# CLINICOPATHOLOGICAL SPECTRUM OF HAEMOPHAGOCYTIC SYNDROME

**DISSERTATION**

**SUBMITTED FOR**

**M.D in PATHOLOGY**

**THE TAMILNADU DR.M.G.R MEDICAL UNIVERSITY, CHENNAI**

**DEPARTMENT OF PATHOLOGY**

**PSG INSTITUTE OF MEDICAL SCIENCE & RESEARCH**

**PEELAMEDU, COIMBATORE- 641 004**

**TAMILNADU, INDIA**

# Certificate

# CERTIFICATE

This is to certify that the dissertation work entitled **"CLINICOPATHOLOGICAL SPECTRUM OF HAEMOPHAGOCYTIC SYNDROME"** submitted by **Dr. Abinaya Sundari A,** is a work done by her during the period of study in this department from 30/06/2015 to 30/06/2017. This work was done under the guidance of **Dr. Prasanna N Kumar,** Professor & HOD, Department of Pathology, PSG IMS&R..

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# CERTIFICATE

This is to certify that the thesis entitled "**CLINICOPATHOLOGICAL SPECTRUM OF HAEMOPHAGOCYTIC SYNDROME"** submitted by **Dr. Abinaya Sundari A** to The Tamilnadu Dr MGR Medical University, Chennai, for the award of the degree of **Doctor of Medicine in Pathology,** is a bonafide record of research work carried out by her under my guidance. The contents of this thesis, in full or in parts, have not been submitted to any other Institute or University for the award of any degree or diploma.

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# DECLARATION

I **Dr. Abinaya Sundari A**, do hereby declare that the thesis entitled **"CLINICOPATHOLOGICAL SPECTRUM OF HAEMOPHAGOCYTIC SYNDROME"** is a bonafide work done by me under the guidance of **Dr. Prasanna N Kumar** , Professor & HOD, Department of Pathology, PSG Institute of Medical Sciences & Research. This study was performed at the PSG Institute of Medical Sciences & Research, Coimbatore, under the aegis of the The Tamilnadu Dr MGR Medical University, Chennai, as part of the requirement for the award of the MD degree in Pathology.

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# PSG Institute of Medical Sciences & Research
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To
Dr A Abinaya Sundari
Postgraduate
Department of Pathology
**Guide:** Dr Prasanna N Kumar
PSG IMS & R
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**Ref:** Project No.15/394

**Date:** December 29, 2015

Dear Dr Abinaya Sundari,

Institutional Human Ethics Committee, PSG IMS&R reviewed and discussed your application dated 18.12.2015 to conduct the research study entitled *"Clinicopathological spectrum of haemophagocytic syndrome"* during the IHEC meeting held on 24.12.2015.

The following documents were reviewed and approved:

1. Project Submission form
2. Study protocol (Version 1 dated 18.12.2015)
3. Confidentiality statement
4. Application for waiver of consent
5. Data collection tool (Version 1 dated 18.12.2015)
6. Current CVs of Principal investigator, Co-investigators
7. Budget

The following members of the Institutional Human Ethics Committee (IHEC) were present at the meeting held on 24.12.2015 at IHEC Secretariat, PSG IMS & R between 10.00 am and 11.00 am:

| Sl. No. | Name of the Member of IHEC | Qualification | Area of Expertise | Gender | Affiliation to the Institution Yes/No | Present at the meeting Yes/No |
|---|---|---|---|---|---|---|
| 1 | Mr. R. Nandakumar | BA., BL | Legal Expert, Chairperson | Male | No | Yes |
| 2 | Dr. S. Bhuvaneshwari (Member-Secretary, IHEC) | MD | Clinical Pharmacology | Female | Yes | Yes |
| 3 | Dr. S. Shanthakumari | MD | Pathology, Ethicist | Female | Yes | Yes |
| 4 | Dr D Vijaya | M Sc., Ph D | Basic Medical Sciences (Biochemistry) | Female | Yes | Yes |

The study is approved in its presented form. The decision was arrived at through consensus. Neither PI nor any of proposed study team members were present during the decision making of the IHEC. The IHEC functions in accordance with the ICH-GCP/ICMR/Schedule Y guidelines. The approval is valid until one year from the date of sanction. You may make a written request for renewal / extension of the validity, along with the submission of status report as decided by the IHEC.

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Following points must be noted:

1. IHEC should be informed of the date of initiation of the study
2. Status report of the study should be submitted to the IHEC every 12 months
3. PI and other investigators should co-operate fully with IHEC, who will monitor the trial from time to time
4. At the time of PI's retirement/intention to leave the institute, study responsibility should be transferred to a colleague after obtaining clearance from HOD, Status report, including accounts details should be submitted to IHEC and extramural sponsors
5. In case of any new information or any SAE, which could affect any study, must be informed to IHEC and sponsors. The PI should report SAEs occurred for IHEC approved studies within 7 days of the occurrence of the SAE. If the SAE is 'Death', the IHEC Secretariat will receive the SAE reporting form within 24 hours of the occurrence
6. In the event of any protocol amendments, IHEC must be informed and the amendments should be highlighted in clear terms as follows:
   a. The exact alteration/amendment should be specified and indicated where the amendment occurred in the original project. (Page no. Clause no. etc.)
   b. Alteration in the budgetary status should be clearly indicated and the revised budget form should be submitted
   c. If the amendments require a change in the consent form, the copy of revised Consent Form should be submitted to Ethics Committee for approval
   d. If the amendment demands a re-look at the toxicity or side effects to patients, the same should be documented
   e. If there are any amendments in the trial design, these must be incorporated in the protocol, and other study documents. These revised documents should be submitted for approval of the IHEC and only then can they be implemented
   f. Any deviation-Violation/waiver in the protocol must be informed to the IHEC within the stipulated period for review
7. Final report along with summary of findings and presentations/publications if any on closure of the study should be submitted to IHEC

Kindly note this approval is subject to ratification in the forthcoming full board review meeting of the IHEC.

Thanking You,

Yours Sincerely,

SECRETARY PSG IMS&R COIMBATORE-641004. INSTITUTIONAL HUMAN ETHICS COMMITTEE

**Dr Sudha Ramalingam**
**Alternate Member - Secretary**
**Institutional Human Ethics Committee**

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November 25, 2016

To
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Postgraduate
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**Guide:** Dr Prasanna N Kumar
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The Institutional Human Ethics Committee, PSG IMS & R, Coimbatore - 4, has reviewed your proposal on 25th November 2016 in its expedited review meeting held at IHEC Secretariat, PSG IMS&R, between 10.00 am and 11.00 am, and discussed your request to renew the approval for the study entitled:

*"Clinicopathological spectrum of haemophagocytic syndrome"*

The following documents were received for review:

1. Request for renewal dated 23.11.2016

After due consideration, the Committee has decided to renew the approval for the above study.

The members who attended the meeting held on at which your proposal was discussed, are listed below:

| Sl. No. | Name of the Member of IHEC | Qualification | Area of Expertise | Gender | Affiliation to the Institution Yes/No | Present at the meeting Yes/No |
|---|---|---|---|---|---|---|
| 1 | Mr R Nandakumar (Chairperson, IHEC) | BA., BL | Legal Expert | Male | No | Yes |
| 2 | Dr. S. Bhuvaneshwari (Member-Secretary, IHEC) | MD | Clinical Pharmacology | Female | Yes | Yes |
| 3 | Dr S Shanthakumari | MD | Pathology, Ethicist | Female | Yes | Yes |
| 4 | Dr D Vijaya | M Sc., Ph D | Basic Medical Sciences (Biochemistry) | Female | Yes | Yes |

The approval is valid for one year (29.12.2016 to 28.12.2017).

This Ethics Committee is organized and operates according to Good Clinical Practice and Schedule Y requirements.

Non-adherence to the Standard Operating Procedures (SOP) of the Institutional Human Ethics Committee (IHEC) and national and international ethical guidelines shall result in withdrawal of approval (suspension or termination of the study). SOP will be revised from time to time and revisions are applicable prospectively to ongoing studies approved prior to such revisions.

Kindly note this approval is subject to ratification in the forthcoming full board review meeting of the IHEC.

Yours truly,

SECRETARY
PSG IMS&R
COIMBATORE-641004.
INSTITUTIONAL HUMAN ETHICS COMMITTEE

**Dr S Bhuvaneshwari**
**Member – Secretary**
**Institutional Human Ethics Committee**

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Sources included in the report:

http://www.uptodate.com/contents/chediak-higashi-syndrome

Instances where selected sources appear:

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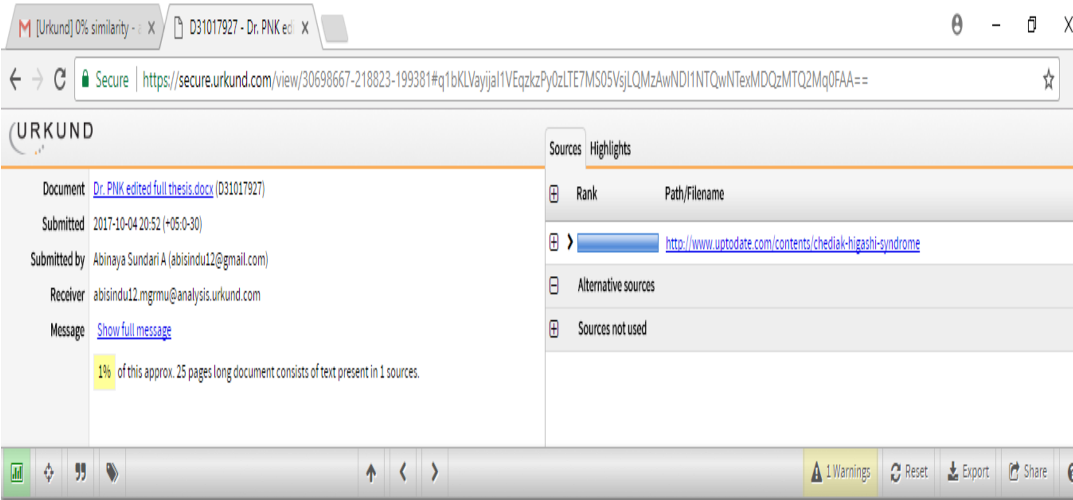


INTRODUCTION Haemophagocytic syndrome (HPS) also known as the Haemophagocytic lymphohistocytosis (HLH) is an aggressive and potentially fatal syndrome that results from inappropriate prolonged activation of lymphocytes and macrophages. The first reported case of HLH was described in 1952 by Farquhar and Claireaux(1) who called the disease as "Familial Hemophagocytic Reticulosis". It is traditionally divided into Primary\ Familial and Acquired\ Secondary. The Familial HLH is due to gene mutations most commonly involving the perforin gene while secondary HLH can occur in a wide variety of conditions including infections, autoimmune disorders to malignancies. The pathological hallmark of the syndrome is aggressive proliferation of macrophages/histiocytes in the reticuloendothelial system that are seen phagocytosing the haematopoietic elements.(2) The incidence is more common in the paediatric population, the highest between birth to 18 months of age. The estimated incidence of HLH is 1.2 cases per million individuals per year. However this is most likely an under estimate because most of the cases results in death before they are diagnosed , some remain undiagnosed while some are not reported. The pathogenetic hallmark for HLH is defective NK cell function. NK cell is a cell of innate immunity which plays an important role in removing stress induced and virus infected cells. In HLH there is a defective NK cell function which can be familial or acquired. Defective NK cell leads to persistent activation of macrophages and T helper cell which results in hypercytokinemia. This hypercytokinemia leads to the various manifestations of HLH. Patients with HPS are commonly very ill at the time of presentation. They usually present with high persistent fever, anaemia, splenomegaly and CNS manifestations. The HLH society-2004 has laid down guidelines for the diagnostic criteria for HLH. Minimal diagnostic parameters are fever, cytopenia, splenomegaly, abnormal liver function tests, elevated serum triglycerides and serum ferritin, low serum fibrinogen and haemophagocytosis in bone marrow aspiration.

# Acknowledgement

# ACKNOWLEDGEMENT

I start in the name of Almighty who has given me more than what I deserve.

This dissertation was brought to life through the constant support from my guide Professor **Dr. Prasanna N Kumar**. I am very grateful to ma'am for her constant support throughout my thesis. She is the best of the teachers any postgraduate could have. Her enthusiasm and her dedication towards the subject and work is an inspiration for everyone in the department.

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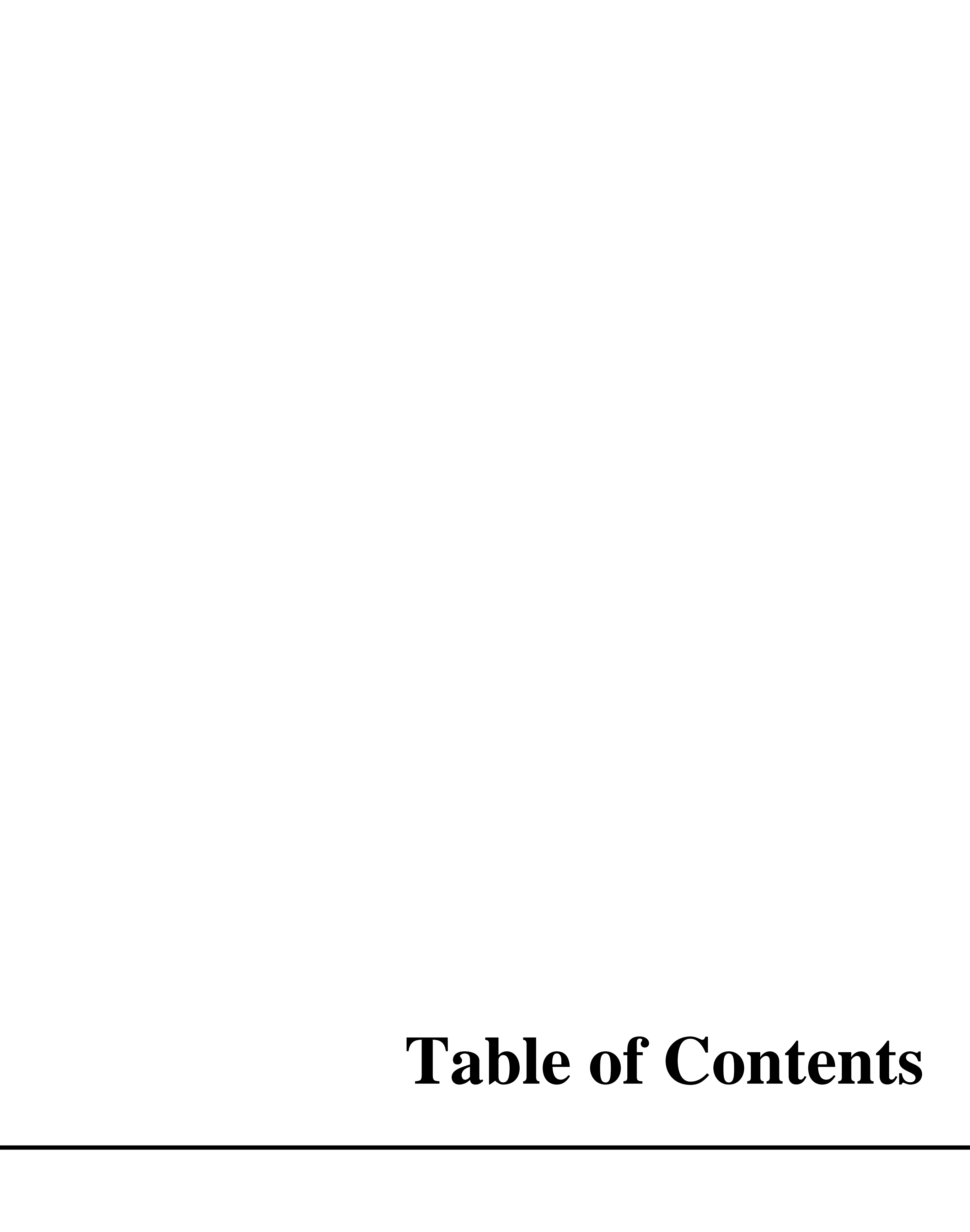
A special mention to Dr. Priya who helped me, figure out what macrophages are!

I am eternally grateful to my parents for their unconditional love and extraordinary sacrifices for my happiness.

I would not be what I am today if not for my husband. He is my friend, my pillar of strength and beyond! Thank you my love for all that you have sacrificed

Last but not the least my little boy Krishiv, my love and my life. I'm sorry for not being there for you as much as I wanted to. Thank you for being strong, kind and for putting up with my tantrums. You are my silver lining, my life, my world and my everything.



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# Introduction

# INTRODUCTION

Haemophagocytic syndrome (HPS) also known as the Haemophagocytic lymphohistocytosis (HLH) is an aggressive and potentially fatal syndrome that results from inappropriate prolonged activation of lymphocytes and macrophages. The first reported case of HLH was described in 1952 by Farquhar and Claireaux[1] who called the disease as "Familial Hemophagocytic Reticulosis". It is traditionally divided into Primary\ Familial and Acquired\ Secondary. The Familial HLH is due to gene mutations most commonly involving the perforin gene while secondary HLH can occur in a wide variety of conditions including infections, autoimmune disorders to malignancies. The pathological hallmark of the syndrome is aggressive proliferation of macrophages/histiocytes in the reticuloendothelial system that are seen phagocytosing the haematopoietic elements.[2]

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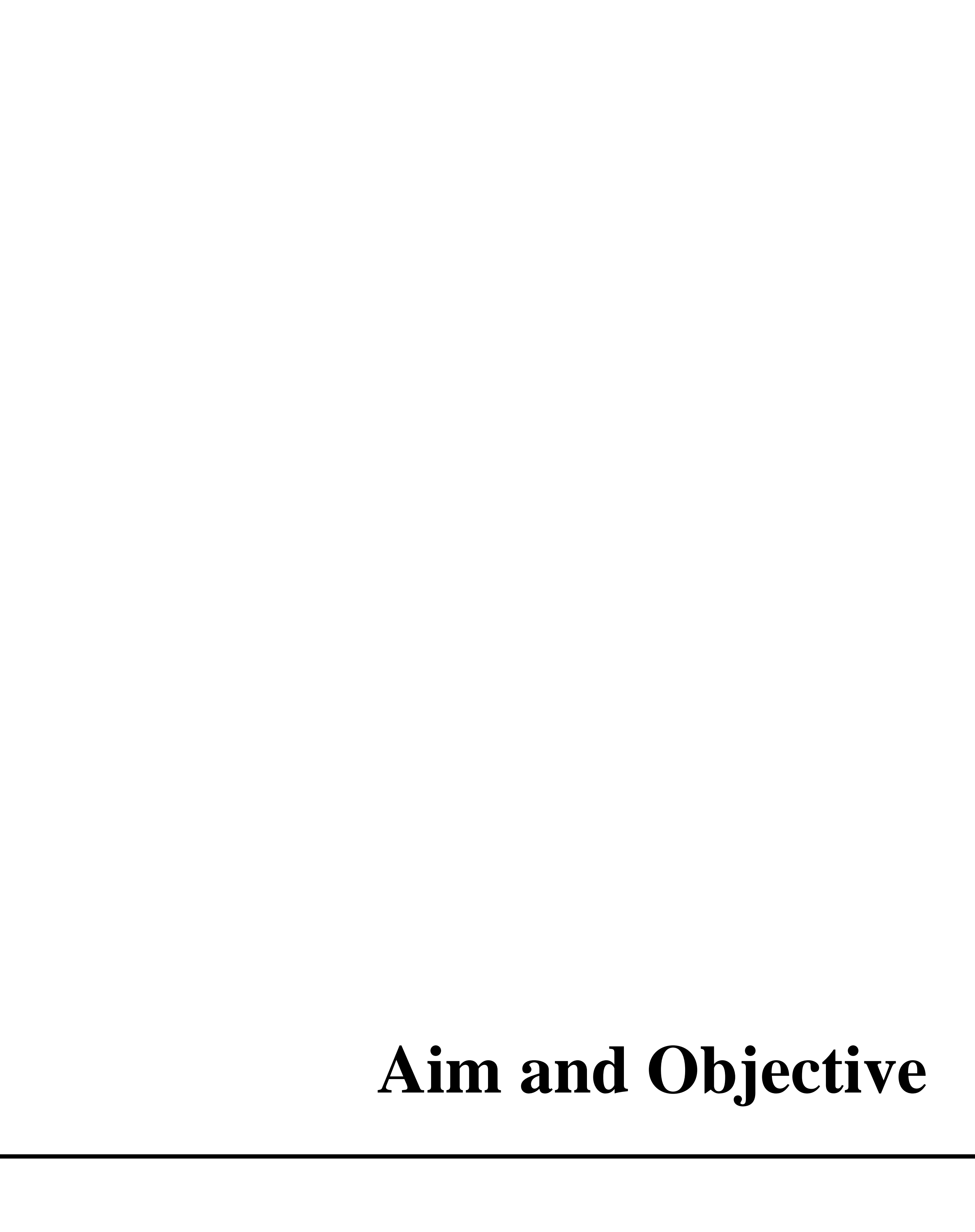
The pathogenetic hallmark for HLH is defective NK cell function. NK cell is a cell of innate immunity which plays an important role in removing stress induced and virus infected cells. In HLH there is a defective NK cell

function which can be familial or acquired. Defective NK cell leads to persistent activation of macrophages and T helper cell which results in hypercytokinemia. This hypercytokinemia leads to the various manifestations of HLH.

Patients with HPS are commonly very ill at the time of presentation. They usually present with high persistent fever, anaemia, splenomegaly and CNS manifestations. The HLH society-2004 has laid down guidelines for the diagnostic criteria for HLH. Minimal diagnostic parameters are fever, cytopenia, splenomegaly, abnormal liver function tests, elevated serum triglycerides and serum ferritin, low serum fibrinogen and haemophagocytosis in bone marrow aspiration. Two highly sensitive diagnostic markers are increased plasma concentration of the alpha chain of soluble IL2 receptor (CD25) and impaired NK cell activity. The HLH society in 1994 has laid down therapeutic guidelines for the treatment of HLH. An improved revised therapeutic criteria came in 2004. This 2004 guideline mainly involves three treatment regimens - initial, continuation and reactivation therapy. However though disease control can be established with these treatment regimens, a complete cure can be established mainly by haematopoitic stem cell transplantation. After the advent of improved conditioning and graft versus host disease regimen the success of HSCT in patients with HLH has dramatically improved.

The Department of Pathology, PSGIMS & R receives bone marrows of patients with a spectrum of various neoplastic and non-neoplastic disorders. In this study we propose to evaluate the spectrum of presentations of haemophagocytic syndrome by identifying haemophagocytic activity in the bone marrows received and correlating with clinical and biochemical parameters that are included in the Haemophagocytic lymphohistocytosis (HLH) diagnostic criteria. Since the overall prognosis depends on early diagnosis and prompt treatment, we in this study propose to elucidate the aetiopathogenesis of this diverse disorder which may play a significant role in the prognosis of the patient.



# Aim and Objective

# AIM AND OBJECTIVE

1. To evaluate the clinicopathological profile of haemophagocytosis diagnosed in the bone marrow in the Department of Pathology during the study period.
2. To correlate the clinical, biochemical and other haematological parameters of these patients with the findings in the bone marrow.
3. To highlight the haemophagocytic activity in bone marrow trephines using CD 68, an immunohistochemical marker for macrophages.
4. To elucidate the aetiopathogenesis of this diverse disorder.

# Review of Literature

# REVIEW OF LITERATURE

## HAEMATOPOIESIS:

Each of the blood cells have a distinctive morphology and a specialized biological function. Erythrocytes are anucleate cells that contains haemoglobin and plays an important role in oxygenation of tissues. Granulocytes and monocytes are phagocytic cells which leave the circulation and migrate into tissues to perform their respective functions. B lymphocytes provide immunity by production of the antibodies while the T lymphocytes have a large variety of immune functions including killing of the cells that bear foreign molecules on their surface. Platelets are also anucleate cells which contains many enzymes that play an important role in haemostasis. Though all these cells have extensive differences in their morphology and function there is strong evidence that they all originate from a single progenitor called the hematopoietic stem cell (HSC).

The process of haematopoiesis begins early during embryogenesis and undergoes many changes during the foetal and neonatal period. Unlike few of the other cells in the body which after maturation do not undergo mitosis or replenishment, the haematopoietic cells are continually replenished by the ongoing process of haematopoiesis. The period and the rate of replenishment of each cell depends on the life span of the particular cell. For example

erythrocytes have a life span of 120 days so the rate of erythropoiesis is less when compared to the WBC's which have a shorter life span.

**SITES OF HAEMATOPOIESIS:**

The process of haematopoiesis begins early in the gastrulation stage of the embryo in the extra embryonic yolk sac and the aorta gonad mesonephros (AGM). The process in the yolk sac is called primitive haematopoiesis since it produces erythrocytes, platelets and macrophages before the development of the circulatory system. From the yolk sac haematopoiesis shifts to liver and finally to bone marrow by the process of mobilisation and homing of stem cells. Although bone marrow is the active site for production of blood cells in children and adults, in adults it is restricted to the marrow of flat bones like vertebrae, ribs, sternum, etc.

**GENES CONTROLLING HAEMATOPOIESIS:**

Gene knockout experiments have opened a new insight into the study of genes controlling haematopoiesis. They are a process by which a particular gene is deleted from an experimental animal like mouse and the consequences of its absence are studied. By such methods genes controlling haematopoiesis like Bmp 4, VEGF,Tal-1/Scl, Gata-2 & RUNX-1 have been detected. In these the Bmp 4 and VEGF genes play important roles in primitive haematopoiesis and the embryos deficient in these genes die at an early gastrulation stage.

**RETENTION AND HOMING OF HSC's:**

As the process of haematopoiesis shifts from yolk sac to placenta then to liver and finally to bone marrow, the HSC's should migrate from one site to the other. The process of egress of HSC'S from one site is called "mobilisation" and the directed movement towards the targeted site and retention in the particular hematopoietic organ is called "homing".

There are several factors which help in the egress and homing of HSC'S. They are selectins, integrins, chemokines and the stroma of the haematopoietic organs.

The selectins are the L, P and E selectins. They like in acute inflammation help in margination, rolling and adhesion of the HSC's to the endothelium.

The integrins help in homing and retention of HSC'S in the extravascular compartment. They are transmembrane receptors that transmit signals from the stroma to the cell and vice a versa.

Chemokines like CXCL12 secreted by the bone marrow stroma bind with the ligand CXCR4 on the HSC's which directs them towards the bone marrow and helps in the process of homing.

## STEM CELL NICHES:

Once the HSC enters the bone marrow by the process of homing it migrates and aggregates in specialised areas called 'niche's. This is a specialised microenvironment in the bone marrow which helps in maintenance of stem cells. The proposed cells which aid in the maintenance of stem cell niche's are the osteoblasts[(3)] , endothelial cells[(4)], mesenchymal lineage cells and non myelinating Schwann cells [(5)].

## GROWTH FACTORS IN HAEMATOPOIESIS

There are several growth factors that play an essential role in division and maturation of cells in hematopoiesis. They can be multilineage that is they enable growth of multiple lineages or lineage specific where they enable the growth of only a single lineage. Examples of growth factors are GM-CSF, G-CSF, CSF-1, IL-3, IL-4, IL-6, IL-11, IL-12 and TPO[(6)].

## PROCESS OF HAEMATOPOIESIS

## COMMITTED HAEMATOPOIETIC PROGENITOR CELLS:

The process of haematopoiesis starts with HSC. The distinctive feature of a HSC is self renewal. But as the stem cells mature, each successive stage has a restricted differentiation potential. The HSC will differentiate to form multilineage progenitors.

## MULTILINEAGE PROGENITORS:

These are the first committed progenitors which lose the property of self renewal. There are multiple commitment steps and in each of these steps there is specific loss of lineage potential in a particular order. In the first step there is division into lymphoid and myeloid. Then myeloid differentiates into erythroid, megakaryocyte (CFU-MK), granulocyte, macrophage (CFU-GM) and so on.

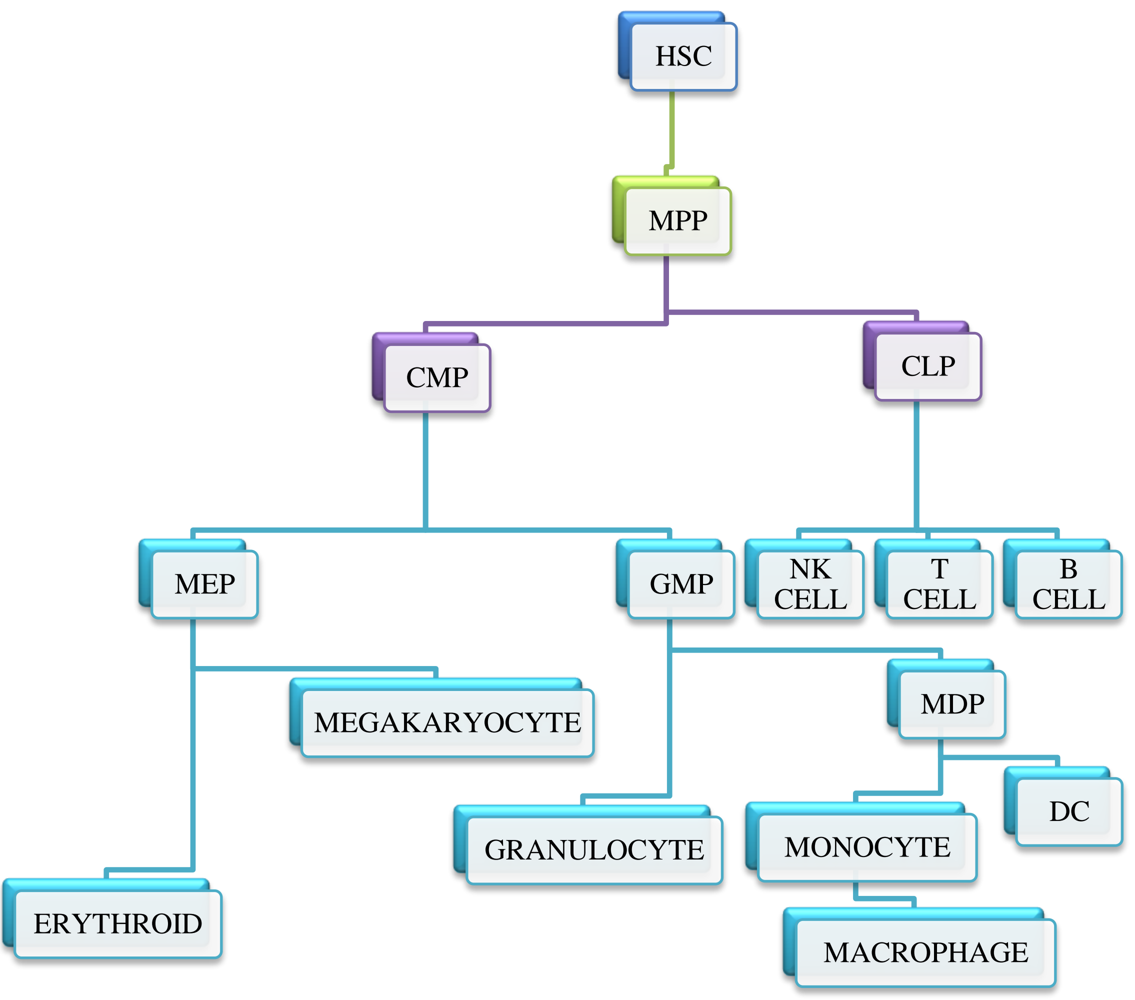
## SINGLE LINEAGE PROGENITORS:

They are descendants of multilineage progenitors and are ultimately related to only one lineage. It is until this stage they are all morphologically similar and differentiation can be made only with immuno histochemical markers.

## TERMINAL PHASES OF DIFFERENTIATION

These cells are sufficiently differentiated that they are morphologically distinct. Although they are capable of only few divisions they undergo dramatic changes which help them differentiate and perform distinct functions.

**HAEMATOPOIESIS**



# NK CELL

Natural killer cells (NK cell) are cells of haematopoietic origin belonging to the lymphoid lineage. Since they lack the antigen receptor rearrangement of lymphocytes, these cells play an important role in innate immunity rather than adaptive immunity. This means that they do not require any pre-activation to recognise antigens to perform their function.

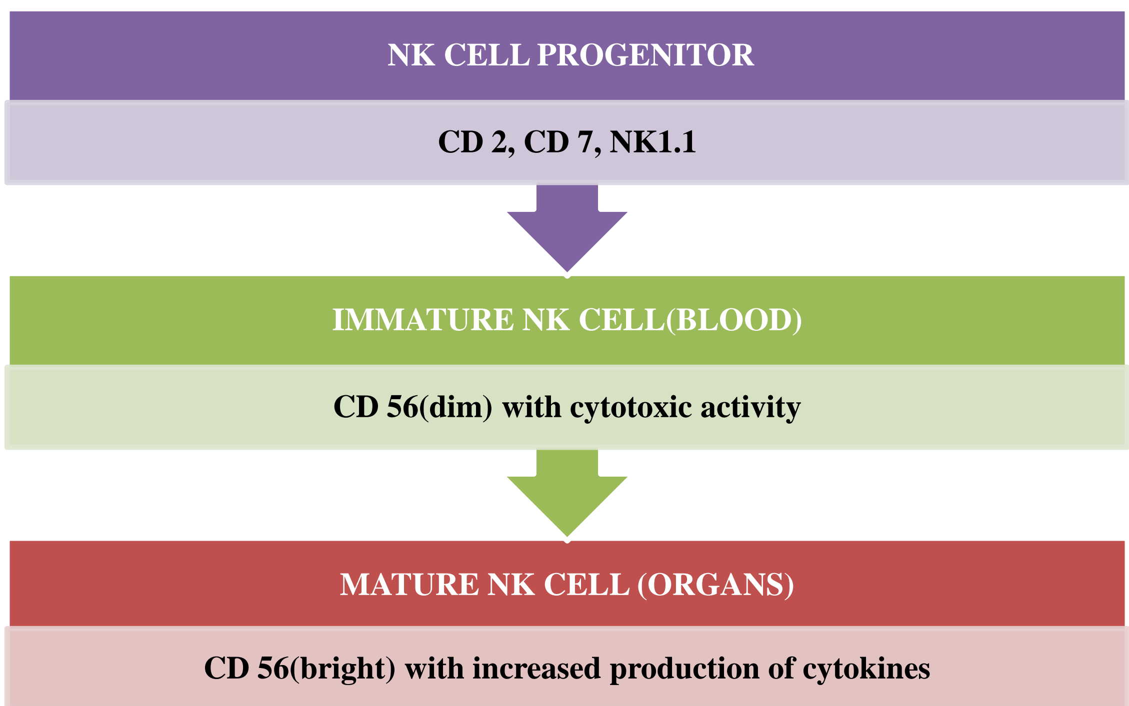
## ORIGIN

NK cells are produced by the process of haematopoiesis. They develop from the common lymphoid progenitor (CLL) [(1)]. The CLL cells then differentiate to form a NK cell progenitor and lymphoid progenitors. The common lymphoid progenitor expresses CD 117, CD 44 and NK1 but as they differentiate in to NK cell progenitors there is loss of CD 117 and there is acquisition of CD 2, CD 7 and CD 56.

Initially the immature NK cells express dim CD56 which is the major component of the blood NK cell constituting 2% to 6% of blood leucocytes and 10% to 15% of peripheral blood lymphocytes.[(7)] . Their main function is cytotoxic activity.

Later the NK cell matures with a bright CD 56 expression. This constitutes the major NK cells in tissues like in lymph node, spleen, thymus and the uterine decidua[(8)]

| CD 56 (dim) | CD 56( bright) |
|---|---|
| **Immature** | Mature |
| Seen in peripheral blood | Seen in lymph node , spleen & thymus |
| Main function is cytolysis | Main function is production of cytokines |




## CYTOKINES FOR PRODUCTION & MATURATION

The cytokines that are involved in NK cell production, maturation and proliferation are IL-15, IL-2, IL-4, IL-12, IL-18 and IL-21. IL2 and IL 4 activates and promotes proliferation of NK cells. IL 12 and IL 18 are secreted by dendritic cell and macrophages. They aid in the cytotoxic activity of the NK cells. IL 21 enhances functional capacity and maturation of NK cell.

## MORPHOLOGY

NK cells are nothing but the large granular lymphocytes (LGL). They have moderate pale blue cytoplasm with fine azurophilic granules. The nuclear cytoplasmic ratio is high with condensed chromatin and inconspicuous nucleoli. These cells like monocytes are positive for alpha naphthyl esterase with a diffuse staining of the cytoplasm in contrast to dot like staining in T lymphocytes.

Ultra structurally they are heterogeneous. The granules are situated close to the golgi apparatus. They have an electron dense centre surrounded by a pale area. The granules as in T lymphocytes contain perforin and granzymes. These play an important role in cytotoxic effects of NK cells.

## NK CELL RECEPTORS

There are three main groups[1] of NK cell receptors called the

1. Leucocyte immunoglobulin like receptors "KIRs"
2. Leucocyte Ig like receptors "LIRs"
3. C- type lectin like receptors

All these receptors have an extracellular ligand binding site and an intracellular cytoplasmic domain. The cytoplasmic domain can be long or short, inhibitory or activating site respectively.

The ligand for KIRs are HLA A, B or C and for C type lectins is HLA E. Whenever there is a binding of the receptors to the corresponding ligands there is either an activating signal or an inhibitory signal. In case of activating signals there is intracytoplasmic activation of immunoreceptor tyrosine based activating motif (ITAM) which enhances NK cell function. When there is an inhibitory signal there is activation of immunoreceptor tyrosine based inhibitory motif (ITIM) which inhibits NK cell activity.

| | **INHIBITORY RECEPTORS** | **ACTIVATING RECEPTORS** |
|---|---|---|
| LIGAND | MHC 1[8] | Viral molecules and stress induced proteins[8] |
| SIGNALS | Via ITIM motifs | Via ITAM motifs |
| FUNCTION | For NK cell licensing | For NK cell cytotoxic activity |

## FUNCTION OF NK CELL

NK cell plays an important role in the immune function of the body.

Immunity is classically defined as “Protection from infection”[9]. There are two broad categories of immune system in human body. They are innate immunity and adaptive immunity.

Innate immunity is immunity which does not require prior exposure. Here the cells are ready to react even before an infection occurs and it is the first line of defense.

Adaptive immunity develops following exposure to a particular pathogen. Since adaptive immunity is highly specific for a particular pathogen it is much more powerful and specific than innate immunity.

The components of innate immunity are:

a. The epithelium of skin, gastrointestinal tract, respiratory and genitourinary tract
b. Monocytes and neutrophils
c. Dendritic cells
d. Complement system and
e. NK cell

Thus the NK cell forms a part of the innate immune system and plays an important role in the body's first line of defense.

**MISSING SELF HYPOTHESIS**

The major breakthrough in the function of NK cell is the proposal of "Missing self" hypothesis. It explains that normally cells express host specific MHC 1 molecule. This is expressed by almost all cells in the body. When a cell expresses MHC 1 it is recognised by the inhibitory receptor of the NK cell

which down regulates its function and prevents destruction. But when there is a loss of MHC 1 molecule as in case of virus infected cell or tumour cells, NK cell recognises these cells as foreign and destroys these cells. This is called the missing self hypothesis.

**SPECIFIC NK CELL FUNCTION**

The cytotoxic function of NK cells plays an important role in two places.

- lysis of virus infected cells
- lysis of tumour cells.

The NK cell performs its function of cytotoxic effect through three main pathways

1) Perforin and granzyme mediated lysis
2) Death receptor mediated lysis
3) Antibody dependent cell mediated cytotoxicity

**1) PERFORIN & GRANZYME MEDIATED LYSIS:**

The NK cell contains membrane bound granules which contain the two important enzymes called granzyme and perforin. Whenever NK cell encounters a virus infected cell or a tumour cell both with loss of MHC 1 molecule, it is activated. The granule contents are released by the process of exocytosis. The perforin present in the granules makes a pore in the target cell

and the granzyme enters the opponent cell to cause cell lysis by the process of apoptosis.

**2) DEATH RECEPTOR MEDIATED LYSIS**

The virus infected cells express the death receptor (Eg; CD 95/FAS). Death receptors belong to TNF family of receptors. They possess an extracellular ligand binding site and a protein-protein interacting cytoplasmic domain called the death domain(9) .These death receptors are recognised by the cognate ligands in NK cell(10). When there is engagement of these receptors three or more molecules of death receptors are brought closer to form FAS-associated death domain ( FAS-DD). This FAS-DD then binds pro-caspase and cleaves it to form activated caspase. Once pro-caspase 8 is activated it sets up an enzymatic cascade in which one enzyme activates another and there is subsequent activation of the successive enzymes. The subsequent caspases which are activated are caspase 3, 6. These caspases act on many of the cellular components like DNA and cause cleavage. It acts on nucleus to cause nuclear fragmentation and finally there is death of cell by the process of apoptosis.

**3)ANTIBODY DEPENDENT CELL MEDIATED CYTOTOXICITY (ADCC)**

The NK cell in addition to CD 56 also expresses CD 16 on its cell membrane. CD 16 is a receptor for Fc portion of the IgG antibody. Hence the

NK cell is able to recognise and bind the IgG coated target cells and cause cell lysis. This is termed as the antibody dependent cell mediated cytotoxicity.

## INTERACTIONS OF NK CELL WITH DENDRITIC CELL & CYTOTOXIC T LYMPHOCYTE (CTL)

NK cell secretes cytokines like IFNγ. This IFNγ stimulates dendritic cells which in turn activate the CTL. Also killing of tumour cells by NK cells and CTL increases the amount of antigen presentation by dendritic cells and hence enhance the adaptive immunity[11]. However the outcome of these reactions depends on the maturity of the dendritic cell. Immature dendritic cells are killed by NK cells but mature dendritic cells are resistant to lysis. These interactions take place mainly at the site of infection. The other place where they encounter each other is in the lymph node.

# MACROPHAGES

Macrophages form a part of the mononuclear phagocytic system. Elie Metchnikoff won the Nobel prize in 1908 for the discovery of macrophages.[11] They play an important role in host defense & also in tissue repair

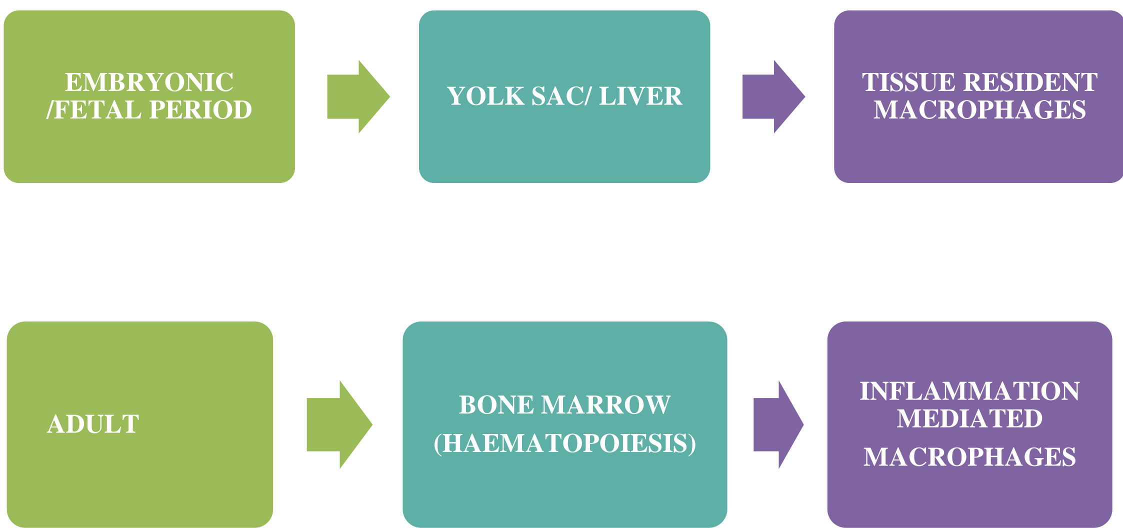
## ORIGIN AND DEVELOPMENT OF MACROPHAGES

Macrophages are also derived through the process of haematopoiesis. Haematopoiesis produces monocytes which are released into the circulation. These circulating monocytes enter tissues to differentiate into macrophages. The life span of macrophages ranges from months to years which is longer than that of the circulating monocytes.[12] There are two different type of macrophages. – the tissue or the resident macrophages and the monocyte derived macrophages.

The tissue macrophages are derived early in the embryonic and foetal period from the yolk sac and liver respectively[9]. These macrophages after they are produced, migrate to the respective tissues to form the permanent or the tissue resident macrophages. Examples are the astroglial cells in brain, Kupffer cells in the liver, osteoclasts in the bone marrow etc.

The monocyte derived macrophages, produced by the process of haematopoiesis subsequently migrate to tissues to form macrophages. This is called steady state production. Conditions like stress upregulate haematopoiesis

through increased production of granulocyte and monocyte stimulating factor which leads to increased production of macrophages.




## MORPHOLOGY

Macrophages have a varied morphology. They are usually large rounded or stellate, with vesicular nucleus and conspicuous nucleoli. The cytoplasm has a well organised cytoskeleton, intracellular organelles and abundant lysosomes.

## CELL SURFACE RECEPTORS(13)

The cell surface receptors help macrophages perform their function. The most important are the Pattern Recognition Receptors (PRR's). There are two types of PPR's. The first type is mannose receptor and scavenger receptor which helps in the process of phagocytosis(14). The second is the Toll like receptors which help in the recognition of microbes or damaged tissues.(15)

**FUNCTIONS**

The main functions of macrophages are

**1) PHAGOCYTOSIS**[9]

The macrophages ingest and eliminate microbes and damaged tissues by the process of phagocytosis. This process of phagocytosis involves three main steps

a) Recognition of microbes or dead tissues: This is done with the help of the receptors like the Toll like receptors which recognise and bind the inciting agent.
b) Engulfment: after the binding of the inciting agent cytoplasm extends to encircle the particle, the plasma membrane then pinches off to form the phagosome.
c) Intracellular destruction: once the phagosome is formed it fuses with the lysosomes to form the phago-lysosome. Inside the phagolysosome the destruction is mediated by numerous factors like reactive oxygen species(ROS), reactive nitrogen species and the lysosomal enzymes, thus finally the inciting agent is destroyed and removed by macrophages

**2) TISSUE REPAIR**

Macrophages helps in tissue repair and fibrosis by production of certain cytokines like transforming growth factor beta ( TGF-β).

### 3) INFLAMMATION

Macrophages secrete mediators of inflammation like tumour necrosis factor (TNF), and interleukin 1(IL 1).

### 4) ANTIGEN PRESENTATION

Macrophages also play a role in innate immunity. They recognise and present the antigens to the T lymphocytes and also respond to signals from the T lymphocyte

## MACROPHAGE ACTIVATION PATHWAYS

There are two pathways of macrophage activation

- Classical pathway &
- Alternate pathway

Accordingly there are three types of macrophages

- M1 macrophages which are activated by the classical pathway
- M2 which are activated by the alternate pathway and
- The regulatory macrophages(16)

## M1 MACROPHAGES

In the classical activation pathway the macrophages are stimulated by two important cytokines, interferon gamma (IFN γ) and tumor necrosis factor (TNF) which are secreted by NK cells and T lymphocytes. Thus they can be

stimulated either by innate immunity or adaptive immunity. The cytokines which are produced activate macrophages to form M1 macrophage. M1 macrophages help in phagocytosis and microbicidal activity. They also produce certain cytokines like IL-1, IL-12 and IL-23 which recruit inflammatory cells and further enhance the inflammatory process.(17)

## M2 MACROPHAGES

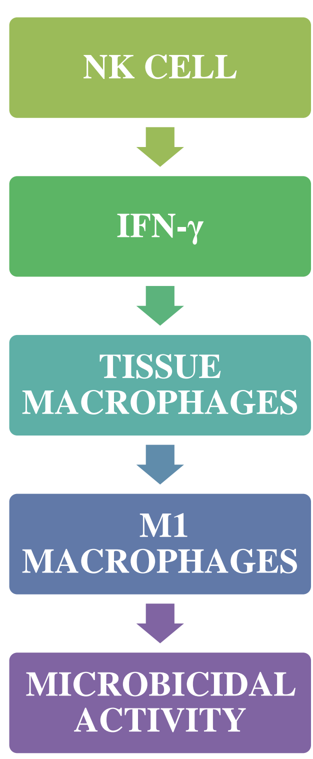
In alternate activation pathway certain cytokines like IL-13 and IL-4(18) secreted mainly by the mast cells and basophils activate macrophages to form M2 macrophages. These macrophages produce enzyme arginase which produces TGF-β that helps in the production of extracellular matrix. Thus these M2 macrophages play an important role in tissue repair and fibrosis.

## REGULATORY MACROPHAGES

These macrophages like the M1 macrophages can be stimulated by both innate and adaptive immune system. (19) TGF-β and immune complexes helps in the switch over to regulatory macrophages. These secrete many cytokines most importantly IL-10. This is an anti inflammatory cytokine which helps to sustain the process of inflammation and prevents tissue damage.

## MACROPHAGE NK CELL INTERACTION

NK cells of the innate immune system secrete cytokines like IFN-γ. This leads to stimulation of macrophages to form M1 macrophages when an antigen is encountered. The M1 macrophages serve to eliminate the antigen by the process of phagocytosis and also produce cytokines which enhances the process of inflammation. When there is defective NK cell function, it is not able to perform its function and there is persistent activation of NK cell and production of IFN- γ. This causes uncontrolled activation of macrophages which leads to untoward action of macrophages like haemophagocytosis. This forms the basis for the pathogenesis of Haemophagocytic syndrome.



# HAEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS

Haemophagocytic lymphohistiocytosis (HLH) also called Haemophagocytic syndrome is not a single disease. It is a syndrome which can manifest in numerous disease conditions. It consists of series of clinical symptoms, signs and laboratory manifestations which occurs due to defective NK cell activity leading to uncontrolled activation of macrophages resulting in haemophagocytosis. Defective NK cell activity and uncontrolled macrophage activation forms the hallmark of Haemophagocytic syndrome.

## INCIDENCE

Estimated incidence of haemophagocytic syndrome is 1.2 cases per million individuals per year [20]. This is most likely an underestimate because many cases remain undiagnosed and few are not reported.

## TYPES

HLH is broadly categorised into two forms: Familial (FHL) or Primary HLH and Secondary HLH(s HLH). This delineation into primary and secondary forms is just an over simplification because there is always a considerable overlap in many cases between the familial and the secondary forms.

## CAUSES [20]

Primary HLH is mainly due to defects in genes involved in normal NK cell activity. It can be any of the genes which are involved in the production, transport or export of perforin granules involved in NK cell mediated lysis of target cells.

Secondary HLH can be due to numerous causes as varied as infections, auto immune disorders and malignancies.

## PRIMARY HLH [21]

### FAMILIAL HLH1 (FHL1)

It was originally discovered in Pakistani families. It is linked to chromosome 9q21.33-22. The defect underlying the mutation is unknown.

### FAMILIAL HLH2 (FHL2)

It was initially discovered in Turkish families. More than 70 different mutations have been identified. The most common mutation is linked to the perforin gene (PRF1) at 10q21-22. This gene is involved in the production of perforin enzymes. Mutation in this gene leads to impaired or block in production of perforin enzymes leading to impaired function of NK cell mediated lysis of infected cells.

## FAMILIAL HLH 3 (FHL3)

This form of HLH is due to defect in gene UNC13D located on chromosome 17q25. Mutations are scattered throughout the gene mainly affecting the mRNA splicing. This mutation results in defective delivery of perforin and granzyme to the target cell resulting in defective NK cell mediated lysis.

## FAMILIAL HLH 4 (FHL4)[22]

This type of HLH was initially described in Turkish and Kurdish families. It is due to defect in the gene STX (syntaxin)11 situated on chromosome 6q24. Defect in STX11 gene leads to defective degranulation of NK cells. However HLH occurring due to defective STX11 gene is milder when compared to FHL1 and FLH 2

## FAMILIAL HLH 5 (FHL5)

Initial reports of this form of HLH were from families of Saudi Arabia. It is due to defect in mutation of syntaxin binding protein 2 (STXBP2) located on chromosome 19p. STXBP2 codes for the protein Munc18-2 which is involved in regulation of transport of vesicle to plasma membrane of the NK cell.

**CHEDIAK HIGASHI SYNDROME (CHS)[23]**

CHS is a rare autosomal recessive disease characterised by recurrent pyogenic infections, oculocutaneous albinism and recurrent respiratory tract infections. In CHS there is defect in the gene LYST which leads to defective NK cell activity resulting in haemophagocytic syndrome. About 85% of patients with CHS syndrome in accelerated phase present with HLH.

**GRISCELLI SYNDROME (GS2)**

Griscelli syndrome is due to defect in gene RAB27A resulting in defective NK cell activity.

**X LINKED LYMPHOPROLIFERATIVE DISEASE (XLP1 & XLP2)**

XLP1 and XLP2 are due to defects in gene SH2D1A and XIAP respectively .

## FAMILIAL HLH

| SYNDROME | INHERITANCE | GENE | PROTEIN |
|---|---|---|---|
| FHL1 | AR | 9q21.3-22 | Not known |
| FHL2 | AR | PRF1 | Perforin |
| FHL3 | AR | UNC13D | Munc13-4 |
| FHL4 | AR | STX11 | Syntaxin11 |
| FHL5 | AR | STXBP2 | Munc18-2 |
| CHS1 | AR | LYST | LYST |
| GS2 | AR | RAB27A | RAB27A |
| XLP1 | XL | SH2D1A | SAP |
| XLP2 | XL | XIAP | XIAP |

## SECONDARY HLH

Secondary HLH can be due to many causes starting from infections, auto immune disorders to malignancies

### 1) MACROPHAGE ACTIVATION SYNDROME (MAS)[24]

MAS is a severe acute inflammatory syndrome which is often under diagnosed. Most of these cases present with a clinical picture of HLH. This form of HLH occurs secondary to autoimmune diseases like systemic juvenile idiopathic arthritis, rheumatoid arthritis, Kawasaki disease, Systemic lupus erythematosus, and Stills disease. Marked decreased expression of perforin in

the CD 8 and NK cell activity forms the pathogenesis of HLH occurring in MAS.

## 2) HLH ASSOCIATED WITH INFECTIONS

### VIRUS ASSOCIATED HLH [25]

#### Epstein Barr Virus (EBV) Associated HLH[26]

EBV is an oncogenic virus which is the most common virus involved in the pathogenesis of HLH. It is common in the East Asian population. The main cell involved in EBV associated HLH is CD 8 T cell. EBV infected CD 8 T cell expresses Latent Membrane Protein (LMP) which makes it resistant to killing by other cytotoxic T cells. These EBV infected T cells produce a wide variety of cytokines which can result in HLH. HLH occurring in EBV virus infected persons is very aggressive and the prognosis is bad when compared to other forms of HLH.

#### Cytomegalovirus (CMV) Associated HLH

Compared to EBV which occurs in immunocompromised individuals, CMV associated HLH occurs in normal healthy individuals. This occurs usually in patients with severe avian influenza ( H5N1).

#### HIV Associated HLH

It can occur in persons with HIV alone or in persons with associated opportunistic infections. It usually occurs late in course of the disease when the

CD4 counts fall below 150cells/cu.mm. Rarely it may occur as an initial manifestation in persons with HIV.

**Parvovirus Associated HLH**

There are many species of parvovirus that can cause HLH of which B19 associated HLH has a better prognosis. It is more common in patients with hereditary spherocytosis who are infected by parvovirus B19.

**Dengue Associated HLH**

Dengue fever virus is a virus of the family Flaviviridae. It causes dengue fever. Dengue associated HLH usually occurs late in the course of the disease. The prognosis is bad therefore early diagnosis and interpretation is essential.

**Other viruses involved in HLH**

Adenovirus, parvovirus, influenza, measles, mumps, enterovirus, pox virus, Heptatitis A, B & C etc

**BACTERIAL ASSOCIATED HLH**

**Tuberculosis associated HLH**

This is the most common bacterial infection associated with HLH accounting for about 25% of infection associated cases.

Other bacterial infections associated HLH:

Campylobacter, Staphylococcus, Fusobacterium, Mycoplasma, Chlamydia, Leigonella, Salmonella, Rickettsia, Brucella, Ehrlichia and Borrelia

**FUNGAL ASSOCIATED HLH**

The fungi which can cause HLH are Candida, Crytococcus, Pneumocystis, Histolplasma and Aspergillus. It's more common in immune compromised individuals like AIDS patients, transplant receipients and lymphoma patients

**PARASITE ASSOCIATED HLH**

The parasitic infections associated with HLH are Leishmania, Toxoplasma, Plasmodium, Pneumocystis Strongyloides, Babesiosis and Scrub Typhus

**3) MALIGNANCY ASSOCIATED HLH[27]**

This HLH are divided into two groups

1. HLH occurring before or during the treatment of malignancy like
    a. Acute lymphoblastic lymphoma
    b. Acute myeloid leukemia
    c. Multiple myeloma
    d. Germ cell tumour
    e. Thymoma
    f. Carcinomas: Hepatocellular, Prostate, Lung

2. HLH with a masked haemato lymphoid malignancy
    a. T/NK cell leukaemia
    b. Adult B cell lymphoma
    c. Large cell Anaplastic lymphoma

**4) IMMUNE DEFICENCY ASSOCIATED HLH**

Common immunodeficiencies associated with HLH are

- Severe combined immunodeficiency
- Common variable immunodeficiency
- Chronic granulomatous disease and
- Stem/ bone marrow transplant

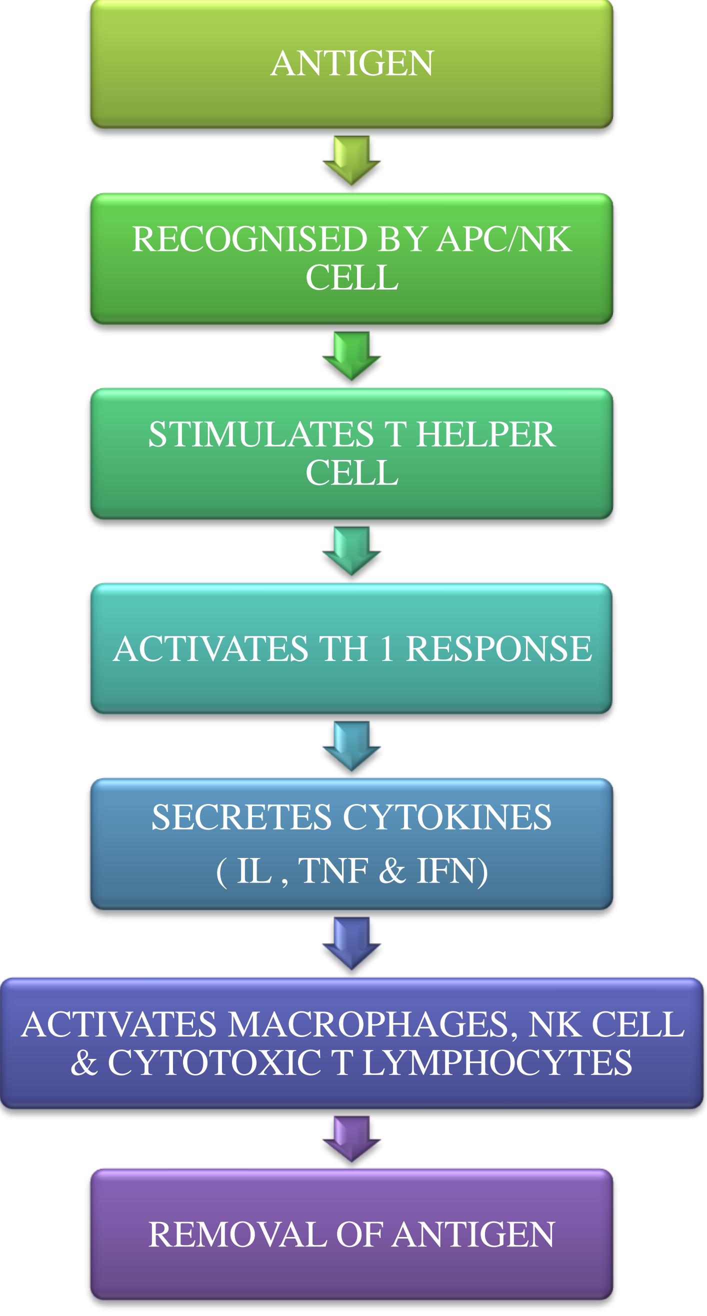
# PATHOGENESIS [28]

Defective NK cell activity and persistent macrophage activation is the hallmark of the pathogenesis of Haemophagocytic LymphoHisticytosis (HLH).

Natural killer cell as already mentioned plays an important role in innate immunity which is the first line of defence. The natural killer cells recognise the virus infected and stressed cells and mediate lysis through perforin and granzyme.

Whenever an antigen enters the human body it is recognised by the antigen presenting cell or the NK cell which secrets cytokines like IL 1. Interleukin 1 causes activation of T cells. The T cells activate and differentiate into T helper cells 1(TH1). TH1 secretes cytokines which further mediates the removal of the antigen by NK cell mediated lysis. It also promotes phagocytosis by the macrophages.

In HLH there is defective NK cell activity which is due to genetic mutations in familial HLH or acquired defects in secondary HLH.

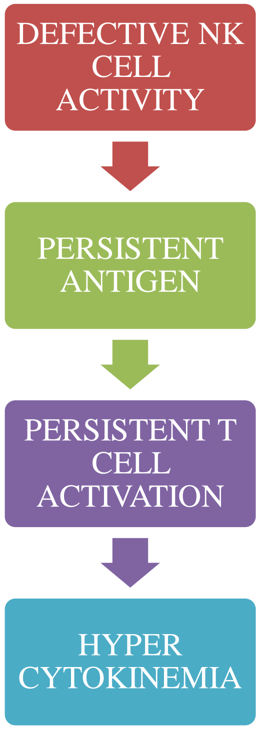



NK cell in its cytoplasm contains membrane bound vesicles containing perforin and granzymes. These enzymes are transported to the plasma membrane and the contents are released by exocytosis. The perforin creates a hole in the plasma membrane of target cells whereas the granzyme enters the cell and causes lysis.

In FLH 1 and FLH 2 there is defective production of these enzymes. In FLH 3 there is defective delivery of these enzymes into target cells. In FLH 4 and FLH 5 there is defective exocytosis of the granules containing these enzymes

In acquired HLH there is an acquired defect in the function of the NK cell, the mechanism of which remains unknown.

When there is defect in NK cell activity there is persistence of the antigen which leads to uncontrolled activation of macrophages and outpouring of the cytokines which leads to hypercytokinemia. The hypercytokinemic state explains the cause for all the various manifestations of the HLH syndrome.



Hypercytokinemic state further leads to accentuation of TH1 response. There is an increase in TH1 cytokines like TNF-α, IFN-γ and IL-18[29]. IL1 and TNF-α are called pyrogens and they cause fever. In addition these cytokines stimulate macrophages to perform the untoward or the non physiologic act, haemophagocytosis which is not normally the function of macrophages. In addition to this untoward action, macrophages start proliferating in the reticuloendothelial system causing organomegaly.

Hypercytokinemic state also leads to suppression of haematopoiesis and apoptosis of haematopoietic elements[29] which along with haemphagocytosis leads to cytopenias.

TNF-α, IFN-γ in addition cause inhibition of lipoprotein lipase. Lipoprotein lipase catalyses the conversion of triglycerides to fatty acids. Inhibition of this enzymes leads to increase in serum triglycerides.[30]

Activated macrophages convert plasminogen to plasmin. Plasmin causes degradation of fibrinogen hence there is a decrease in serum fibrinogen levels[31]. The inflammatory cytokines and the endotoxins causes upregulation of heme oxygenase which is a heat shock protein. Heme oxygenase causes increased production of ferritin leading to hyperferritinemia[32].

In response to accentuated TH 1 response there is increase in soluble CD25.

## CLINICAL FEATURES[33]

### SYMPTOMS AND SIGNS

### CONSTITUTIONAL SYMPTOMS

The cardinal clinical features of HLH are fever, cytopenias and organomegaly most commonly splenomegaly. Although these are the cardinal features they are not specific since there are numerous conditions which can present with such symptoms and signs.

The patients are generally very ill and require urgent intervention and treatment. The first presentation is fever which is persistent and prolonged. This is mediated by the two cytokines IL-1 and TNF-α. The temperatures are usually very high and do not respond to any form of treatment. Usually children have a history of preceding upper respiratory tract infection or enteric infection. However instead of resolution of the infection, it proceeds to produce HLH which is characterised by more constitutional symptoms.

### ORGANOMEGALY

There is usually organomegaly most commonly splenomegaly because of which the person presents with abdominal distension. Palpation of the abdomen reveals an enlarged non tender spleen and sometimes an enlarged liver. There can be associated ascites and derangement of liver functions causing nausea, vomiting and loss of appetite.

## NEUROLOGICAL MANIFESTATIONS [34,35]

Central nervous system manifestations are common in HLH. Patients present with a range of CNS symptoms ranging from irritability, lethargy, stupor, seizures, cranial nerve palsies, hypotonia, ataxia, meningismus, focal deficits, decreased level of consciousness or loss of consciousness. There can be diffuse peripheral neuropathy secondary to infiltration by macrophages. There can also be loss of vision owing to retinal haemorrhage or infiltration of optic nerve by the macrophages.

## CUTANEOUS MANIFESTATIONS

Cutaneous manifestations like petechiae, purpura or a generalised maculopapular rash, erythroderma and panniculitis can occur. The incidence of cutaneous manifestations ranges from 6% to 65%.[36]

## PULMONARY MANIFESTATIONS

Patients can have respiratory manifestations like breathlessness, chest tightness and dyspnoea. Severe cases can present with acute respiratory failure with interstitial opacities in the radiograph. It is estimated that there is a high mortality (88% of cases) in patients presenting with respiratory failure and interstitial opacities.[37]

## LABORATORY FINDINGS:

### CYTOPENIAS:

The most common and consistent laboratory finding is cytopenias, that is decrease in blood cell counts which may be anaemia, leucopenia or thrombocytopenia. Of these, thromobocytopenia is very common and usually there is bicytopenia involving atleast two of the cell lineages. The diagnostic criteria for HLH involves bicytopenia with any two of the following: haemoglobin <10g/dl, platelets $< 100 * 10^9/L$ or absolute neutrophil count less than $<1.0 * 10^9/L$.

### LIVER FUNCTIONS:

Most patients present with deranged liver functions. Neonates with HLH can present with hydrops foetalis and acute liver failure. Deranged liver function can be attributed to the primary cause like infections or it can also be due to proliferation of macrophages in the hepatic sinusoids which can compress the hepatic parenchyma, secrete cytokines and cause inflammation of liver producing hepatitis. There is an increase in liver alanine and aspartate amino transferases.

### COAGULOPATHY

Derangement of coagulation is called coagulopathy. About 95% of HLH patients present with disseminated intravascular coagulation (DIC).[35]. There is

usually a decrease in the level of fibrinogen due to excess activation of plasminogen which in turn degrades fibrin causing hypofibrinogenimia. The patient can present with bleeding or thrombosis. Laboratory investigations reveals decreased fibrinogen levels and a prolonged thrombin time. Fibrinogen levels <1.5g/L is diagnostic. When there is an added DIC, there can be prolonged thrombin time, activated partial thromboplastin time and even low platelet count.

**LIPID PROFILE**

There is increase in serum triglycerides causing hypertriglyceridemia . This is due to inactivation of the enzyme lipoprotein lipase by the cytokines. Serum triglyceride level of > 265mg/L is diagnostic.

**FERRITIN**

Ferritin levels are increased in HLH patients owing to upregulated function of the enzyme heme oxygenase. Hyperferritinemia is an important hallmark of infection related HLH or secondary HLH. Serum ferritin of > 500microgram/ L is diagnostic. Since ferritin is an acute phase reactant, high ferritin levels can be seen in other acute and also chronic inflammatory conditions. Therefore high ferritin levels are not specific but can be used as a reliable indicator to assess the disease activity during diagnosis and treatment. However ferritin level of > 2,000ng/ ml is considered to be specific. [38]

## CSF ANALYSIS

Analysis of cerebrospinal fluid shows elevated CSF protein and cell count of > 5 cells/μL which is considered diagnostic. Cytological examination of CSF fluid may also show evidence of haemophagocytosis.

## BONE MARROW EXAMINATION

Examination of bone marrow may reveal haemophagocytosis. Bone marrow macrophages are seen engulfing the haematopoietic elements which can either be erythroid precursors or myeloid precursors or even platelets. While the normal function of a bone marrow macrophage is to phagocytose the nucleus of the erythroid precursors and to remove the apoptotic haematopoietic elements, the exaggerated function of macrophages in haemophagocytosis in engulfing even normal haematopoietic elements is considered as untoward and pathological.

Haemophagocytosis can be more readily recognised in a bone marrow aspirate when compared to a bone marrow trephine. However it can be highlighted in the trephine with immunohistochemical stains for macrophages like CD 68. The immunohistochemical marker CD 68 increases the sensitivity of detecting haemophagocytosis because there have been cases where HLH was not detected in aspiration but was diagnosed in trephine biopsy.[39]

The main differentiating feature of haemophagocytosis from normal phagocytosis is that in phagocytosis there is immediate destruction of the

engulfed material. On the other hand, in heamophagocytosis the engulfed elements remain inside the cytoplasm for some time before they are destroyed.

Haemophagocytosis may be observed in reticuloendothelial system like liver, spleen, lymph nodes and also in fluids, peripheral nerves etc

**SOLUBLE CD25**

CD 25 is an important cytokine secreted by the activated TH1. Since there is accentuated activation of TH1 in HLH there is dramatic rise in sCD25 levels. This is otherwise called soluble interleukin 2 receptor. A value of > 2,400U/ml is diagnostic. This usually helps to differentiate HLH from infectious mononucleosis and pseudomonas infection [40]

**NK CELL ACTIVITY [41]**

Low or absent NK cell activity is the hallmark of HLH. It can either be hereditary where it is due to mutations in genes responsible for the NK cell function or secondary. In secondary HLH the cause for defective NK cell function remains unknown.

NK cell activity can be measured using

1. Cytotoxic assays
2. Flow cytometry
3. Degranulation assays
4. Genetic diagnosis

**1) Cytotoxic assays**

In this assay, chromium labelled NK cell sensitive target cells are incubated with the peripheral blood mononuclear cells obtained by Ficoll gradient separation. Lysis of target cells by the NK cells leads to release of chromium which can be quantified. However when there is a reduced number of NK cells this test becomes less specific and can give false positive results.

**2) Flow cytometry**

It is based on the detection of intracellular perforin in NK cells. This can be highlighted by immunostaining of the perforin granules. Reduced expression of perforin in the cytoplasm indicates defective perforin enzyme and hence defective NK cell function.

**3) Degranulation assays**

These are based on the fact that NK cell cytotoxicity requires exocytosis of the granule content in the lysosomes. During this process of exocytosis the lysosomal membrane fuses with the plasma membrane. The activated or functional NK cell expresses the lysosomal membrane protein CD 107. Expression of CD 107 indicates normal functioning NK cell.

In this test initially the NK cells are treated with K562 or IL2 which stimulate the NK cell and cause exocytosis. The exocytosis can be detected using IHC marker CD 107.

**4) Genetic diagnosis**

There are certain mutations which are specific for certain familial HLH which can be detected by molecular diagnosis.

## DIAGNOSTIC CRITERIA

The first diagnostic criteria for HLH were proposed in 1991 and later were modified in 2004. The current guidelines for the diagnosis of HLH is based on the recent 2004 revision.

## GUIDELINES 1991

In 1991, diagnostic guidelines for HLH were presented by the Histiocyte Society. In 1991 a proposal was based on the common clinical laboratory and molecular presentations of HLH. Since HLH can have varied and even have atypical presentations, the criteria have been revised and a new 2004 criteria has been proposed. [42]

In the 1991 proposal even the molecular diagnosis by a specific mutation had to be supported by an extra clinical manifestation for a confirmatory diagnosis. In the 2004 diagnostic criteria, the presence of one specific mutation is confirmatory for diagnosis of HLH.

The category of supportive criteria in the 1991 classification has been deleted from the 2004 classification. In addition the 2004 classification spells out specific cut off values for the various laboratory parameters.

**GUIDELINES 2004**

In the 2004 guidelines, either the presence of a molecular pathology consistent with HLH or the presence of five out of the eight proposed criteria is diagnostic of HLH. The molecular mutations specific for HLH include PRF1, UNC13D, STX11 and UNC18B at a minimum. When X linked lymphoproliferative disease is suspected additional mutations like SH2D1A/SAP and BIRCA4 should also be considered. Specific molecular testing for diseases like Chediak Higashi syndrome, Hermansky Pudlak syndrome or Griscelli syndrome 2 should also be looked for.

The eight diagnostic criteria for HLH are fever, splenomegaly, bicytopenia, hyperferritinemia, hypertriglyceridimia, evidence of haemophagocytosis, increased soluble CD 25 and low or absent NK cell activity.

But applying these criteria for cases of Macrophage Activation Syndrome (MAS) occurring secondary to auto immune diseases like systemic juvenile idiopathic arthritis resulted in delay in diagnosis. So the histiocytic society has set up a separate criteria for MAS syndrome where they have cut down few criterias and have increased the cut off values for certain laboratory parameters like serum ferritin.

| HLH DIAGNOSTIC CRITERIA, 1991 |
|---|
| Molecular diagnosis of Haemophagocytic Lymphohistiocytosis or X linked Lymphoproliferative syndrome(XLP) |
| OR AT LEAST 3 OF 4 |
| Fever |
| Splenomegaly |
| Cytopenias (minimum 2 cell lines ) |
| AND AT LEAST 1 0F 4 |
| Haemophagocytosis |
| Increased ferritin |
| Increased sILR alpha |
| Absence or decreased NK cell activity |
| OTHER INVESTIGATIONS SUPPORTIVE OF HLH DIAGNOSIS |
| Hypertriglyceridimia |
| Hypofibrinogenimia |
| Hyponatremia |

**Diagnosis of HLH can be established if either A or B is fulfilled**[43]

A. **Molecular diagnosis consistent with HLH**

B. **Diagnostic criteria for HLH - 5 out of 8 criteria**

1. **Persistent fever**
2. **Cytopenias (affecting two or more lineages in the peripheral blood) haemoglobin $<10$g/dl, platelets $< 100 * 10^9$/L or absolute neutrophil count less than $<1.0 * 10^9$/L**
3. **Splenomegaly**
4. **Ferritin $\geq$ 500ng/mL**
5. **Hypertriglyceridemia and /or hypofibrinogenemia**

   **Fasting triglycerides $\geq$ 265mg/dl**

   **Fibrinogen $\leq$ 150mg/dL**
6. **Haemophagocytosis in bone marrow , spleen or lymph node**

   **No evidence of malignancy**
7. **Low or absent NK cell activity**
8. **Soluble IL-2 receptor $\geq$ 2400U/Ml**

**Classification of MAS in Systemic juvenile idiopathic arthritis (sJIA)[44]**

**A febrile patient with sJIA is diagnosed with MAS if the following criteria are met**

**Ferritin > 684ng/mL and any two of the following**

1. **Aspartate transaminase >48U/L**
2. **Platelet count ≤ 181000/µL**
3. **Fibrinogen ≤ 360mg/dL**
4. **Triglycerides > 156mg/ Dl**

## DIFFERENTIAL DIAGNOSIS AND PITFALLS

The main differential diagnosis of HLH is Systemic Inflammatory Response Syndrome (SIRS) due to other causes where the clinical presentation is almost similar. However demonstration of haemophagocytosis in bone marrow, liver or spleen establishes the diagnosis of HLH.

In addition neonatal haemochromatosis, or metabolic diseases of children presenting with massive organomegaly and hypertriglyceridemia can be differentials – here also the evidence of haemophagocytosis in the lymphoreticular system can be helpful. [45]

HLH can be difficult to diagnose in patients with Kawasaki disease. [46] The other differentials are Langherhan cell histiocytosis but there the proliferating histiocytes compared to HLH are monoclonal [47] and Leishmania

donovani where one sees Leishmania donovani bodies in bone marrow rather than haemophagocytosis.

## TREATMENT[48]

### THERAPEUTIC BACKGROUND

The first major achievement in the treatment of HLH came in 1994 when etoposide and immunosuppressive agents were added in the treatment regimen of HLH. For patients with CNS complications intrathecal therapy was introduced. Following this there was drastic improvement in the prognosis of patients with HLH. Even though there was improvement in the clinical condition of the patient complete cure was established only after Haematopoietic Stem Cell Transplantation (HSCT). In 2004 a new regimen was introduced which added a few more drugs suggesting a symptom based approach.

### HLH- 94 TREATMENT PROTOCOL:

HLH 94 protocol involved three stages of therapy:

#### 1) INITIAL THERAPY( 1-8 WEEKS)

During first 8 weeks of therapy a combination of etoposide and dexamethasone were given. It was found that even with this regimen deaths were reported. Hence an intensive 8 weeks therapy with immunosuppressive agents was recommended.

**2) CONTINUATION THERAPY (9- 24 weeks)**

Continuation therapy included the same regimen of immunosuppression with dexamethasone along with CSF analysis every four weeks. This was added because most of the deaths during continuation therapy were due to CNS complications.

**3) INTRATHECAL THERAPY**

If the CSF analysis during continuation therapy showed abnormalities intra-thecal medications were given.

**4) HSCT**

If patient remained unresponsive to treatment, haematopoietic stem cell transplantation was considered.

**HLH- 2004 THERAPEUTIC DESIGN**

The revised 2004 treatment regimen has few modifications compared to the 1994 regimen. It includes four therapies.

**1) INITIAL THERAPY**

Initial therapy involves using etoposide and dexamethasone for 8 weeks along with monitoring of CSF every 4 weeks .If the CSF analysis shows pleocytosis, intrathecal administrations are considered.[49,50]

## 2) CONTINUATION THERAPY

In patients without a molecular mutation and patients without a family history of HLH in whom there is improvement, the treatment can be stopped at this point. However, others are administered the continuation therapy until HSCT.

## 3) REACTIVATION THERAPY

Reactivations are common in HLH. The recurrence of HLH in patients with a previous history of HLH who were declared cured after treatment is called reactivation. Reactivation therapy is very intense compared to the initial therapy. It includes frequent intrathecal administrations.

## 4) SALVAGE THERAPY

Salvage therapy includes a combination of steroids, immunosuppressives and antithymocyte globulin.

## 5) STOPPING THERAPY

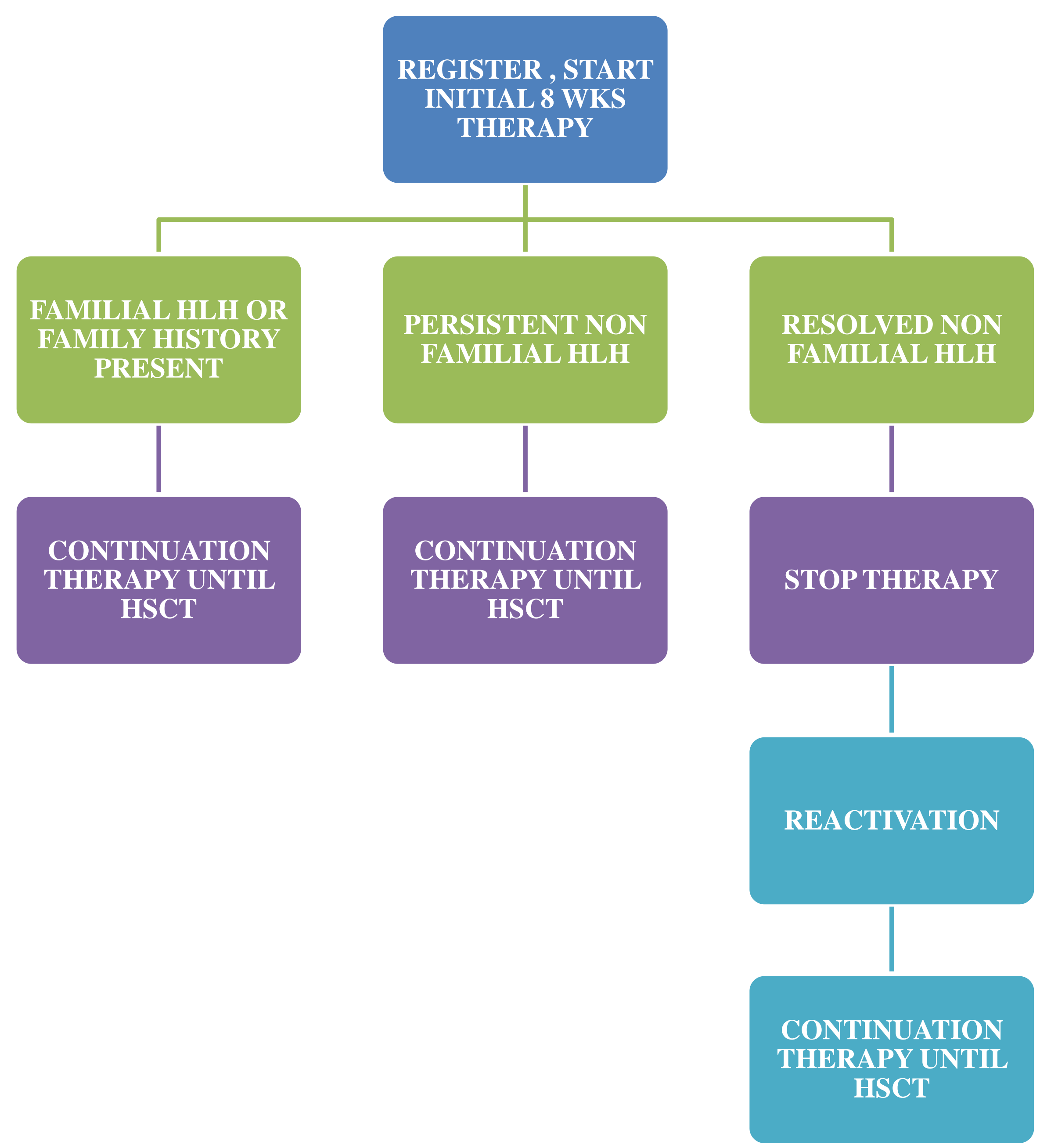
Stopping therapy is only recommended in patients with complete cure. But even then close monitoring of these patients for fever, blood counts and organomegaly is suggested.

## HAEMATOPOIETIC STEM CELL TRANSPLANTATION

The choice of the donor depends on the clinician. HLH matched sibling donor is preferred but even they should be investigated for HLH mutations before transplant. If no matched donor is available a haplo-identical family donor is suggested.[51]

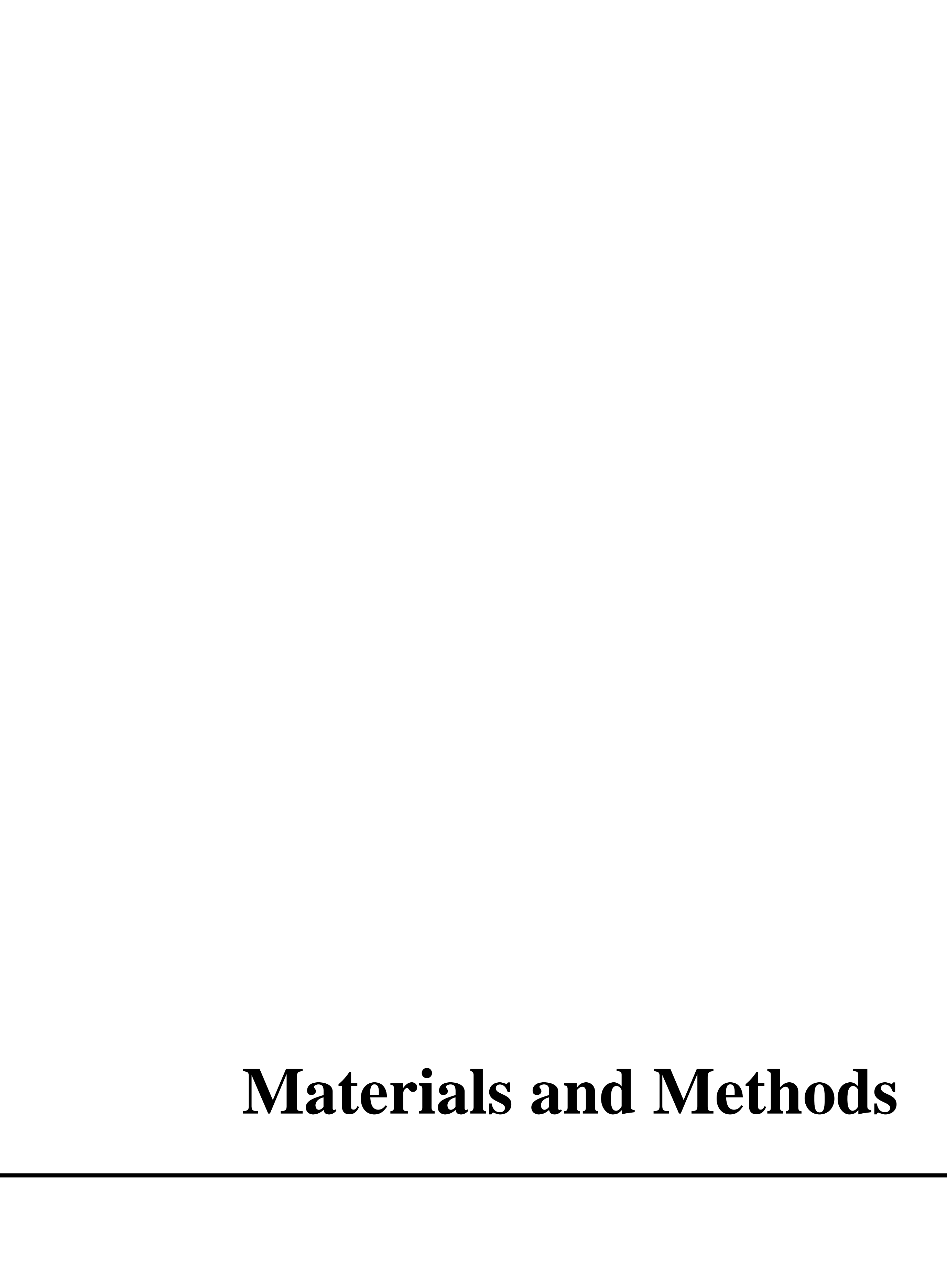
The preparative regimen and GVHD prophylaxis proposes using etoposide, busulphan and cyclophosphamide.

The recommended marrow infusion is $>3*10^8$nucleated cells/kg and non T cell depleted. It is recommended to use non T cell depleted because the donor T cells and NK cells are required for curing HLH. However when haplo-identical donors are used T cell depletion is a must to prevent GVHD.


REGISTER , START INITIAL 8 WKS THERAPY
FAMILIAL HLH OR FAMILY HISTORY PRESENT
PERSISTENT NON FAMILIAL HLH
RESOLVED NON FAMILIAL HLH
CONTINUATION THERAPY UNTIL HSCT
CONTINUATION THERAPY UNTIL HSCT
STOP THERAPY
REACTIVATION
CONTINUATION THERAPY UNTIL HSCT

**PROGNOSIS [48]**

Survival for patients with HLH has drastically improved in the last decade after the above treatment regimens were put into practice and the advent of HSCT. The overall 3 year survival for HLH patients after HSCT is 64%. With HLA matched donors it is 71%, with matched unrelated donors 70%, with haplo-identical family donors it is 50% and with mismatched unrelated donors 54%. In order to further improve the prognosis of patients with HLH clinical trials in the field of pathogenesis, diagnostic and therapeutic guidelines are required and recommended for the future.



# Materials and Methods

# MATERIALS & METHODS

This is a retrospective study. Cases of Haemophagocytosis reported in the bone marrows received in the Department of Pathology, PSGIMS & R during the period starting from January 2010 to May 2017 were taken.

Information regarding the age, sex and clinical presentation of the patients were derived from the requisition forms sent along with the bone marrow aspirates and trephine biopsies as well as from the hospital information system.

## METHODS

## MATERIALS REQUIRED:

- Salah's bone marrow aspiration needle
- Jamshidi trephine biopsy needle
- Clean glass slides of size 7.5*2.5* 0.1 cm
- Methanol for fixation of smears
- Giemsa staining solution

## SITE OF BIOPSY [52]:

- The posterior superior iliac spine is the preferred site.
- In case of obese patients the anterior superior iliac spine or the sternum was taken.

## PROCEDURE

Under aseptic precautions and local anaesthesia the posterior superior iliac spine was palpated and the Salah's needle was inserted with a boring movement. The entry of the needle into the marrow was identified by the giveaway feel. Once inside the marrow the cannula was removed, a syringe was attached and the marrow contents were aspirated.

After the process of aspiration, the bone marrow trephine biopsy needle (Jamshidi needle) was inserted and a core of bone marrow trephine was obtained. The specimen was placed in a container containing 10% neutral buffered formalin.

## PREPARATION OF SMEARS

There are two methods for preparing a bone marrow aspiration smear

1. Squash technique
2. Smear technique

In our institution we use the squash technique for preparing the smear. The bone marrow aspirate is placed on one end of the slide and with a help of another slide the material is squashed between the two slides to obtain the smear.

## STAINING OF BONE MARROW ASPIRATE SMEARS

## PROCEDURE:

1. Fix smears in methanol for 30 minutes
2. Air dry the smears
3. Mix 1 part Giemsa solution with 3 parts buffered water of pH 7
4. The smears are flooded in the staining solution for 20 minutes
5. The stained slides are washed in running tap water
6. The smears are allowed to dry
7. Dipped in xylene
8. Mounted using DPX

Once the slides are stained, the bone marrow aspirate is studied.

## PROCESSING OF BONE MARROW TREPHINE BIOPSY

The bone marrow trephine biopsies were sent to histopathology laboratory in 10% neutral buffered formalin. They were decalcified using aqueous EDTA of pH 7.6 . The blocks were processed and sections taken for H & E staining and immuno histochemical marker (IHC) studies.

## HEMATOXYLIN & EOSIN STAINING[53]

## REAGENTS USED

1. Xylene
2. Iso propyl alcohol
3. 1% acid alcohol
4. Eosin Y
5. Harris hematoxylin
6. DPX

## PROCEDURE:

1. Deparaffinisation – xylene three changes 5 minutes each
2. Hydration – graded concentration of iso propyl alcohol followed by water – 2 minutes each
3. Progressive staining- Harris haemtoxylin for 15 minutes
4. Bluing- running tap water for 5 minutes
5. Differentiation - 1% acid alcohol for 10 seconds,
6. Bluing – tap water for 10 minutes
7. Counter staining- Eosin Y for 2 minutes
8. The stained slides are then washed in running tap water for three minutes
9. Dehydration- increasing gradient of isopropyl alcohol 2 minutes each
10. Clearing- Xylene 1 dip
11. Mounting- with cover slip using resinous mounting media DPX.

The stained slides were then studied.

## IMMUNOHISTOCHEMICAL STAINING [54]

The blocks were cut at 5 micron thickness and the sections were taken onto Poly L lysine coated slide.

## REAGENT USED

| Antibody reagent | Clone |
|---|---|
| CD 68 | Monoclonal mouse antihuman antibody<br>Clone: KP1 |

## PRINCIPLE

**The staining process involves two steps:**

1. Binding of the specific primary antiboby to the target epitope.
2. Identification of the primary antibody using a secondary antibody by a colorimetric reaction.

## ANTIGEN RETRIEVAL

Fixation in formalin leads to masking of certain antibodies due to cross linking of proteins. These antigenic sites can be exposed using the antigen retrieval technique.

There are three methods for antigen retrieval:

1. Pressure cooker method
2. Microwave method
3. Proteolytic digestion method

The antigen retrieval for the material in this study were done using the pressure cooker method. Before antigen retrieval, the slides were dewaxed with xylene and hydrated in graded alcohols. The slides were then placed in a silver trough containing Tris-EDTA buffer at an alkaline pH of 9 which was preheated to boiling point using a pressure cooker. The lid was then sealed and the slides were incubated in the pressure cooker for 15 minutes.

**REAGENTS USED**

1. Ethylene Diamine Tetra Acetic acid ( EDTA) buffer at ph 9
2. 0.01M Phosphate Buffered Saline (PBS) at a pH of 7.6. It is prepared by mixing di-basic sodium phosphate- anhydrate 17.5 g, sodium chloride 17.0 g and monobasic potassium phosphate- anhydrate 2.5 g in one litre of distilled water.
3. 3% hydrogen peroxide which blocks the endogenous peroxidase and hence prevent background staining.
4. Blocking reagent- Caesin with 15mM sodium azide which helps in blocking the non specific protein binding.
5. Primary antibody- CD68

6. Horse Radish Peroxidase enzyme
7. Chromogen- DAB ( 3,3’ DiAmino Benzidinetetrachloride) which is responsible for the brown colour.
8. Harris haematoxylin – counter stain
9. DPX- mountant

**PROCEDURE**

1. Dewax – xylene
2. Hydration- in graded alcohols
3. Antigen retrieval- in EDTA buffer at an alkaline pH 9 in the pressure cooker for 15 minutes.
4. Washed in running tap water
5. Washed in PBS for 5 minutes
6. Immersed in hydrogen peroxide for blocking of the endogenous peroxidase.
7. Washed in PBS three times for 5 minutes.
8. Diaminio Benzidine used for eight minutes.
9. Incubated in blocking solution for ten minutes.
10. Washed in PBS buffer three times 5 minutes each.
11. Slides incubated with CD 68 primary antibody for one hour.
12. Washed with PBS buffer three times for 5 minutes
13. DAB for eight minutes
14. Washed with PBS buffer three times for 5 minutes.

15. Counter stained with Harris hematoxylin for 15 minutes.
16. Washed in running tap water
17. Cleared with xylene
18. Mounted using DPX

The stained slides are then screened for macrophages which demonstrate haemophagocytosis.

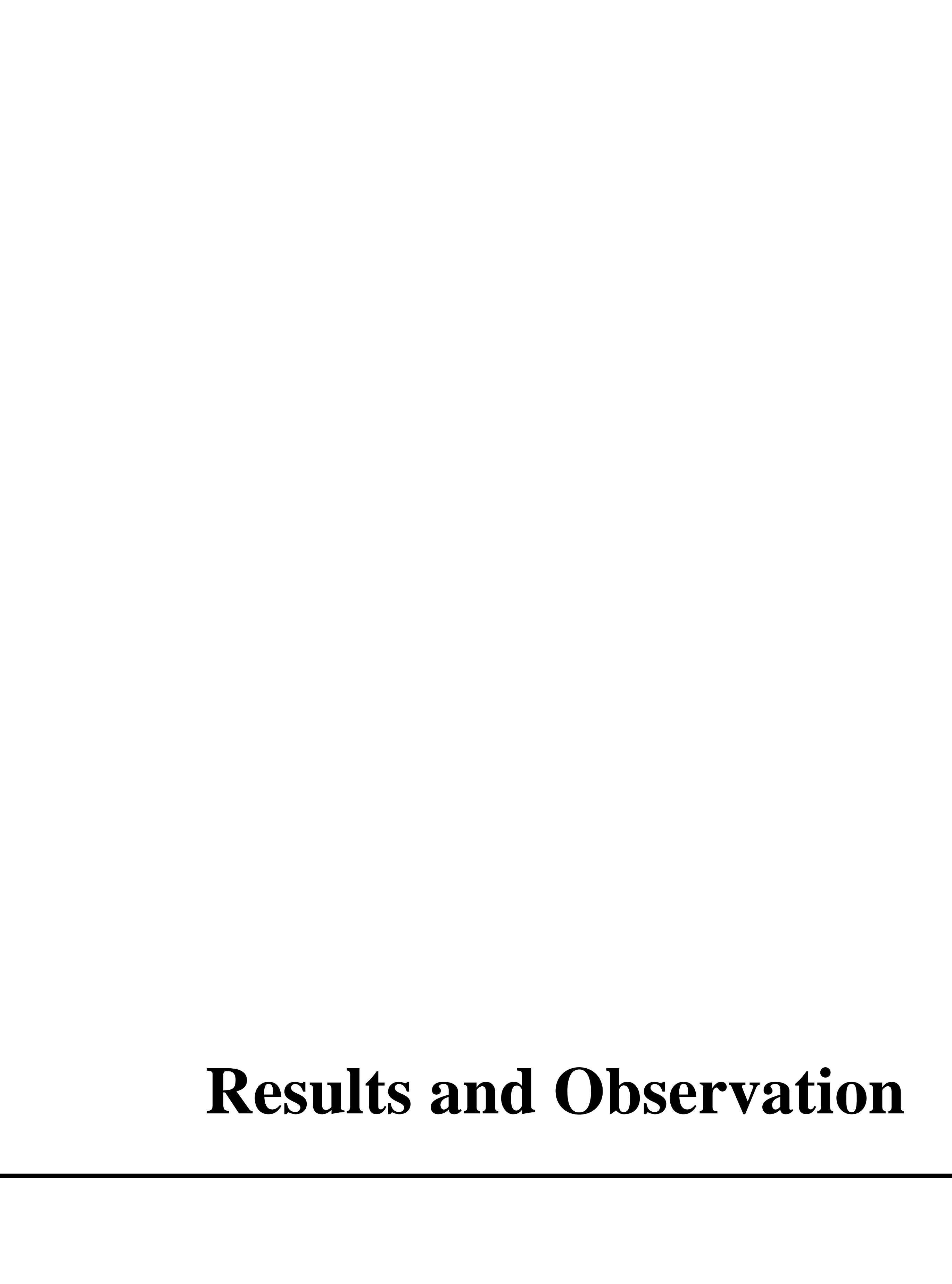
**DATA ANALYSIS**

The data for this study was collected using the patient requisition forms, hospital information system and discharge summary of the patients.

**The parameters documented were:**

1. Age of the patient
2. Fever
3. Splenomegaly
4. Blood counts
5. Triglycerides levels
6. Fibrinogen levels
7. Ferritin levels
8. Bone marrow findings
9. Investigations relevant to the pathology underlying the haemophagocytosis.

The information was entered into a Microsoft Excel worksheet and extrapolated into statistical package.



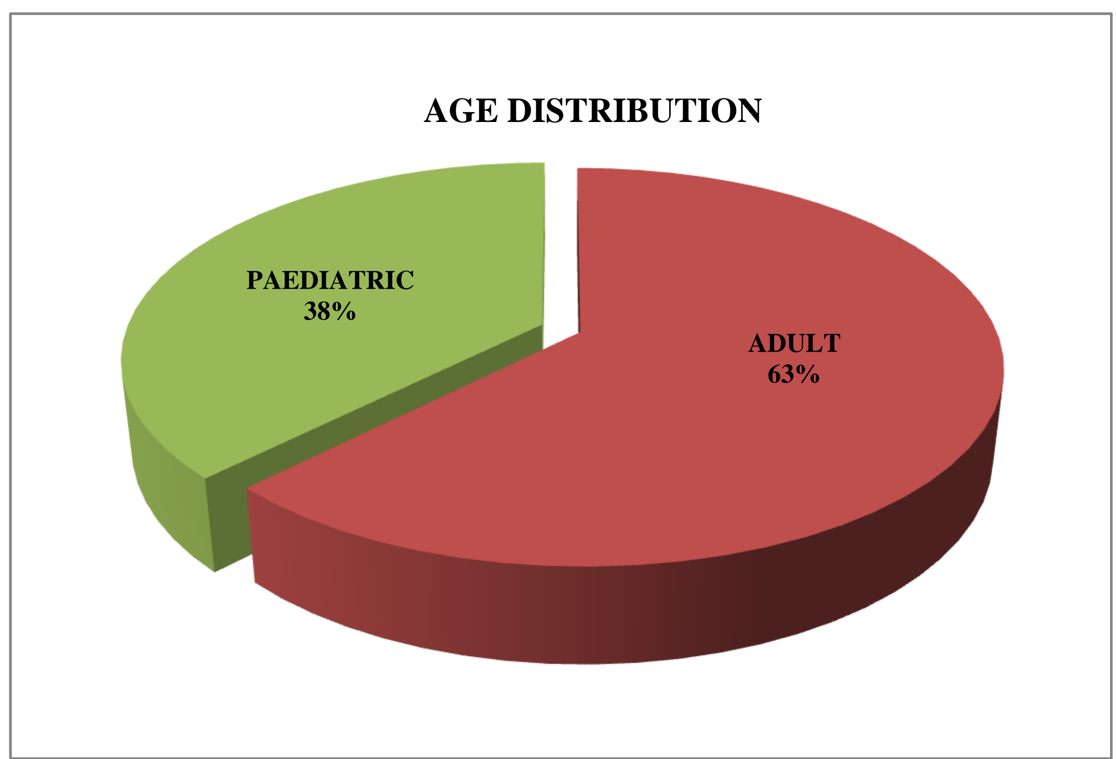
# Results and Observation

# RESULTS AND OBSERVATIONS

32 cases that presented with Haemophagocytosis in the bone marrow during the period January 2010 to May 2017 were studied. The age, clinical presentation like fever, splenomegaly, laboratory parameters like blood counts, ferritin levels, triglyceride levels and fibrinogen levels were analysed.

## AGE DISTRIBUTION

Of the 32 cases in our study 62% of cases were in the adult age group (>18 yrs) and 38% were from the paediatric age group.

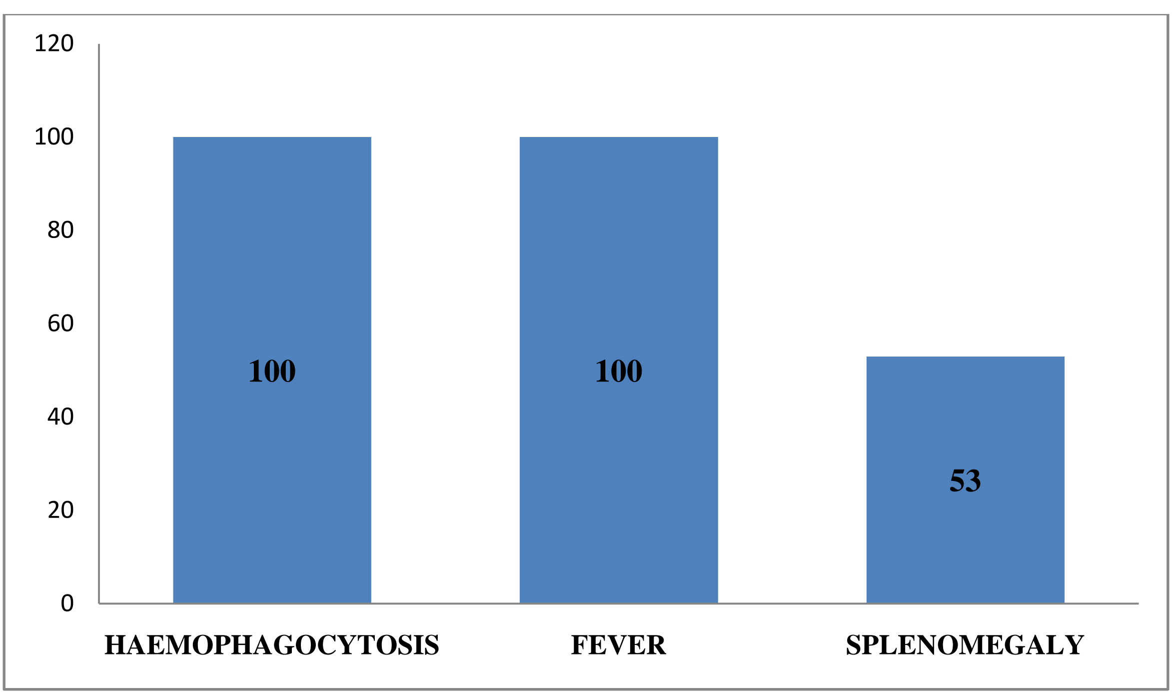


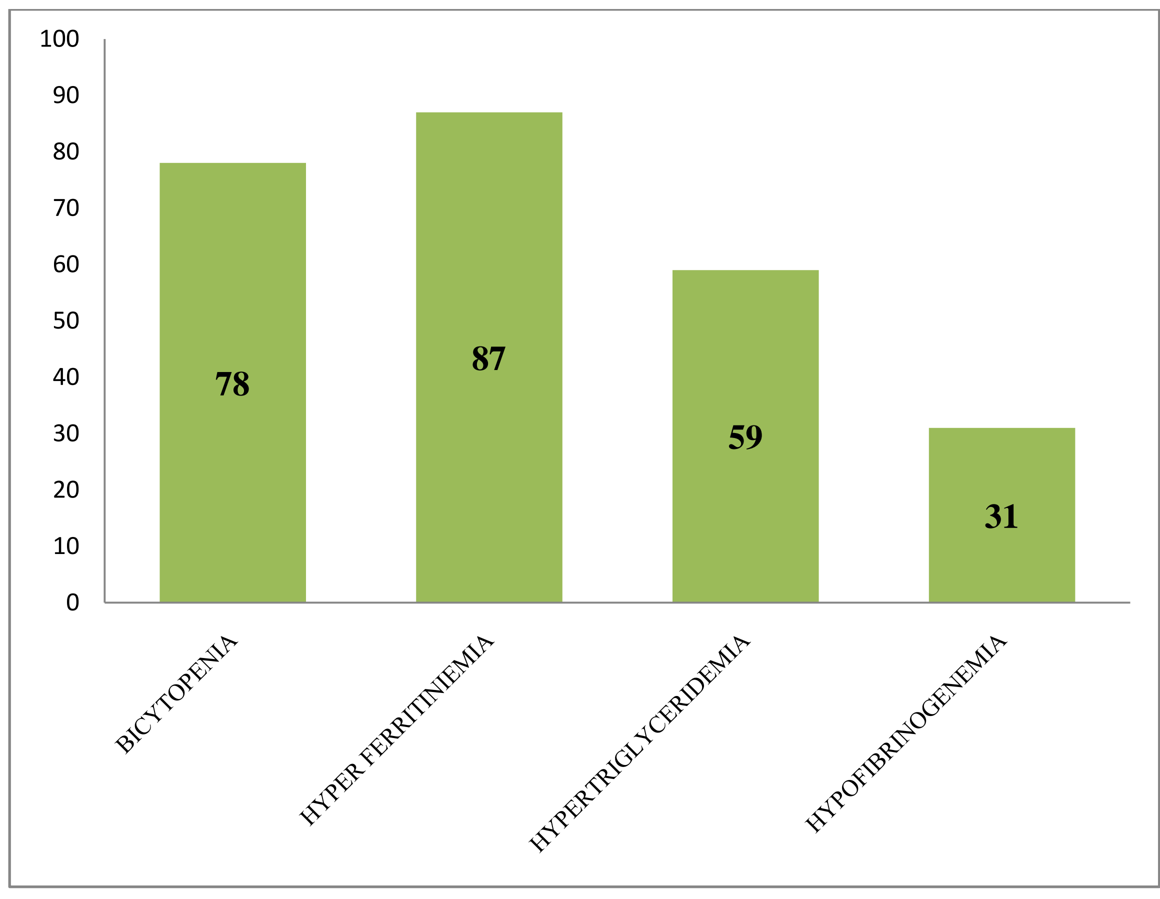
## DIAGNOSTIC CRITERIA

Of the eight criteria for diagnosis of HLH, CD 25 levels and NK cell activity were not performed in our laboratories. The other criteria like fever, splenomegaly, blood counts, ferritin levels, triglyceride levels and fibrinogen levels were analysed.

The presence of haemophagocytosis in the bone marrow was taken as the criteria for selecting cases, hence 100% of the cases had morphologic evidence of haemophagocytosis. Where the rest of the criteria are concerned, 100% of cases had fever, 53% had splenomegly, 78% had bicytopenia, 87% had hyperferritinemia, 59% had hypertriglyceridemia and 31 % had hypofibrinogenemia.

## DIAGNOSTIC CRITERIA



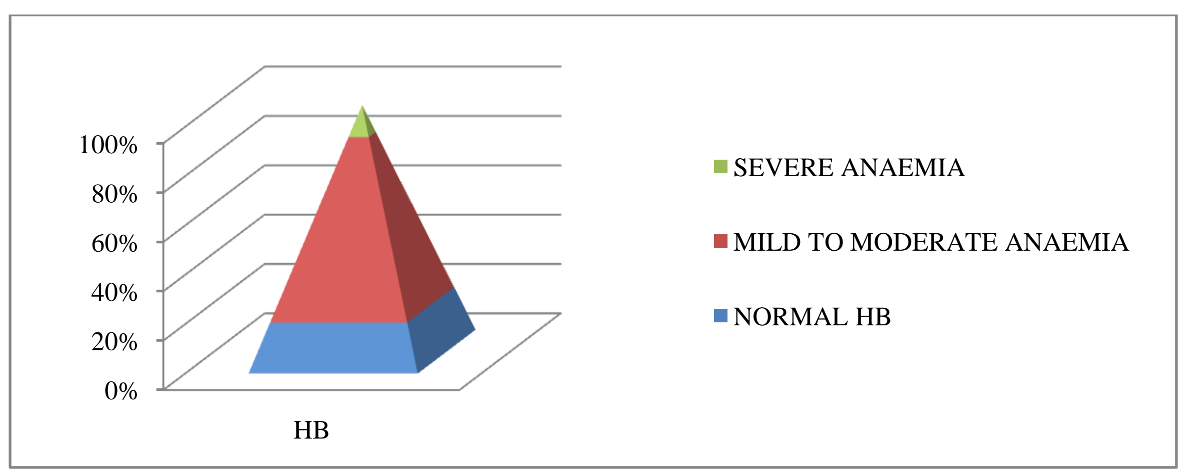
**CYTOPENIAS**

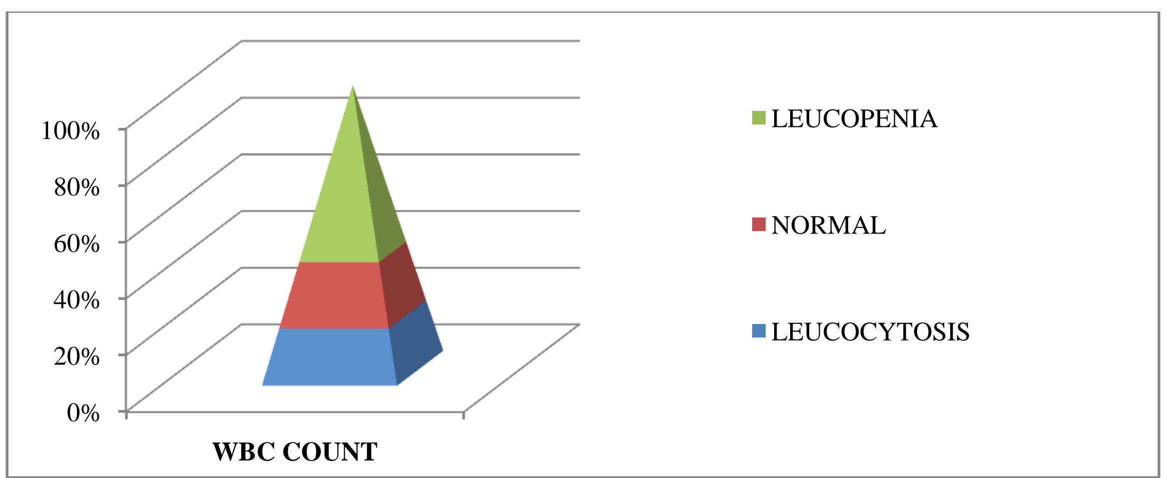
78% of cases in our study presented with bicytopenia. The haematological parameters are again graded based on the severity [54]. Haemoglobin levels are graded into normal (>12 g/dl), mild to moderare anaemia (7-12 g/dl %) and severe anaemia (<6g/dl). Total count is graded into those with leucopenia (<4* $10^9$/l), normal WBC count (4- 12 *$10^9$/l) and leucocytosis (>12*$10^9$/l). Platelet counts are graded as severe thrombocytopenia (<10*$10^3$/µl), mild to moderate thrombocytopenia (10-150*$10^3$/µl), normal platelet count (150-450*$10^3$/µl) and thrombocytosis (>450*$10^3$/µl).

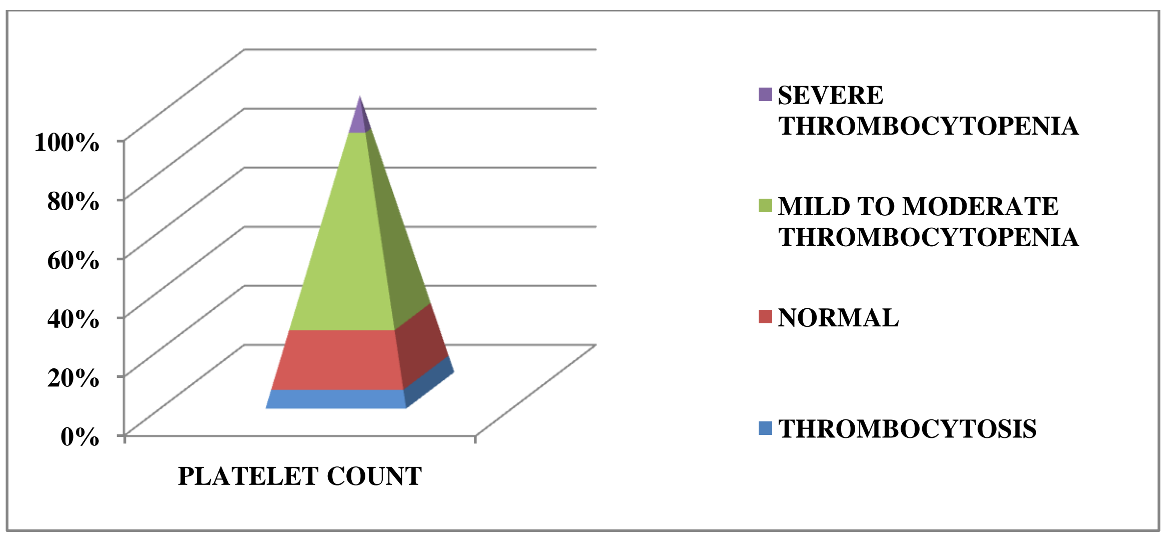
The results of the patients studied along with the grade of severity of the haematological parameters:

| | **Hb (g/dl)** | **Percentage of patients** |
|---|---|---|
| **Severe anaemia** | < 6 | 12 % |
| **Mild to moderate anaemia** | 7-12 | 69% |
| **Normal** | >12 | 19% |
| | **WBC count (*$10^9$/l)** | **Percentage of patients** |
| **Leucopenia** | <4 | 59% |
| **Normal leucocyte count** | 4-12 | 22% |
| **Leucocytosis** | >12 | 19% |
| | **Platelet count(*$10^3$/µl)** | **Percentage of patients** |
| **Severe thrombocytopenia** | <10 | 12% |
| **Mild to moderate thrombocytopenia** | 10-150 | 63% |
| **Normal** | 150-450 | 19% |
| **Thrombocytosis** | >450 | 6% |

## BLOOD COUNTS

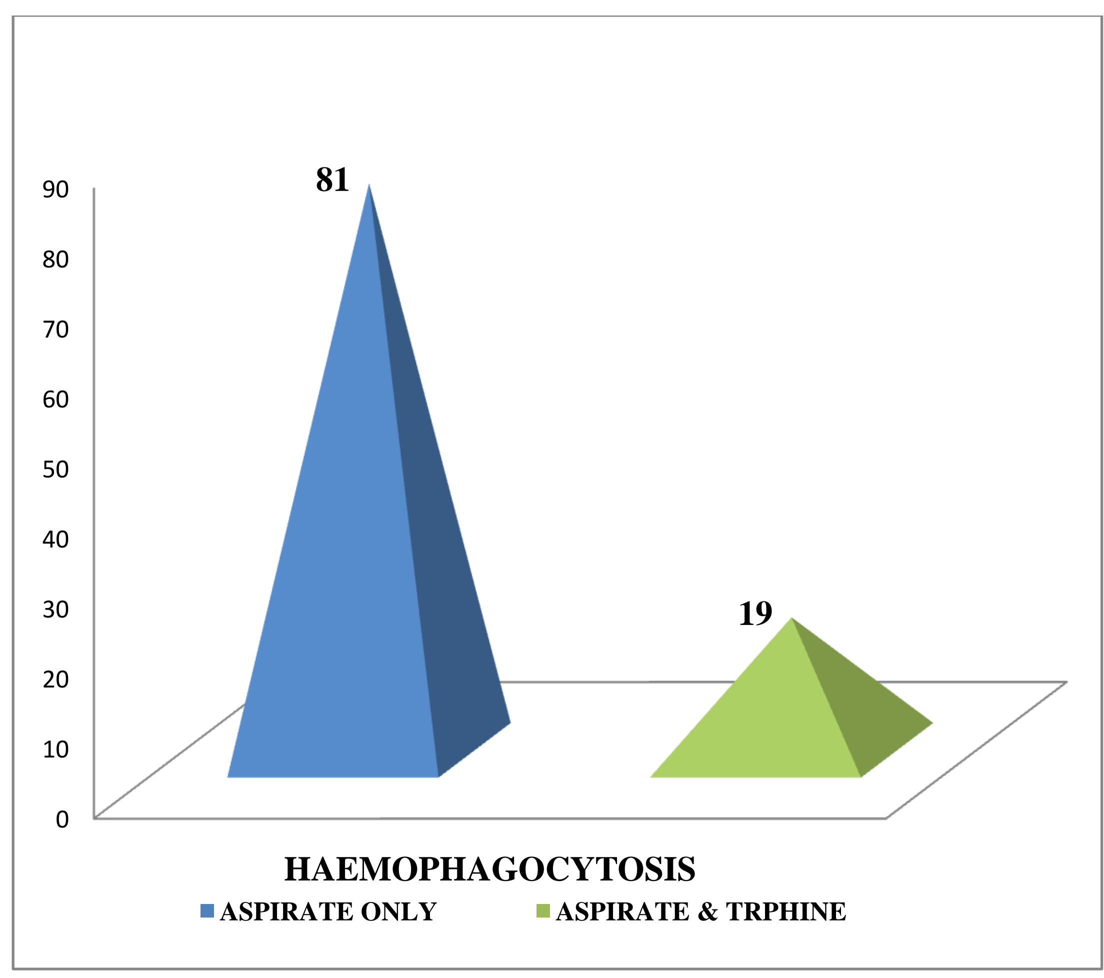


## BONE MARROW TREPHINE

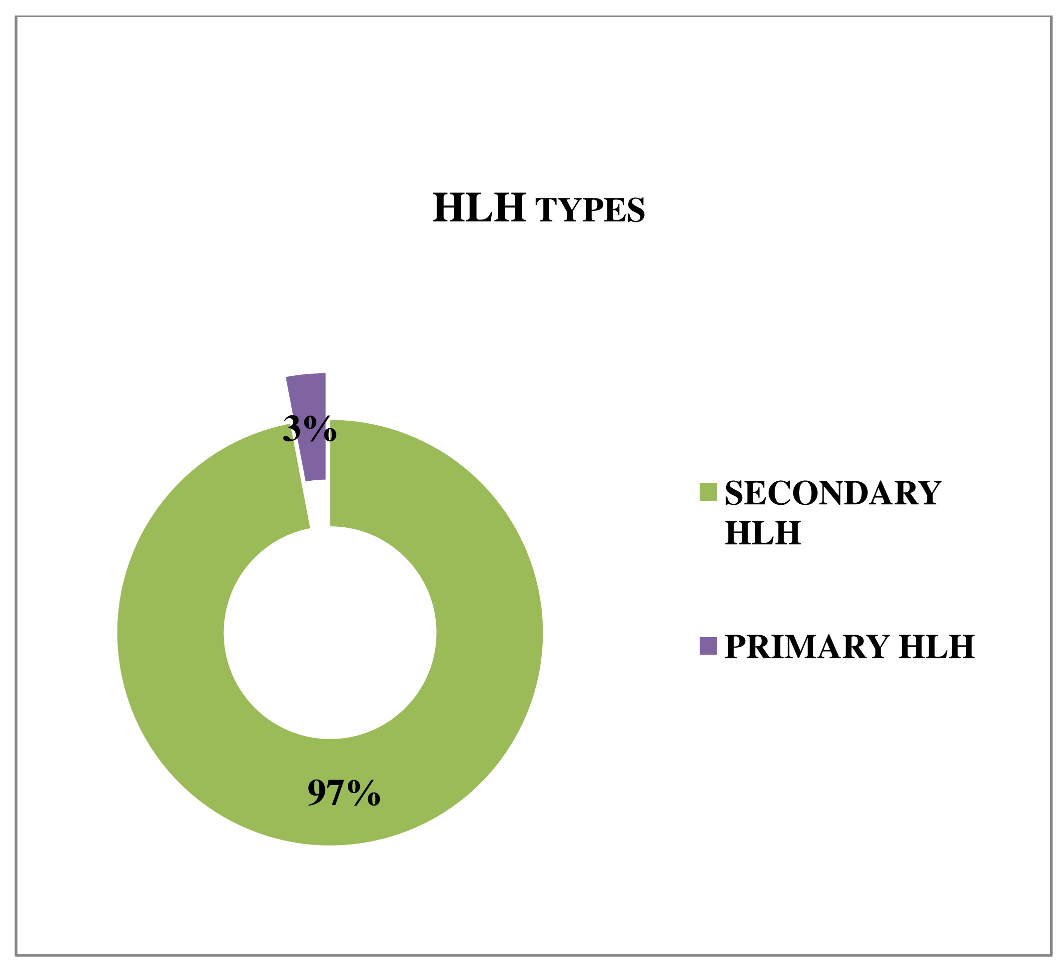
Of the 32 cases, 19% of cases presented with haemophagocytosis in both bone marrow aspirate and trephine, while the remaining 81% demonstrated haemophagocytosis only in bone marrow aspirate. There were no cases that were trephine positive but bone marrow aspirate negative. The haemphagocytic activity in the bone marrow trephine was highlighted using IHC marker CD 68.



**TYPES OF HLH**

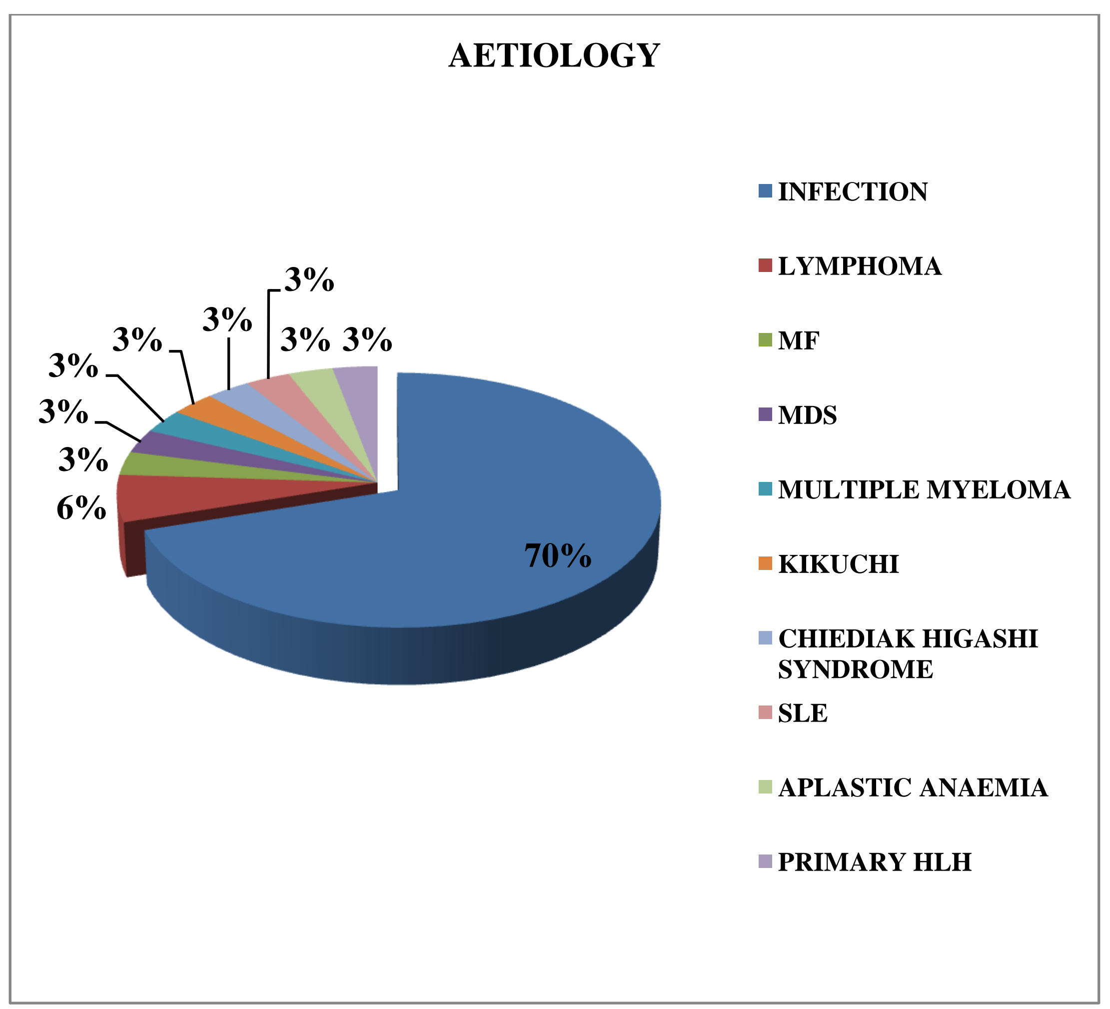
HLH is divided into two types - Primary HLH and Secondary HLH. Primary HLH includes cases which have a diagnostic molecular mutation. Secondary HLH includes cases which occur secondary to causes like infections, inflammatory conditions and even malignancies.

Of the 32 cases in our study, 3 % cases were of Primary HLH and the remaining 97% were Secondary HLH.



## AETIOLOGY

The most common aetiology for HLH in this study was infection which accounted for about 70 % of the cases. This was followed by cases of Non Hodgkins lymphoma which accounted for about 6 %. The rest of the cases were due to Myelofibrosis, Multiple myeloma, Myelodysplastic syndrome, Aplastic anaemia, Kikuchi Fujimotos disease, Chediak Higashi disease, and Systemic lupus erythematosus HLH each of which accounted for about 3% of the case. The remaining cases were primary HLH.

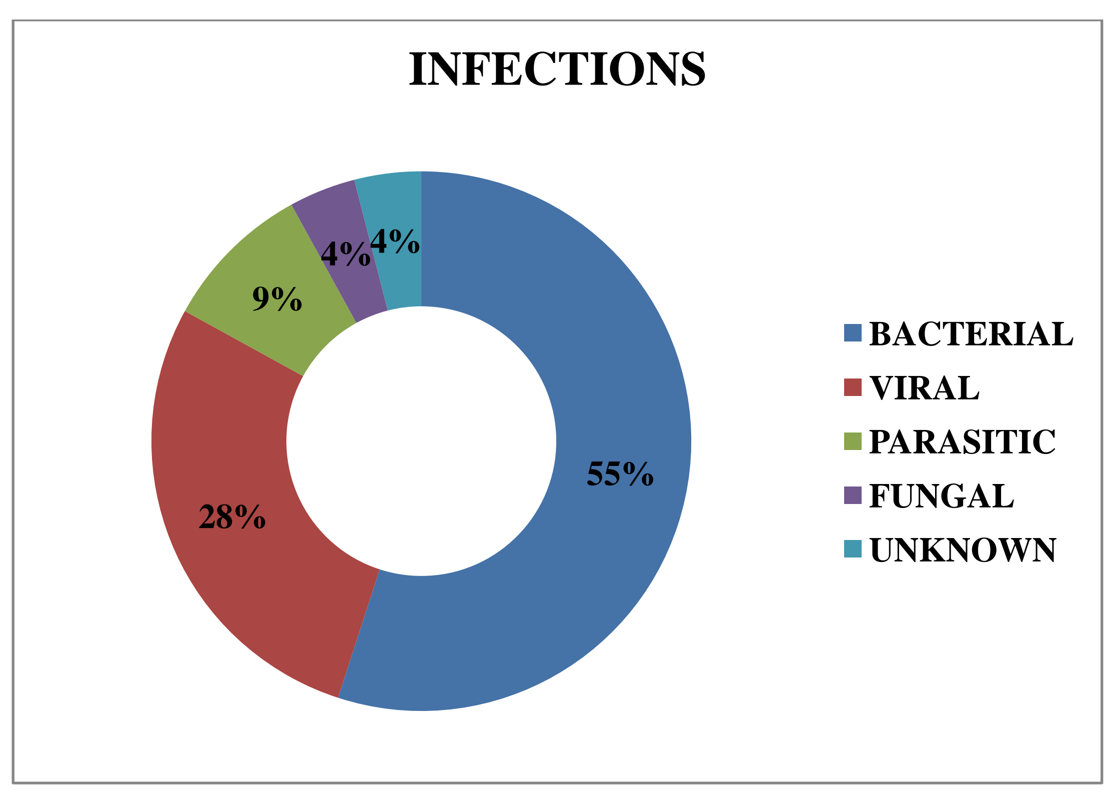


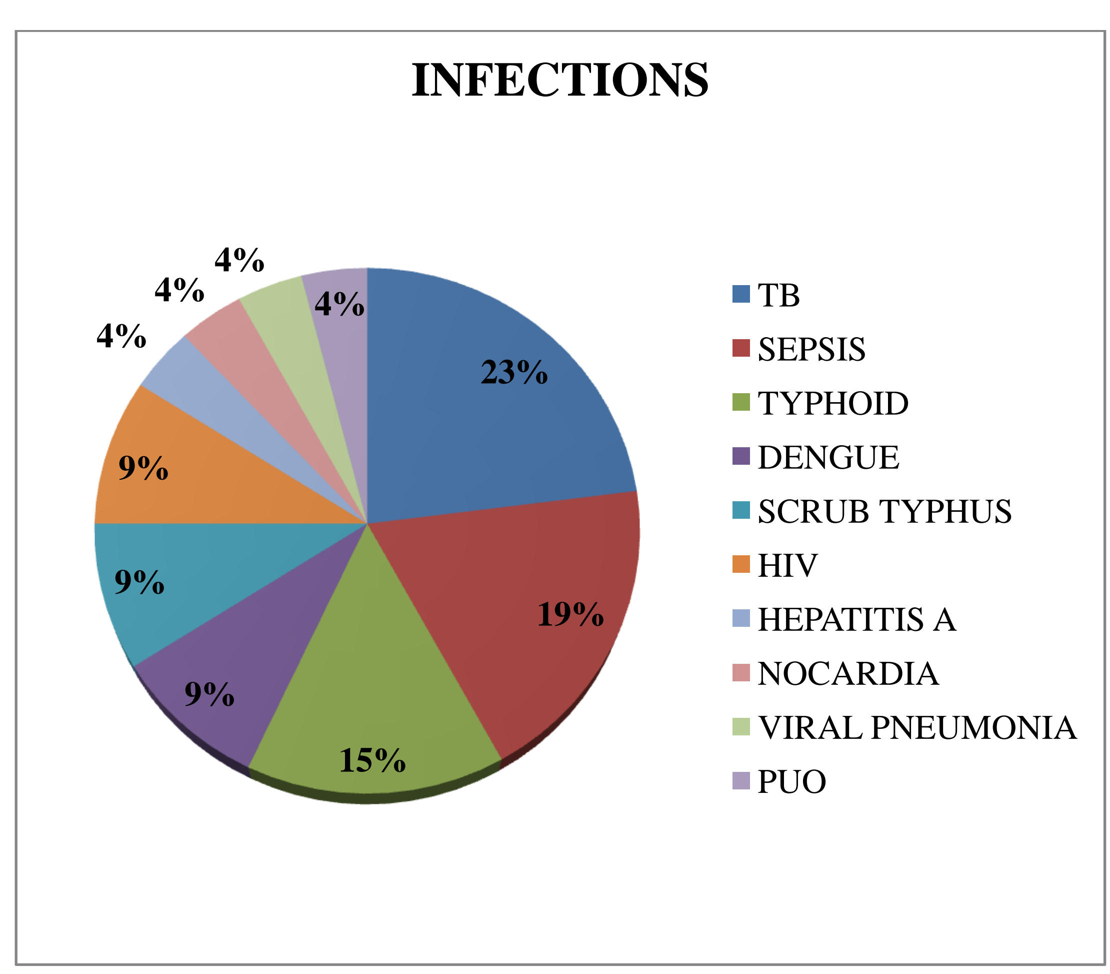
## INFECTIONS

Infection was found to be the most common cause for the HLH accounting for 70% of the cases.

Bacterial infections accounted for 55%, viral infections were 28%, parasitic infestations 9% and fungal 4%. In the remaining 4% the causative organism could not be isolated.

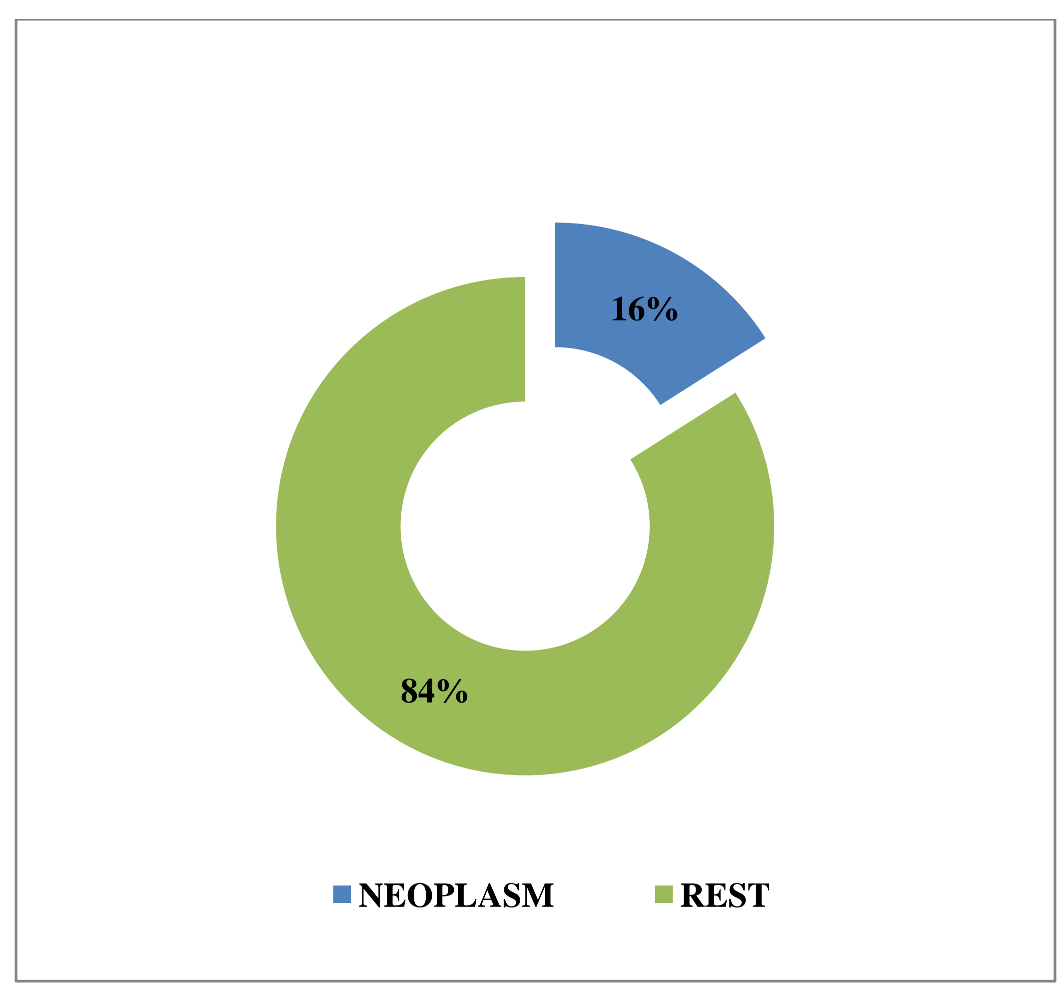
Tuberculosis was the most common infection accounting for 23% of the cases due to infections. The second most common cause was disseminated sepsis which accounted for 19%. Typhoid accounted for 15%, Dengue fever, Scrub typhus and HIV accounted for 9% totally. Hepatitis A, Nocardial infection,Viral pneumonia and PUO were 4% each.


INFECTIONS
55%
28%
9%
4%
4%
BACTERIAL
VIRAL
PARASITIC
FUNGAL
UNKNOWN

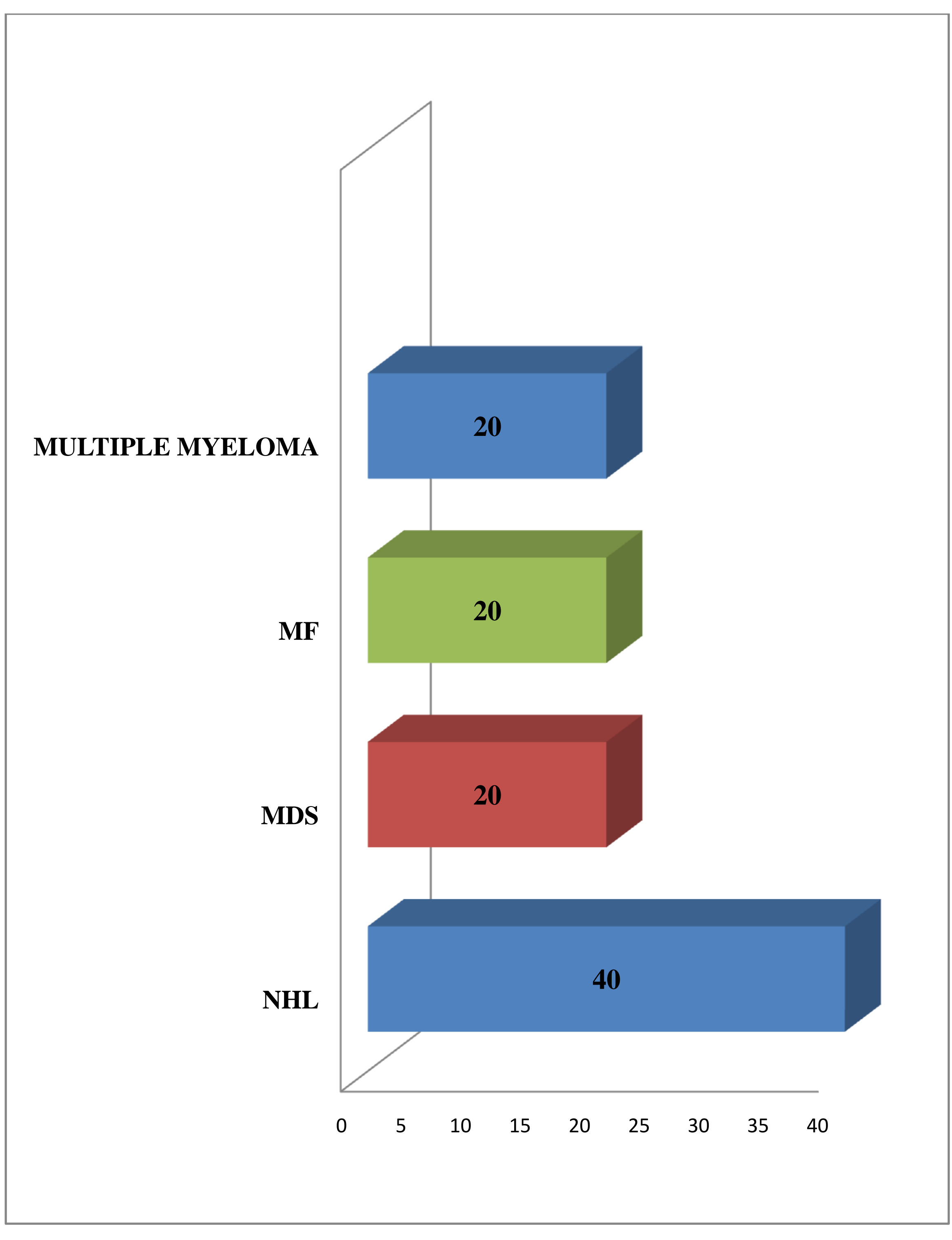

INFECTIONS
23%
19%
15%
9%
9%
9%
4%
4%
4%
4%
TB
SEPSIS
TYPHOID
DENGUE
SCRUB TYPHUS
HIV
HEPATITIS A
NOCARDIA
VIRAL PNEUMONIA
PUO

## NEOPLASMS ASSOCIATED WITH HLH

After infections, the most common cause for haemophagocytosis was neoplasm associated HLH accounting for 16% of the total cases. Lymphomas accounted for 40% of the neoplasm associated HLH. 20% of the cases had a diagnosis of Multiple myeloma The rest were Myelodysplastic syndrome and Myelofibrosis each accounting for 20 %.



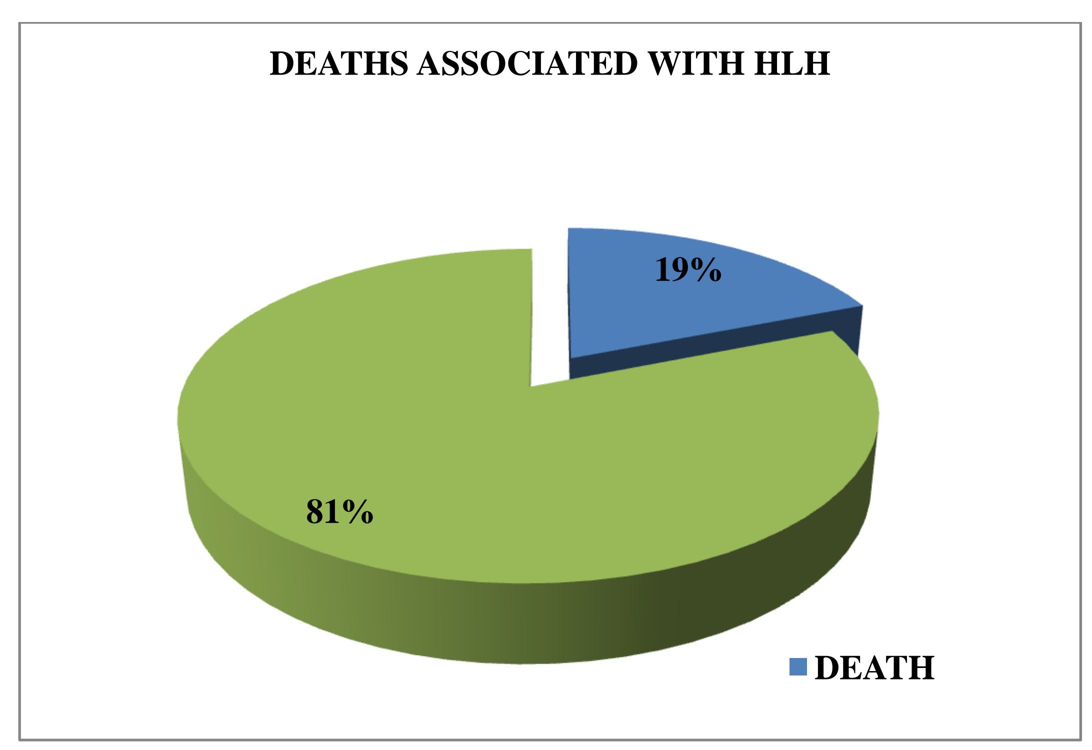
## NEOPLASM ASSOCIATED HLH

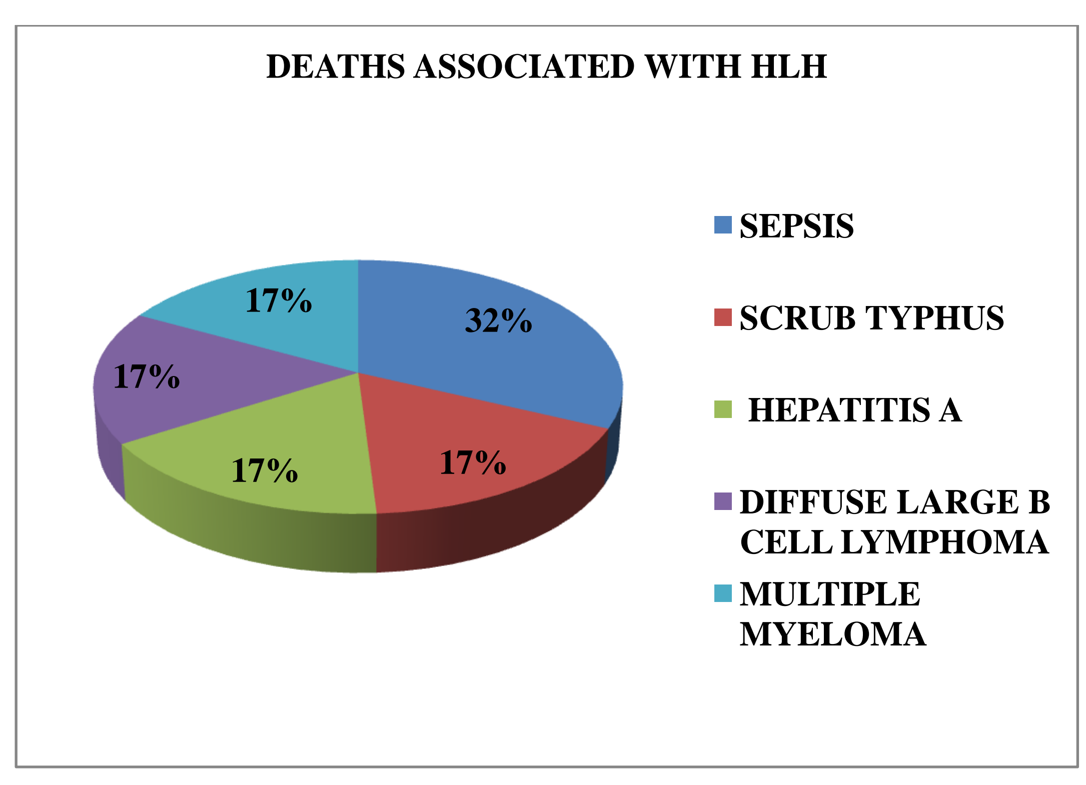


## DEATHS ASSOCIATED WITH HLH

Of the 32 cases, 19 % of cases had a very turbulent clinical course which resulted in the death of these patients.

Of the deaths associated with HLH 32% were due to sepsis. Scrub typhus, hepatitis A, diffuse large B cell lymphoma & multiple myeloma contributed to 17% each.


DEATHS ASSOCIATED WITH HLH
19%
81%
DEATH


DEATHS ASSOCIATED WITH HLH
17%
32%
17%
17%
17%
SEPSIS
SCRUB TYPHUS
HEPATITIS A
DIFFUSE LARGE B CELL LYMPHOMA
MULTIPLE MYELOMA

# Pictures

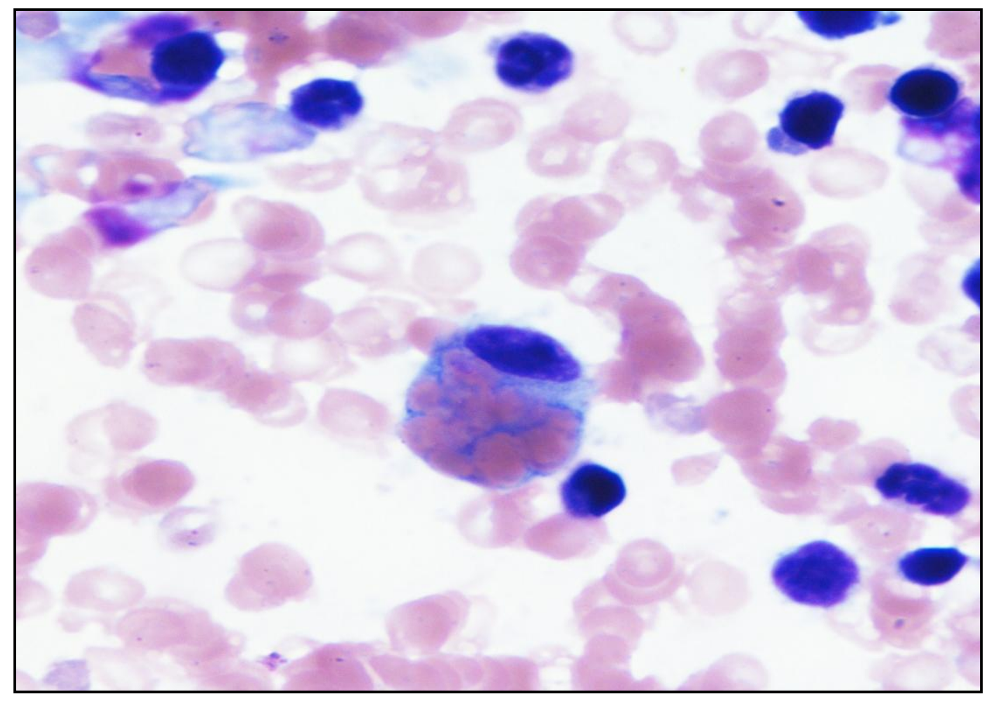


**Fig 1: Macrophages phagocytosing RBC’s**

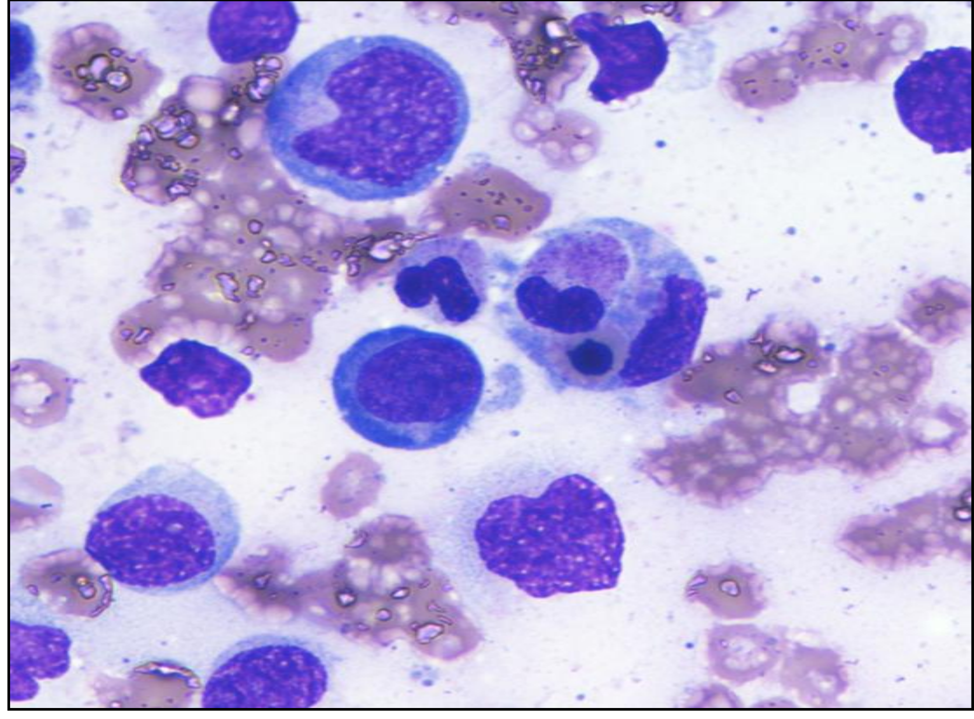


**Fig 2: Macrophages phagocytosing normoblasts & metamyelocytes.**

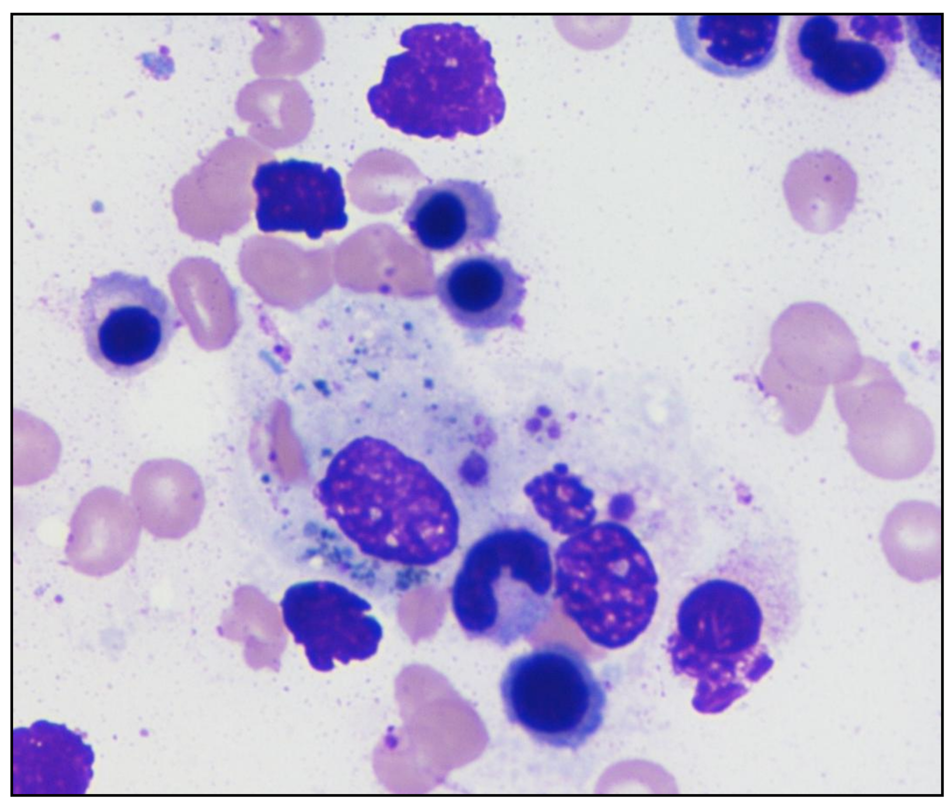


**Fig 3: Macrophages phagocytosing platelets.**

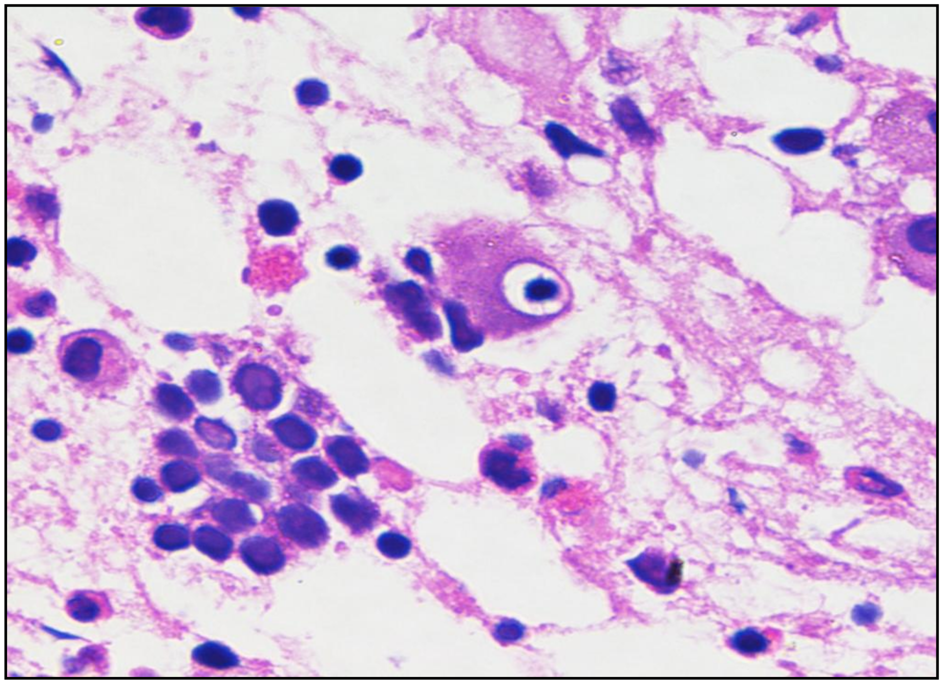


**Fig 4: Haemophagocytosis in bone marrow trephine biopsy.**

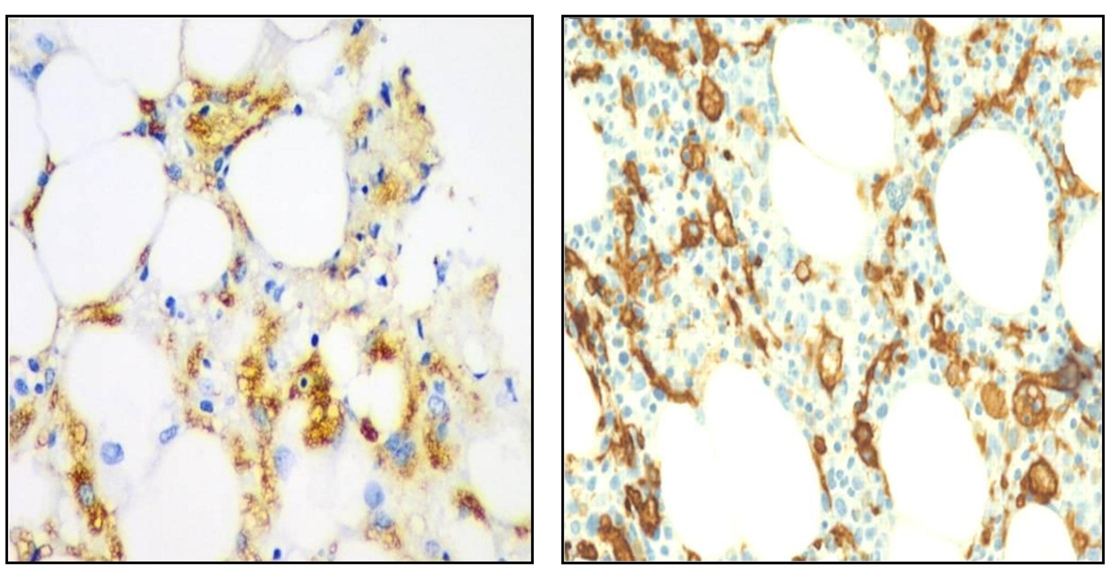


**Fig 5: Haemophagocytosis in trephine highlighted using Immunohistochemical marker CD 68.**

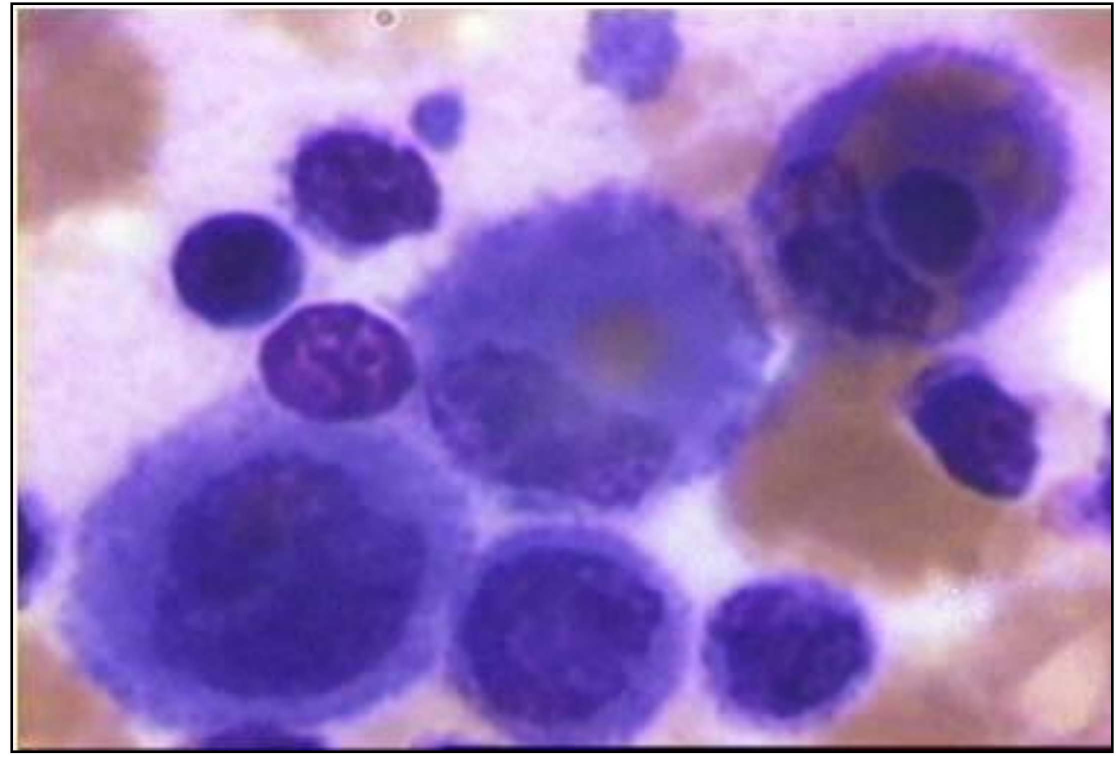


**Fig 6: Haemophagocytosis in a patient with primary HLH.**

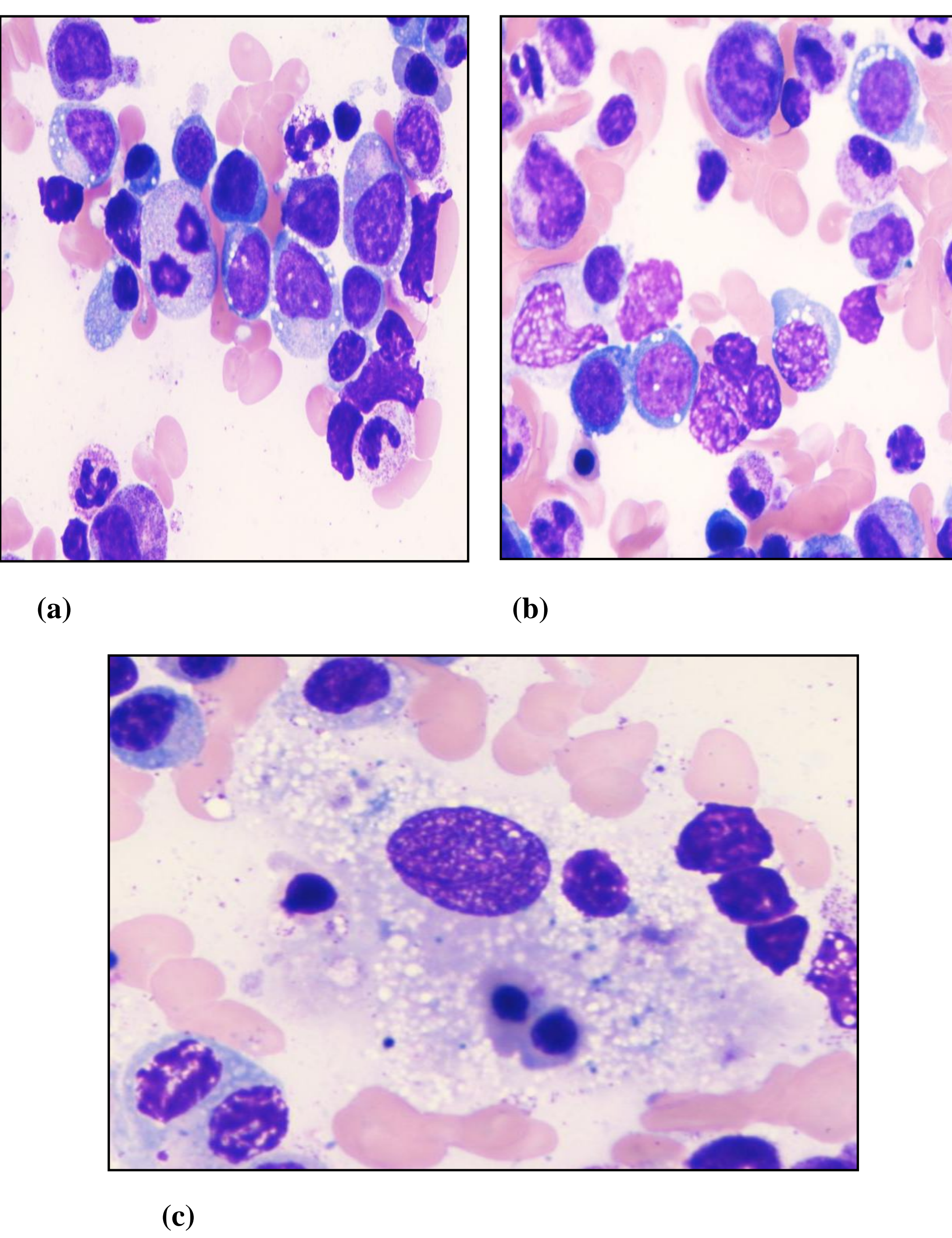
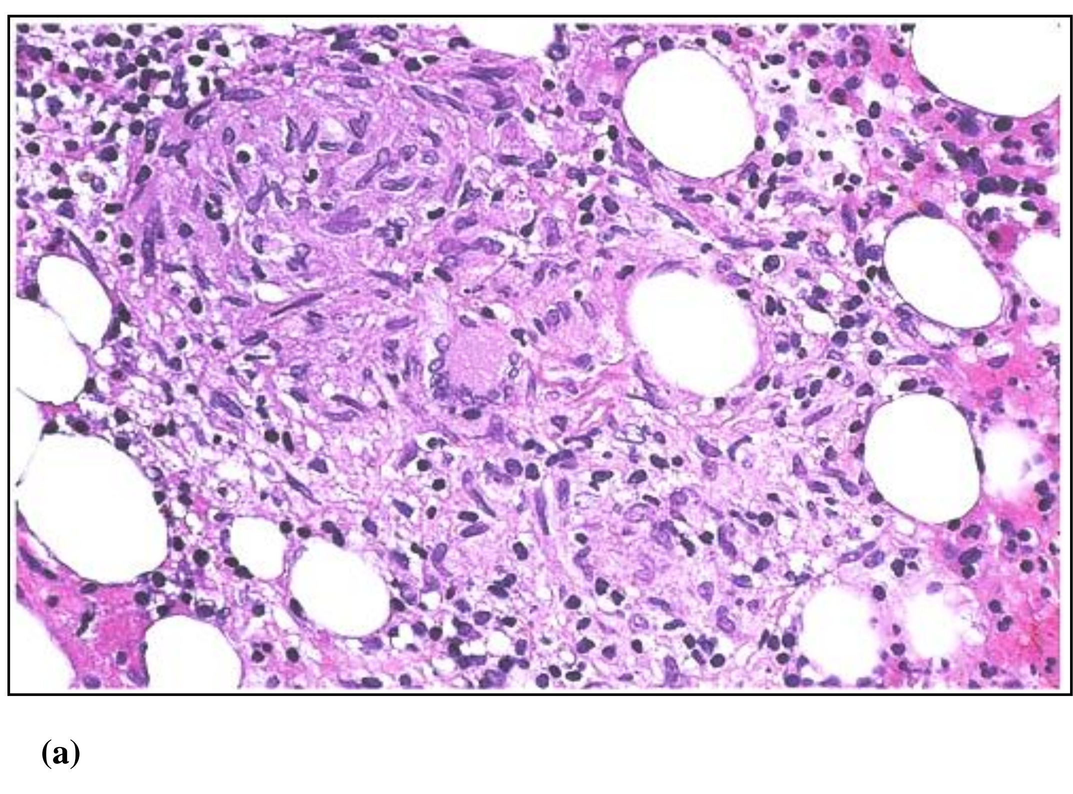
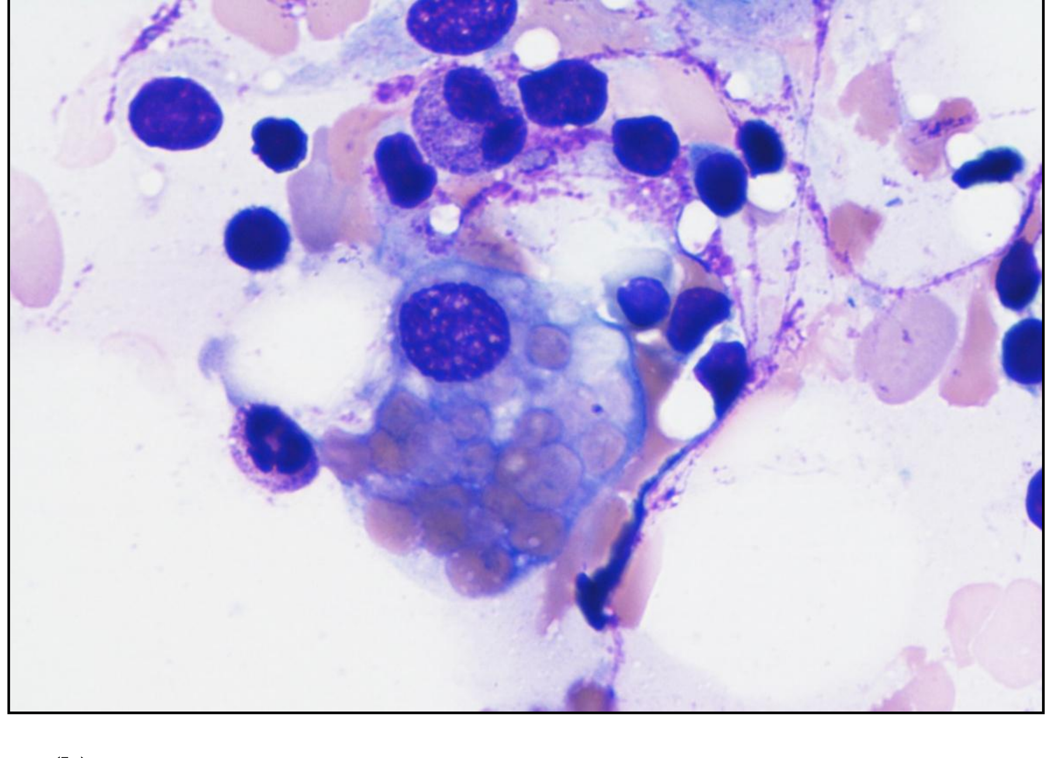


**Fig 7: (a & b) Cytoplasmic vacuolations in the erythroid precursors in bone marrow of an alcoholic patient with Hepatitis E infection.**
**(c) Haemaophagocytosis in the bone marrow of same patient.**

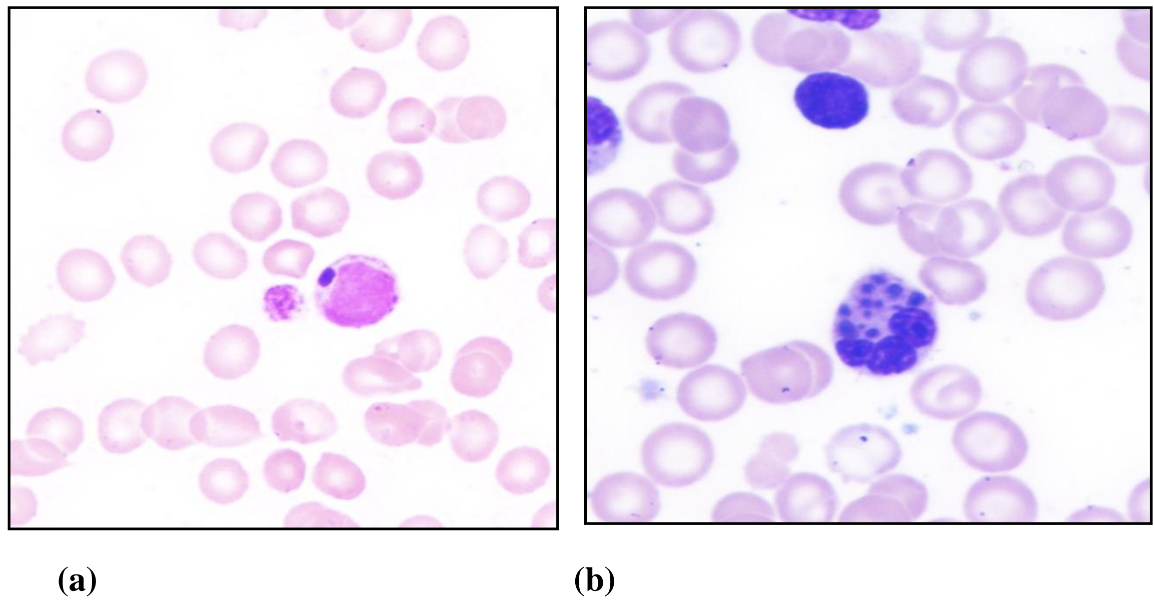


**(a)**



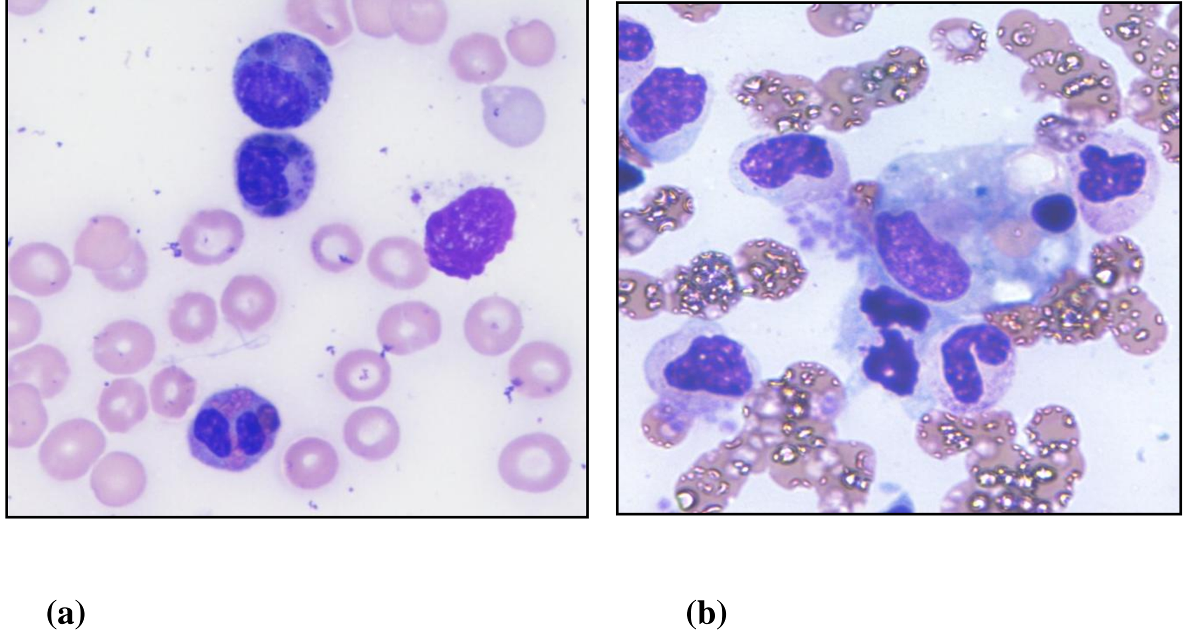
**(b)**

**Fig 8: (a) Granulomas in bone marrow of a patient with tuberculosis. (b) Erythrophagocytosis in bone marrow of the same patient**



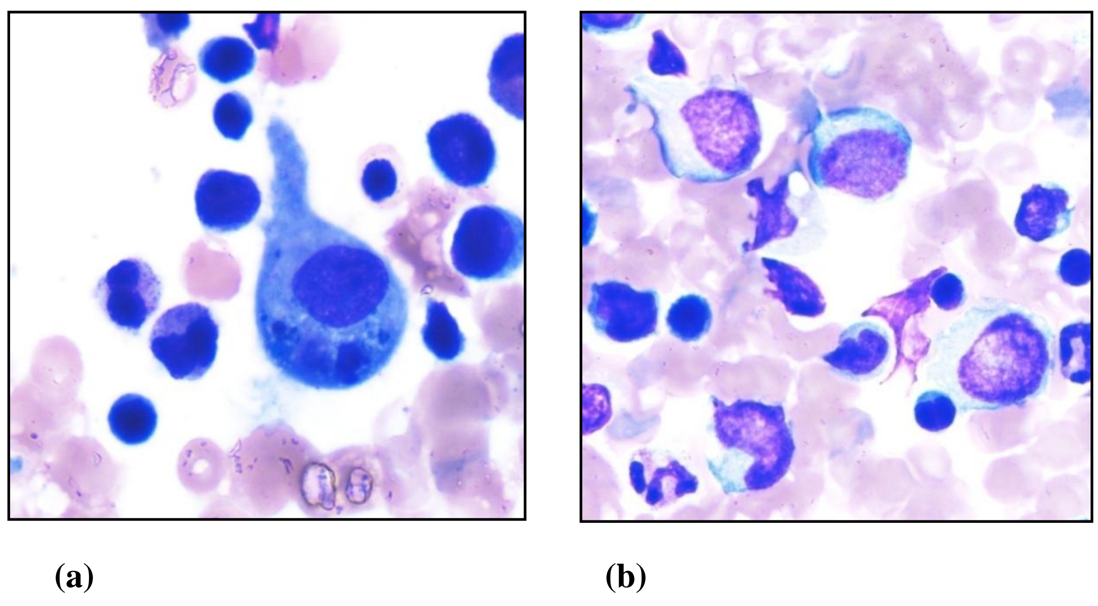
(a) (b)

**Fig 9: Abnormal granules in the cytoplasm of WBC's in peripheral smear of a patient with Cheidiak Higashi syndrome (CHS). (a) within lymphocytes (b) within neutrophils**



(a) (b)

**Fig 10: (a)Abnormal granules in the cytoplasm of myelocytes in bone marrow of a patient with CHS. (b) Haemophagocytosis in the bone marrow of the same patient**



(a) (b)

**Fig 11: Haemophagocytosis(a) in a patient with Atypical lymphoid cells(b) in bone marrow diagnosed to be DLBCL.**

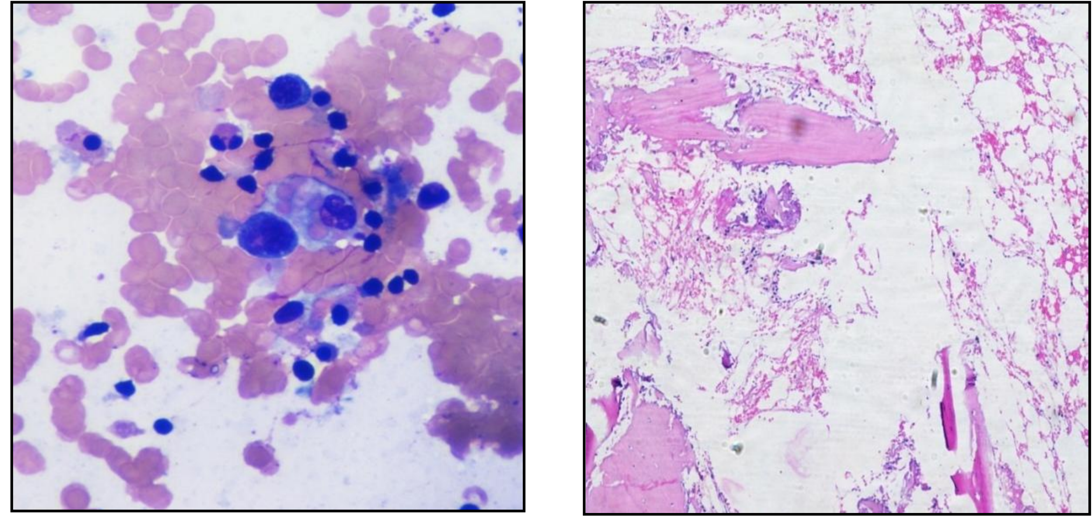
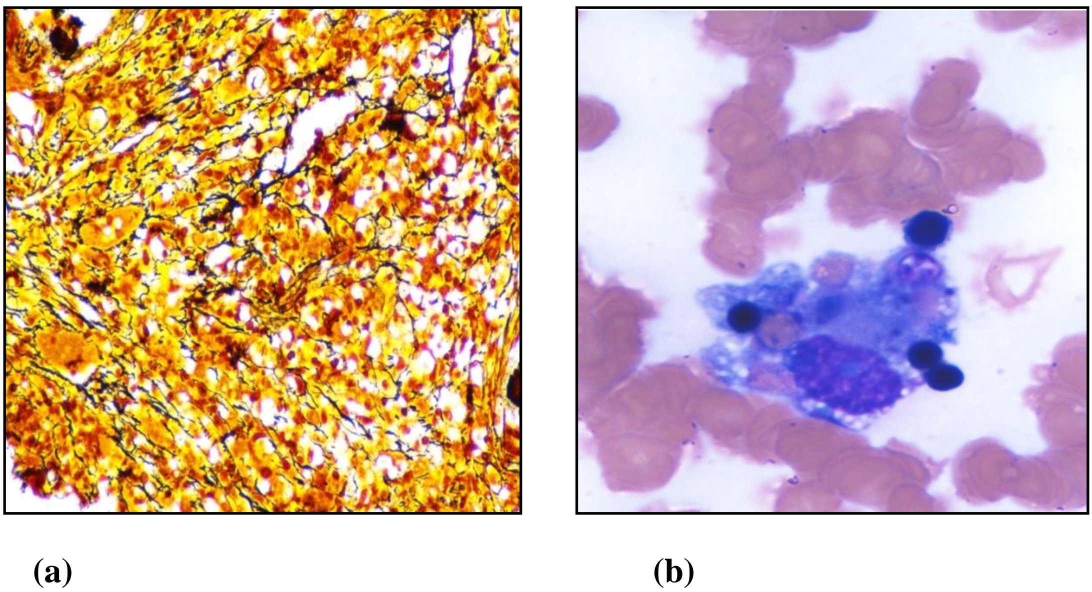
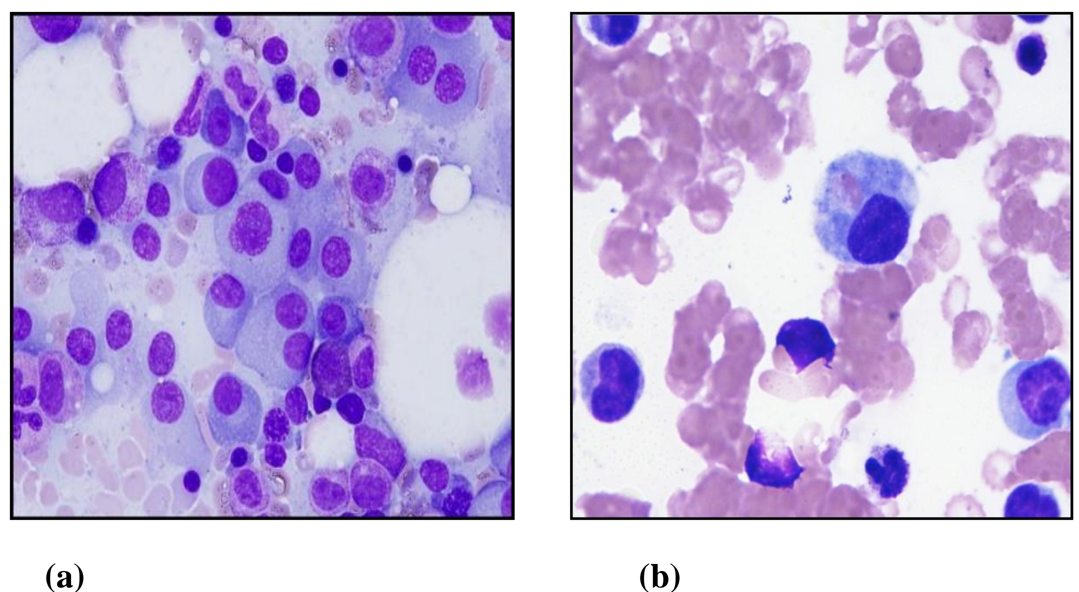


**Fig 12: Haemophagocytosis in a patient with aplastic anaemia showing acellular marrow spaces.**



**(a)** **(b)**

**Fig 13: (a) Bone marrow trephine of a patient with myelofibrosis. (b) Haemophagocytosis in the bone marrow of the same patient**



**(a)** **(b)**

**Fig 14: (a) Increased plasma cells in bone marrow of a patient with multiple myeloma (b) Erythrophagocytosis in bone marrow of same patient.**

# Discussion

# DISCUSSION

Haemophagocytic lymphohistiocytosis is a syndrome rather than a single disease which can occur in numerous disease conditions. The estimated incidence of HLH is 1 in 1,00,000 live births.(55) But this is just an under estimation because many of the cases are under recognised. The primary HLH is more common in childhood while secondary HLH can occur at any age.

This study was done with the aim of elucidating the various aetiopathogenesis of HLH. Awareness of the various aetiologies of this important entity will go a long way in helping to make an early diagnosis and promptly treat the patient.

In the 32 cases we analysed 62% were of the adult age group (>18yrs) and the remaining 38% of the paediatric age group (<18 years) with a mean age of presentation of 30.7 yrs. This was in correlation with a study by Iqbal et al who found the mean age of presentation of HLH to be 30.8 years. (56)

The difference between the presentations in males and females was not found to be significant. This is similar to what has been stated by Iqbal et al.

Taking in to account the diagnostic criteria, fever was the most common clinical presentation in our study. Melissa et al observed that almost 100% of HLH cases presented with fever(55) while Iqbal et al stated that 65.2% of cases presented with fever.

Our study was in correlation with Melissa et al where all 100% of cases presented with fever thus concluding that the most common presentation of a patients with HLH is prolonged and persistent fever unresponsive to therapy.

The diagnostic criteria of splenomegaly was observed by Iqbal et al in 37.2% of patients while Laurence et al observed splenomegaly in 65% of cases [57]. Melissa et al stated that an enlarged spleen was present in 100% of patients with primary HLH and 80-90% in secondary HLH.

In our study splenomegaly was present in 53% of cases. This result approximately correlates with what was seen by Laurence et al.

Melissa et al observed cytopenias in 80% of cases, Iqbal et al have graded the cytopenias and have given percentages accordingly.

**HAEMOGLOBIN LEVELS**

| | **Hb (g/dl)** | **Iqbal et al** | **This study** |
|---|---|---|---|
| Severe anaemia | < 6 | 29 % | 12 % |
| Mild to moderate anaemia | 7-12 | 66 % | 69% |
| Normal | >12 | 11% | 19% |

## WBC COUNT

| | WBC count ($*10^9/l$) | Iqbal et al | This study |
|---|---|---|---|
| Leucopenia | <4 | 34% | 59% |
| Normal leucocyte count | 4-12 | 26% | 22% |
| Leucocytosis | >12 | 55% | 19% |

## PLATELET COUNT

| | Platelet count($*10^3/\mu l$) | Iqbal et al | This study |
|---|---|---|---|
| Severe thrombocytopenia | <10 | 2.4% | 12% |
| Mild to moderate thrombocytopenia | 10-150 | 70% | 63% |
| Normal | 150-450 | 64% | 19% |

## COMMON PRESENTATION

| | Iqbal et al | This study |
|---|---|---|
| Hb levels | Mild to moderate anaemia | Mild to moderate anaemia |
| WBC count | Leucocytosis | Severe leucopenia |
| Platelet count | Mild to moderate thrombocytopenia | Mild to moderate thrombocytopenia |

Mild to moderate anaemia was common in both the above studies. Leucocytosis was found to be common in HLH cases studied by Iqbal et al. In contrast to this we observed severe leucopenia in our study. As was in the case of the haemoglobin levels mild to moderate thrombocytopenia was found to be common in both the studies.

Ferritin levels are an important indicator of the degree of phagocytic activity of macrophages[38]. Melissa,et al observed hyperferritinemia in70-90%of cases, while Chandra et al observed this abnormal parameter in only 40% of cases[58].In our study hyperferritinemia was found in 87% of cases which correlates with the observations of Melissa et al.

Laurence et al observed that ferritin levels of >2,000ng/ml were more diagnostic of HLH which was also observed in our study. Neelima et al in their study observed that ferritin levels more than being a diagnostic criteria acts better as a prognostic indicator in patients on treatment with the HLH regimen. Hyperferritinemia of >2000ng/ml therefore appears to be diagnostic of HLH and also serves better as a prognostic factor in patients on treatment.

Melissa et al observed hypertriglyceridemia in 40% of cases while Chandra et al observed abnormal lipid levels in 45% of cases. Our study had 59% of HLH cases presenting with hypertriglycerdemia.

Melissa et al and Chandra et al both observed 40% of cases presenting with hypofibrinogenemia. Similar results were observed in our study which had 31% of the cases presenting with low fibrinogen levels.

Caleb Ho et al used the IHC marker CD 68 to highlight haemophagocytosis in bone marrow biopsies. This helped in increasing sensitivity and enabled quantification of haemophagocytic activity in trephine biopsies.[39]. However, in our study, using the marker CD 68 on trephine biopsies did not help to increase the sensitivity of detection of abnormal phagocytic activity.

The HLH essentially is divided into primary HLH and secondary HLH. Primary HLH is more common in children usually less than one year of age. Ling et al have stated that primary HLH accounted for 25% of the HLH presenting in the paediatric age group[59]. In our study HLH in the paediatric age group was 38%. However the percentage of primary HLH amongst these cases was only 3%.This can be attributed to the our lack of facilities for the diagnosis of molecular mutations and the economic constraints of the study population.

In our study the most common cause for secondary HLH was infection which accounted for 70% of the total cases. Melissa et al stated 50% while Chandra et al observed 13% of infection associated HLH. Though there is a

difference in the percentage of cases, in all three studies infection associated HLH was the most common cause for secondary HLH.

Among infections Ling et al observed bacterial infections to be the most common cause. While Chandra et al observed equal presentations amongst bacterial and viral infections, Melissa et al observed an increased incidence in viral infections contributing to HLH.

| | **Percentage of cases** | | |
|---|---|---|---|
| | **Ling et al** | **Chandra et al** | **This study** |
| Bacterial | 46% | 30% | 55% |
| Viral | 41% | 30% | 28% |
| Fungal | 13% | 20% | 4% |
| Parasitic | - | 20% | 9% |

Amongst the bacterial infections tuberculosis was found to be the most common. This aetiological factor was observed in all the studies. ( Ling et al, Chandra et al & Tseng, et al).

| | **Percentage of cases** | | | |
|---|---|---|---|---|
| | **Ling, et al** | **Chandra, et al** | **Tseng, et al[(60)]** | **This study** |
| Tuberculosis | 23% | 20% | 25% | 23% |

Patel R et al stated typhoid as a very rare cause for HLH [61]. In our study we had 15% of the infection associated HLH cases associated with typhoid.

Ling et al observed EBV as the most common viral infection for HLH accounting for about 18% while in our study we had only 3% of EBV associated cases (one patient) who also had a coexsisting diffuse large B cell lymphoma.

Sara et al observed 5% of HIV associated HLH[62]. Dengue and HIV associated viral infections was found to be more common in our study accounting for 9 % each of the cases of haemophagocytosis.

**Percentage of Viral Infection Associated HLH**

| | **Ling et al** | **Ramachandran et al[63]** | **This study** |
|---|---|---|---|
| Common infection | EBV( 18%) | Dengue virus(12%) | HIV & Dengue(9% each) |

Chandra et al found malaria as the most common (20%) parasitic infection associated with HLH. In contrast in our study Scrub typhus was the most common parasitic infestation (9%).

Micheal et al [64] , Ling et al, Melissa et al observed that the second most common cause for secondary HLH is neoplasm associated HLH (both haematological and non- haematological). Among the malignancies lymphoma was the most common in all studies.

In our study 16% of cases were due to malignancies (both haematological and non- haematological). Among them lymphoma was the most commonly observed (40%) diagnosis. This finding confirms to what has been observed above.

**MALIGNANCY ASSOCIATED HLH[64]**

| STUDY | No. of patients with malignancy associated HLH | Most frequent associated malignancy |
|---|---|---|
| Tamamyam et al[65] | 35 | AML/MDS |
| Parikh et al[66] | 32 | T cell lymphoma |
| Otrock & Eby et al[67] | 21 | B cell lymphoma |
| Lehmberg et al[68] | 21 | T cell lymphoma |
| Shabbier et al[69] | 6 | T cell lymphoma |
| This study | 5 | B cell lymphoma |

Chandra et al observed 3% of multiple myeloma patients presenting with HLH which was in correlation with our study also in which we found that 3% of cases with myeloma and haemophagocytosis.

Elena et al stated perforin gene mutations in aplastic anaemia can act as a aetio-pathogenetic factor for the subsequent development of HLH(70). Chandra et al observed 3% of aplastic anaemia cases presenting with HLH which was in correlation with our study which also had 3% of aplastic anaemia associated HLH.

Many immunodeficiency syndromes like Cheidiak Higashi syndrome, Hermansky Pudlak syndrome, Griscelli syndrome are all found to be associated with HLH. Amina et al states that 85% of Chediak Higashi syndrome patients in their accelerated stage present with HLH(23). In our study we had 3% of HLH cases secondary to Chediak Higashi syndrome.

Cesar et al observed that HLH secondary to autoimmune diseases requires special diagnostic criteria for its identification because of the varied presentations in such diseases. (42).

**Classification of HLH in autoimmune conditions(42)**

**A febrile patient with a known auto immune disorder is diagnosed with HLH if the following criteria are met**

**Ferritin > 684ng/mL and any two of the following**

5. **Aspartate transaminase >48U/L**
6. **Platelet count ≤ 181000/µL**
7. **Fibrinogen ≤ 360mg/dL**
8. **Triglycerides > 156mg/ Dl**

Using the above parameters as criteria for diagnosis Cesar et al observed that the most common autoimmune condition associated with HLH was SLE (15% )[24]. In our study we had 3% of SLE associated HLH cases.

Nishiwaki et al states Kikuchi Fujimoto's disease as a rare cause for HLH. These patients present with lymphadenopathy. In all such cases, an underlying lymphoma associated HLH has to be ruled out before diagnosis [71]. In our study we had a paediatric patient presenting with HLH secondary to Kikuchi Fujimoto's disease.

Jan-Inge et al states that the three year survival for patients with HLH following haematopoietic stem cell transplants is 64%[47]. Melissa et al & Michael et al observed that recurrences are common and prognosis is bad for malignancy associated HLH. Likewise in our study 34% of deaths in HLH were due to malignancy followed by deaths due to sepsis which accounted for about 32%.

The conclusions drawn from the studies are as follows

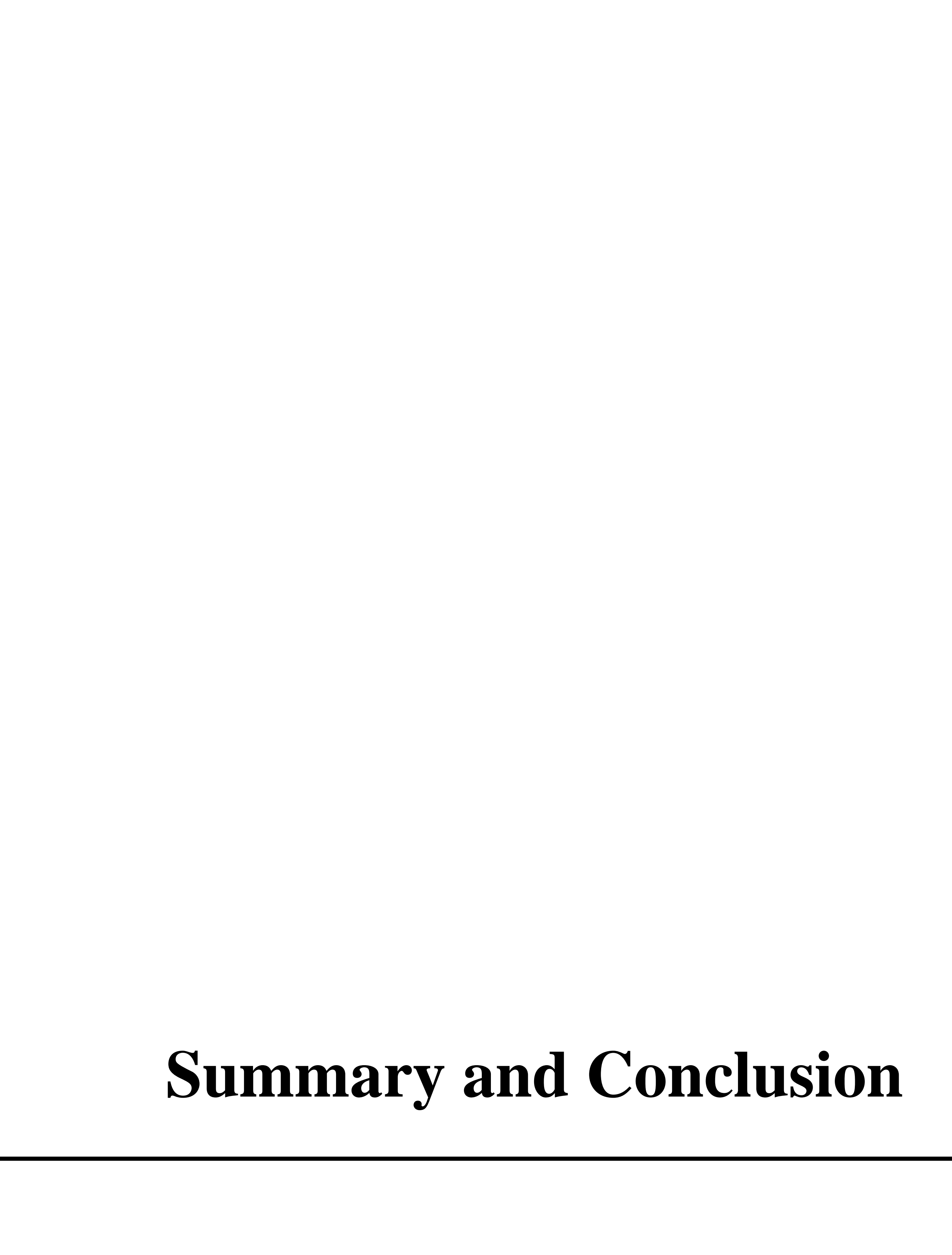
| Entity compared | Authors of the study | Conclusion | Conclusion from this study |
|---|---|---|---|
| **Mean age of presentation** | Iqbal et al | 30.8 yrs | 30.7yrs |
| **Sex distribution** | Iqbal et al | Insignificant | Insignificant |
| **Fever** | Melissa et al | Most common presentation(100%) | Most common presentation(100%) |
| **Hb levels** | Iqbal et al | Mild to moderate anaemia common | Mild to moderate anaemia common |
| **WBC count** | Iqbal et al | Leucocytosis common | Severe leucopenia common |
| **Platelet count** | Iqbal,et al | Mild to moderate thrombocytopenia common | Mild to moderate thrombocytopenia common |
| **Splenomegaly** | Laurence et al | 65% | 53% |
| **Hyperferritinemia** | Melissa et al | 70-90% | 87% |
| **Hypertriglyceridiemia** | Chandra et al | 45% | 59% |
| **Hypofibrinogenemia** | Chandra et al Melissa et al | 40% | 31% |

| Entity compared | Authors of the study | Conclusion | Conclusion from this study |
|---|---|---|---|
| **CD 68 for trephine** | Caleb Ho et al | Increased sensitivity & enabled quantification | Did not increase sensitivity |
| **Primary HLH** | Ling et al | 25% | 3% |
| **Most common cause for secondary HLH** | Melissa et al & Chandra et al | Infections | Infections |
| **Infection associated HLH** | Ling et al<br>Chandra et al<br>Melissa et al | Bacterial > others<br>Bacteria=viral<br>Viral> others | Bacterial > others |
| **TB associated HLH** | Tseng et al | 25% | 23% |
| **Common viral associated HLH** | Ramachandran et al<br>Ling et al | Dengue ( 12%)<br>EBV(18%) | HIV &Dengue(9%) |
| **Malignancy associated HLH** | Ling et al, Melissa et al, Michael et al | Second most common HLH | Second most common HLH |
| **Common Lymphoma with associated HLH** | Parikh et al,Lehemberg et al & Shabbir et al<br>Otrock & Eby et al | T cell lymphoma<br>B cell lymphoma | B cell lymphoma |

| Entity compared | Authors of the study | Conclusion | Conclusion from this study |
|---|---|---|---|
| **HLH in Aplastic anaemia** | Elena et al | 3% | 3% |
| **HLH in SLE** | Cesar et al | 15% | 3% |
| **Kikuchi with HLH** | Nishiwaki et al | Rare presentation | 3% of cases |
| **HLH with poor prognosis** | Michael et al | Malignancy associated HLH | Malignancy associated HLH |

**LIMITATIONS OF THIS STUDY**

- The techniques for measuring NK cell activity and CD 25 levels were not available in our institution so those parameters were not analysed in our study
- The facilities for molecular diagnosis of mutations associated with primary haemophagocytic lymphohistiocytosis were also not available which was probably responsible for the decreased incidence of primary HLH (3%) in our study when compared to other studies.
- Information regarding therapeutic response and prognosis of the patients were not available to us as many patients were lost to follow up.



# Summary and Conclusion

# SUMMARY & CONCLUSION

- The mean age of presentation of HLH is 30.7 years
- Prolonged and persistent fever is the most common presentation of patients with HLH
- Mild to moderate anaemia, severe leucopenia and mild to moderate thrombocytopenia are common
- Ferritin levels of > 2000ng/ml are more diagnostic of HLH than any other parameter. In our study almost 100 % of cases with ferritin levels of > 2000ng/ml were specific for HLH.
- CD 68 can be used to highlight haemophagocytosis in trephine biopsy. However in our study it did not help to increase the sensitivity of detection.
- The incidence of primary HLH is only 3% which can be an underestimate due to our limited facilities for molecular diagnosis.
- The most common cause for secondary HLH is infections followed by malignancies.
- Tuberculosis is the most common bacterial infection while Dengue is the most common viral infection associated HLH
- Lymphoma is the most common malignancy associated HLH.
- We had rare cases of Kikuchi Fujimoto disease & Diffuse large B cell lymphoma presenting as HLH.
- Prognosis of malignancy associated HLH was bad.

Thus, HLH is a diverse disorder which can present with varied clinical manifestations. There are many factors which contribute to the aetiopathogenesis of HLH. Because of the complex pathway of pathogenesis and different clinical presentations many of the HLH cases go under recognised. This remains the main cause for increased morbidity and mortality in persons with HLH.

In our study we have made conclusions regarding the clinical manifestations and aetiologies of HLH which will aid in the early diagnosis. Hereby we recommend that all patients with the clinical suspicion of HLH have to be carefully evaluated for the possible aetiology. A thorough and careful screening of bone marrow of these patients suspected to have HLH is essential for early diagnosis and prompt treatment. In spite of emergence of many treatment regimens for HLH, haematopietic stem cell transplantation remains the final option for treatment of patients with HLH. Hence more studies are required to raise awareness of this syndrome and improve the effectiveness of the existing treatment regimen.

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# Master Chart

# MASTER CHART

| S.NO | AGE | SEX | FEVER | SPLENOMEGALY | BICYTOPENIA | HYPER FERRITINEMIA | HYPER TRIGLYCERIDIEMIA | HYPOFIBRINOGENI MIA | ETIOLOGY |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 21 | MALE | YES | YES | NO | YES | - | - | SCRUB TYPHUS |
| 2 | 10 | FEMALE | YES | NO | YES | YES | YES | NO | KIKUCHI FUJIMOTO |
| 3 | 50 | MALE | YES | YES | YES | - | - | YES | NON HODGKINS LYMPHOMA |
| 4 | 39 | MALE | YES | NO | NO | YES | YES | - | TUBERCULOSIS |
| 5 | 20 | MALE | YES | YES | YES | YES | NO | YES | SEPSIS |
| 6 | 8 | FEMALE | YES | YES | YES | YES | YES | - | SEPSIS |
| 7 | 48 | MALE | - | - | YES | YES | NO | YES | TUBERCULOSIS |
| 8 | 3 MONTHS | FEMALE | YES | YES | YES | YES | YES | - | SEPSIS |
| 9 | 35 | MALE | TES | YES | YES | YES | YES | NO | HIV |
| 10 | 13 | MALE | YES | NO | YES | YES | YES | YES | SCRUB TYPHUS |
| 11 | 36 | FEMALE | YES | NO | YES | NO | YES | - | TUBERCULOSI- ABDOMEN |
| 12 | 11 | MALE | YES | NO | YES | YES | YES | NO | TYPHOID |
| 13 | 15 DAYS | MALE | YES | NO | NO | YES | NO | NO | PRIMARY HLH |
| 14 | 34 DAYS | MALE | YES | YES | YES | YES | YES | YES | PNEUMONIA |
| 15 | 60 | MALE | YES | NO | YES | YES | - | - | SEPSIS |
| 16 | 16 | FEMALE | YES | YES | NO | YES | YES | YES | TYPHOID |
| 17 | 51 | MALE | YES | YES | YES | YES | YES | - | DENGUE |
| 18 | 69 | FEMALE | YES | NO | YES | - | - | NO | DENGUE |

| S.NO | AGE | SEX | FEVER | SPLENOMEGALY | BICYTOPENIA | HYPER FERRITINEMIA | HYPER TRIGLYCERIDIEMIA | HYPOFIBRINOGENI MIA | ETIOLOGY |
|---|---|---|---|---|---|---|---|---|---|
| 19 | 13 | FEMALE | YES | NO | NO | YES | YES | - | TYPHOID |
| 20 | 2 MONTHS | MALE | YES | YES | YES | YES | YES | NO | CHEIDIAK HIGASHI SYNDROME |
| 21 | 29 | MALE | YES | YES | YES | YES | YES | - | TUBERCULOSIS |
| 22 | 42 | MALE | YES | NO | NO | - | - | YES | TUBERCULOSIS |
| 23 | 16 | FEMALE | YES | NO | YES | YES | - | - | APLASTIC ANAEMIA |
| 24 | 50 | FEMALE | YES | YES | YES | YES | - | - | MYELOFIBROSIS |
| 25 | 31 | FEMALE | YES | YES | YES | YES | NO | - | DIFFUSE LARGE B CELL LYMPHOMA |
| 26 | 41 | MALE | YES | YES | YES | YES | YES | - | HEPATITIS A |
| 27 | 63 | FEMALE | YES | YES | YES | YES | YES | NO | MYELODYSPLASTIC SYNDROME |
| 28 | 50 | MALE | YES | YES | NO | YES | - | - | HIV |
| 29 | 70 | FEMALE | YES | NO | YES | YES | NO | - | MULTIPLE MYELOMA |
| 30 | 19 | FEMALE | YES | - | YES | YES | YES | YES | VIRAL FEVER |
| 31 | 52 | MALE | YES | YES | YES | YES | YES | YES | NOCARDIA |
| 32 | 17 | MALE | YES | - | YES | YES | YES | YES | SYSTEMIC LUPUS ERYTHEMATOSIS |