrations	2.6710	42	5.35440	.82620
Pair 2	Stress scores	19.0952	42	8.17733	1.26179
	Cortisol Concentrations	1.8548	42	1.41632	.21854
Pair 3	IgA concentrations	2.6710	42	5.35440	.82620
	Cortisol Concentrations	1.8548	42	1.41632	.21854

Line graphs showing comparism for the Mean Stress scores, Mean IgA concentrations and Mean cortisol concentrations fig 8



4.6 Results for Health questionnaires of the Subjects for the six-month of study.

The health questionnaire used in this study was designed to asses the health conditions of the subjects every month. The basic idea of the questionnaire is to keep a detailed account of what kind of infections or disease they had encountered within the month. There is a great emphasis on whether the subjects had suffered from infections involving the respiratory system and the oral cavity.

Table 16 Showing the Response to the Health Questionnaire by the subject studied indication any infection or recurrent infection.

Health Status over Six-month Period						
Subjects	**Months**					
	1	**2**	**3**	**4**	**5**	**6**
4	Well	Well	Well	Well	Well	Well
5	Well	Well	Well	Well	Well	Well
7	Well	Well	Well	Well	Well	Dental Infection G.I Infection
10	Well	Well	Well	Well	Well	Well
14	Well	Well	Dental Infection	Dental Infection	Well	Well
19	Dental Infection	Dental Infection	Flu Infection	Well	Well	Flu Re occurring Infection
26	Well	Flu infection	Well	Well	Well	Well

CHAPTER 5: Discussions

5.0 Discussions

5.1 Discussions of Correlations for Stress and IgA concentrations

The subjects recruited in this study were 7, the total number of samples obtained were 42 samples. Each subject had 6 samples, one for each month. The subjects were all healthy individuals and a mix of males and females from different ethnicity. The results were analysed statistically by three different correlations, the Pearson's correlations, spearman's correlations and the Kendall tau_b correlations.

Pearson's correlations had a significant p value, where P= 0.03 for the correlations between stress and IgA. The relationship was inverse where the value of r, coefficient of correlation was –0.33, meaning as one of the variables increases the other variable decreases. Therefore the null hypothesis 1, H0 can be rejected. The other correlations also showed an inverse relationship between stress scores and IgA concentrations, although their p values were not significant. (Spearman's P=0.94, Kendall's tau_b p=0.83). The total correlation no, n=42

The correlations for Mean stress and IgA also showed an inverse relationship (Spearman's r = -0.75, Kendall's r = -0.55, Pearson's r = -0.69), but the p values were not significant. (Spearman's p=0.13, Kendall's P=0.13, Pearson's P= 0.08) The total correlation no, n=6.

5.2 Discussions of Correlations for Stress and Cortisol concentrations

The correlations for stress and cortisol showed a linear correlation, (Spearman's r = 0.16, Kendall's r = 0.11, Pearson's r = 0.17), where as one variable increases, the other variable also increases. The correlations from all the analysis were not significant with a $p > 0.05$, (Spearman's p=0.32, Kendall's P=0.26, Pearson's P= 0.29) The total correlation no, n=42.

The correlations for Mean stress and Mean Cortisol concentrations also showed a linear correlation, (Spearman's r = 0.62, Kendall's r = 0.55, Pearson's r = 0.69), the p values of the correlations were also not significant. (Spearman's p=0.19, Kendall's P=0.13, Pearson's P= 0.13) The total correlation no, n=6.

This analysis means that the correlations deducted were not significant for any of the variables since $p > 0.05$ for all the correlations. The null Hypothesis 2 would be accepted that there is no significant increase in Cortisol concentrations as stress increases.

5.3 Discussions of Correlations for IgA concentrations and Cortisol concentrations

The correlation analysis by spearman's and Kendall's tau_b correlations showed a significant p value for Mean IgA concentrations and Mean Cortisol concentrations (Spearman's p=0.04, Kendall's P=0.02). The correlation coefficient r showed an inverse relationship between the two variables, so as one variable increases the other decreases. (Spearman's r = -0.73, Kendall's r = -0.88, Pearson's r = -0.76). The Pearson's r showed an inverse relationship, the P= 0.13 was not significant, the total correlation no, n=6.

The analysis has shown some significant correlations, so the null hypothesis 3, H0 would be rejected.

The correlations for Cortisol concentration and IgA concentrations also showed an inverse relationship (Spearman's r = -0.01, Kendall's r = -0.03, Pearson's r = -0.17), but the p values were not significant. (Spearman's p=0.94, Kendall's P=0.83, Pearson's P= 0.27) The total correlation no, n=42.

5.4 Discussions of Results for the health Questionnaire

The health questionnaire response showed subjects who had recurrent infections or who suffered infections within the month. The questionnaire response showed subject 19 and 26 as having infections at the first two months of the study. These individuals also had high stress scores for those months and this is verification to Hypothesis 4.

The null hypothesis can be rejected for hypothesis 4.

5.5 Comparism of results to previous studies:

The results from this study were able to prove three hypotheses true out of the four hypotheses put forward. There has been varied research in the area of Stress that has also shown similar conclusions to the ones in this study.

In a related study of IgA and Cortisol, it was observed that the level of IgA measured in saliva was down regulated during periods of chronic stress.
In this study, 30 healthy day active young adults were studied using the awakening cortisol response. The measure of antibody IgA showed a rapid fall (lowering levels of IgA).

The results of the study were:

- A Marked elevation of cortisol, from first awakening level over the succeeding time.

- Antibody IgA showed the opposite response with a marked fall from the highest first awakening concentration in the same samples over same period.

- The cortisol rise was significantly correlated with the Antibody IgA fall (r=0.42).

- These bring about a possibility for vulnerability to infection, since the Antibody IgA is a major mucosal immunity.

(Hucklebridge.F.et al……..1998)

In another study of hormones, brain and stress, it was observed that there are two pathways to the stress system, which is called the CRH1-immediate response mode,

and the CRH2- the slow mode, these two have receptors, which are co-localised in the limbic neural circuitry.

Balance in both systems is essential for cell homeostasis, mental performance and health. Chronic stressors that would change specific neural signalling pathways can induce imbalance. These pathways are underlying psychic domains of cognition, emotion, anxiety and aggression, which lead to cortisol-induced stress-related disorders e.g. severe depression.

(de Kloet ER……Hormones, brain and stress...2003)

This was another study conducted to review the effect of Psychological stress and Antibody response:

It was stated that there is supporting evidence to conclude that psychological stress alters immune status in human. Although this evidence were based on mostly data from in vitro studies. These studies involved the removal of immune cells or tissues, and assessing their functional capabilities.

This established a proof that stress-induced immune changes do occur and this has clinical implications on altering response to immunization.

(Sheldon Cohen et al….2000)

All these studies show correlations between Stress, IgA and how it affects the immune system. The major emphasis has been the mucosal immunity defence, immunoglobulin A. This can support the change in health status seen by some subjects.

6.0 Conclusion

A quality control was used in this experiment for the analysis of IgA and also cortisol. This helps to validate the results obtained. The assay for IgA and cortisol were run separately and the results obtained were all within the standard curves for each assay. This study was able to validate a significant correlation between lowered IgA levels and High stress levels over time.

There is still a necessity to investigate further on Cortisol levels during chronic stress; this is due to the fact that this study could not validate any significant increase for cortisol over the six-month period. The investigation could be done for a longer time than six months and also the number of subjects could be increased.

Another area that would require more investigations would be in the effect of blood pressure and heart rate readings. This study had those readings taken but did not check for any correlations between these variables and stress.

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8.0 References

1. Angela Clow, PhD; Cortisol as a biomarker of Stress, Journal of Holistic Healthcare, Volume 1 issue 3, November 2004.

2. Armelle Queyras, Monica Carosi, Non-invasive techniques for analysing hormonal indicators of stress, volume 40, 2004.

3. Brodal P. The neurobiology of pain, volume 125, September 2005.

4. Carmine M. Pariante, Dr R John Bicknell; Depression, Stress and the Adrenal Axis, Neuro-endocrinology Briefings 19, 2003.

5. Chien-Ming Yang, Coping with sleep disturbances among young adults: A survey of first year college students in Taiwan.

6. de Kloet ER, Hormones, brain and Stress. Endocrinology regulations, volume 37, June 2003.

7. Evalotte Moreliusm RN, MS, Elvar Theodorsson, MD, PhD and Nina Nelson, MD, PhD: Salivary Cortisol and Mood Pain during Skin-Skin care for an unselected group of Mothers and infants in Neonatal Intensive care. PEDIATRICS Vol 116, November 2005.

8. Femina P, Varghese, B.A and E. Sherwood Brown, Ph.D, M.D: The Hypothalamic-Pituitary-Adrenal Axis in Major Depressive Disorder: A Brief Primer for Primary care Physicians. Primary Care Companion Journal of Clinical Psychiatry, Vol 3, 2001

9. Edward Lindop, PhD BA: A comparative study of Stress between pre- and post-project 2000 students. Journal of Advanced Nursing, Vol 29, April 1999.

10. Gregory.E.Miller, Sheldon Cohen, A. Kim Ritchey, Chronic Psychological Stress and the regulation of Pro-inflammatory Cytokines: A glucocorticoid-Resistance Model. Health Psychology Journal Vol 21, 2002.

11. Hucklebridge F, Clow A, endocrine regulations: Hormones, Brain and Stress, Division of Medical pharmacology, Leiden University. June 2003.

12. John McBeth, Yee H Chiu, Alan J Silman, David Ray, Richard Morriss, Chris Dickena, Anindya Gupta and Gary J Macfariane: Hypothalamic-Pituitary-Adrenal stress axis function and the relationship with chronic widespread pain and its antecedents. Arthritis, Research and therapy Vol 7, June 2005.

13. Katrina Woznicki, Zalman S Agus: Recurrent Yeast Infections associated with Chronic Stress.

14. Kugler J, Hess M, Haake D: Secretion of Salivary Immunoglobulin A in relation to age, saliva flow mood states, secretions of albumin, cortisol and

catecholamines in saliva. Journal of Clinical Immunology volume 12, January 1992.

15. Melissa Kane, M. McGulgan: Alteration of immune function and Muscular Power in College Student-Athletes and College Student. Journal of Undergraduate Research, Vol 7, 2004.

16. Sheldon Cohen, Gregory E. Miller and Bruce S Rabin: Psychological Stress and Antibody Response to Immunization; A critical Review of the Human Literature. Psychosomatic Medicine Journal Vol 63. July 2000.

17. Tafet G.E, Sindovich.J: Psychoneuroendocrinological studies on chronic stress and Depression. Annals of the New York academy of Science, December 2004.

18. Nalini Ranjit, Elizabeth A Young and George A Kaplan: Material hardship Alters the diurnal rhythm of Salivary Cortisol. International Journal of Epidemiologist Vol 34, 2005.

19. S R Lee, HK kwon, KB Song and YH Choi: Dental Carries and salivary immunoglobulin A in Down syndrome children. Journal of Paediatrics and Child health Vol 40, September 2004.

20. Vivian Ng, David Koh, Betty Y.Y. Mok, Sin-Eng Chia, Lum-Peng Lim. Salivary Biomarkers Associated with Academic Stress among Dental Undergraduates. Journal of Dental Education, October 2003.

Books

21. Immunology Text by Goldsby, Kuby 2000.

22. Understanding Chronic Pain by Angela. J, 2002

23. Stress and Coping an Anthology by Richard, Lazarus. 1977.

24. Stress and Disease Processes by Andrew, B. 1992

Other Articles not fully cited;

Dusselier et al 2005,

Teeuw et al ….2004,

Lourdes et al...2001

25. Websites:

www.providentmedical.com

www.biodia.com

http://nceph.anu.edu.au

http://www.upliftprogram.com

http://evenstaronline.com

TABLES