AUTOPSY FINDINGS OF THE THYMUS IN CASES OF FATAL PAEDIATRIC SEVERE ACUTE RESPIRATORY CONDITIONS AT THE KENYATTA NATIONAL HOSPITAL FAREWELL HOME.

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In accordance with The University of Nairobi plagiarism policy statement that re-iterates the institution’s commitment towards upholding ethical standards and ensuring honesty and responsible research practice, I hereby declare that this dissertation is my original work under the guidance of the supervisors listed below and has not been submitted to the University of Nairobi or any other higher learning institution for review and approval.

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ABBREVIATIONS

AIDS - Acquired Immune Deficiency Syndrome.
AM- acute malnutrition
CMV – Cytomegalovirus
CD- cluster of differentiation
CDC- center for disease control
ENT - Ear, nose and Throat
ERC - Ethics and research committee.
FFPE - Formalin fixed and paraffin embedded.
HIV - Human Immune Deficiency Virus.
H&E- hematoxylin and eosin
IP - In patient.
IUGR - Intra-Uterine Growth Restriction.
KNH - Kenyatta National Hospital.
MAM- moderate acute malnutrition
MUAC - mid upper arm circumference.
MHC- major histocompatibility complex
N.K cells- Natural Killer Cells.
PALS - Peri-arteriolar Lymphoid Sheath
PJP- Pneumocystis Jirovecii Pneumonia
PID - Primary Immune Deficiency.
SAM- severe acute malnutrition
SOP - Standard Operating Procedures
PRESS - Paediatric respiratory etiology surveillance study.
SARI - Severe acute respiratory infection
T.B - Tuberculosis.
UON - University of Nairobi
WBC- white blood cells
DEFINITION OF TERMS

Atrophy – gradual deterioration in function and size.

Autopsy- complete surgical examination post-mortem to determine cause of death.

Cluster of differentiation- surface markers used in the identification and characterization of cells.

Immunohistochemistry- process of selective cellular antigenic assay using highly specific antibodies.

Malnutrition- deficiencies or imbalances in a person’s intake of energy and nutrients

Tissue microarray- histologic processing of multiple small tissue samples on a single slide.

Positive control- specimen of known result that is run concurrently with the test sample thus ensuring quality of the test.

Quality control- system of maintaining standards by testing against set specifications
ABSTRACT

**Background:** The thymus, together with the bone marrow plays a major role in primary lymphopoiesis. It is a retrosternal organ that weighs up to 15 grams in the paediatric population. Physiologically the thymus is responsible for T-helper and T-cytotoxic cell maturation together with their immune regulatory functions. It then gradually reduces in size and function from teenage with complete atrophy in adulthood. Thymus size and function is influenced by various factors i.e. genetics, nutritional status and infections.

In thymic pathology, paediatric health is adversely affected as the thymus plays a critical role in immune homeostasis from birth to adolescents; particularly involving infectious agents.

A paediatric respiratory aetiology surveillance study that was completed in 2016 at The Kenyatta National Hospital demonstrated thymic hypoplasia in up to 60% of the autopsies conducted. Thus begging the need evaluate the thymic anatomy and affection of function in children dying from infections and malnutrition.

**Objectives:** The main objective of this study was to describe the thymic autopsy findings and clinical correlations among fatal paediatric severe acute respiratory infections at the Kenyatta National Hospital farewell home from 2014-2016.

**Study design:** Cross-sectional descriptive study using autopsy samples from 64 decedents that were obtained from a previous paediatric respiratory aetiology surveillance study that was carried out at The Kenyatta National Hospital from 2014-2016.

**Setting:** The University of Nairobi’s Anatomic Pathology Core Histopathology laboratory.

**Methodology:** The formalin fixed and paraffin embedded thymic and splenic tissue blocks were sectioned and stained using routine hematoxylin/eosin stain. The cellular populations were
assessed by staining for the distribution and quantity of T lymphocytes, B lymphocyte and NK cells using CD3, CD5, CD20, CD79a, PAX5 and CD56 immunohistochemical stains. Slides were examined with the help of my supervisors. Standard operating procedures was applied in specimen handling and processing.

Specific clinical data i.e. age, weight, I.P number, mid-upper arm circumference and lymphocyte differential counts were obtained from the previous PRESS study.
Results: Sixty decedents were included in this study, where 29 were females and 31 were males. We found that 33.3% of these decedents had severe acute malnutrition, 28.3% had moderate acute malnutrition, 10% were at risk of acute malnutrition. Only 28.3% were well nourished. Of the decedents’ thymi, 96.7% showed features of thymic atrophy which included lympho-depleted and shrunken lobules, fibrosis with fat replacement, increased thymic epithelial cells and increased macrophage infiltration.

From this study, 73% of the thymic tissues had high CD3 T-marker expression while 74% of the thymic tissue had high CD20 B-cell marker expression; 85% of the splenic tissues had high CD3 expression while 73.3% of the splenic tissues had high CD20 expression.

Complete blood count results findings were, 61% of the decedents had normal lymphocyte counts, 22.2% had lymphopenia and 16.7% had lymphocytosis.

Conclusion: Thymic involution occurs early in childhood infectious states, with significant findings on histology.

T and B cell populations in the thymus and secondary lymphoid organs were significantly affected by malnutrition states of the decedents.

Peripheral lymphocyte counts on complete blood count were not affected by the atrophic thymi.

Recommendation: Clinicians could be advised to assess thymus size ultrasonographically, as a marker of morbidity/mortality in children who present with chronic infection plus malnutrition.
1.0 INTRODUCTION

The thymus, being a major lymphoid organ in children plays an important role in primary lymphopoiesis, in conjunction with the bone marrow. It’s a restrosternal organ that closely borders the thyroid and parathyroid glands. Grossly, it’s composed of two lobes while ultrastucturally it’s divided into lobules within which is a cortex and medulla. From the 10th to 14th weeks of embryogenesis, it receives T lymphoid precursor cells from the bone marrow(1). In the under 5 pediatric age group, it is the largest lymphoid organ and is responsible for T-helper and T-cytotoxic cell maturation together with their immune regulatory functions. Cells of the B lineage and macrophages also play an important role in thymic physiology, as well as infectious disease surveillance(2).

The high rates of infectious disease in sub-Saharan Africa has previously been attributed to overcrowding, poverty, poor health systems and malnutrition(3). An emerging risk factor in this region is immune deficiency, which could be primary or secondary, as a result of malnutrition and infections. The contribution of immune deficiency to infectious disease morbidity and mortality among children in Africa is unknown(4).

A recent paediatric respiratory aetiology autopsy study at the Kenyatta National Hospital in Kenya, demonstrated a high proportion of thymic hypoplasia among fatal paediatric severe acute respiratory infections. Severe thymic hypoplasia was observed in children with multiple infections including *Pneumocystis Jirovecii* pneumonia and *Klebsiella*, reminiscent of HIV associated pathology(5).

Africa has the largest infectious disease burden and accounts for 50% of global under five mortality(3). Kenyan demographics document under five mortality rate of 52/1000 with the leading cause of death in this age group being pneumonia at 15%(6). Paediatric patients who suffer from chronic respiratory tract, ear, nose and throat (ENT) and skin infections; or have poor response to antibiotic therapy for severe respiratory tract infections, could be having an underlying immune deficiency(7).

Paediatric autopsy studies have proved useful in assessing accuracy of diagnoses, clarifying differential diagnoses and providing feedback regarding therapeutic outcomes(5). Other autopsy
studies in African children dying from respiratory diseases have shown that most children who
die from respiratory diseases have preventable and treatable aetiological agents such as acute
pyogenic pneumonia, PJP, TB and CMV(5). It’s therefore likely that thymic hypoplasia
representing primary or secondary immune deficiency could be a common cause of infectious
disease related deaths in Africa. Compounded with the paucity in documented cases, this is often
overlooked both ante- and post-mortem.

2.0 LITERATURE REVIEW:

2.1 Thymic anatomy, embryology, agenesis, hypoplasia and atrophy:

The thymus is a bi-lobed organ that lies anterior to the mediastinum and extends into the neck.
On embryology, is derived from elements of the 3rd and 4th pharyngeal pouches. Thymic
epitheliocytes are derived from endodermal and ectodermal elements from these arches and
interaction with the associated mesenchyme, which is derived from the neural crest cells, triggers
thymus development. From 10th to 14th weeks of embryogenesis, the T, B and monocyte cell
lineages start to migrate to the organ. By the 17th week of embryogenesis, the thymus is
completely differentiated. Thymic weights at birth range from 10-15 grams, and continual
growth occurs to reach maximum of 30-40 grams at puberty(1).

On histomorphology, each lobe of the normal thymus is composed of multiple lobules within
which is a cortex and medulla. The cortex being primarily composed of thymocytes, epithelial
cells and mesenchymal cells. The medulla has more epithelial cells, which form the thymus
framework and the characteristic Hassal’s corpuscle(8).

Thymic atrophy is normally observed from adolescent age, but premature atrophy is determined
by genetics, nutrition, pre- and post-natal factors including: infections, inflammation, bacterial
and viral infections, exposure to allergic and/or toxic substances, malnutrition, corticosteroid
therapy, chemotherapy, radiotherapy, hypoxia etc. These cause thymic atrophy through either
increased cortisol levels or direct injury to the tissues, leading its involution and transformation
into retrosternal fat and fibrous connective tissue. Premature thymic atrophy results in disruption
in the thymic micro-environment thus compromising the cell mediated immune system with
resultant susceptibility to infections(9).
Ultra-sonographic assessment of 278 pediatric patients aged less than 2 years for thymus sizes in Guinea Bissau showed that all deaths due to infectious diseases were directly associated with small thymus sizes(10).

### 2.2 Splenic anatomy and physiology:

The spleen is composed of red and white pulp. The red pulp filters blood removing foreign material and damaged, senescent erythrocytes. The white pulp has three sub-compartments: the periarteriolar lymphoid sheath (PALS), the follicles, and the marginal zone. The PALS is mainly composed of T-lymphocytes, the lymphoid follicles and mantle zones are mainly composed of B-lymphocytes, while the marginal zone is composed of antigen presenting cells-macrophages(11).

The spleen is the largest lymphoid organ and accounts for up to 25\% of total number of lymphocyte population in the body(12).

### 2.3 Aetiology and Pathology:

In thymic hypoplasia of primary immune deficiency, histology has shown absence of cortico-medullary demarcation, reduced thymic epithelial cells and Hassall’s. Disruption of this thymic micro-environment eventually results in thymic dysfunction with disruption of immune homeostasis(2).

Secondary immune deficiency would be caused by systemic or localized infection i.e. bacterial, viral, fungal or parasitic with resultant thymic epithelial cells damage, generation of anti-thymocyte antibodies resulting in thymocyte apoptosis and depletion. This eventually leads to alteration in disease physiology and immune homeostasis(13).

Histomorphological changes in thymic atrophy, as documented by a study that was conducted on 29 patients who died of AIDS between ages 3-9 years, includes loss of Hassal’s corpuscles, reduced thymic lobules, alteration in thymic epithelial cells, thymocyte depletion and loss of normal corticomedullary demarcation with fibrosis(13). Lipid laden macrophages in the cortex and epithelial cells with densely granular vacuoles have also been described in AIDS related thymic atrophy. Effects of advanced HIV infection described in the spleen include lymphocyte depletion in perivascular sheaths with general reduction of plasma cells(2).
In the recent past, an autopsy case report in China cited a 9-month old girl with failure to thrive and marked thymic involution with associated secondary immunodeficiency. The girl died from systemic *P. aeruginosa* infection with complications of pneumonia, lung abscesses, eczema gangrenosum and bacterial endocarditis(14).

Complete autopsy studies on 20 spontaneous abortions and 10 preterm and term neonates that had histologically proven placental chorioamnionitis, showed histologic evidence of thymic hypoplasia which included extensive cortical lymphoid depletion, loss of cortico-medullary distinction, reversed cortico-medullary ratio and decrease in thymic volume(15).

A case control study by Cromi et. al involving in-vivo ultra-sonographic assessment of the thymus in 60 expectant women with IUGR vs appropriate for gestation fetuses, showed a significant reduction of size of the thymus as compared with controls. The ratio of the thymic circumference to the fetal length, bipolar diameter and abdominal circumference was significantly lower in the intrauterine growth restricted fetuses as compared with the appropriately grown fetuses(16). A similar series that involved 923 6-months old infants, who were identified during measles vaccination concluded that reduced thymic volume is a strong risk factor for all-cause mortality in children who did not receive early vaccination (17).

Autopsy studies have demonstrated significant reduction in CD3+, CD4+, CD8+ T-lymphocytes and CD20+ B lymphocytes in primary immune deficiency with thymic hypoplasia(18).

The size of the thymus is thus determined by intrinsic and extrinsic factors and in cases of hypoplasia, the overall immune homeostasis is greatly affected.

### 2.4 Epidemiology of primary immune deficiency:

Primary immune deficiency may be more common than previously thought. A worldwide study done in 2014 estimated that the worldwide prevalence of PID is at 6 million but only 27,000-60,000 have been identified. In Africa about 902,631 people live with primary immune deficiency but only 1,016 having been identified(4). PID has been seen to affect both adults and children. Di George syndrome is a PID that presents with thymic aplasia, congenital malformations and hypocalcemia(7).
**2.5 Assessment of thymus function:**

Autopsy studies play a critical role in diagnosis as evidenced by a recently documented Kenyan study of a 4-month old child who was diagnosed with primary immune deficiency at autopsy. The child had positive family history, previous sibling death and recurrent respiratory infections. At necropsy, she had an atrophic thymus and reduced T and B cell populations on splenic immunohistochemistry(19).

Certain immunohistochemical antibodies can be used to detect presence of the T, B and natural killer cells in both the spleen and thymus. CD3 antigen which is expressed on all T-cells during their various stages of development, recognizes MHC and assists with the downstream signal transmission of T- cell receptors during antigen binding. It is expressed as a cytoplasmic and membrane glycoprotein and has thus proved useful in T-cell identification(20).

CD5 is a T-cell surface protein that attenuates activating signals from T-cell receptors so that these cells can only be activated by very strong signals such as bacterial proteins and not normal tissue proteins. Some B-cells have been shown to express it as well, making it less sensitive than CD3. A combination of the two immunomarkers thus increases their sensitivity in T-cell identification(21).

CD20 is a pan-B cell membrane protein which is retained even as the B-cells mature, but is lost at the plasma cell stage. It regulates calcium transport cross B-cell membranes and has been shown to play a key role in B cell activation and proliferation(22). CD79a is a B cell marker that is expressed as a pre-B cell membrane protein and is present in all B-cells throughout their stages of development. It is a cytoplasmic protein that mediates intracellular signal transduction. Since it’s is expressed at the earliest stage of B-cell development and due to its specificity to B-cells only, it has been considered a more sensitive marker of B-cells(23). Pax5 is a nuclear transcription factor that is necessary for lymphoid progenitor cells commitment and maturation to B-cells. It is lost at the plasma cell stage. It can thus be detected in all states of B-cell development as a nuclear stain(24). CD56, a glycoprotein that’s expressed in various cells is a prototypic marker of Natural Killer cells (NK) and has proved very useful in detecting the N.K cells in both healthy and disease states(25). Immunohistochemical markers in certain combinations has been used in quantification of T and B-cells in the spleen for the diagnosis of primary immune deficiency(26)
3.0 STUDY JUSTIFICATION.

Evolutionarily, the thymus decreases in size from teenage years with ultimate atrophy and replacement by fat tissue at old age(27). While thymus size and functions is determined by numerous intrinsic and extrinsic factors, thymic dysfunction can thus be classified as being of either primary or secondary etiology(8). Thymic involution, whether primary or secondary, has been associated with progressive deterioration in thymopoiesis with resultant ineffective immune homeostasis and increased morbidity from infectious diseases(8).

Secondary thymic atrophy with lymphocyte depletion has commonly been associated with infectious diseases such as HIV, measles, and malnutrition(2). However, there is paucity of information on effects of common infectious organisms such as *Streptococcus pneumonia* and *Haemophilus influenza* on the thymus, yet these are known to cause significant morbidity and mortality in the pediatric age groups. In Sub-Saharan Africa, malnutrition is a frequent contributor to early childhood mortality and thus infectious disease states with co-morbid malnutrition invariably have adverse outcomes(28). No studies have been conducted documenting the impact of malnutrition on the immune status of Kenyan children.

The diagnosis of immunodeficiency is based on clinical evaluation, lymphocyte subtype enumeration by flow cytometry, molecular diagnostics and functional lymphocyte evaluation(29). At autopsy, gross evaluation, conventional histology and immunohistochemistry may be useful for the diagnosis of immunodeficiency(19). Combination of these techniques is feasible and might identify opportunity for preventive clinical interventions.

Histopathological evaluation of the thymus in these cases of fatal severe infections could establish the pathogenesis of thymic hypoplasia and its contribution to SARI risk. This study should be able to demonstrate the importance of active diagnosis and treatment of malnutrition in pediatric patients who present with chronic infections, or who are not responding to treatment for acute infections.
4.0 STUDY QUESTION:

What are the autopsy findings in the thymus in cases of fatal SARI?

5.0 BROAD OBJECTIVE:

To describe the thymic autopsy findings and clinical correlations among fatal paediatric severe acute respiratory infections at the Kenyatta National Hospital farewell home.

5.1 SPECIFIC OBJECTIVES:

1) To describe the morphological changes on gross pathology and histology of thymi derived from fatal pediatric severe acute respiratory infections.

2) To determine the populations of T lymphocytes, B lymphocytes and Natural Killer Cells in the thymic tissue and respective splenic tissue using immunohistochemistry.

3) To describe the association of above findings with nutrition status and lymphocyte count on complete blood count report.

6.0 METHODOLOGY

6.1 Study design:

This was a cross-sectional descriptive study using autopsy samples obtained from a previous pediatric respiratory aetiology surveillance study that was carried out at The Kenyatta National Hospital from 2014 to 2016.

6.2 Study area:

The University of Nairobi’s Anatomic Pathology Core Histopathology laboratory. Located at the Kenyatta National Hospital histopathology department, off Ngong road. The laboratory stores and processes specimens for research and academic purposes. It serves mainly the U.o.N students fraternity by processing few clinical referral specimens as well as performing special stains. It is under the management of University of Nairobi department of pathology. There are two histotechnologists who are well versed with immunohistochemical techniques.
6.3 Study population
Sample size calculation

The sample size was determined using a sample size formula for cross sectional studies with finite population correction (1).

\[
n' = \frac{N(z^2)P(1 - P)}{(d^2)(N - 1) + (z^2)P(1 - P)}
\]

Where:

- \(n'\) = Sample size
- \(N\) = Finite population (estimated as 6 patients per month for 1 year = 72)
- \(Z\) = Z value (1.96 at 5% type 1 error \([P < 0.05]\) at 95% confidence level)
- \(P\) = Expected proportion of children under the age of 5 years with thymic pathology.
- \(d\) = Margin of error = 5%

Substitution into the formula gives the sample size as 61.

A total of 64 autopsy specimens obtained from fatal paediatric severe acute respiratory tract infections who received care at Kenyatta National Hospital, got enrolled in the PRESS study but unfortunately died during treatment. Autopsies were subsequently conducted on these decedents with the autopsy specimens being archived at the University of Nairobi’s Anatomic Pathology Unit’s Core Histopathology Laboratory.

6.4 Inclusion criteria
1) Well preserved tissue blocks containing adequate tissue with no evidence of autolysis.
2) Thymic and splenic specimens that were retrieved from decedents aged 01-59 months of age who met the SARI case definition criteria during the PRESS study period i.e. history of reported fever >38°C and cough that required hospitalization during the PRESS study period.
6.5 Exclusion criteria
Where specimens were unavailable for analysis.

Autolyzed samples on gross inspection.

6.6 Data collection procedures

6.6.1 Retrieval of tissue blocks:
Permission was sought from the relevant U.o.N- Pathology Departmental authority for retrieval of 64 thymic and splenic formalin fixed paraffin embedded tissue specimens. Tissue blocks retrieved from the repository were assigned a unique study number sequentially.

6.6.2 Slide preparation:
All FFPE tissues were sectioned, mounted on slides and stained with hematoxylin and eosin. Standard operating procedures were applied. (appendix)

Histological examinations assessed for the following features:

Thymic atrophy: using a pre-established criteria in malnutrition and infection(2)

- hyper cellular medulla with hypo cellular cortex,
- reduced number of cortical lymphocytes;
- increased number of tingible body macrophages with apoptotic bodies,
- Shrinkage of thymic lobules,
- increased prominence of interlobular septae,
- blurring of normal cortico-medullary demarcation,
- epithelial cell proliferation with development of glandular structures containing eosinophilic material.

During the study, we found it necessary to categorize the degree of thymic involution (Toti et al) (15) as Grade 1- normal category; Grade 2- reversal of cotrico-medullary ratio, increase tingible body macrophages and moderate increase in thymic epithelial cell; Grade 3- markedly diminished lobules with increase in fibrous septae and thymic epithelial cell; Grade 4 –
predominance of fibrous septae with fat replacement of thymic lobules/replacement by fibroblastic cells.

**Immunohistochemical quantification of T, B and N.K cells:**

Subsequent immunohistochemical staining of thymic and respective splenic tissues for CD3, CD5, CD20, CD79, Pax5 and CD56 quantitative and distributive assessment was done as per the SOP (Appendix) using the tissue micro-array technique and recorded in a data capture sheet (appendix)

6.6.3 Retrieval of clinical data:

Upon obtaining permission from KNH/UON-ERC to proceed with this study, the principle investigator liaised with the previous PRESS study principle investigator who is based at CDC-influenza program department. Each case was matched with ante-mortem clinical file number, then the principle investigator proceeded to retrieve MUAC measurements, age of child, weight of child and the lymphocyte count on complete blood count.

During the PRESS study period, complete blood count studies were carried out at the KNH hematology laboratory with permission from the KNH administration (appendix for IRB authorization agreement).
FLOW CHART ILLUSTRATING SPECIMEN BLOCK RETRIEVAL, PROCESSING, DATA COLLECTION AND ENTRY

64 FFPE study Blocks retrieval and assignment of study number

Processing of retrieved blocks and Slide preparation as per SOP

Hematoxylin and eosin staining of all slides and selection of best thymic blocks for giemsa staining and examination

Respective CD3, CD5, CD20, CD79a, Pax5, CD56 immunohistochemical staining of all thymic and splenic slides

Histological, histochemical and immunohistochemical staining features analyzed and data entered into a data capture sheet
6.7 QUALITY ASSURANCE

6.7.1 Pre-analytical: -

The retrieved FFPE tissue blocks were clearly assigned unique research number and matched for respective block number. A trained technologist on histologic and immunohistochemical tissue processing was engaged in sample sectioning and staining. The retrieved blocks were processed adhering to both histologic and immunohistochemical S.O.P. for immunohistochemical staining, H&E slides were scrutinized and only best preserved blocks containing adequate tissue selected for immunohistochemical assays.

Lymphocyte counts were retrieved from complete blood count records which were previously run at the KNH hematology laboratory. As standard operating procedure, the hematology laboratory ran daily internal quality control procedures with periodic external quality control checks thus guaranteeing quality of results.

6.7.2 Analytical:

The principal investigator and a pathologist (supervisor) reviewed the histological and immunohistochemical features. In cases of lack of consensus, the slides were reviewed by a second experienced blinded pathologist as the tie-breaker.

Immunohistochemical stains were acquired only from reputable distributor. Each immunohistochemical stain was run in tandem with the manufacturers’ recommended positive control samples. These were acquired from the existing clinical tissues that were routinely used as controls in routine clinical tissue processing. Internal controls were utilized in negative control assessment and interpretation.

Every tenth study slides were reviewed by the second blinded pathologist.

6.7.3 Post analytical:

Data was entered immediately into respective data capture sheets to avoid any post-analytical errors.
6.8 VARIABLES

The independent variables included: age of the child, sex and nutritional status of the child.

The dependent variables included: weight of the thymus, lymphocyte count, the histomorphologic patterns of the thymus and functional T, B and NK cell populations on IHC (no, mild, moderate or marked expression CD3, CD5, CD20, CD79a, Pax5, and CD56 cell populations in the thymus and spleen).

6.9 DATA COLLECTION INSTRUMENTS:

Clinical, histology, histochemical and immunohistochemical data were collected using predesigned data capture sheet (appendix).

6.10 ETHICAL CONSIDERATIONS:

Permission to conduct this study was sought from KNH/UON-ERC.

Study Specimens had been obtained only from decedents whose parents/guardians had consented to the postmortem procedures during the previous 2014-2015 PRESS study. (consent form in appendix).

Permission to conduct the PRESS study was sought from the Institutional Review Board at KNH (appendix for IRB agreement).

The retrieved blocks were returned to their corresponding archives after processing.

6.10.1 Privacy protection:

Data collected from each case was identified using unique research case numbers.

Results from the study were not linked to ante-mortem participants by name.

In accordance with the current guidelines for patient records, privacy and confidentiality of medical records collected during this study was strictly maintained.

Confidentiality was maintained whereby only the principle investigator, supervisors and statistician were allowed to view the data.

The personal computer that was used to store the data is password protected.
6.11 DATA MANAGEMENT AND ANALYSIS:

Data was collected in a standardized data capture sheet and verified before entry into a Microsoft Excel worksheet for storage. Stata Statistical Software (Release 14) was used for all statistical analyses.

The clinical characteristics of the study population were summarized using measures of central tendency and presented in tables and charts. Morphological changes on gross pathology and histology of thymi and populations of T lymphocytes, B-lymphocytes and Natural Killer Cells in respective splenic tissue using immunohistochemistry were described using descriptive statistics and presented as proportions in a graphical format.

Nutrition status was classified as severe acute malnutrition (SAM), moderate acute malnutrition (MAM), at risk of AM and well-nourished using the previously collected MUAC and weight measurements.

Correlation between independent variables like nutritional status, lymphocyte counts and the thymic histologic findings was determined using fisher’s exact test. Similarly, the relationship between these variables on the cell populations (T lymphocytes, B-lymphocytes and Natural Killer Cells) was determined using bivariate and multivariate analysis.
7.0 RESULTS

7.1 Demographic Characteristics of the Decedents

Out of the 64 decedents, 60 met the inclusion criteria. 4 were rejected due to presence of tissue autolysis. The characteristics of the decedents were as outlined The age distribution of the decedents was right skewed, with a median (IQR) age of 8 (10) months. The youngest was 1 month old, and the oldest was 48 months. The females were 48.3% (n=29) and males were 51.7% (n=31). The male to female ratio was 1.07:1.

Table 1: Demographic characteristics of the decedents

<table>
<thead>
<tr>
<th>Variable</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>31 (51.7%)</td>
</tr>
<tr>
<td>Female</td>
<td>29 (48.3%)</td>
</tr>
<tr>
<td>Nutritional status</td>
<td></td>
</tr>
<tr>
<td>SAM (MUAC&lt;110mm)</td>
<td>20 (33.3%)</td>
</tr>
<tr>
<td>MAM (MUAC110-125mm)</td>
<td>17 (28.3%)</td>
</tr>
<tr>
<td>At risk of AM (MUAC 125-135mm)</td>
<td>6 (10%)</td>
</tr>
<tr>
<td>Well nourished (MUAC&gt;135mm)</td>
<td>17 (28.3%)</td>
</tr>
</tbody>
</table>

Figure 1: Density plot showing age distribution of the decedents

N= 60
Mean= 11.48
SD= 10.99
Median= 8
IQR=10
7.2 Nutritional status:

Majority of the decedents had malnutrition, 20 (33.3%) had SAM with a MUAC<110mm, 17 (28.3%) had MAM with MUAC of 110-125mm, 6 (10%) were at risk of AM with MUAC 125-135mm. Only 17(28.3%) of the decedents were well nourished with MUAC >135mm. This is depicted in table 1 (page 15).
7.3 Thymus morphology:
As shown in figure 3, the mean thymic weight from the 60 decedents was 6.06 grams with a median of 4.35 grams. 78.3% of the thymi weighed less than 10 grams while 21.67% had normal thymic weight of more than 10 grams.

**Figure 3: Thymic weights**

![Graph showing thymic weights](image)

**Table 2: Distribution of microscopic findings of 60 specimens.**
As shown in table 2 below, only 2 (3.3%) of the decedents had normal (grade 1) thymi on histology. 16 (26.7%) had features of fibrosis (grade 4), 24 (40.0%) were grade 3 and 18 (30.0%) had grade 2.

<table>
<thead>
<tr>
<th>Microscopic findings</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 1</td>
<td>2 (3.3%)</td>
</tr>
<tr>
<td>Grade 2</td>
<td>18 (30%)</td>
</tr>
<tr>
<td>Grade 3</td>
<td>24 (40%)</td>
</tr>
<tr>
<td>Grade 4</td>
<td>16 (26.7%)</td>
</tr>
</tbody>
</table>

N = 60
Mean = 6.02 gm
SD = 5.5
Median = 4.35 gm
IQR = 6.8
Majority of the thymic tissues had marked (49.2%) and moderate expression (23.7%) of CD3. This is comparable to respective splenic specimens where 45% had marked expression while 40% of the splenic tissues has moderate expression. CD 5 was almost similar, where 30.5% of the thymic tissues showed marked expression while 35.6% exhibited moderate expression of the marker. In the splenic tissues majority had marked and moderate CD5 expressions of 38.3% and 43.3% respectively.

Figure 4: Comparison of T-lymphocytes in Thymus and Spleen.
Majority of the thymi had mild (39.0%) and moderate (35.6%) CD20 expression; mild (52.5%) and moderate (28.8%) CD79a expression. Contrary to this, majority of the splenic tissues showed moderate (40%) and marked (33.3%) CD20 expression; moderate (28.8%) and marked (40%) CD79a expression.

**Figure 5: B lymphocytes in Thymus and Spleen**
Figure 6: NK cells in thymus and spleen

![NK Cell Population Diagram]

CD56

- No Expression: Thymus 27.1%, Spleen 5%
- Mild Expression: Thymus 49.2%, Spleen 43.3%
- Moderate Expression: Thymus 20.3%, Spleen 51.7%
- Marked Expression: Thymus 3.4%, Spleen 0%

7.4 Lymphocyte Count on Complete Blood Count

Figure 7: Lymphocyte count in $\times 10^9$/L on CBC

- N=54
- Mean = 4.98
- SD = 6.3
- Median = 3.05
- IQR = 3.5
Figure 8: Distribution of lymphocyte categories based on lymphocyte count on 54 specimens,

Fifty four decedents had complete blood count results documented from previous PRESS study. The mean lymphocyte count was $4.98 \times 10^9$ with a median of 3.05. Age specific reference range for lymphocyte count (1.5-7$ \times 10^9$/L) was used. 61.1% of the decedents had normal lymphocyte counts, 22.2% had lymphopenia and 16.7% had lymphocytosis.
Table 3: Association between nutrition status and microscopic findings

<table>
<thead>
<tr>
<th>Nutrition status</th>
<th>Grade 1</th>
<th>Grade 2</th>
<th>Grade 3</th>
<th>Grade 4</th>
<th>Total</th>
<th>Fishers exact test</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAM</td>
<td>1</td>
<td>2</td>
<td>10</td>
<td>7</td>
<td>20</td>
<td>0.1</td>
</tr>
<tr>
<td>MAM</td>
<td>0</td>
<td>6</td>
<td>6</td>
<td>5</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>At risk of AM</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Well nourished</td>
<td>1</td>
<td>8</td>
<td>7</td>
<td>1</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>2</td>
<td>18</td>
<td>24</td>
<td>16</td>
<td>60</td>
<td></td>
</tr>
</tbody>
</table>

Fisher’s exact test was performed to examine the association between nutrition status and microscopic findings. There was no significant association between the nutrition status and microscopic findings (P>0.05) as shown on table 6 above.

Table 4: Association between lymphocyte count and microscopic findings.

<table>
<thead>
<tr>
<th>Lymphocyte count</th>
<th>Grade 1</th>
<th>Grade 2</th>
<th>Grade 3</th>
<th>Grade 4</th>
<th>Total</th>
<th>Fishers exact test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphopenia</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>6</td>
<td>12</td>
<td>0.219</td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
<td>11</td>
<td>14</td>
<td>8</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Lymphocytosis</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1</td>
<td>15</td>
<td>22</td>
<td>16</td>
<td>54</td>
<td></td>
</tr>
</tbody>
</table>

A Fishers exact test was performed to examine the relationship between lymphocyte count and microscopic findings. There was no significant association between the lymphocyte count and microscopic findings (P>0.05) as shown on table 7 above.
Table 5: Association between nutrition status and CD3 (Spleen):

<table>
<thead>
<tr>
<th>Nutrition status</th>
<th>Thymus CD3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Expression</td>
</tr>
<tr>
<td>SAM</td>
<td>2</td>
</tr>
<tr>
<td>MAM</td>
<td>1</td>
</tr>
<tr>
<td>At risk of AM</td>
<td>1</td>
</tr>
<tr>
<td>Well nourished</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>5</td>
</tr>
</tbody>
</table>

A Fishers exact test was performed to examine the association between nutrition status and CD3 (Splenic T-lymphocytes). There was a significant association between the nutrition status and CD3 (Spleen) (P<0.05) as shown on table 8 above.

Table 6: Association between nutrition status and CD20 (Spleen):

<table>
<thead>
<tr>
<th>Nutrition status</th>
<th>Thymus CD20</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Expression</td>
</tr>
<tr>
<td>SAM</td>
<td>6</td>
</tr>
<tr>
<td>MAM</td>
<td>0</td>
</tr>
<tr>
<td>At risk of AM</td>
<td>1</td>
</tr>
<tr>
<td>Well nourished</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
</tr>
</tbody>
</table>

A Fishers exact test was performed to examine the association between nutrition status and CD20 (Splenic B lymphocytes). There was significant relationship between the nutrition status and CD20 (Spleen) (P<0.05) as shown in table 9 above.
Table 7: Association between lymphocyte count and CD3 (Thymus)

<table>
<thead>
<tr>
<th>Lymphocyte count</th>
<th>CD3 IHC staining in thymus</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Fisher's exact</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Expression</td>
<td>Mild expression</td>
<td>Moderate expression</td>
<td>Marked expression</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>Lymphopenia</td>
<td>2</td>
<td>5</td>
<td>3</td>
<td>1</td>
<td>11</td>
<td>0.016</td>
</tr>
<tr>
<td>Normal</td>
<td>2</td>
<td>5</td>
<td>8</td>
<td>18</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Lymphocytosis</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>7</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>5</td>
<td>11</td>
<td>11</td>
<td>26</td>
<td>53</td>
<td></td>
</tr>
</tbody>
</table>

A Fishers exact test was performed to examine the association between lymphocyte count and CD3 (Thymus). There was significant association between the lymphocyte count and CD3 (Thymus) (p<0.05)

Table 8: Association between lymphocyte count and CD20 (Thymus)

<table>
<thead>
<tr>
<th>Lymphocyte count</th>
<th>CD 20 IHC staining in thymus</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Fisher's exact</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Expression</td>
<td>Mild expression</td>
<td>Moderate expression</td>
<td>Marked expression</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>Lymphopenia</td>
<td>3</td>
<td>5</td>
<td>3</td>
<td>0</td>
<td>11</td>
<td>0.769</td>
</tr>
<tr>
<td>Normal</td>
<td>5</td>
<td>14</td>
<td>10</td>
<td>4</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Lymphocytosis</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>21</td>
<td>17</td>
<td>5</td>
<td>53</td>
<td></td>
</tr>
</tbody>
</table>

A Fisher’s exact test was performed to examine the association between lymphocyte count and CD20 (Thymus). There was no significant association between the lymphocyte count and CD20 (Thymus) (P>0.05)
7.5 Histomorphological photomicrographs:

H/E staining of the spleen on TMA showing PALS, white and red pulps.

CD3 positive control using appendicular lymphoid tissue.
Marked CD3 (>50% cells staining) expression in the spleen. Higher magnification showing cytoplasmic staining

Marked CD5 expression in normal thymic lobules
Moderate CD3 (10-50% cells staining) expression in thymic lobules.

Mild CD5 (<10% cells staining) expression in the thymus- few scattered positively staining cells.
No cells took up CD20 stain on markedly atrophic thymic lobules.

Grade 4 thymic atrophy with predominantly fibrous septae and replacement of lobules by fat tissue.
Marked thymic atrophy (grade 3) with C:M ration reversal, shrunken thymic lobules, prominent interlobular septae and prominent thymic epithelial cells.

Grade 2 thymic involution showing reversal of cotrico-medullary ratio and moderate increase in thymic epithelial cell.
Normal grade 1 thymus- lobular preservation with hypercellular cortex and a hypocellular medulla, thinned out interlobular septae and few small epithelial cells.
8.0 DISCUSSION

The thymus plays a critical role in primary T-cell lymphopoiesis and several factors have been documented to affect its function. Malnutrition, infections and other acute stressors contribute to thymic involution with resultant secondary immune deficiency. This was an autopsy study which included 60 decedents, between 1-48 months of age. A similar autopsy study was done by Abe et al that included 40 children who had been exposed to various stressors (30). In the current study, the proportion of decedents that had thymic involution and malnutrition was significantly high.

We found that 78.3% of the study population had thymus weights below 10 grams, with a mean thymus weight of 6 grams which is considerably lower than the normal documented weight of 10-15 grams. This result was similar to a study done by Abe et al that found reduced thymic/body weights ratios on autopsy, in infants and neonates who were exposed to various stressors i.e injury, child abuse, severe infection and congenital disease (30). 96.7% of the decedents in this current study had significant features of thymic involution, whereby marked predominance of stromal extracellular matrix was classified as grade 4; scant thymic lobules with increased tangible body macrophages plus epithelial cells and prominent interlobular septae was classified as grade 3; intact lobules but with reversal in cortico-medullary ratio and mild increase in tangible body macrophages was termed as grade 2. 26.7% of thymi showed grade 4, 40% showed grade 3 and 30% exhibited grade 2 features. Only 3.3% had normal histologic findings (grade 1). Findings that were similar to a study done by Toti et al, where grade 2 and grade 3 thymic involution were present in spontaneously aborted fetuses due to chorio-amnionitis (10). Abe et al in his study found higher grades of involution i.e. 2,3 and 4; in the thymic tissue from stressor positive infants and neonates as compared to the control group which exhibited normal histology (30). A study by Garly et al concluded that small thymus size at 6 months of age is a strong and independent risk factor for mortality in cases of childhood infections. Similarly, a study done by Billard et al showed atrophic changes in the thymus as
early as 24 hours - 4 days in mice that were exposed to lipopolysaccharide induced endotoxemia(31).

In infectious states, the thymus is directly affected by localized infectious agents or through systemic mediators such as increased glucocorticoids and pro-inflammatory agents from distant infectious sites (32). Rice et al reported that malnutrition is strongly associated with increased risk of mortality from acute lower respiratory tract infections and pneumonia (33). In a separate study done by Bosire et al, it was found that 75.9% of the 60 decedents who were included in the current study died within 4 days of hospital admission with majority of the deaths occurring within the first day of admission. An unusual finding in this study was one case of disseminated tuberculosis to the thymus, with marked tissue fibrosis, this is contrary to the fact that there exists a blood-thymus barrier that restricts activated T-cells entry and cytokine generation within the thymus (34). Features of localized infections that we saw included vascular channel proliferation with hemorrhage, thrombotic microangiopathy, vascular hyalinization within lobules, frequent plasma cells, hemosiderin laden and granular macrophages. Other additional finding seen was fat replacement which has mainly been documented in thymic atrophy related to ageing (9).

Despite the high prevalence of thymic involution, there was significant expression of T-cell markers where majority of thymic tissue had moderate-marked expression (72.9% CD3 staining) of the T-cell markers. This scenario was replicated in the spleen (85% CD3 staining). Contrary to this, majority of the thymi had mild-moderate expression (74.5% CD20 staining) of the B-cell markers with moderate-marked B-cell expression (73.3% CD20 staining) in the spleen. This finding is similar to the Toti et al study which did not show difference in T, B and NK cellular populations of atrophic thymi from fetuses with chorioamnionitis as compared to the normal group. A study by Nobrega et al showed that homing of peripheral T-lymphocytes in an attempt to mediate protection against infection resulted in significant T-cell population within the affected thymi (32). The increased lymphocyte expression in the thymus can thus be explained by peripheral lymphocyte infiltration.
Malnutrition has been associated with escalated childhood mortality rates due to various factors such as impaired gut barrier functions, reduced plasma immunoglobulin and complement levels and diminished thymus sizes as a result of decreased thymocyte proliferation and increased thymocyte apoptosis(2). During an infectious process, the high metabolism associated with the immune response further worsens the malnutrition by consumption of proteins and energy(35). In this study, 81.7% of the decedents were malnourished, with 33.3% having severe acute malnutrition, 28.3% having moderate acute malnutrition and 10% were at risk of malnutrition. Only 28.8% of the decedents were well nourished. Fishers exact test was performed to determine the relationship between malnutrition and thymic involution, no association was found. This is contrary to a study by Chromi et al where prenatal fetuses with IUGR had disproportionately small thymus size as a result of intrauterine starvation(16). Similarly, Sante et al documented marked thymic involution - on ultrasonography, in children who were followed up and treated for malnutrition. However, there was documented immune recovery that was greatly accelerated by additional zinc supplementation(36). A study by Abe et. al on stressor positive children found increased leptin receptors on the thymic epithelial cells in malnourished children. Thymi that had higher histologic grade of involution, had higher expression of the leptin receptors(30). The inclusion criteria for this current study was history of cough and reported fever, the pre-existing infection could therefore have acted as a confounding factor affecting the association between thymic involution and malnutrition in this case(30).

Although there was no significant relationship found between the microscopy of the thymus and nutritional status, we found that the relationship between CD3 T-cell populations and CD20 B-cell populations in the thymus with nutritional status to be significant (P<0.05). These findings are similar to a study done by Shushimita et al who reported significant reduction in immature and mature B-cells in lymphoid organs of mice that were subjected to dietary restriction and fasting. Similarly, their thyme exhibited T-cell depletion with arrested thymopoiesis(37).

In this study, we found that 61.1% of the decedents had normal lymphocyte counts while 16.7% had lymphocytosis. Only 22.2% of these decedents had lymphopenia; despite the high prevalence of malnutrition and thymic involution in this study population. This finding compares with a previous systemic review article by Maren et.al that reported high lymphocyte counts in malnourished children(38).
Fisher’s exact test performed to examine the relationship between thymus involution and peripheral blood lymphocyte count found no significant relationship between the two. This is contrary to a study done by Varga et.al that found a positive correlation between thymus size and peripheral lymphocyte count(39). A study done on mice by Shushimita et.al showed increased T and B cell production from the bone marrow despite these mice’s thyme showing T-cell depletion due to dietary restriction and fasting(37). Savino et al found that children who had severe protein malnutrition had lymphopenia, under-developed secondary lymphoid organs with an increase in immature CD4/CD8 double negative/positive in the periphery (28).

9.0 CONCLUSION:
Thymic involution occurs early in childhood infectious states. The common histologic changes in the under five children presenting with acute respiratory infections; include fibrosis, marked reduction in lobular sizes, increase in thymic epithelial cells, fat replacement, increased interlobular extracellular matrix, increase in macrophages. Changes related to infection included vascular channel proliferation with hemorrhage, thrombotic microangiopathy with vascular hyalinization within lobules, frequent plasma cells, hemosiderin laden and granular macrophages.
Cellular populations of B and T and NK cells in the secondary lymphoid organs were not adversely affected by thymic involution.
T and B cell populations in the thymus and secondary lymphoid organs were significantly affected by malnutrition states of the decedents.
Peripheral lymphocyte counts on complete blood count were not affected by the atrophic thymi. Majority of these under-fives had malnutrition and thymic involution which may have contributed to these childhood mortalities.

10.0 LIMITATIONS:
Lack of control of the sampling process may not have given the true histologic picture as we relied entirely on samples that had been previously collected and archived.
Some samples were poorly fixed, leading to difficult histologic interpretation.
We relied on pre-established thymic weight ranges from western countries as there are no documented normal weights from our local population. This might have affected interpretations of thymic weights.

Not all decedents had their clinical data readily available, this could have led to the negative clinical correlations.

11.0 RECOMMENDATION:

Clinicians could be advised to assess thymus size ultrasonographically, as a marker of morbidity/mortality in children who present with chronic infection plus malnutrition. Aggressive clinical management of malnutrition in children is recommended to avoid preventable fatalities from SARI. Future studies that target a wider study population that incorporate C4/CD8 markers, infectious etiologic agents, immunoglobulin levels could be done to further analyze thymic function, and the effects of malnutrition and infections on immunologic status of these children.
12.0 Bibliography:


29. Improving cellular therapy for primary immune deficiency diseases.pdf.

30. Abe S. Stressors increase leptin receptor-expressing thymic epithelial cells in the infant / child thymus. 2018;

31. Billard MJ, Gruver AL, Sempowski GD. Acute endotoxin-induced thymic atrophy is


13.0 APPENDIX:

13.1 DATA CAPTURE SHEET

AUTOPSY FINDINGS IN THE THYMUS IN SARI CASES

**Biodata**

DATE:

RESEARCH STUDY NUMBER:

AGE OF CHILD:

MUAC MEASUREMENT:

WEIGHT OF CHILD:

HEIGHT OF CHILD:

1. Is the child malnourished?
   1. SAM MUAC < 110
   2. MAM MUAC 110-125
   3. At risk of AM MUAC 126-135
   4. Well nourished MUAC >135

Clinical information: lymphocyte count-

0. No date
1. Lymphopenia:
2. Normal.
3. Lymphocytosis

**Histomorphology**

2. MACROSCOPY: weight in grams
3. MICROSCOPIC DESCRIPTION:-
   i) hypercellular medulla with hypocellular cortex, YES ☐ NO ☐
   ii) reduced number of cortical lymphocytes; YES ☐ NO ☐
   iii) increased number of tangible body macrophages with apoptotic bodies,
iv) Shrinkage of thymic lobules, YES NO
v) increased prominence of interlobular septae, YES NO
vi) blurring of normal corticomedullary demarcation, YES NO
vii) Epithelial cell proliferation with development of glandular structures containing
eosinophilic material. YES NO

1. Fibrotic
2. Severly atrophic
3. Mildly atrophic
4. Normal
4. IMMUNOHISTOCHEMISTRY:
   I. Thymus
      +3 – >50% cellular staining (marked expression), +2 – 10-50% cellular staining
      (moderate expression), +1- < 10% cellular staining (mild expression), 0 – no cells
      staining (no expression).

<table>
<thead>
<tr>
<th></th>
<th>+3</th>
<th>+2</th>
<th>+1</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD20</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CD5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD79a</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CD56</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>PAX 5</td>
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</tr>
</tbody>
</table>

   II. Spleen
      +3 - marked expression, +2 - moderate expression, +1- mild expression, 0 - no expression

<table>
<thead>
<tr>
<th></th>
<th>+3</th>
<th>+2</th>
<th>+1</th>
<th>0</th>
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<td>CD79a</td>
<td></td>
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13.2 SOP FOR RETRIEVED SPECIMEN BLOCKS HANDLING AND PROCESSING.

1. The retrieved blocks shall be sectioned using microtome [3-5 microns] and the sections floated in warm water to remove wrinkles.
2. The sections will then be picked on a slide and placed in a warm oven for 15 minutes so to adhere to the slide.
3. The sections will then be deparaffined by dipping them in xylene to alcohol and then water.
4. Staining will be done using standard Haematoxylin and Eosin techniques which entails:
   I. Staining in Harris Haematoxylin for 5 minutes. Then,
   II. Washing in running tap water for 1 minutes, Then,
   III. Dipping 3 to 5 times in 1% Acid Alcohol, Then,
   IV. Wash in running tap water for 1 minute. Then,
   V. Rinse in 95% alcohol 10 dips
   VI. Stain in working eosin Y, making sure stain covers slides completely.
   VII. Wash in running tap water for 30 seconds
   VIII. Dehydrate in ascending alcohols levels and Clear in three changes of xylene
5. The quality of staining will be confirmed first before mounting.
6. A cover slip will be applied to the slide.
7. Upon drying microscopic examination will be done.
13.3 SOP FOR IHC STAINING.

Quantitative immunohistochemical staining of splenic tissues for CD3, CD20 and CD56 shall be done as per the SOP below.

- Sectioned slides shall be heated at 100°C for 1 hour then cooled.
- Dewaxing will be done in xylene 3 changes × 2 minutes each. Hydration will be done in alcohol 3 changes × 2 minutes each.
- The slides will be air-dried and novapen used to outline tissue sections.
- The slides shall be flooded with citric acid buffer pH 6.0 or EDTA buffer pH 8.0.
- The slides shall be put in a microwave or steamer for 20 minutes and checked at 5 minutes to confirm they are flooded by the buffer.
- The slides shall be cooled for 10 minutes then rinsed with distilled water.
- Peroxidase block shall be applied for 5 minutes then rinsed with Tris/PBS buffer.
- Primary antibody shall be applied for 30 minutes then washed thoroughly with Tris/PBS buffer.
- A post-primary conjugate shall be applied for 30 minutes then washed thoroughly with Tris/PBS buffer.
- A polymer shall be applied for 30 minutes then washed with Tris/PBS buffer.
- DAB chromogen diluted at 1:20 with substrate buffer shall be applied for 2-5 minutes then washed with Tris/PBS buffer.
- Counterstaining shall be done using hematoxyllin for 1-2 minutes then washed with distilled water.
- Differentiation will be done in 1% acid-alcohol then washed with distilled water.
- The slides shall be blued in scott’s tap water then washed with distilled water.
- The slides shall then be hydrated with alcohol 3 changes × 2 minutes then cleared with xylene 3 changes × 2 minutes.
- The slides shall then be mounted with DPX and then examined in comparison to the H&E respective counterpart.
- Normal splenic tissues subjected through similar staining techniques shall be used for positive Quality Control.
KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713949, 07225-05401, 0733-400000. Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1

January 24, 2014

TO: DR. HENRY NJENGA NJUGUNA (PRINCIPAL INVESTIGATOR)

THROUGH: DR. STEPHEN MUNGA,
ACTING DIRECTOR, CGHR,
KISUMU

Dear Sir,

RE: SSC PROTOCOL NO. 2692 (RESUBMISSION): ETIOLOGY OF PEDIATRIC RESPIRATORY MORTALITY AT KENYATTA NATIONAL HOSPITAL, NAIROBI, KENYA

Reference is made to your letter dated 16th January, 2014. The ERC Secretariat acknowledges receipt of the revised document on 22nd January, 2014.

This is to inform you that the Ethics Review Committee (ERC) reviewed the document submitted, and is satisfied that the issues raised at the 221st meeting of 26th November 2013, have been adequately addressed.

This study is granted approval implementation effective this January 24, 2014. Please note that authorization to conduct this study will automatically expire on January 23, 2015. If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the ERC secretariat by December 12, 2014.

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the SSC and ERC for review prior to initiation.

You may embark on the study.

Yours faithfully,

[Signature]

DR. ELIZABETH BUKUSI,
ACTING SECRETARY,
KEMRI/ETHICS REVIEW COMMITTEE

Name of Institution or Organization Providing IRB Review (Kenyatta National Hospital):

IRB Registration #: __IORG0001873__ Federal wide Assurance (FWA) #, if any: __FWA00002173__

Name of Institution Relying on the Designated IRB (Institution B):
__KENYA MEDICAL RESEARCH INSTITUTE (KEMRI)__

FWA #: __FWA00002066__

The Officials signing below agree that __KENYA MEDICAL RESEARCH INSTITUTE__ may rely on the designated IRB for review and continuing oversight of its human subjects research described below:

☐ This agreement applies to all human subjects research covered by Institution B’s FWA.

☐ This agreement is limited to the following specific protocol(s):

Name of Research Project: ____________________________
Name of Principal Investigator: ______________________
Sponsor or Funding Agency: __________________________ Award Number, if any: ______________________

☐ Other (describe): describe All protocols submitted and reviewed and approved by the Kenyatta National Hospital: IRB will be accepted and reliance on the said IRB granted approval by the Kenya Medical Research Institute IRB on request of the investigator.

The review performed by the designated IRB will meet the human subject protection requirements of Institution B’s OHRP-approved FWA. The IRB at Institution/Organization A will follow written procedures for reporting its findings and actions to appropriate officials at Institution B. Relevant minutes of IRB meetings will be made available to Institution B upon request. Institution B remains responsible for ensuring compliance with the IRB’s determinations and with the Terms of its OHRP-approved FWA. This document must be kept on file by both parties and provided to OHRP upon request.

Signature of Signatory Official (Institution/Organization A):

[Signature]

Print Full Name: PROF. A.N. GUANTAI, Ph.D
Institutional Title: Dean, College of Health Sciences, University of Nairobi, Department of Pharmacology & Pharmacognosy

Date: __3rd__ April 2014

NOTE: The IRB of Institution A must be designated on the OHRP-approved FWA for Institution B.

Signature of Signatory Official (Institution B):

[Signature]

Print Full Name: DR. ELIZABETH A. BUKUSI
Institutional Title: Deputy Director (Research & Training)

Date: __3rd__ April 2014

45
Njuguna, Henry Njenga (CDC/CGH/DGHP)

May 11 (4 days ago)

to me

Dear Dr. Cathy Samo

This email is to confirm that you have been granted permission to use data collected as part of the Pediatric Respiratory Etiology Surveillance Study (PRESS) located at the University of Nairobi/Kenyatta National Hospital pathology department for your thesis work and publications related to it.

I would also like to wish you great success in your studies.

Yours sincerely,

Dr. Henry N. Njuguna
Principal investigator
PRESS study
Dear Sir/Madam

The Influenza Program, at the Centers for Disease Control and Prevention (CDC) office in Kenya, is aware of Dr. Cathy Samo’s study proposal that aims to describe thymus autopsy findings among children <5 years who were hospitalized with respiratory illness at the Kenyatta National Hospital and died during hospitalization. She is proposing to use pre-existent data and clinical specimens collected as part of a CDC study looking at postmortem evaluation of causes of deaths in children hospitalized at the Kenyatta National Hospital, in Nairobi [namely the Pediatric Respiratory Etiology Surveillance Study – PRESS (KEMRI SSC no 2692)].

We are in agreement of the use of thymus and splenic tissue blocks kept at the hospital for her study proposal. We are also in agreement of sharing data already collected as part of PRESS on demographics, clinical presentation and outcomes to facilitate her study. We can also share clinical assessment of cause of death and the gross pathology autopsy findings. Once her proposal is approved, we will prepare a dataset to share with Dr. Samo.

The only data we would not share pertains to research tests done in clinical specimens collected post-mortem and the final cause of death and etiology of disease that was part of the main objective of our study and are currently being analyzed (as of September 8, 2017). Until we can understand the laboratory results done as part of our research, we are constraint on our ability to share with other researchers. This being our only restriction to the use of the data. We were able to enroll 64 subjects in our study and are currently summarizing our findings for peer-reviewed publication.

Please let me know if this email suffice in order to have her proposal accepted. I will be glad to provide further clarifications if needed.

Best regards,

Sandra

Sandra S. Chaves, MD, MSc
Influenza Program Director
Centers for Disease Control and Prevention, Kenya Office
CONSENT FORM FOR POSTMORTEM SPECIMEN COLLECTION DURING SARI
STUDY: 2014-2015

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<th>Today’s date</th>
<th>Decedents unique identification number</th>
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Name of decedent:

Name next of kin:

File num:

Receive our condolences on the death of (name). (Name) had an illness affecting his/her lungs which may have led to his/her death. Lung illnesses are one of the leading causes of sickness and death in our country. We would like to find out the cause of the lung illness in (name) that may have led to his/her death. This will enable us to know the leading germs that cause lung illnesses. This knowledge will then help us choose vaccines and other prevention and treatment options that might help others avoid similar deaths in future.

What we will do

We will take some lung specimen using a needle and syringe. As is normally done in an autopsy we will open the body and take samples of the lungs and other organs that may be damaged or have disease causing germs. These specimens will be sent to laboratories in KNH and KEMRI/CDC in Nairobi, and to CDC Laboratories in Atlanta, Georgia, USA for analysis so as to identify germs that may have led to your child’s illness and death. For each specimen, we will place a number that uniquely identifies the samples. This number is similar to the one on questionnaires that collected clinical data when the child was in the ward. In addition, we will collect some blood and test it for HIV infection and influenza. If you would like, we will let you know the HIV test result. If it is positive, we will recommend to you and your spouse (if blood relatives with the deceased) to have an HIV test.

We will let you know the results of tests done once they are ready and the cause of death. If the results of tests reveal TB infection, we will recommend that household contacts be screened for TB as it is highly infectious.

Benefit from being in this study:

This study will let you know the cause of death of your child, based on studies performed. We will call you to inform you on the preliminary cause of death. At a later date when all test results are available, we will call you and inform you on the confirmed cause of death. Otherwise the study will provide no direct benefit to you. In general, the study will help us to learn more about causes of lung illnesses that lead to death in our country so that we can provide better care to prevent similar deaths in the future. If you would like we will call you and tell you what we think was the cause of death for your child. In addition, if you would like to know, we will let you know your child’s HIV test result, if positive you will be referred to a testing and counseling center for follow-up. It may take up to six months for us to determine the final cause of death.
Risks from being in this study:
The body may have cuts which will be sewed together after the post mortem.

Confidentiality
No names shall appear on samples collected. Instead, numbers will be used to identify the samples. Most of the samples collected will be tested here in the hospital and at KEMRI/CDC laboratory. However, for some tests that cannot be done here, part of the samples may be sent to special laboratories in CDC in the United States.

All the study records will be kept secretly and securely. There will be people involved in the study who will need to see the deceased’s health records. These people may include members of the study team, the study monitors, and members of the ethics committees that oversee the study. In addition, the information collected about the deceased will be shared with our data team, who are located at KEMRI/CDC offices in Nairobi. The information shared with the data team will not contain names or any other personal identifying information. Reports and publications from this study will not contain decedent’s name or any other personal identifying information.

What happens if I do not want a post mortem for the deceased?
You can choose not to have the deceased participate in this study. The body will still receive the usual care at the mortuary.

Will it cost anything?
It will not cost you anything to have the deceased participate in this study. We will meet the post-mortem costs, costs of any laboratory tests done and mortuary fees for a period of up to 7 days since death.

Who do I call if I have questions or problems?

- If you have questions or complaints about this study, you can call the principal investigator, Henry Njuguna, at phone: 020-271-3008 x176.
- If you have questions about rights as a study participant, call the Ethical Review Committee for Research in Human Subjects. You should contact the ethics committee if you feel you have not been treated fairly or if you have other concerns. The ethics committee contact information is: The Secretary, KEMRI Ethics Review Committee, P.O. Box 54840-00200, NairobiTelephone numbers: 020-2711541, 0722205901, 0733400003. Email address: ERCadmin@kemri.org

What does your signature (or initials/mark) on this consent form mean?

Your signature (or initials/mark) on this form means:

- You have been informed about this study’s purpose, procedures, possible benefits and risks.
- You have been given the chance to ask questions before you sign.
- You have voluntarily agreed to the statements below where you answer yes.

I have read or had the consent form read to me and had a chance to ask questions.

Please indicate Yes or No:
I agree to have the deceased body be used in this study ☐ Yes  ☐ No
If No why?
I agree to allow the deceased medical records to be reviewed by study staff and ethics committee members:
☐ Yes  ☐ No
I agree to allow specimens from the deceased to be stored and possibly used after this study is over to test for things related to respiratory disease:
☐ Yes  ☐ No
I agree to specimens and data being sent outside the country for research:
☐ Yes  ☐ No
I agree to be informed about the child’s HIV test results:
☐ Yes  ☐ No

Consent agreement:

I understand that I am free to choose not to have his/her body take part in this study and that saying “NO” will have no effect on post-mortem care

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<td>kin</td>
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<tr>
<td>Witness*</td>
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*Next of kin can sign or make a mark and have his/her consent confirmed by the signature of a witness