DISSERTATION

TITLE OF STUDY:

INTEREUKIN 6 LEVELS IN ADENOTONSILLAR HYPERPLASIA AND CHRONIC RECURRENT TONSILLITIS

Principal researcher: DR. ELAINE AGONO YUKO
M. Med ENT HEAD AND NECK SURGERY
REG: H58/72205/08

Supervisors: DR. MAJOR (RTD) GONTIER C.S.
CONSULTANT PATHOLOGIST, SENIOR LECTURER,
DEPARTMENT OF PATHOLOGY, UNIVERSITY OF NAIROBI.

DR. OBURRA H.O
ASSOCIATE PROFESSOR,
OTORHINOLARYNGOLOGY, HEAD AND NECK SURGERY, DEPARTMENT OF SURGERY
UNIVERSITY OF NAIROBI

A study submitted in part fulfilment of the requirements for the degree of Master of Medicine in Ear, Nose and Throat- Head and Neck Surgery, at the University Of Nairobi

DECLARATION

This is my original work and has not been presented for a degree in any other university.

Signed_____ Date_____

Dr. Elaine Agono Yuko

This thesis will be supervised by:

Professor Oburra H.O

Associate Professor, Otorhinolaryngology, Head and Neck Surgery, Department Of Surgery
University Of Nairobi

Signed_____ Date_____

DR. MAJOR (RTD) GONTIER C.S.

Consultant Pathologist, Senior Lecturer, Department Of Pathology, University Of Nairobi.

Signed_____ Date_____

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I dedicate this work to Joyce Yuko, my mum.

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ACRONYMS AND ABBREVIATIONS:

AH	ADENOID HYPERTROPHY
ATH	ADENOTONSILLAR HYPERPLASIA
ATS	ADENOTONSILLAR SURGERY
CRP	C REACTIVE PROTEIN
CRT	CHRONIC RECURRENT TONSILLITIS
ELISA	ENZYME LINKED IMMUNOSORBENT ASSAY
ENT	EAR NOSE AND THROAT
GCSF	GRANULOCYTE COLONY STIMULATING FACTOR
HIV	HUMAN IMMUNODEFICIENCY VIRUS
IL	INTERLEUKIN
INF	INTERFERON
KNH	KENYATTA NATIONAL HOSPITAL
OSA	OBSTRUCTIVE SLEEP APNEA
ORL HNS	OTORHINOLARYNGOLOGY HEAD AND NECK SURGERY
PHA-P	PHYTO-HEMOAGGLUTINATION P IMMNUNOGEN
SPSS	STATISTICAL PACKAGE FOR SOCIAL SCIENCES (SPSS)
TNF-α	TUMOR NECROTIC FACTOR ALFA
TNF-β	TUMOR NECROTIC FACTOR BETA
WBC	WHITE BLOOD CELLS

INTERLEUKIN 6 LEVELS IN ADENOTONSILLAR HYPERPLASIA AND CHRONIC RECURRENT TONSILLITIS

ABSTRACT

BACKGROUND

The primary etiology of adenotonsillar hyperplasia and chronic recurrent tonsillitis is inflammation which is mediated by cytokines such as Interleukin-6 which is both a pro and anti-inflammatory agent. In this study the IL-6 levels in adenotonsillar tissue of patients with AH, ATH and CRT were measured.

OBJECTIVE

To measure the levels of interleukin 6 in adenotonsillar tissue in adenotonsillar hyperplasia and chronic recurrent tonsillitis.

STUDY DESIGN

Prospective cross-sectional study

MATERIALS AND METHODS

83 patients undergoing adenotonsillectomy for ATH and CRT were recruited and IL-6 assays carried out and correlated with the use of medications such as antihistamines, topical steroids and antibiotics prior to surgery.

DATA ANALYSIS

Data was entered into preformatted sheets and analyzed with the SPSS17.0. Means, percentages and statistical significance were calculated. Independent t-test and Analysis of variance (ANOVA) with LSD Post Hoc multiple comparisons were used.

RESULTS

The male to female ratio was 53.01:46.99. Patients with ATH and AH were72.3% with grade 3 and 4 tonsils as the majority at 66.2%. The highest level of IL-6 was 1.029 while the lowest was 0.104 with a mean of 0.4347. Patients who had tonsillectomies had higher IL- 6 levels compared to those who had adenoidectomies with a mean difference which was significant ($p<0.001$). The mean difference in IL6 levels of patients who were on antihistamines versus those who were not was not significant ($p= 0.444$). The mean difference of IL-6 of patients on topical steroids and those who were not was significant ($p<0.001$)

CONCLUSION

IL- 6 levels were more elevated in patients with both CRT and ATH than in patients with AH only. Thus elevated IL-6 levels may be a mark of disease chronicity

The use of topical nasal steroids leads to reduced IL6 levels thus are an effective medical treatment for AH. The use of oral antihistamines did not significantly affect IL6 levels.

INTRODUCTION:

Adenoidectomy and tonsillectomy are the most commonly performed procedures in Otorhinolaryngology, Head and Neck Surgery (ORL-HNS) practice in the world and the worldwide picture mirrors the situation in the ORL HNS practice in Kenya.[1, 2.] The primary indications for adenotonsillectomy are chronic infection and upper airway obstruction which becomes more pronounced during sleep when the oropharyngeal musculature is relaxed.[3, 4.] There may be significant advantage in pursuing medical therapy for ATH and CRT as this may limit the surgical risks and complications especially for poor surgical candidates and reduce the psychological and financial burden to the patients and their families.

Ongoing research on adenotonsillar disease include the role of atopy, tonsillar tissue as a reservoir for Human Immunodefiency Virus (HIV), the topical use of Leukotriene inhibitors and topical steroids on tonsil tissue and the role of biofilm forming bacteria.[5,6] ATH and CRT are both sequelae of inflammation. Recent studies indicate that local inflammation and mucosal immunity function independently from the systemic response. Inflammation is primarily mediated by cytokines which are peptides involved in regulation of both cellular and humoral immune response by relaying information between cell signalling molecules. Cytokine levels may differ in ATH and CRT.[7]

Adenoids and tonsils are most immunologically active from four to ten years of age with involution by adolescence, with continued growth of the skull base growth plates adenoids and tonsils are rarely obstructive after this period. The exact mechanisms underlying follicular lymphoid proliferation and hyperplasia remain poorly understood.[8]

Previously it has been widely assumed that tonsillar and adenoidal tissue enlarge at a rate faster than the bony structure of the nasopharynx during early childhood thus reducing the airway diameter. Recent studies in normal children showed that the adenotonsillar growth is proportionate to the somatic growth of the airway, and that any deviation would be abnormal.[9]

Research indicates that localized inflammation in the nasopharyngeal area is involved in the pathophysiology of upper airway obstruction and subsequently obstructive sleep apnoea in children. Assessment of adenotonsillar tissues from children with obstructive sleep apnoea

(OSA) has shown markedly increased inflammatory cell proliferation especially T-cell lymphocytes and increased expression of pro-inflammatory cytokines and other inflammatory mediators, such as TNF-α, IL-6 and IL-1α, when compared with adenotonsillar tissues surgically removed in the treatment of children with CRT.[10] It is postulated that in OSA, respiratory viruses and recurrent vibration of the upper airway from snoring will promote localized inflammation with subsequent mucosal swelling and over-expression of inflammatory cytokines.[11] Studies examining exhaled breath condensate and induced sputum in children with OSAS have revealed the upregulation of localized inflammatory processes in upper airway tissues.[12]

ANATOMY

Nasopharyngeal tonsils also called adenoids and palatine tonsils are part of the Waldeyer's ring of lymphoid tissue within the nasopharynx and oropharynx respectively. The other members of the Waldeyer's ring include the tubal tonsils, lingual tonsils and the lateral pharyngeal band. These are immunoreactive organs that located within the gateway of the aerodigestive tract and contain immunologically active cells which are directly exposed to inspired or ingested antigens and maximize the development of immunologic memory.

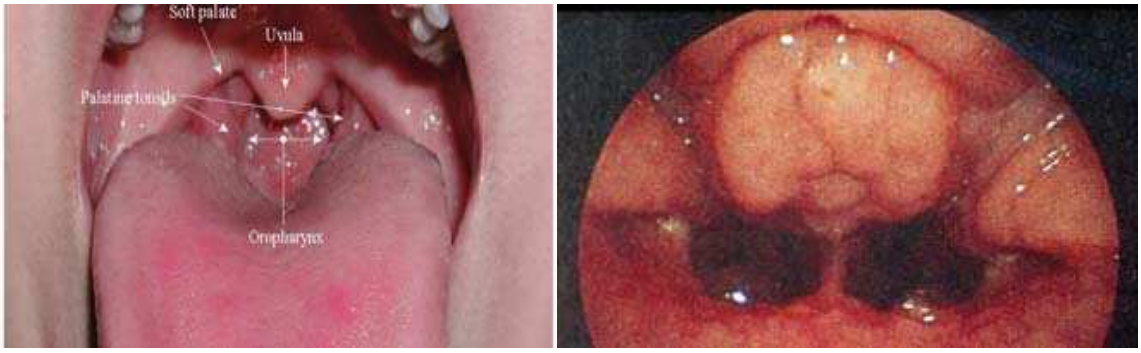

Figure1. Image of tonsils and adenoids Adapted from Anatomy & Physiology of the Pharynx Emad A. Magdy, Department of Otorhinolaryngology, Faculty of Medicine, Alexandria University. 1/13/2009

The palatine tonsils are the largest component of the ring and are located within the palatopharyngeal arch. Their lymphoid tissue is more compact in its normal state and has crypts lined with stratified squamous epithelium that extend deeply into the tonsillar tissue. They maximize the exposure of tissue to surface antigens but can also harbor debris and bacteria and may be the cause of recurrent tonsillar infections. They have a distinct fibrous and very adherent capsule which binds the deep surface of the tonsil. The capsule limits inflammation thus tonsillar tissue swelling extends medially into the oropharyngeal airway. The loose connective tissue between the capsule and the muscles of the tonsillar fossa form a potential space which is the usual site for peritonsillar abscesses.[13]

The adenoids or pharyngeal tonsil is a single mass of pyramidal tissue with its base on the posterior nasopharyngeal wall and the apex pointed toward the nasal septum. The surface is invaginated in folds with few crypts. The epithelium is pseudostratified ciliated epithelium infiltrated by lymphoid follicle. [13]

TONSIL SIZE AND POSITION

The size and the position of the tonsils significantly affect the diagnosis and symptomatology of upper airway obstruction secondary to tonsillar hyperplasia. Tonsils may be bi-lobed with extension into the hypopharynx, or more rarely into the nasopharynx. Inferior extension is associated with a history of obstruction and relatively normal appearing tonsils.Brodsky classification is a useful determinant for tonsillectomy and is a grading system of tonsillar size that is expressed as the estimated percentage of patent oropharyngeal airway between the palatine tonsils.[14]

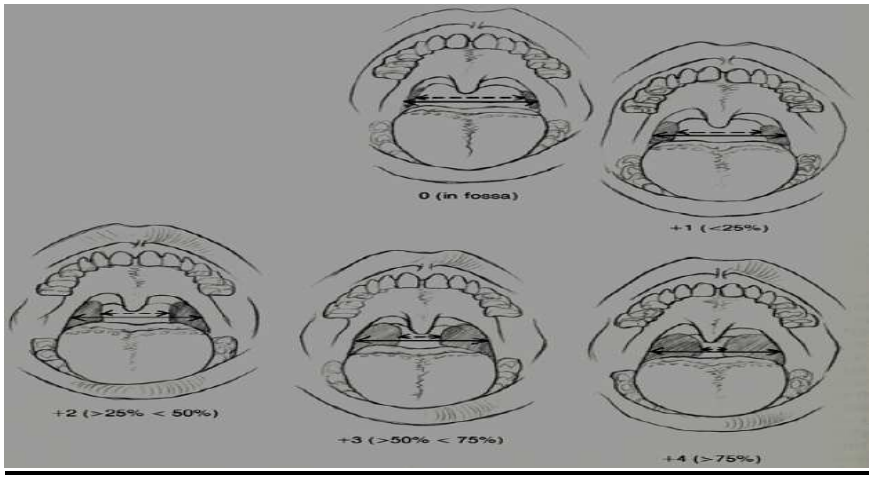

Figure 2: Brodsky classification of tonsillar size, Adapted from Lawrence Elikan, M.D. Seckin Ulualp, M.D.University of Texas Medical Branch Department of Otolaryngology. Special situations in management of tonsil and adenoid disorders, January 11, 2006

FUNCTION AND IMMUNOLOGY

The immunologic structure of the tonsils and adenoids is divided into four compartments from the surface in; reticular crypt epithelium, extra follicular area, mantle zone of the lymphoid follicle, and the germinal centre of the lymphoid follicle. Without afferent lymphatics the lymphoid nodules in these structures are exposed to antigen only in the crypts of the palatine tonsils and the folds of the adenoids. Membrane cells and antigen presenting cells are involved in transporting the antigen through the epithelial layer via transcytosis and presenting them to T-helper cells. This stimulates the B cells in the germinal zone of the lymphoid follicle to differentiate and produce antibodies. Palatine tonsils and adenoids are involved in the

production of mostly secretory IgA, which is transported to the surface and provides mucosal immune protection and assists in preventing bacteria and viruses from adhering to pharyngeal mucosa[15]

CYTOKINES

Cytokines are soluble intercellular signalling molecules that act via specific receptors to regulate host cell function. Cytokines can act in an autocrine, paracrine, or endocrine fashion via lymph or plasma and have local and systemic actions. Different cell types may secrete the same cytokine while a single cytokine can exhibit pleiotropy thus acts on several different cell types. Redundancy implies that similar functions can be stimulated by different cytokines. They are often produced in a cascade and can act synergistically or antagonistically.

Cytokines regulate immunity, inflammation, and hematopoiesis. They are produced in response to an immune stimulus and are very potent biological molecules at very low concentrations and generally though not exclusively act over short distances and short time spans. They bind to specific membrane receptors, which signal the cell via second messengers to alter gene expression. Responses to cytokines include increasing or decreasing expression of membrane proteins (including cytokine receptors), proliferation, and secretion of effector molecules. There are six major types of cytokines chemokines, colony-stimulating factors, interferons, interleukins, transforming growth factors, and tumour necrosis factors (TNF). Cytokines may also be classified by the cell of production such as lymphokines by lymphocytes and monokines by monocytes. Chemokines have chemotactic activities while interleukins are produced by leukocytes.[16]

CYTOKINE ACTIVITIES

Cytokines are made by many cell populations, but the predominant producers are T helper lymphocyes (Th) and macrophages. The largest group of cytokines is that which stimulates immune cell proliferation and differentiation. This includes Interleukin 1 (IL-1), which activates T cells; IL-2 which stimulates proliferation of antigen-activated T and B cells; IL-4, IL-5, and IL-6, which stimulate proliferation and differentiation of B cells, Interferon gamma (IFNg), which activates macrophages and IL-3, IL-7 and Granulocyte Monocyte Colony-Stimulating Factor (GM-CSF), which stimulate hematopoiesis. IFN alpha and IFN beta inhibit virus

replication in infected cells, while IFN gamma stimulates antigen-presenting cell MHC expression.[16]

TH1 AND TH2 BALANCE, REGULATION, AND INVOLVEMENT IN DISEASE

T lymphocytes are a major source of cytokines and they bear antigen-specific receptors on their cell surface to allow recognition of foreign pathogens. The two main subsets of T lymphocytes are distinguished by the presence of cell surface molecules CD4 and CD8. T lymphocytes expressing CD4 are also known as Helper T cells and can be further subdivided into Th1 and Th2 cells and produce Th1-type cytokines and Th2-type cytokines.

Th1 cells promote cell mediated immunity while Th2 cells induce humoral immunity. Cellular immunity directs Natural killer T cells and macrophages to attack abnormal cells and microbial agents at the sites of infection inside the cells. Humoral immunity results in the production of antibodies used to neutralize antigens outside of the cells. Th1 cells secrete INF-gamma and IL-2, which activate macrophages and cytotoxic T-cells to kill intracellular organisms; Th2 cells secrete IL-4, IL-5, IL-6 and IL-10, which in turn lead B cells to secrete protective antibodies.[17] The immune system relies on both Th1 and Th2 cells to exist in a balanced and regulated manner, thus an inadequate Th1 response leads to chronic infection and cancer while an overactive Th2 response can contribute to allergies and autoimmune diseases.[17]

TH-1 CELLS

TH-1 cells produce pro-inflammatory cytokines like IFN-γ, IL-2, and TNF-β and are involved in cell-mediated immunity. These cytokines stimulate phagocytosis and destruction of microbial pathogens via macrophages which produce toxic forms of oxygen which destroy the microorganisms within the phagosomes and lysosomes. Several chronic inflammatory diseases are described as Th1 dominant diseases such as multiple sclerosis, diabetes, and rheumatoid arthritis.

TH2 CELLS

TH2 cytokines counteract the effects of the TH1 cytokines – they have an anti-inflammatory action but they also help kill extracellular pathogens by stimulating the production of antibodies and eosinophilic response directed toward large extracellular parasites. Atopy and allergy are

Th2 dominant conditions. Th2 cells produce IL-4, IL-5, IL-9, IL-10, and IL-13. Pro-inflammatory cytokines are produced primarily by activated macrophages and are involved in the up-regulation of inflammatory reactions. Anti-inflammatory cytokines belong to the T cell-derived cytokines and are involved in the down-regulation of inflammatory reactions.[18]

The main cytokines involved in inflammation and fever are tumour necrosis factor-α, IL-1, and IL-6 in their order of secretion. IL-1, TNF-α and IL-6 are pro-inflammatory. IL-6 then inhibits the secretion of TNF-α and IL-1, In normal settings it limits the inflammatory reaction thus it is both pro-inflammatory and anti-inflammatory. TNF-α, IL-1ß, and IL-6 lead to monocyte-macrophage activation, due to repeated stimulation by the pathogenic agents. These mediators induce the activation and proliferation of endothelial cells and fibroblasts, which may eventually lead to progressive replacement of immunologically active tissue with fibrotic tissue in chronic recurrent tonsillitis and hyperplasia.[18]

INTERLEUKIN 6

Interleukin-6 (IL-6) is a cytokine of approximately 26 kDa that is synthesized by T-cells, macrophages, B-cells, fibroblasts, endothelial cells, and epithelial cells. It is also known as interferon-B2, cytotoxic T-cell differentiation factor, and B-cell stimulatory factor-2, among others. Depending on the particular condition it may be an anti-inflammatory or a pro-inflammatory mediator as shown above.

It is among the primary mediators of the clinical manifestations of tissue injury. These include fever, cachexia, leukocytosis, thrombocytosis, increased plasma levels of acute-phase proteins, and decreased plasma levels of albumin. IL-6 also stimulates plasmacytosis and hypergammaglobulinemia. It is required to regulate cell growth as well as immune functioning. Receptor sites on the surface of different cells of the body mediate three major signal transduction pathways: protein kinase C, cAMP/protein kinase A, and calcium release.

The circulating interleukin-6 stimulates the acute-phase reaction which leads to production and release of acute-phase proteins such as C-reactive protein which increase phagocytosis and destroy invading bacteria and other pathogens. This results in an acute-phase response, such as fever. Repeated inflammation from the acute phase reaction leads to hyperplastic adenoids and

tonsils. This may be due to the recurrent viral upper respiratory tract infections that occur in patients with OSA or acutely during adenoiditis or tonsillitis due to specific bacteria. On resolution of infection the tissue does not fully revert to its previous normal size. The specific role of IL-6 in hyperplastic adenoids and tonsils and OSA in children is still under investigation.

Impaired or uncontrolled interleukin-6 gene expression can produce unwanted immune responses leading to autoimmune disorders such as rheumatoid arthritis. Thus interleukin-6 therapy by its stimulation or inhibition is under investigation for the treatment of obesity, diabetes type II and rheumatoid arthritis. Research is currently underway on ways of preventing IL-6 binding to its receptors. However, it has been found somewhat more effective to bind IL-1 and TNF, and thus reduce the secretion of IL-6. An example is the anti IL- 6 receptor antibody (MRA), used as one of the new therapeutic approaches in rheumatic arthritis.

Summary of the Actions of lnterleukin-6 [19]
Hematologic
Proliferation of multipotential hematopoietic progenitors
Myeloma and plasmacytoma cell growth
Immunologic
Differentiation and maturation of B cells (B-cell stimulating factor-2)
Production of immunoglobulin by B cells
Proliferation and differentiation of T cells
Hepatic
Hepatocyte stimulation
Induction of various genes of the acute-phase response (CRP, haptoglobin, fibrinogen)
Neurologic
Nerve cell differentiation
Gliosis (in transgenic mice)
Cardiac
Myocardial hypertrophy
Endocrine
Induction of thermogenesis (endogenous pyrogen)
Stimulation of the hypothalamic-pituitary-adrenal axis
Stimulation of vasopressin secretion
Stimulation of growth hormone secretion
Suppression of the thyroid axis
Suppression of serum lipid levels
Osteoporosis (postmenopausal or due to hypogonadism)

LITERATURE REVIEW:

Locally there are a few studies that have been conducted on adenotonsillar tissue. These include studies on tonsil immunology [20], complications of adenotonsillectomy [4] and cardiopulmonary effects of adenotonsillar hyperplasia.[21.] A prospective study in KNH in 1977 by Mulimba (20) on histological, microbiological and immunological study of patients undergoing tonsillectomy for recurrent sore throats showed that tonsils are immunologically active and competent as shown by their response to phyto-hemoagglutination P immnunogen (PHA-P) stimulation. The levels of immunoglobulin were generally lower than the international standard, though not too low as to be used as explanation for frequent sore throats in these patients. There were no local figures comparable for both age and sex comparison and a study to determine the local normal level was thus recommended.[20]

A study by Desiderio et al (7) analysed structural and immunological aspects of tonsils and adenoids in subjects who underwent adenotonsillectomy because of recurrent inflammatory episodes with fever. Histological studies and analyses of the cytokine patterns were carried out in palatine tonsils and adenoid samples from 105 patients who underwent adenoidectomy and bilateral extracapsular tonsillectomy for chronic inflammatory hyperplasia of these organs; 46 of the 105 cases examined presented hyperkeratosis of the crypt epithelium; in the remaining 59, the epithelium was hyperplastic with no signs of keratosis. Titration of interleukin-1ß and tumor necrosis factor alpha in serum and tissues demonstrated higher concentrations in the adenotonsillar specimens, whereas the rise in interleukin-6 was more modest. [7]

Sugiyami et al did a study on the influence of IL-6 on proliferation and differentiation of tonsillar lymphocytes and detection of IL-6 producing cells in the tonsils. Tonsillar B cells were cultured in the presence of exogenous interleukin-6, a small portion of them differentiated into plasma cells which seemed to have IL-6 receptors on their surfaces. The number of plasma cells tended to be greater in the tonsils of children than in the tonsils of adults. When tonsillar cells collected by the Ficoll-Conray's method were cultured in medium containing IL-6, T cells differentiated and the number of activated T cells increased. Of the four subsets of T cells, this effect of IL-6 was strongest on cytotoxic T cells. IL-6-mediated proliferation and activation of cytotoxic T cells tended to be greater in the tonsils of children than in those of adults and the number of

macrophages producing IL-6 in tonsils of children tended to be greater than the number in tonsils of adults. [22]

Kheirandish-Gozal et al (23) did an invitro study on adenoid and tonsil tissue removed post adenoidectomy. In the study the tissue was cultured in corticosteroids and the levels of cytokines Tumour necrotic factor α, interleukins 6,and 8 measured. The study found that tonsils and adenoids obtained from children with obstructive sleep apnoea undergoing tonsillectomy and adenoidectomy displayed increased proliferative rates and proinflammatory cytokine production. Furthermore, treatment with corticosteroids resulted in marked dose-dependent reductions in proliferative rates, increased cellular apoptosis and diminished cytokine release. The relative potency of the three corticosteroids used in the current study was highest for fluticasone and the lowest for dexamethasone. Its findings supported the use of tonsillar or adenoidal tissue cell cultures as a potentially useful approach for in vitro assessment of therapeutic efficacy of corticosteroids and other candidate drugs in the treatment of the lymphoid hypertrophy that underlies obstructive sleep apnoea in children.[23]

Rania Esteitie et al(24) studied the effect of fluticasone furoate on interleukin 6 secretion from adenoid tissues in 24 children between the ages of 2 and 12 years who were undergoing adenotonsillectomy for polysomnogram-documented obstructive sleep apnea syndrome. The study was a randomized, prospective, exploratory study. The children were randomized to either no treatment or treatment with fluticasone furoate nasal spray, for 2 weeks before adenotonsillectomy. The study showed a reduction of IL-6, , in adenoid tissue obtained from children with obstructive sleep apnea syndrome treated with fluticasone furoate nasal spray. The aurthors believe that interleukin 6 an important predictor of cardiovascular risk.[24]

Krystal Revai et al (25) did a study on the association between cytokine gene polymorphisms and risk for URTI and Acute Otitis Media in children. Two hundred and forty two children between 6 to 35 months were prospectively followed for occurrences of URTI and AOM. Blood or buccal mucosa samples were collected for DNA extraction to determine cytokine genotypes. Active and passive surveillance was used to capture all URTI episodes during the one-year follow-up period in order to study the rate of AOM following URI. Children who had IL-6 and

TNFα polymorphism had a higher susceptibility to URTI during the study period and were more likely to meet established otitis susceptibility criteria ($p<0.01$). [25]

Murat Ünal et al (26) did a study on serum interleukins (IL)-1β, IL-4, IL-6, IL-8 and tumor necrosis factor (TNF)-α levels in 17 children aged 5–12 years (mean 7) with chronic tonsillitis before and after tonsillectomy. Cytokine concentrations were measured by ELISA. IL-1β and IL-6 levels were significantly higher than the control levels ($p < 0.05$) in preoperative serum samples. Other cytokine levels were within normal limits. After tonsillectomy, IL-1β and IL-6 levels were significantly reduced ($p < 0.05$). It is suggested that IL-1β and IL-6 may be mediators which have a role in chronic tonsillitis.[26]

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None of the patients in this study were on topical antihistamines or leukotreine inhibitors but it has recently been reported that montelukast, a cysteinyl leucotriene receptor antagonist, induced considerable reductions in adenoidal size and respiratory-related sleep disturbances in children with OSA.[27] Currently there is ongoing research into the role of topical antihistamines on adenoid tissue.

From the above literature review and paucity of published data, it is imperative that more work be done on the molecular events that propagate the chronicity of andenotonsillitis and hyperplasia. This will open new treatment strategies for proper targeted medical therapy as an option before surgery.

JUSTIFICATION OF STUDY:

Adenotonsillar hyperplasia and chronic recurrent tonsillitis are known to be caused by chronic inflammation which is mediated by cytokines at the local tissue level. The study has provided a record of local interleukin 6 levels in adenotonsillar tissues in children. The study is a pioneer study that will aid future studies aiming to modulate inflammmatory cytokines and develop new less invasive non-surgical modes of treatment.

NULL HYPOTHESIS

Interleukin 6 does not play a role in adenotonsillar hyperplasia and chronic recurrent tonsillitis.

ALTERNATE HYPOTHESIS

Interleukin 6 plays a role in adenotonsillar hyperplasia and chronic recurrent tonsillitis.

AIMS AND OBJECTIVES:

BROAD OBJECTIVE:

The broad objective of the study was to determine the level of interleukin 6 in Adenotonsillar tissue of patients obtained at adenotonsillectomy at KNH.

SPECIFIC OBJECTIVES:

1. To measure the level of the inflammatory cytokine interleukin 6 from adenotonsillar tissue.
2. To determine differences in Interleukin 6 in patients with obstructive symptoms only (adenotonsillar hyperplasia) and in those with chronic recurrent tonsillitis, or both of the above
3. To correlate the level of interleukin 6 in adenotonsillar tissue of patients who have been on medications such as antibiotics, topical nasal steroids and antihistamines within one month prior to surgery.

STUDY METHODOLOGY

STUDY DESIGN:

The study was a prospective descriptive study

STUDY SITE

This study was conducted in Kenyatta National Hospital ENT department.

STUDY POPULATION

The patients recruited were sourced from the ENT department at KNH. This included patients on follow up for adenotonsillar hyperplasia causing obstructive symptoms and patients on follow up for chronic recurrent tonsillitis.

INCLUSION CRITERIA

This consisted of the following

1. Patients twelve years and below whom were to undergo adenoidectomy, tonsillectomy or adenotonsillectomy for adenoid hyperplasia, adenotonsillar hyperplasia or chronic recurrent tonsillitis at the satellite theater in the ENT department at KNH.
2. Patients whose parents or guardians gave informed consent to participate in the study.

EXCLUSION CRITERIA

This included the following;

1. Patients whose guardians declined to participate in the study
2. Patients undergoing tonsillectomy as a biopsy for suspected neoplastic lesions.
3. Patients older than 16 years of age were excluded as adenoids and tonsils involute by puberty which varies from 12years to 16 years.
4. Patients who were found to have fever, neutrophilia of $12x10^9$/l, and patients with respiratory tract infections at least five days pre-operatively as most patients have hemograms done between five days to seven days pre-operatively.
5. Patients with comorbidities such as rickets, heart disease sickle cell and overt asthma who did not qualify for surgery in the satellite theatre
6. Patients undergoing branchial sinus surgery where tonsillectomy was done.

SAMPLE SIZE CALCULATION:

Yamen formula:

$$n = \frac{N}{1+N(e)^2}$$

N is the population prevalence.

e is the error margin.

At a confidence interval of 95% and an error margin of 5% the sample size (n) was based on a previous study by Desiderio et al[8] where 105 cases will be the N (population size).

$$n = \frac{105}{1+105(0.05)^2}$$

$$n = 83.16$$

SAMPLING METHOD:

Consecutive sampling method was carried out to recruit 83 children who underwent adenotonsillar surgery.

STUDY DURATION:

The study was conducted over a period of two months from March to April 2012.

PROCEDURES

The patients were recruited from the ENT clinic in KNH. Informed consent for participation in the study was sought and participants who consented were recruited into the study. All patients had a hemogram done pre-operatively as part of their routine pre-op work up.

The adenotonsillar tissue was collected after adenoidectomy by sharp curettage and extra-capsular blunt dissection tonsillectomy. The adenotonsillar tissue was then homogenized in 2 milliliters of normal saline. All samples were centrifuged at 3,000 rpm, for 10 minutes and the supernatant collected and refrigerated at minus 80^0C.

Levels of interleukin-6 were determined in the supernatant, with a quantitative enzyme-linked immunosorbent assay which was collectively done by a single technician once the sample size was achieved. The lowest detectable values in the standard curve were determined according to the manufacturer.

CLINICAL EVALUATION:

Patient selection was done from the ENT consultant clinics at the Kenyatta National Hospital. The relevant clinical history was be taken and this mainly include determining if the patient had ATH causing features of OSA or CRT, any recent URTI or antibiotic use with the last month and

any medication the patient was on for the condition such as topical nasal steroids and antihistamines. A physical examination included a general exam and a local ENT examination of was carried out and the findings were recorded in the proforma.

MATERIALS AND EQUIPMENT:

The materials and equipment used for assessing the patients included interleukin-6 assay kit, pippetes, ionized or distilled reagent water a centrifuge and refrigeration facilities.

QUALITY CONTROL:

All specimens were processed in the same laboratory at Kenya Aids Vaccine Initiative (KAVI) and standard operating procedures for specimen handling, processing and analysis were followed to ensure standardization. Guidelines on tissue handling followed the University of Nairobi Laboratory testing protocols which are based on ISO certification.

ETHICAL CONSIDERATIONS:

1. The study was carried out only after approval of the KNH ethics and research committee.
2. Those included in the study were required to give informed consent either personally or by their guardians in pediatric patients
3. Patients incurred no extra financial costs and their confidentiality was be maintained at all times.
4. Participants reserved the right to withdraw from the study at any time without any healthcare penalties.
5. There was no monetary gain by the primary investigator from the study.
6. During the screening period patients requiring further referral such as patients with cormobidities such as cardiac disease were referred accordingly.

DATA ENTRY:

The biodata of the patient, relevant history and the findings on the screening tests were recorded in the proforma.

The data was then separated into different preformatted data sheets under the following headings; age; sex; indication for surgery; adenotonsillar hyperplasia and CRT..

ANALYSIS:

Data was exported to SPSS17 and the cleaning was done in SPSS17. Independent t-test and Analysis of variance (ANOVA) with LSD Post Hoc multiple comparisons were used. Independent t-test was used to compare mean difference whenever a variable had two categories for example IL-6 levels in patients with topical nasal steroids and those who were not (yes, no) and interleukin level, ANOVA was used when the variable of interest had more than two categories e.g. surgery and interleukin levels.

DATA PRESENTATION:

Data has been presented in form of tables and bargraphs and piecharts.

COMFOUNDING FACTORS

Topical steroids are given nasally and have maximal effect on the nasopharyngeal mucosa rather than oropharyngeal mucosa thus affecting exposure of the tissue and achieving an adequate dose within the adenoid and tonsil tissue. The effect of this factor between adenoid and tonsil tissue is not ascertained in the study.

RESULTS

The study recruited a total of 83 children who underwent adenotonsillar surgery. The male to female ratio was 1:1.1

Figure 1: Gender

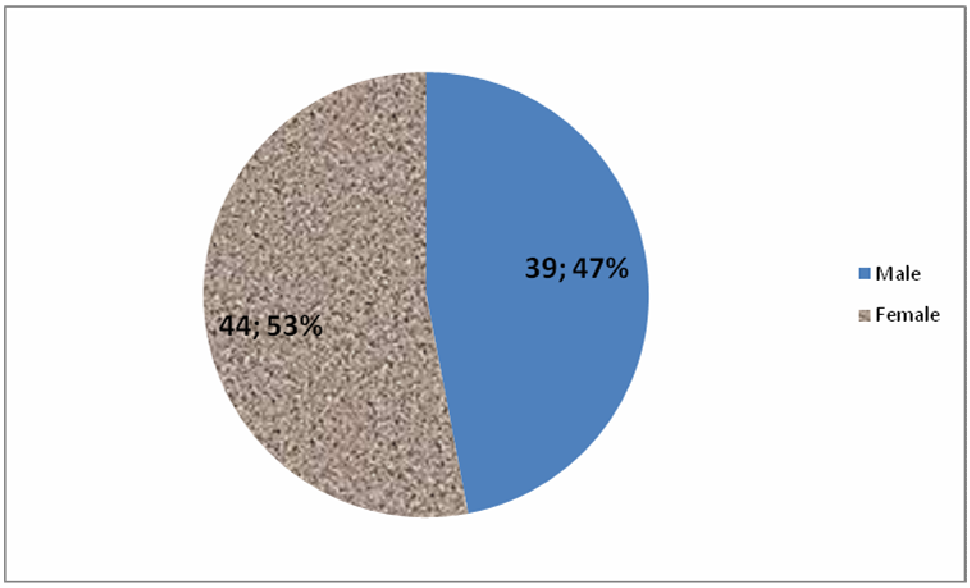

Table 1: Age and interleukins

	Age	Interleukin level (pg/ml)
N	83	79
Mean	4.96	0.43
Median	4	0.23
Std. Deviation	2.62	0.26
Minimum	1.50	0.104
Maximum	11.00	1.029

Table 1: Age and interleukins

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Tonsillar Grade

Figure 2: Tonsil grade

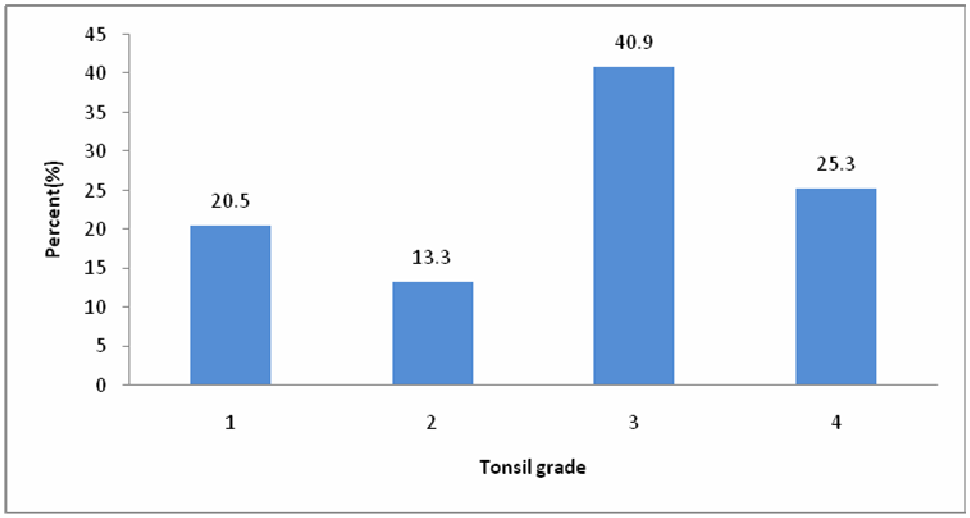
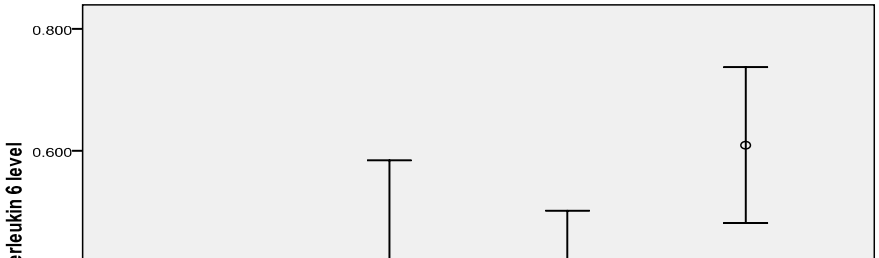


Table 2: Tonsillar Grade

Grade	N	Mean(SD)	Median	95% C.I. Mean	Minimum-Maximum
1	16	0.27 (0.13)	0.231	0.196-0.34	0.157-0.67 pg/ml
2	10	0.40(0.26)	0.324	0. 21-0.58	0.139-0.89 pg/ml
3	33	0.42 (0.23)	0.340	0.33-0.50	0. 104-0.87 pg/ml
4	21	0.61(0.28)	0.589	0.48-0.74	0.242-1.029 pg/ml

Figure 3: Mean plot of interleukin level by tonsillar grade



The different tonsillar grades and the level of IL-6 were compared and the p values were as follows: Grade 1 versus grade 3 (p=0.044); grade 1 versus grade 4(p<0.001), grade 2 versus grade 4(p=0.022) and grade 3 versus grade 4 (p=0.005)

DIAGNOSIS AND SURGERY

Figure 4: Diagnosis

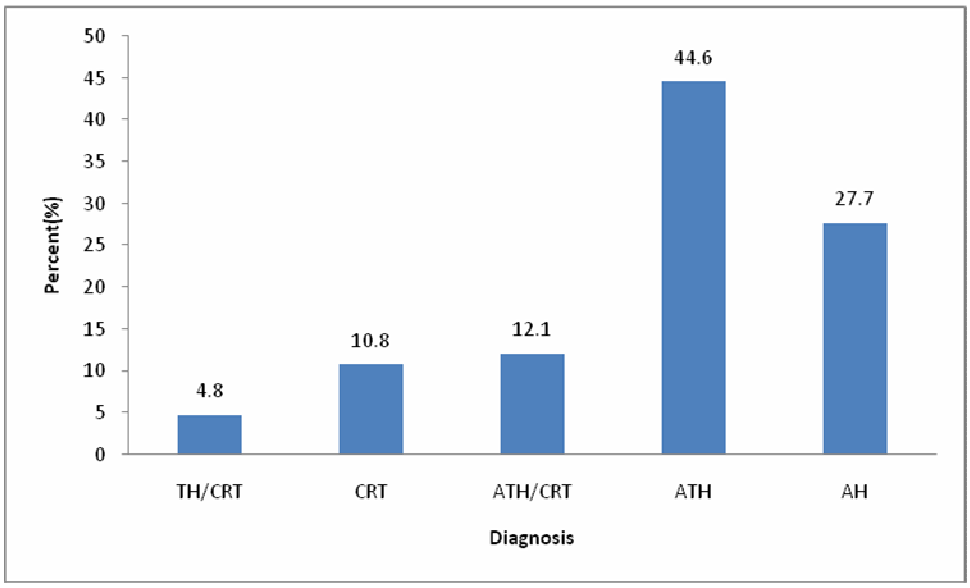
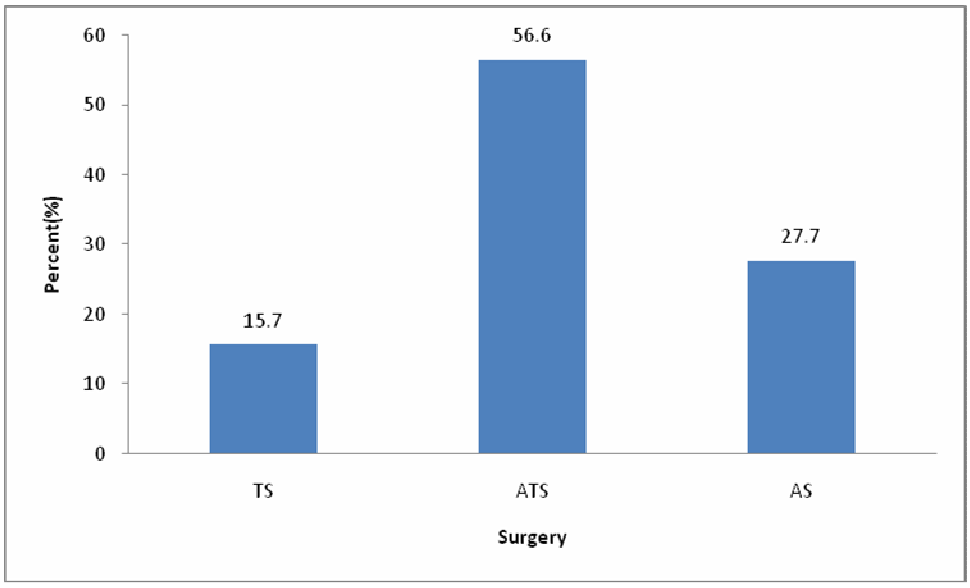


Figure 5: Surgeries

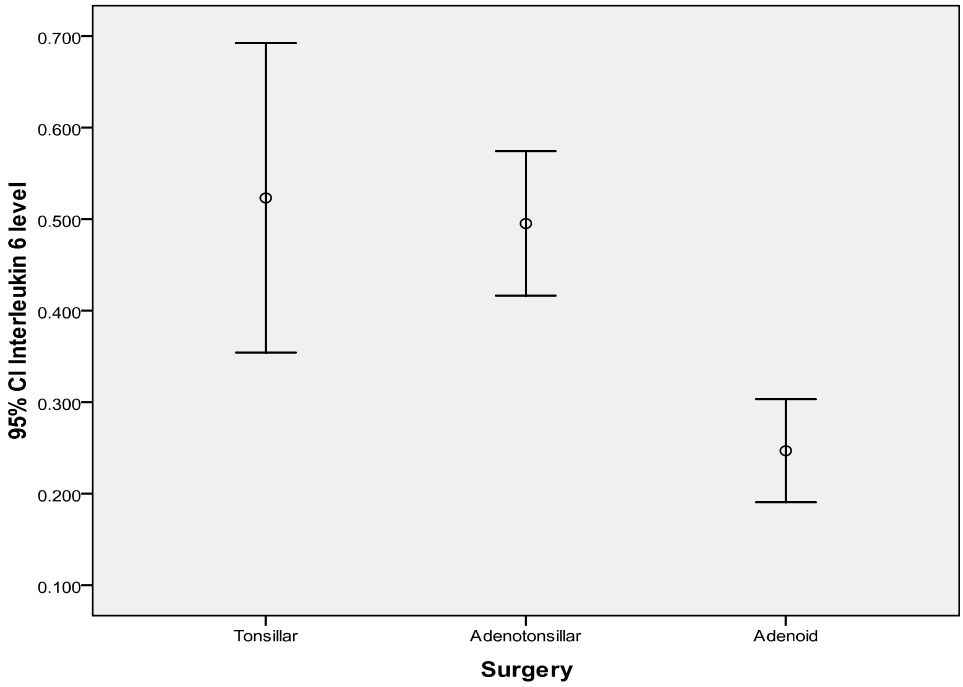


Key-: TS: Tonsillectomy ATS: Adenotonsillectomy AS: Adenoidectomy

Table 3: Summary of interleukin level by surgery

Surgery	N	Mean(SD)	Median	95% C.I. Mean	Minimum-Maximum
Tonsillar	13	0.523(0.280)	0.492	0.354-0.692	0.227-1.029 pg/ml
Adenotonsillar	46	0.495(0.265)	0.449	0. 417-0.574	0.104-1.022 pg/ml
Adenoid	21	0.247(0.124)	0.218	0.191-0.303	0.139-0.665 pg/ml

Figure 6: Mean plot of interleukin 6 level by surgery



The mean difference between Tonsillar Surgery and Adenoid Surgery was significant (p=0.002). Mean interleukin levels for patients who had tonsillar Surgery was 0.52pg/ml compared to those who had adenoid Surgery whose mean interleukin level was 0.25. The mean difference of the above two which was 0.028 is statistically significant. Patients who had adenotonsillar surgery had higher interleukin compared to those who had adenoid Surgery only. The Mean difference (0.063) between Adenotonsillar Surgery and Adenoid Surgery is significant (p<0.001).

MEDICATIONS AND URTIs

Figure 7: Use of Topical steroids

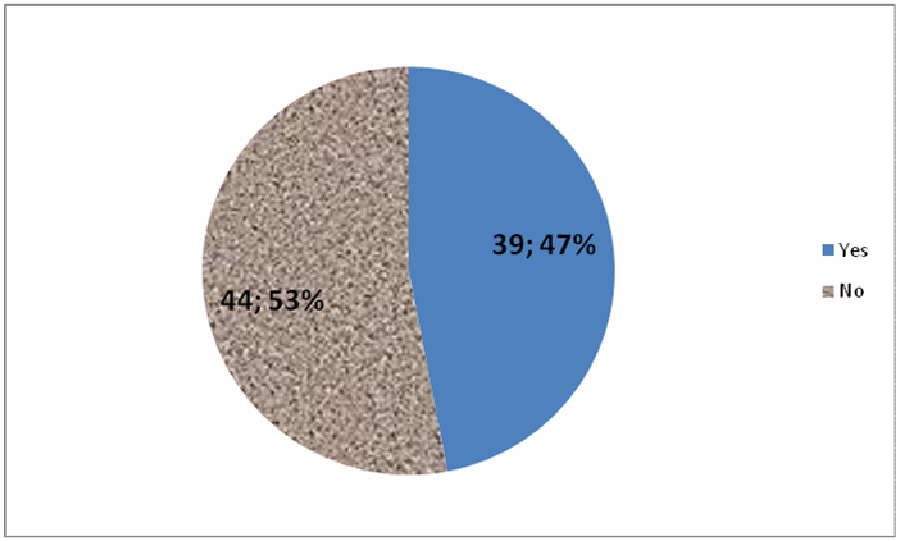


Table 3: Topical Steroids Statistics

Topical steroids	n	Mean(SD)	Median	95% C.I. Mean	Minimum-Maximum
Yes	38	0.285(0.177)	0.243	0.226-0.343	0.104-0.798
No	42	0.571(0.253)	0.532	0.492-0.649	0.218-1.029

Mean difference is significant ($p<0.001$)

Figure 8: Mean plot of interleukin 6 level by use of topical steroids

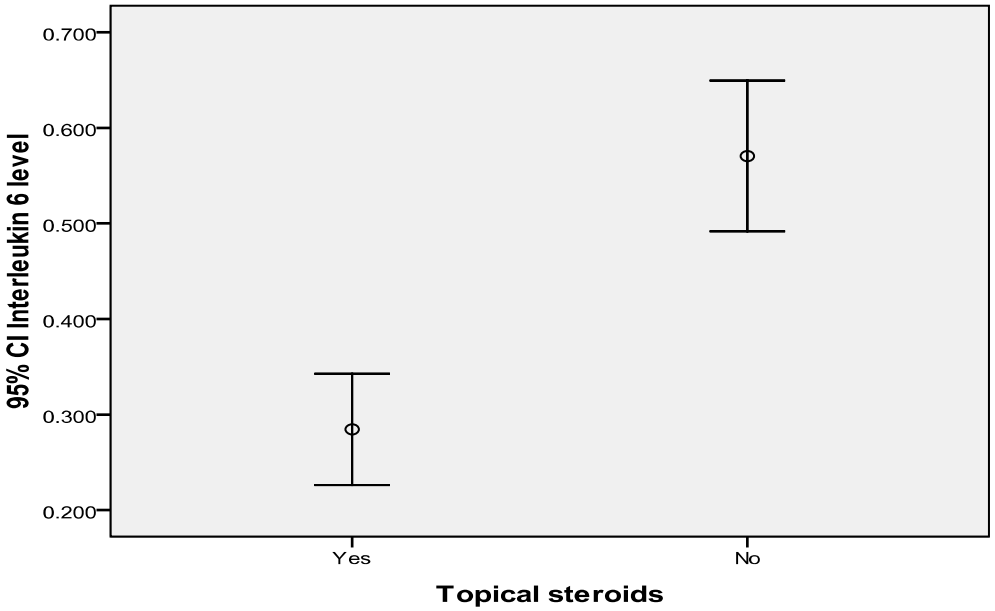


Figure 9: Use of Antihistamines

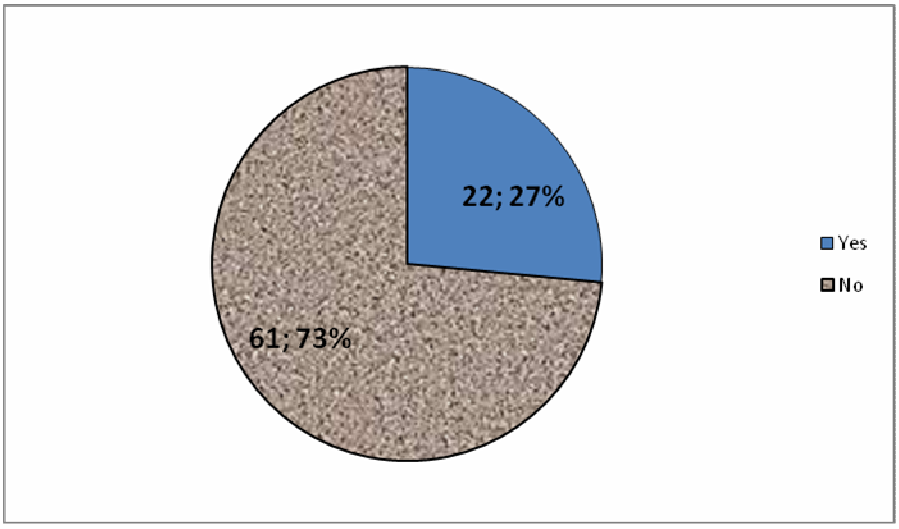


Table 4: Statistics of Patients on Antihistamines with the last one month

Antihistamines	n	Mean(SD)	Median	95% C.I. Mean	Minimum-Maximum
Yes	22	0.395(0.24)	0.31	0.29-0.50	0.10-0.97
No	58	0.45(0.269)	0.32	0.38-0.52	0.14-1.029

The mean difference of IL- 6 levels in patients who were on antihistamines and those who were not on antihistamines was not significant (p= 0.41)

Figure 10: Mean plot of interleukin 6 level by use of antihistamines

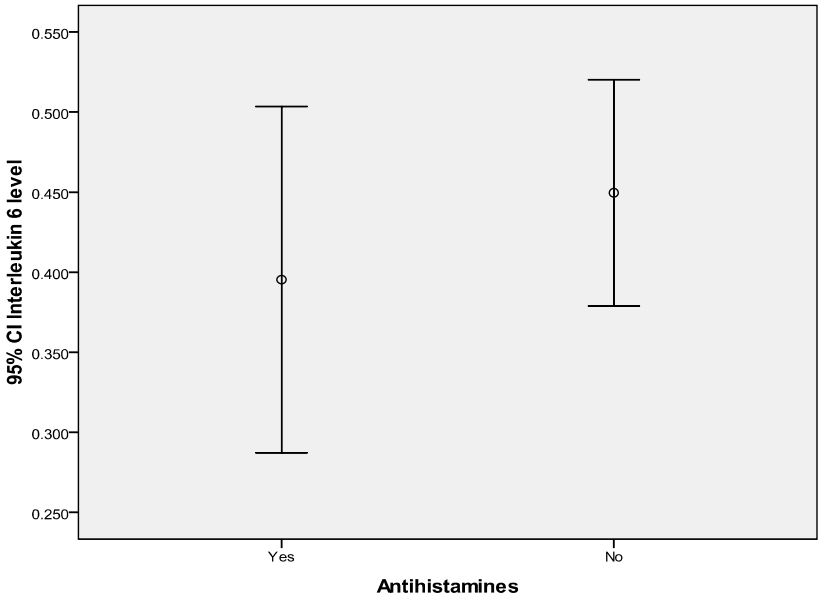


Figure 11: Use of Antibiotics within last 1month

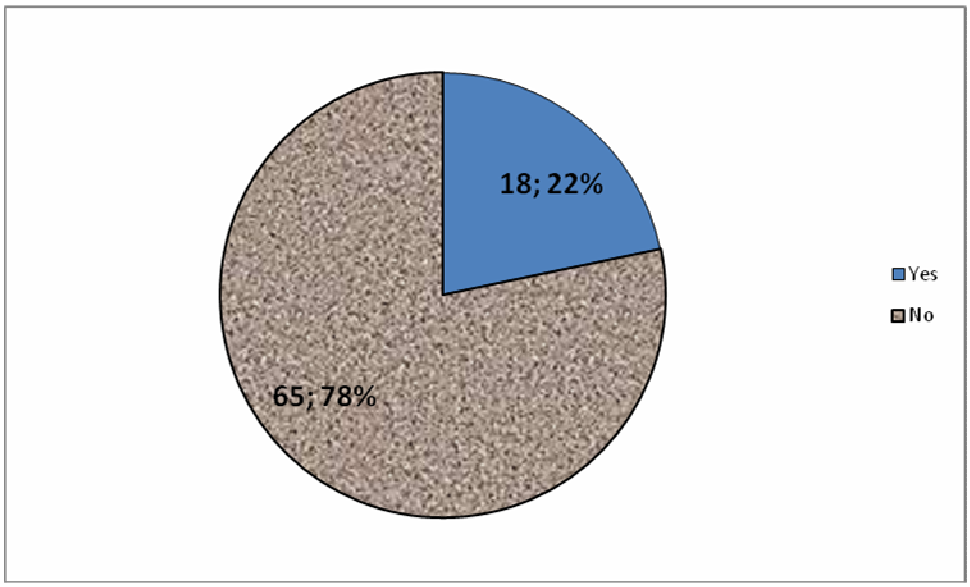
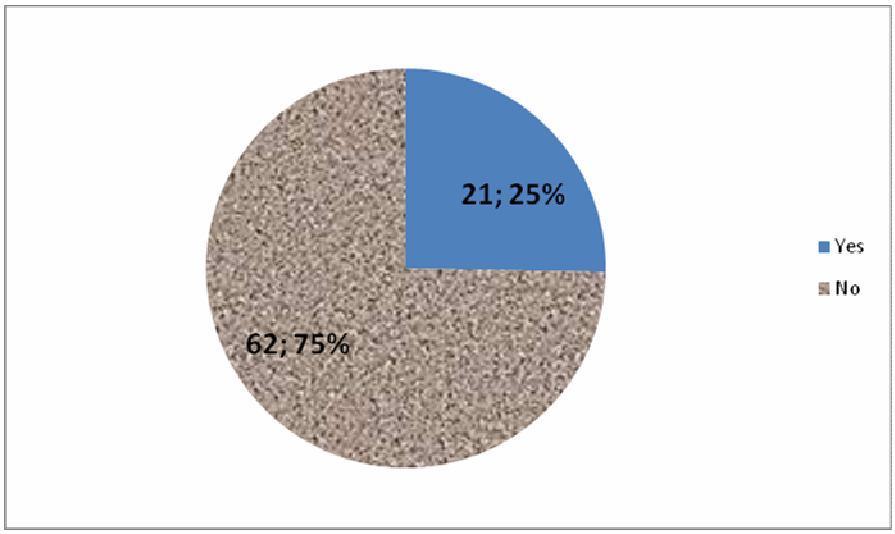


Figure 12: Patients who had URTIs within the last 1month



21 children representing 25% of the children were reported to have had URTIs within the month prior to surgery but only 18 (22%) were treated with antibiotics. 3 of the patients were managed as URTI of viral etiology and were not treated with antibiotics.

Table 5: URTIs within last 1month and IL-6 levels

	URTI last 1 month- IL-6 level pg/ml	No URTI last 1 month- IL-6 level pg/ml
Median	0.59	0.23
Std. Deviation	0.25	0.14
Mean	0.62	0.37
Minimum	0.24	0.10
Maximum	1.029	0.97

DISCUSSION

In the descriptive analysis a slight female preponderance is noted. This was not found to be statistically significant and research does not support any consistent findings of a particular preponderance of sex in children with adenotonsillar hyperplasia and chronic recurrent tonsillitis. In prepubertal children the size of the upper airways are similar in males and females after puberty the upper airways become longer in boys than girls partially due to laryngeal descent leading to higher prevalence of OSA in post-pubertal boys and adult men.[28]
Age associated changes in levels of serum IL-6 have only been studied in adults especially geriatrics in healthy subjects, increased production of IL-6 is not a normal consequence of aging but there is no research into the changes in IL-6 levels in children.[29]

The mean age of children who had only adenoid hyperplasia was 2.5 years while the mean age of children with adenotonsillar hyperplasia and adenotonsillar hyperplasia with CRT was 4.6 and 6.1 years respectively. These finding correlate with studies using upper airway endoscopy under anesthesia that have shown that in younger children the anatomic region of maximal narrowing is at the level of the adenoids and soft palate. The tonsils proliferate between 4 to 10 years of age thus the maximal narrowing of the upper airway within this age group occurs in the retropalatal region, where the soft palate, adenoids and tonsils overlap in the three-dimensional space.[30]

In the study patients 62.6% of the patients had tonsillar grade 3 and 4 and those with higher tonsillar grades had higher IL-6 levels as compared to the lower tonsillar grades that is 1 and 2. This is due to the more severe obstructive symptoms associated with grade 3 and 4 tonsils. Previous studies have shown IL-6 levels to be higher in severe as compared to mild OSA.[31] Research indicates that IL-6 mRNA production in upper airway tissues is higher in patients with severe compared to mild OSA. [31] The higher IL-6 levels with grade 3 and 4 tonsils may also be due to increased tissue bulk as hyperplastic tissue maintains most of its immunologic function but this is a postulation not supported by fact.

In the study most of the patients had surgery due to obstructive symptoms secondary to adenoid hyperplasia and adenotonsillar hyperplasia rather than recurrent infections due to chronic recurrent tonsillitis. 86.4% of the patients cumulatively had adenoid hyperplasia and

adenotonsillar hyperplasia Patients with adenoid hyperplasia tended to have lower levels of IL-6 as compared to those with adenotonsillar hyperplasia. This may be due to the duration of the disease. The higher IL-6 levels may a mark of disease chronicity as the mean IL6 level in AH, ATH, ATH and CRT was 0.25, 0.42 and 0.78 respectively. Patients with CRT had a mean age of 9.34 years and a mean IL6 level of 0.52. Thus patients who had a combination of obstructive symptoms and recurrent infections had higher IL6 than those with recurrent tonsillitis only. It is postulated that the mode of inflammation and cytokine production differs in obstructive disease versus recurrent adenoiditis and tonsillitis thus cumulatively leading to higher tissue IL-6 levels.

Patients who were on steroid nasal sprays had significantly lower IL6 levels than those who were not on IL6 with a mean of 0.285 and 0.569 respectively and a $p<0.001$ which was significant. This correlates with a study by Kheirandish-Gozal et al which was a invitro study on adenoid and tonsil tissue removed post adenoidectomy and cultured in corticosteroids. In that study treatment with corticosteroids resulted in marked dose-dependent reductions in proliferative rates, increased cellular apoptosis and diminished cytokine release. The relative potency of the three corticosteroids used in the current study was highest for fluticasone and the lowest for dexamethasone[23].A study by Rania Esteitie et al on the effect of fluticasone furoate on interleukin 6 secretion from adenoid tissues in children who were undergoing adenotonsillectomy for polysomnogram-documented OSA showed a reduction of IL-6 in adenoid tissue treated with fluticasone furoate nasal spray[24].

It is possible that some of the patients especially those with adenoid hyperplasia only might have benefited from optimum medical therapy and avoided surgery. This would involve a delicate balancing act of patient education to improve compliance on medications and patient follow up to monitor response to medication so as to avoid the dangerous sequelae of adenotonsillar hypertrophy and OSA such as pulmonary hypertension.

In this study the difference in IL6 levels in patients on oral antihistamines and those who were not on them was no significant with a p value of 0.44.There were no patients on topical antihistamines or oral leukotreine inhibitors. There are no studies showing the effect of oral antihistamines on cytokines in adenotonsillar tissues.

Mean IL6 levels in patient who had URTIs was 0.62 and mean IL6 levels in patient who did not have URTIs was 0.37. Recurrent inflammatory episodes with fever have been found to lead to higher cytokines levels including IL6[8.] This finding correlates with an experimental study by Gentile D.A et al that intranasally inoculated a safety-tested clinical isolate of RV-39 into healthy patients. Nasal lavages were submitted for viral culture and assayed for cytokine protein levels by ELISA. During infection, significant increases in mean levels of nasal IL-6) and IL-1 were observed in symptomatic but not asymptomatic subjects.

CONCLUSION

Interleukin 6 levels in adenotonsillar tissue was more elevated in patients with both chronic recurrent tonsillitis and adenotonsillar hypertrophy causing obstruction than in patients with adenoid hypertrophy. Thus elevated interleukin 6 levels are a mark of chronicity of the disease.

The use of topical nasal steroids leads to reduced IL6 levels thus are an effective medical treatment for adenoid hypertrophy. The use of oral antihistamines did not significantly affect IL6 levels. The study has introduced local levels of interleukin 6 in adenotonsillar tissue that can be used as for future reference.

RECOMMENDATIONS

A follow up study should be carried out with several proinflamatory and anti-inflamatory cytokines to get a wider picture of the subcellular events to allow for future immunomodulation that would lead to alternative medical treatment and reduced number of patients undergoing surgery. A follow up study on the relationship between atopy and adenotonsillar hyperplasia and chronic recurrent tonsillitis should be carried out possibly using tissue and serum inflammatory cytokines and skin prick tests. In this study pre and post operative tissue and serum cytokine levels should be measured to evaluate the effect of adenotonsillectomy on cytokine levels and local imunity.

Studies should be carried out on inflammation in the tonsils and the specific effect of medication to tonsillar size separate from adenoid hypertrophy as nasal steroids are most effective on

adenoid tissue rather than palatine tonsils. This might pave the way for topical medication for tonsillar hypertrophy and chronic recurrent tonsillitis.

Studies have shown that intranasal antihistamines such as azelestamine and leukotrine inhibitors such as monteleukast are effective in treating adenoid hypertrophy. These medications should be made more widely available and patients educated on their benefits.

APPENDIX 1

CLIENT INFORMATION

Adenoids are tissue located behind the nose while tonsils are found inside the mouth. They are useful in protecting the body against infection and allergies. Their surgical removal is necessitated when they significantly increase in size causing difficulty in breathing which is referred to as adenotonsillar hyperplasia. They may also get repeated infections which is referred to as chronic recurrent tonsillitis.

1. **Why is the study being done?**

   The study is being undertaken to help us to better understand the factors that contribute to the causation and worsening adenoid and tonsillar enlargement and recurrent tonsillitis. Interleukin 6 is a substance produced by cells that aids in fighting constant tissue swelling due to recurrent infection and allergies. The data will contribute to research activities that may one day contribute to preventive treatment and earlier diagnosis of patients for surgical treatment and those in whom medical treatment is sufficient.

2. **Who will participate in the study?**

   Patients undergoing adenotonsillar surgery due to obstructive symptoms or Chronic recurrent tonsillitis.

3. **How many people will participate?**

   83 patients will be enrolled in the study

4. **What does this study involve?**

   If you agree to participate in the study; you will be required to answer a few questions by an ENT resident and undergo physical examination. After this you will undergo the surgery; adenoidectomy, adenotonsillectomy or tonsillectomy as indicated. You will be allotted a study number. The extracted tissue will then be used to assess the level of interleukin 6 and its role in adenotonsillar enlargement and recurrent tonsillitis analyzed.

5. **What are the risks of the study, and will your participation affect your child?**

   .The study does not affect the child negatively in any way because:

   1. All the information you give will be confidential

2. The study does not reveal individual identity

3. The conclusions drawn from the study shall be useful to improve the management adenotonsillar enlargement and recurrent tonsillitis.

Are there any hidden dangers in your participation or non-participation?

1. None whatsoever

2. Objecting to any part or whole of this study will not affect the quality of care you receive.

What do we do with the information we get

1. The information we get may not be of immediate benefit to you but it will help us in the long run in managing the condition better.

2. Like all scientific information we will seek to share our findings with other people undertaking similar studies. Therefore we may publish our findings in scientific journals or present them in scientific meetings.

3. If you require discussing this matter with the family or friend you are free to do so and we will be ready to answer any questions. If you are satisfied with our explanation and willing to participate, then please sign the consent form below

4. In any case a child is found with some issues related to his health,this matter will be taken into consideration. A recommendation to the Doctor specialist will be given for close follow-up.

6. What are the benefits of participating in the study?

The study may not benefit you directly but it will enable researchers to develop improved less invasive treatment modalities for adenotonsillar enlargement and recurrent tonsillitis.

Participants in the study are entirely voluntary and you can get out at any stage of the study without you having to justify your decision. No victimization of any participant who withdraws from the study will be permited.

APPENDIX 3: KIELEZO

Madhumuni ya utafiti huu

Utafiti huu unafanywa kuangazia chanzo cha kuvimba kwa tonsils na adenoids na haswa maaambukizi yake sugu. Interleukin 6 inazalishwa na vyembe vya tonsils na adenoids ili kusuaidia kupambaae na uvimbe unaosababishwa na virusi na vijidudu nyingine vinavyohusika.Utafiti huu utasaidia kuchangia kwa kuelewa chanzo na kiini cha maambukizi haya na kuangaza njia ya kutambua mapema na kuzuia ugonjwa wa maambukizi ya tonsils na adenoids.

Watu gani wanapaswa kushiriki kwa utafiti huu?

Wale ambao wanakisiwa kushiriki kwa utafiti huu ni wale wanaougua mara kwa mara au kisugu na ugonjwa wa tonsils na adenoids. Lakini kushiriki kwao ni kwa hiari yao.

Kiwango Cha watu watakaoshiriki kwenye utafiti huu

Ni watu wasiopungua 83 watakaohitajiwa kushiriki kwenye utafiti huu lakini itakuwa ni kwa hiari yao.

Sera Za Utafiti huu

Ukikubali kushiriki katika utafiti huu kwa kutoa kibali chako mwenyewe au kwa hisani ya mwana, utatatarajiwa kujibu maswali kadhaa yatakayoulizwa na daktari. Utatazamwa kiafya kikamilifu na kufanyiwa ukaguzi wa kawaida wa damu wa wale wanaotarajiwa kushiriki upasuaji. Baada ya upasuaji wa adenoids na tonsils, vipande hivyo vya nyama vilivyongolewa vitaelekezwa kwa chumba cha ukaguzi cha laboratory na kupekuliwa na kukaguliwa kuhusu kiwango cha IL6 na uhusiano wake na ugonjwa wa tonsils na adenoids.

Je, kuna dhara au faida yoy yote inayohusika na utafiti huu ?

Hakuna yoyote atakayo adhiriwa na sera na mikakati ya utafiti huu. Kili mhusika anaweza kujiondoa kwenye utafiti wakati wowote, au kutoshiriki kamwe. Isitoshe, hakuna lawama au adabu yo yote itakayomkabili ye ypte atakaye thubutu kupinga utafiti huu. Licha ya haya, kila mgonwa awe au asiye mshiriki atapata matibabu sawa na ya ugonwa wa tonsils na adenoids na matibabu haya yatakuwa sare kwa kila mtu ahusike asihusike.

Habari za uvumbuzi wo wote zitakazotokea kwenye utafiti huu pengine haitakufaidi kibinafsi lakini itawapa madaktari maarifa zaidi yatakayoboresha na kuendeleza matibabu ya kuvimba

kwa tonsils na adenoids, kukoroma na hata kushindwa kupumua wakati walioadhiriwa wakiwa usingizini.

Kuna uwezekano kwamba matokeo ya utafiti huu utachapishwa kwa majarida ya kisayansi au kujadiliwa kwenye kongamano ya madaktari. Licha ya hayo, washika dao wote, kama wewe, wanapata fursa ya kudokezwa kibinafsi au kwenye magazeti ya kawaida, kwa njia ta radio, televisheni au mtandao wa internet, au barua pepe kuhusu matokeo haya.

Ukihitaji majadiliano zaidi na jamaa yako, familia au marafiki zako kuhusu utafiti huu,una uhuru wa kufanya hivyo kwani kushiriki kwenye utafiti huu ni kwa hiari yako na dhati na niko tayari kujibu maswali yo yote yatakayotokea. Ukiridhika na maelezo yangu na kuamua kushiriki, utapaswa kunipa idhini ya kukuhusisha kwenye utafiti huu kwa kuweka sahiihi kwenye form spesheli iliyowekwa kwa madhumini ya huu utafiti.

Igundulikana kwamba mshiriki kwa bahati mbaya amepatikana na shida ingine ya afya usiyotarajiwa, shida hilo, liwalo lile, litashugulikiwa na daktari gwiji wa ugonjwa huo mpya bila ubaguzi wowote.

APPENDIX: 4

CONSENT FORM:

Patient number:……

CONSENT BY PATIENT:

I……of……hereby give consent to be included in this study. I understand that the material collected may be used for future research. I have understood the objectives of this study as well as my role as a participant. They have been explained to me and I have had the opportunity to ask questions and any concerns I had been adequately explained to me by Dr. ……..

Date……. Signed……..

I Dr……..confirm that I have explained to the patient the nature of the study and that the material collected may be used for future research.

Date……..signed……

KUKUBALI KWA MGONJWA:

(Watu wazima) Mimi……kutoka……..

(Watoto) Mimi ……..mzazi wa ……

nakubali kushirikishwa kwa utafiti huu ambao lengo lake nikufanya utafiti kwa viongo vya adenoid na tonsil. Nimeelezewa kiunaga na daktari. Nimeelewa wazi kwamba vipande vya nyama vitakavyotolewa kutoka koo vitatumiwa kwa utafiti. Nimepewa fursa ya kuuliza maswali yanayohusu utafiti huu na kafafanuliwa wajibu wangu na haki zangu katika utafiti huu.
Nime elezewa na dakatri…….

Tarehe:……..sahihi…….

Mimi daktari……. nahakikisha ya kwamba nimemelezea mgonjwa juu ya utafiti huu.

Tarehe……. Sahihi…….

APPENDIX 3: HISTORY AND EXAMINATION

A. BIODATA

1. Initials:…….. ID number

2. Sex male ☐ female ☐

3. Age

B. HISTORY:

1. Recent URTI 1 week prior to surgery YES ☐ NO ☐

2. Medication use last 1 month prior to surgery:

a) Topical nasal steroids:

YES ☐ NO ☐

b) Antibiotics:

YES ☐ NO ☐

c) Antihistamines

YES ☐ NO ☐

1. General examination

Good condition ☐ ☐

Pallor

YES ☐ NO ☐

Jaundice

YES ☐ NO ☐

Cyanosis

YES ☐ NO ☐

Edema

YES ☐ NO ☐

2. Tonsil grade: 1 ☐ ☐ ☐ ☐

INDICATION FOR SURGERY:

A) adenoid hyperplasia ☐ adenotonsillar hyperplasia ☐ chronic recurrent tonsillitis ☐

APPENDIX 4

IMPLEMENTATION TIMETABLE:

PERIOD	**ACTIVITY**
September to November 2011	Proposal writing
November to December 2011	Presentation of the proposal/ethical approval
January 2012 to April 2012	Data collection, analysis and report writing
May 2012	Presentation of results and submission.

BUDGET:

CONSIDERATION	**UNIT**	**QUANTITY**	**UNIT COST (Ksh)**	**TOTAL COST (Ksh)**
Biostatician				25,000/=
Printing paper		20	400	10000/=
Immunoserological tests			700$	90000
Contingency				25000/=
Total				150000

APPENDIX 5

AssayMax Human Interleukin-6 (IL-6) ELISA Kit

Principal of the Assay

The AssayMax Human IL-6 ELISA kit is designed for detection of IL-6 in human plasma, serum or cell culture supernatants. This assay employs a quantitative sandwich enzyme immunoassay technique that measures IL-6 in less than 5 hours. A murine monoclonal antibody specific for human IL-6 has been pre-coated onto a microplate. IL-6 in standards and samples is sandwiched by the immobilized antibody and a biotinylated polyclonal antibody specific for human IL-6, which is recognized by a streptavidin-peroxidase conjugate. All unbound material is then washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color is measured.

Caution and Warning

• Prepare all reagents (working diluent buffer, wash buffer, standards, biotinylatedantibody, and SP conjugate) as instructed, prior to running the assay.

• Prepare all samples prior to running the assay. The dilution factors for the samples are suggested in this protocol. However, the user should determine the optimal dilution factor.

• Spin down the SP conjugate vial and the biotinylated-antibody vial before opening and using contents.

• This kit is for research use only. • The kit should not be used beyond the expiration date. • The Stop Solution is an acid solution.

Reagents

• **IL-6 Microplate:** A 96 well polystyrene microplate (12 strips of 8 wells) coated with a murine monoclonal antibody against IL-6.

- **Sealing Tapes:** Each kit contains 3 pre-cut, pressure-sensitive sealing tapes that can be cut to fit the format of the individual assay.
- **IL-6 Standard:** Human IL-6 in a buffered protein base (2 ng, lyophilized).
- **Biotinylated IL-6 Antibody (50x):** A 50-fold concentrated biotinylated polyclonal antibody against IL-6 (120 µl).
- **MIX Diluent Concentrate (10x)**: A 10-fold concentrated buffered protein base (30 ml).

- **Wash Buffer Concentrate (20x)**: A 20-fold concentrated buffered surfactant (30 ml, 2 bottles).
  - **Streptavidin-Peroxidase Conjugate (SP Conjugate):** A 100-fold concentrate (80 µl).
- **Chromogen Substrate**: A ready-to-use stabilized peroxidase chromogen substrate tetramethylbenzidine (8 ml).
  - **Stop Solution**: A 0.5 N hydrochloric acid to stop the chromogen substrate reaction (12 ml).

Storage Condition

- Store components of the kit at 2-8^{0}C or -20^{0}C upon arrival up to the expiration date.
- Store SP Conjugate and Biotinylated Antibody at -20^{0}C
- Store Microplate, Diluent Concentrate (10x), Wash Buffer, Stop Solution, and Chromogen Substrate at 2-8^{0}C
- Opened unused microplate wells may be returned to the foil pouch with the desiccant packs. Reseal along zip-seal. May be stored for up to 1 month in a vacuum desiccator.
  - Diluent (1x) may be stored for up to 1 month at 2-8^{0}C.
- Store Standard at 2-8^{0}C before reconstituting with Diluent and at -20^{0}C after reconstituting with Diluent.

Other Supplies Required

- Microplate reader capable of measuring absorbance at 450 nm
- Pipettes (1-20 µl, 20-200 µl, 200-1000 µl and multiple channel)
- Deionized or distilled reagent grade water

Sample Collection, Preparation and Storage

- **Plasma:** Collect plasma using one-tenth volume of 0.1 M sodium citrate as an anticoagulant.

  Centrifuge samples at 2000 x g for 10 minutes and assay. The undiluted samples can be stored at -20^{0}C or below for up to 3 months. Avoid repeated freeze-thaw cycles. (EDTA or Heparin can also be used as anticoagulant.)
- **Serum:** Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2000 x g for 10 minutes. Remove serum and assay. Store serum at -

20^0C or below. The undiluted samples can be stored at -20^0C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

- **Cell Culture Supernatants:** Centrifuge cell culture media at 2000 x g for 10 minutes to remove debris. Collect supernatants and assay. Store samples at -20^0C or below. Avoid repeated freeze-thaw cycles.

Reagent Preparation

- Freshly dilute all reagents at room temperature before use.

MIX Diluent Concentrate (10x): If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the MIX Diluent Concentrate 1:10 with reagent grade water. Store for up to 1 month at 2-8^{0}C.

- **Standard Curve:** Reconstitute the 2 ng of human IL-6 Standard with 2 ml of MIX Diluent to generate a solution of 1 ng/ml. Allow the standard to sit for 10 minutes with gentle agitation prior to making dilutions. Prepare duplicate or triplicate standard points by serially diluting the IL-6 standard solution 1:2 with equal volume of MIX Diluent to produce 0.5, 0.25, 0.125, 0.063, 0.031, 0.016 and 0.0078 ng/ml. MIX Diluent serves as the zero standard (0 ng/ml). Any remaining solution should be frozen at -20^0C.

Standard Point	Dilution	[IL-6] (ng/ml)
P1	Standard (1 ng/ml) + 1 part MIX Diluent	0.500
P2	1 part P1 + 1 part MIX Diluent	0.250
P3	1 part P2 + 1 part MIX Diluent	0.125
P4	1 part P3 + 1 part MIX Diluent	0.063
P5	1 part P4 + 1 part MIX Diluent	0.031
P6	1 part P5 + 1 part MIX Diluent	0.016
P7	1 part P6 + 1 part MIX Diluent	0.008
P8	MIX Diluent	0.000

- **Biotinylated IL-6 Antibody (50x):** Spin down the antibody briefly and dilute the desired amount of the antibody 1:50 with MIX Diluent. Any remaining solution should be frozen at -20^0c
- **Wash Buffer Concentrate (20x):** Dilute the Wash Buffer Concentrate 1:20 with reagent grade water.
- **SP Conjugate (100x):** Spin down the SP Conjugate briefly and dilute the desired amount of

the conjugate 1:100 with MIX Diluent. Any remaining solution should be frozen at -20^{0}C.

Assay Procedure

- Prepare all reagents, working standards and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature (20-30^{0}C).
- Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccant inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
- Add 50 µl of Standard or sample per well. Cover wells and incubate for two hours. Start the timer after the last sample addition.
- Wash five times with 200 µl of Wash Buffer manually. Invert the plate each time and decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid.
  If using a machine wash six times with 300 µl of Wash Buffer and then invert the plate, decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid.
  - Add 50 µl of Biotinylated IL-6 Antibody to each well and incubate for two hours.
  - Wash the microplate as described above.
- Add 50 µl of Streptavidin-Peroxidase Conjugate per well and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.
  - Wash the microplate as described above.
- Add 50 µl of Chromogen Substrate per well and incubate for approximately 12 minutes or till the optimal blue color density develops. Gently tap the plate to ensure thorough mixing and break the bubbles in the well with pipette tip.
  - Add 50 µl of Stop Solution to each well. The color will change from blue to yellow.
- Read the absorbance on a microplate reader at a wavelength of 450 nm **immediately**. If wavelength correction is available, subtract readings at 570 nm from those at 450 nm to correct optical imperfections. Otherwise, read the plate at 450 nm only. Please note that some unstable black particles may be generated at high concentration points after stopping the reaction for about 10 minutes, which will reduce the readings.

Data Analysis

- Calculate the mean value of the duplicate or triplicate readings for each standard and

sample.

- To generate a Standard Curve, plot the graph using the standard concentrations on the x-axis and the corresponding mean 450 nm absorbance on the y-axis. The best-fit line can be determined by regression analysis of the linear portion of the curve.
- Determine the unknown sample concentration from the Standard Curve and multiply the dilution factor.

Standard Curve

A standard curve should be generated each time the assay is performed.

Performance Characteristics

- The minimum detectable dose of IL-6 is typically ~ 0.008 ng/ml.
- Intra-assay and inter-assay coefficients of variation were 4.9% and 7.5% respectively.
- This assay recognizes both natural and recombinant human IL-6.

Linearity

	Average Percentage of Expected Value	
Sample Dilution	**Plasma**	**Serum**
No Dilution	96%	97%
1:2	99%	102%
1:4	102%	103%

Recovery

Standard Added Value	0.01 – 0.2 ng/ml
Recovery %	89-1112%
Average Recovery %	97 %

Cross-Reactivity

Species	**% Cross Reactivity**
Canine	None
Bovine	< 2
Monkey	None
Mouse	None
Rat	None
Swine	None
Rabbit	None

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