Aspects of infection and leukemia in Rwanda

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Cover illustration: by Belson Rugwizangoga
Top left, a diagram on pathogen clearance according to the host IFNLA genotypes; top right, a diagram on interaction between *P. falciparum*-infected erythrocyte and EBV-infected B-lymphocytes in B cell expansion; bottom left, a diagram on disease incidence; bottom right, a diagram on DNA. The last three diagrams show background microphotographs of ALL (right) and AML (left) cells (peripheral blood film). Microphotography courtesy of the Butaro Hospital Pathology Laboratory – modified by the author.
ABSTRACT

A first part of this thesis addressed the potential impact of variants of genes encoding interferon-λ4, which is a cytokine that participates in protection against pathogens at epithelial surfaces, for the resolution of upper respiratory tract infections in Rwandan children. In a study of 480 subjects (≤5 years old), where follow-up samples were available from 161 subjects, it was observed that IFNL4 genotypes were associated with clearance of RNA viruses from upper airways. Our results thus suggest that IFNL4 variants that are overrepresented among subjects of African descent, such as TT at rs12979860, entail reduced clearance of respiratory RNA viruses, in particular ss(+)RNA viruses (Paper I). A second part aimed at determining the epidemiology, subtypes and outcome of acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) in Rwanda using contemporary western world databases for comparison. In Paper II, which comprises observations made in 180 Rwandan AML cases diagnosed in 2012-17, we show that AML occurs less frequently and at a younger age in Rwanda than in Sweden. The outcome of AML in terms of survival is distinctively poor in Rwanda, which is likely explained by the shortage of AML therapy with curative intent and, possibly, by the accumulation of somatic gene aberrations that have been shown to predict poor prognosis for survival. Similarly, the results presented in Paper III imply that the incidence of ALL, based on a study comprising 318 Rwandan cases, was lower in Rwanda than in Sweden with a lower peak age at diagnosis. Although protocols for ALL treatment are available in Rwanda, the survival in ALL was clearly inferior to that of patients in the western world, in particular among children. We observed an apparent accumulation of T-ALL subtypes in Rwandan patients along with genomic abnormalities associated with poor survival outcome, including somatic mutations of NOTCH1. We also noted that serological signs of recent EBV infection and malaria, which have been associated with Burkitt leukemia/lymphoma in regions where malaria is holoendemic, were more common in ALL than in AML patients. Analysis of the genetic profile and morphology of Rwandan EBV/malaria-related ALL cases suggested the existence of a lymphoproliferative disorder distinct from Burkitt leukemia/lymphoma. In Paper IV, we investigated factors of potential relevance to the low incidence of and poor outcome of ALL and AML in Rwanda and identified the contribution by low awareness, financial constraints and an insufficiently efficacious referral system along with suboptimal diagnostic and treatment capacities. In conclusion, this work may spark further studies and interventions aiming to improve healthcare in Rwanda and similar developing countries.

Keywords: interferon-λ, respiratory infection, nucleotide polymorphism, acute leukemia, Rwanda, Epstein-Barr virus, malaria
LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals:


IFNLA genotypes predict clearance of RNA viruses in Rwandan children with upper respiratory tract infections.


*Equal contribution


Incidence, subtypes and outcome of acute myeloid leukemia in Rwanda.

*In Manuscript.*


Aspects of incidence, subtypes, and outcome of acute lymphoblastic leukemia in the Rwandan population.

*In Manuscript.*


Experience and perception of acute leukemia in Rwanda by patients and healthcare professionals.

*Submitted.*

*Equal contribution
ADDITIONAL PAPER

Additional publication not part of this thesis:

SI. Marie Francoise Mukanyangezi, Belson Rugwizangoga, Olivier Manzi, Stephen Rulisa, Kristoffer Hellstrand, Gunnar Tobin, Anna Martner, Emile Bienvenu, Daniel Giglio.
Persistence rate of cervical human papillomavirus infections and abnormal cytology in Rwanda.
CONTENT

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<tr>
<td>Ab</td>
<td>Antibody</td>
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<td>Ag</td>
<td>Antigen</td>
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<td>ALL</td>
<td>Acute lymphoblastic leukemia</td>
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<td>AML</td>
<td>Acute myeloid leukemia</td>
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<td>BL</td>
<td>Burkitt lymphoma/leukemia</td>
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<td>CD</td>
<td>Cluster of differentiation</td>
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<td>CMV</td>
<td>Cytomegalovirus</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>EBV</td>
<td>Epstein-Barr virus</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>ELN</td>
<td>European LeukemiaNet</td>
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<td>FAB</td>
<td>French-American-British classification</td>
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<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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<td>HBc</td>
<td>Hepatitis B core</td>
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<td>HBs</td>
<td>Hepatitis B surface</td>
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<td>HCV</td>
<td>Hepatitis C virus</td>
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<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HRP-2</td>
<td>Histidine-rich protein 2</td>
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<td>IFNL4</td>
<td>Interferon lambda 4</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>INDEL</td>
<td>Insertion/deletion</td>
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<tr>
<td>OHSU</td>
<td>Oregon Health and Science University</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PfEMP1</td>
<td><em>P. falciparum</em> erythrocyte membrane protein 1</td>
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<tr>
<td>RT-PCR</td>
<td>Real-time polymerase chain reaction</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>SE36</td>
<td>Serine-repeat antigen protein</td>
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<tr>
<td>SNV</td>
<td>Single nucleotide variation</td>
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<tr>
<td>TARGET</td>
<td>Therapeutically Applicable Research to Generate Effective Treatments</td>
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<tr>
<td>TCGA</td>
<td>The Cancer Genome Atlas</td>
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<tr>
<td>WES</td>
<td>Whole exome sequencing</td>
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<td>WHO</td>
<td>World Health Organization</td>
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1. INTRODUCTION

1.1 PREAMBLE

Infection is the leading cause of morbidity and mortality in low-income countries, most of which are located in Africa [1]. Interferon-λs (IFN-λ) are antiviral and immunomodulatory proteins that participate in the innate immune defense against infections at mucosal surfaces. This thesis uses the case of Rwanda, an east-central African country, to explore the role of variants of the gene encoding IFN-λ4 (IFNL4) for the course of acute respiratory tract infections in sub-Saharan African children. The thesis also comprises a study of the incidence, subtypes and outcomes of acute leukemia in Rwanda versus western countries with special reference to the potential implication of malaria (P. falciparum) and Epstein-Barr virus (EBV) infection for the occurrence of acute lymphatic leukemia (ALL). Additionally, this thesis explores the healthcare services utilization in Rwanda with focus on ALL and acute myeloid leukemia (AML). The context of healthcare in Rwanda is presented to highlight opportunities and challenges that are likely shared by other low-income countries.

1.2 HEALTHCARE IN RWANDA

1.2.1 Background

The healthcare in Rwanda has faced significant challenges in the past decades. For example, the emergence of HIV/AIDS in Rwanda from 1983 and onward [2, 3], accompanied by opportunistic infections and AIDS-associated neoplasms, provided a substantial burden on healthcare [4]. The genocide in 1994, in which more than one seventh of the country's population perished, drastically reduced healthcare functionality. The rebuilding of Rwanda was accompanied by an increase in the urban population, and the trend towards urbanization is still growing [5]. Revitalizing the health system with improved quality of, and access to healthcare services has resulted in improved diagnosis of non-communicable diseases. For example, the yearly reported number of cancer cases rose from less than 300 in 2004 [6] to approximately 3,000 in 2018 [7], reflecting increased awareness and diagnostic capacity. Determining health metrics is likely a vital aspect of paving the way for the implementation of efficacious anti-cancer therapy in Rwanda.

1.2.2 Achievements in the Rwandan healthcare sector

Rwanda may serve as a model for other developing countries to address health-related challenges. The most significant achievements in Rwanda in recent years relate to improved maternal and child health and halting the incidence of HIV/AIDS-related complications [8] along with the implementation of several vaccination programs. Moreover, there was a 50% reduction of malaria mortality between 2010 and 2017 [9], which is likely attributable to the use of insecticide-
treated mosquito nets and the introduction of artemisinin-based drugs in 2006 [10]. Rwanda has also designed and implemented programs, policies and guidelines to control non-communicable diseases [11, 12]. In cancer, improved diagnostics has been introduced [13, 14] alongside the inauguration of Rwanda’s first cancer treatment center in 2012 [15]. These and other aspect of improved health in Rwanda have translated into an increased life expectancy from 47 and 51 years for males and females, respectively, in 1990, to 66 versus 71 years in 2017 [16].

Utilization of existing health services depends on the geographic and financial accessibility as well as their acceptability by the population, among other factors [17, 18]. In order to improve the availability of health services in Rwanda, the number of health facilities has increased and the quality of services offered has improved over time [19]. Aiming to improve the accessibility to healthcare services, Rwanda has initiated a community-based health insurance program [20] that is utilized by 70-90 % of Rwandans [21, 22]. In parallel with the community-based health insurance, Rwanda has developed a community-based system called Ubudehe in Kinyarwanda, through which the poorest households are subsidized when encountering health-related issues [22].

1.2.3 Health challenges in Rwanda

In 2000 the United Nations established goals, to be achieved by 2015, for reducing poverty and improving the health in developing countries. These goals were originally denoted Millennium Development Goals (MDGs) and are currently being replaced by the sustainable development goals (SDGs) [23]. Rwanda has achieved several health-related MDGs [8, 24] and is now endeavoring to achieve SDGs. Figure 1 shows the current SDG index of Rwanda using Sweden as the comparator [25]. Sweden was reported in 2017 as the top performer worldwide in achieving SDGs [26]. Whilst Figure 1 shows successful achievements in Rwanda in areas such as vaccination coverage (99%) and birth attendance by skilled healthcare professionals (93%), it also points to areas for improvement, for example the control of infection (malaria, current index at 11%) and deaths related to non-communicable diseases.
Figure 1. Indexes for achievement of health-related SDGs
Rwanda (left, overall index of 30%) and Sweden (right, overall index of 84%) in 2020. The percentages refer to the extent to which the targets have been achieved by the country so far. Source: [25]. Used with written permission. Creator: Institute for Health Metrics and Evaluation (IHME); Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International

Infection-related cancers are prevalent in Rwanda and a significant cause of cancer mortality [27]. Vaccination programs against potentially carcinogenic viruses such as human papillomavirus (HPV) and the hepatitis B virus (HBV) were recently introduced in Rwanda. While these efforts likely will translate into a reduction of infection-related cancers, the apparently insufficient detection of several forms of cancer poses a significant challenge. Thus, the WHO has estimated approximately 10,000 cancer cases in Rwanda per year [28], but only 3,000 are currently captured by the health system [7]. This may be surprising as Rwanda has invested significantly in granting the population with access to healthcare [21, 29, 30] along with training health professionals, including those who diagnose cancer [14, 31], and the efforts should continue. Factors of relevance to the insufficient utilization of modern healthcare services and to the dismal prognosis of cancer patients in Rwanda need to be elucidated and addressed.

1.2.4 Prioritized non-communicable diseases in Rwanda

The compilation of data on a specific disease, such as a type of cancer, adopting a uniform method of evaluation such as a registry, is currently unavailable in Rwanda, even for cases diagnosed in health facilities. The population-based cancer registry that was operational in the former Butare Prefecture from 1985 [27] was not restarted after the genocide. Consequently, retrieving epidemiological data on cancer at the national level is challenging. Nevertheless, there are five public hospitals with cancer diagnostic capacity, and some of those are delivering cancer therapy at various levels of distinction. In the recent national strategic plan (2015-2019) on non-communicable diseases, 13 types of cancer were prioritized for treatment in Rwanda; the selection was based primarily on types of cancer likely to
have favorable prognosis at a realistic level of therapeutic intervention [11]. Hodgkin lymphoma, large B-cell lymphoma, Burkitt lymphoma and chronic myeloid leukemia (CML) are the only hematological malignancies that are currently on the list of priorities in Rwanda [11].

Little is known about the demographics, subtypes and survival of patients with acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) in Rwanda and other sub-Saharan countries. The treatment of ALL implemented in Rwanda comprises schemes of low-intensity chemotherapy designed for low-income countries, based on a protocol composed of 4 treatment regimens (sometimes called Hunger 1-4, named after the person proposing these protocols) [32]. The regimens within the protocols of low-intensity chemotherapy for low-income countries are discussed in section 1.4.4 of this thesis. There is no curative treatment for AML in the Rwandan public healthcare system, and patients are offered only palliative treatment.

1.3 HUMAN IMMUNE SYSTEM AND INFECTION

1.3.1 Human immune system

**Overview of immunity**

Immunity (from the Latin word *immunitas* from *immunis* means “exempt” or “protected from” [33]) comprises host mechanisms for protection primarily against infectious pathogens. The human immune system is composed of two principal parts, the innate system, which acts directly upon encountering a pathogen, and the adaptive immune system, which acts with a delay when facing a pathogen for the first time, but with high specificity and memory. Figure 2 is a diagram illustrating the main components of the immune system in humans.
Figure 2. Components of the immune system in humans

The innate immune system comprises external physical and chemical barriers and internal barriers (phagocytes and soluble factors). Adaptive immunity is composed of internal barriers consisting of T- and B-cells and soluble factors released and/or affecting the functions of these cells. Dendritic cells serve as a bridge between innate and adaptive immunity, while natural killer cells and γδT cells share features of both systems. CD, cluster of differentiation; IFNs, interferons; IL, interleukins

Innate immunity

Once a pathogen has crossed external barriers and reached a tissue, innate immune cells constitute the first line of defense [34]. These cells are equipped with an array of germline-encoded pattern recognition receptors (PRRs), which recognize a fixed set of pathogen-associated molecular patterns (PAMPs) that are shared and conserved among microbes, as well as danger-associated molecular patterns (DAMPs) that are expressed or exposed by injured cells [35]. Sentinel cells, such as tissue-resident macrophages, sense danger via their PRRs and respond by producing cytokines and chemokines that orchestrate the recruitment of additional phagocytes, including neutrophils and monocytes, to the site of infection. The phagocytes combat infections via phagocytosis, by which they engulf and degrade invading pathogens. Dendritic cells and macrophages that have taken up pathogens can also process and present antigens on major histocompatibility complex (MHC) class I and II, and thereby initiate activation of antigen-specific responses by CD8+ and CD4+ T cells, which are part of the adaptive immune system.

Natural killer (NK) cells are non-phagocytic cells that belong to the innate immune system. NK cells are lymphocytes endowed with constitutive (“natural”) and inducible cytotoxic capacity against aberrant cells. They express a variety of
activating and inhibitory receptors and survey their surrounding for altered (foremost virus-infected or malignant) cells. The balance between inhibitory and activating stimuli from a potential target cell determines the outcome of the interaction. Thus, if a target cell expresses more activating than inhibitory ligands, the NK cell may eliminate it [36, 37]. In addition to direct killing of pathogens and altered cells by phagocytes and NK cells, innate immune cells produce a multitude of cytokines and other mediators that aim at containing and eliminating the invading agent along with repairing the invaded and inflamed tissue. One such mediator that is induced upon PRR activation consists of interferons (IFN) that may directly interfere with the replication of viruses and regulate immunity, as discussed in detail below. In addition, there are natural antibodies whose action does not depend on exogenous antigenic stimulation [38].

**Adaptive immunity**

T cells and B cells constitute the adaptive immune cells. They recognize antigens using their T cell receptors (TCR) and B cell receptors (BCR), respectively. While innate immune cells recognize a fixed set of antigens, adaptive immune cells recognize an almost unlimited range of antigens due to the genetic recombination of their TCRs and BCRs in individual somatic cells. Hence, each individual carries naïve T and B cells expressing millions of different TCRs and BCRs, albeit at very low frequencies. For a T cell or B cell to participate in immune defense, these cells must become activated, multiply and differentiate into effector cells. This process takes days to weeks, which is the reason for the delayed adaptive immune response after the initial encounter with an antigen.

A T cell becomes activated once its specific antigen is presented by an antigen-presenting cell (APC) within the context of MHC I (for CD8$^+$ T cells) or MHC II (for CD4$^+$ T cells) [39]. Cytokines and costimulatory molecules expressed by the APC will modulate the amplitude of T cell activation and direct the T cell polarization. Some of these T cells will become memory T cells that ensure a swift response to future pathogen exposure. A B cell that encounters a specific antigen via its BCR engulfs this antigen to process and present it on MHC II to CD4$^+$ T cells. With assistance from antigen-specific CD4$^+$ T cells, the B cell becomes activated and differentiates into plasma cells that produce specific antibodies, while other B cells become memory B cells [40]; this is the humoral component of adaptive immunity.

The distinction between innate and adaptive immunity is not absolute. For example, antigen-presenting dendritic cells bridge these aspects of immunity [39, 40] and NK cells and γδT cells have features of both types of immunity [39, 41]. There is also an overlap between soluble mediators, such as cytokines and complement factors, that govern functions of innate and adaptive immune cells [42, 43]. Figure 3 displays an overview of innate and adaptive mediators of immunity.
**Figure 3. Example of innate and adaptive mediators of immunity**

**Left section.** An invading pathogen expresses PAMPs that are recognized by PRRs on a host macrophage (in this example). The macrophage releases mediators that initiate immunological cascades.

**Right section.** A dendritic cell (DC) processes and presents antigens to CD8+ cells that, under IL-2 autocrine stimulation, differentiates into CTL. DCs also present antigens to naïve CD4+ cells, triggering differentiation of T cells with different polarizations. For example, Th2 cells contribute in activating B cells that differentiate into antibody-producing plasma cells. Based on information from [34, 42-47]. Abs, antibodies; ADCC, antibody-dependent cellular cytotoxicity; CD, cluster of differentiation; CTL, cytotoxic T lymphocyte; DC, dendritic cell; FcR, fragment crystallizable (Fc) receptor; IFN, interferon; IL, interleukin; mem, memory; MHC-I/-II, major histocompatibility complex class I/II; MΦ, macrophage; NK, natural killer cell; PAMPs, pathogen-associated molecular patterns; PMN, polymorphonuclear neutrophil; ROS, reactive oxygen species; PRRs, pattern recognition receptors; TCR, T-cell receptor; Th, T helper cell

### 1.3.2 Interferon λ

Interferons (IFN) are cytokines produced by host cells in response to microbial, in particular viral, stimulation. IFNs comprise three predominant classes, type I, type II and type III IFNs. All classes of IFNs are assumed to participate in defense against viruses and act by inducing an antiviral state in neighboring cells along with enhancing protective immune responses [48-51]. This thesis has mainly focused on type III IFN or IFN-λ, which signal by binding to a receptor complex consisting of IL10R2 and IFNLR1 that is mainly expressed by epithelial cells [49, 52-54]. IFN-λ is thus assumed to function to protect respiratory and digestive epithelial mucosae against infectious pathogens [55-59]. Four types of IFN-λ (IFN-λ1-4) are known in humans [55] and are encoded on chromosome 19 (19q13) [49, 60-62]. Variation at IFNLA is implicated in the clinical course of hepatitis C virus (HCV) infection. Several studies thus demonstrated that specific single nucleotide polymorphisms (SNP) within IFNLA, such as rs12979860 and rs368234815, predict spontaneous
clearance of HCV and a sustained viral responses to therapy in infected patients [62-68].

The SNP rs12979860, which is located within the first intron of IFNLA, is in strong linkage disequilibrium with the adjacent genetic variant rs368234815 located within the IFNLA exon [62, 64]. Individuals who carry the C allele at rs12979860 or the TT allele at rs368234815 are more likely to resolve primary HCV infection than those carrying rs12979860 T or rs368234815 ΔG alleles [49, 64, 66, 69-72]. This may seem counter-intuitive as rs368234815 ΔG carriers express functional IFNLA and thus produce IFN-λ, while those carrying rs368234815 TT allele do not. The TT allele thus creates a frameshift that causes premature termination of the IFN-λ4 protein [63].

Similar to other IFNs, IFN-λ4 is endowed with antiviral activity, although it appears to be poorly secreted in vivo [73]. The mechanisms that link production of IFN-λ4, and other aspects of IFN-λ function, to reduced clearance of HCV are not fully elucidated. A prevailing hypothesis is that IFN-λ4 induces interferon-stimulated genes (ISGs), and that carriers of rs368234815ΔG alleles thus may have an exhausted interferon-mediated antiviral response, although other mechanisms are conceivable [73].

The allele frequency of favorable (in terms of clearance of HCV) IFNLA genotypes is higher in East-Asians (90 %) and Caucasians (70 %) than in Africans (30 %) [49, 70, 71, 74-76]. These previous results inspired us to initiate studies in Rwandan children with acute respiratory infections aiming to clarify whether or not IFNLA variation may determine the efficiency of elimination also of other viruses.

1.3.3 Infection in humans

Malaria

The World Health Organization (WHO) recently reported that 92% of all malaria cases occur in Africa, with >99% of cases in Africa being caused by P. falciparum [77]. In humans, P. falciparum typically causes a more severe form of malaria than other species (P. vivax, P. ovale, P. malariae) [78]. Malaria is holoendemic in tropical African regions and has two obligate hosts, the Anopheles mosquito (also serving as its vector) and humans [79].

Malaria is transmitted by the female Anopheles mosquito, which inoculates sporozoites in the subcutis or blood stream of humans. These sporozoites proceed toward the liver where they migrate through hepatocytes to invade and develop in these cells (exo-erythrocytic schizonts) [80]. Several thousands of merozoites develop from each sporozoite, forming schizonts. Upon rupture of schizonts inside hepatocytes [81], the merozoites are released into the blood stream and invade red blood cells (RBCs) [78]. It is this blood stage of the life-cycle of P. falciparum that causes symptoms [78, 81]. The asexual cycle multiplication of merozoites (ring-stage trophozoites) inside the RBCs (erythrocytic schizogony) results in the formation of erythrocytic schizonts [81]. During this asexual multiplication, it is estimated that in
48 hours, each parasite produces approximately 20 new merozoites, which in turn may infect new RBCs [78]. Only a small proportion of the asexual merozoites develop into sexual gametocytes that, once ingested by the mosquitoes during bite, develop within the vector into sporozoites that may be transmitted to humans [78, 81]. During the erythrocytic cycle, mature trophozoites multiply within erythrocytic schizonts that rupture into the blood stream and cause an increase in parasitemia [81].

Several molecular pathways are in play in the pathophysiology of *P. falciparum* malaria. The *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which is expressed on the surface of infected mature RBCs, is assumed to mediate parasite-host interaction, resulting in severe malaria [78, 82, 83]. Figure 4 illustrates the main pathogenic effects mediated by PfEMP1. Briefly, an infected RBC rolls on endothelial cells and then adheres to the vascular wall by attaching intercellular adhesion molecule 1 (ICAM-1) receptor. Infected RBCs cluster with platelets using receptors such as platelet-endothelial cell adhesion molecule 1 (PECAM-1 or CD31), E-selectin and others. Also, an infected RBC binds to non-infected RBCs (rosetting) via receptors such as CD36, complement receptor 1 (CR1) and others. The consequence of these scenarios is the sequestration of RBCs that results in microvascular occlusion and evasion of spleen-dependent killing of infected RBCs [78]. The binding of infected RBCs to dendritic cells impairs the functions of the latter, resulting in the downregulation of the host immune system [78]. Moreover, the binding of infected RBC to EBV-infected B lymphocytes, via the PfEMP1’s cysteine-rich interdomain region 1α (CIDR1α), triggers a cascade of events that may induce the expansion of B-cells [82]. The role of PfEMP1 in the carcinogenesis of B-cell malignancies is further discussed in section 1.3.4. In addition, PfEMP1 induces antigenic clonal variations which results in evasion of the antibody-dependent immunity against the *P. falciparum* [78].
Figure 4. Pathogenic effects mediated by PfEMP1

The diagram illustrates, from right to left within the vascular lumen, infected RBCs binding to a migrating dendritic cell, an infected RBC rolling on the endothelial surface, infected RBCs adhering to the endothelial cells, non-infected RBCs rosetting around an infected RBC, and infected RBCs interacting with an EBV-infected B-cell. Cell receptors involved in these events as well as possible consequences, are presented. Based on information from [78, 82, 83]. Ags, antigens; CD, cluster of differentiation; CIDR1α, cysteine-rich interdomain region 1α; CR1, complement receptor 1; CSA, chondroitin sulfate A; DCs, dendritic cells; EBV, Epstein-Barr virus; HS, heparin sulphate; ICAM-1, intercellular adhesion molecule 1; IgM, immunoglobulin M; RBC, red blood cell; TSP, thrombospondin; VCAM-1, vascular cell adhesion molecule 1

Consequences
- B-cell malignancy
- Suppression of EBV-specific T-cell immunity
- EBV reactivation in B cells → genomic instability
- Downregulation of the host immune response

Receptors in play
- CIDR1α domain
- CD36

Events
- Binding of *P. falciparum*-infected RBC to EBV-infected B lymphocyte
- Binding of *P. falciparum*-infected RBCs to DCs

Post-capillary venule
- Lymphatic capillary
- Parenchymal cells

Arteriole
- Rosetting
- Clumping
- Adhesion
- Rolling
- Selectins

Consequences
- Sequestration of RBCs (infected and non-infected)
- Evasion of spleen-dependent killing
- Microvascular occlusion resulting in severe (cerebral, placental, ...) malaria

Epstein-Barr virus infection

Epstein-Barr virus (EBV) is a double-stranded DNA virus of the *Herpesviridae* family. EBV is referred to as human herpes virus 4 (HHV4) [84]. EBV was first described by Epstein, Achong, and Barr in Burkitt lymphoma biopsies from Africa. It spreads through bodily fluids such as saliva (the main route), blood or genital secretions. EBV is implicated in diseases such as infectious mononucleosis, B- and T-, and NK cell malignancies, nasopharyngeal carcinomas and gastric carcinoma [85-99]. It is estimated that, globally, about 90% of adults have IgG antibodies against
EBV [100-102] indicating a past EBV infection. The primary EBV infection occurs early in life in children from low socioeconomic groups and developing countries but may appear in adolescence or in young adults in developed countries [101, 103]. In addition, the primary infection may be asymptomatic in children, while it typically causes infectious mononucleosis in adolescents and adults [101, 104].

Various host cell and viral gene products play role in the pathophysiology of EBV infection, as shown in Figure 5. Upon primary infection, EBV binds to host B cells via the viral glycoprotein gp350/220 that attaches to CD21 (C3d complement receptor); its entry into the B cell is mediated by other viral glycoproteins such as gH, gL and gp42 [101, 105]. Thereafter, the viral genome enters into the cell nucleus and circularizes to form episomes [101, 105, 106]. The circularization is followed by activation of the viral growth program that stimulates the infected B cells to become proliferating blasts (polyclonal expansion of infected B cell). The proliferation stage is, however, transient as viral proteins trigger a differentiation of the infected B cells, via the germinal-center reaction, into a resting memory B cell phenotype. Memory B cells proliferate slowly, but rarely die meaning that EBV may remain within the infected B cells infinitely [105-107]. In memory B cell, EBV no longer activates its growth program, but switch into another pattern of transcription, denoted the default program. In the default program, three latent viral proteins are expressed; LMP-1 and LMP-2 that are involved in germinal center formation, and EBNA-1 that is expressed when latently infected memory cells divide, and allows viral DNA replication by binding to the viral origin of replication [105, 106]. EBV may remain in the latency phase within B cells life-long, but occasionally EBV-infected B cells differentiate into plasma cells and viral production is reactivated. During EBV reactivation, linear double-stranded viral DNA is produced which is packaged into new virions that are released into bodily fluids as shown in Figure 5. These viruses may infect new B cells that are transformed into proliferating blasts. In immunocompetent individuals, the newly infected B cells are controlled by EBV-specific immune responses, but in immunocompromised individuals the proliferating blasts may give rise to symptomatic disease or, occasionally, lymphoproliferative disorders [47, 105, 106, 108].

In addition to B cells, accumulating data suggest that EBV also infects other cell types. Hence, during mononucleosis, EBV-positive T cells, NK cells and epithelial cells of Waldeyer's ring have been detected [105].
Figure 5. Life-cycle of Epstein-Barr virus within an infected B lymphocyte

EBV binds to and enters into B cells, and its genome forms an episome in the nucleus. EBV induces a number of processes that may result in expansion and immortalization of infected cells. Under certain circumstances, ZEBRA protein expression is induced, followed by upregulation of genes favoring viral replication, and shedding of new viruses. Based on information from [103, 105, 106]. CD, cluster of differentiation; CTLs, cytotoxic T lymphocytes; EBV, Epstein-Barr virus; EBERs, EBV-encoded RNAs; EBNA, EBV nuclear antigen; gp, glycoprotein; ICAM, intercellular adhesion molecule; LFA, lymphocyte function-associated antigen; LMP, latent membrane protein; VCA, viral capsid antigen; ZEBRA, Z EBV replication activator.

The current serological tests for EBV include analyses for antibodies against the viral capsid antigen (VCA) and the anti-EBNA-1 IgG [109]. VCA IgG is rapidly formed in primary EBV infection and is detectable at onset of the disease and throughout life, whereas presence of VCA IgM indicates recent or ongoing infection [103, 108]. The combination of serological tests is generally useful for diagnosis, but can in some cases be complemented by molecular biology methods [109]. Polymerase-chain reaction (PCR) for nucleic acid detection is used for the detection of viral load [108], or as a complement to serological tests in acute infections and reactivation [109]. To determine which B cells that are EBV-infected e.g. in B cell malignancies, EBV may be detected within B cells using in situ hybridization for EBV-encoded RNAs (EBERs).
1.3.4 Infection and cancer

The link between infection and cancer was established more than a century ago by the identification of an avian cancer virus [110, 111]. Since then, several infectious agents have been found to be associated with, or causal agents of, specific cancers in humans. It is estimated that infection is the cause of approximately 20% of human cancers [100], the majority being caused by viruses [112]. Table 1 outlines pathogens and their associated cancers in humans.

Table 1. Examples of pathogens associated with carcinogenesis

<table>
<thead>
<tr>
<th>Pathogen group</th>
<th>Pathogens</th>
<th>Associated cancer</th>
<th>Early described association</th>
<th>Mechanism of association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viruses</td>
<td>Epstein-Barr virus</td>
<td>Burkitt lymphoma (BL), other B- and T-cell cancers, Hodgkin’s disease, and CNS lymphomas, PTLPD</td>
<td>1958 (for BL) [113]</td>
<td>t(8;14); expansion B-cell precursors; suppression of T cell-mediated immunity; genomic instability [82]. Unclear mechanisms for T- and NK cell cancers. Unclear mechanism for smooth muscle tumors, but immune deficiency is a factor [115].</td>
</tr>
<tr>
<td></td>
<td>Bacteria</td>
<td>H. pylori</td>
<td>1988 [132]</td>
<td>Translocation of H. pylori CagA in epithelial cells [133]</td>
</tr>
<tr>
<td></td>
<td>Parasites</td>
<td>P. falciparum</td>
<td>1961 [79, 137]</td>
<td>PfEMP1 promotes expansion of EBV-infected B cells [82]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S. haematobium</td>
<td>1970 [138, 139]</td>
<td>Inflammation induces genotoxic products [140]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C. sinensis; O. riverrini</td>
<td>1900 [141, 142]</td>
<td>Replication and fixation of damaged DNA [143]</td>
</tr>
<tr>
<td></td>
<td>Fungi</td>
<td>Aflatoxin (A. flavus product)</td>
<td>1985 [144]</td>
<td>AFB1-guanine adducts induce mutations (p53) [145]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human gammaherpesvirus 8 (HHV-8) (Kaposi's sarcoma-associated herpes virus, KSHV)</td>
<td>Kaposi sarcoma</td>
<td>1994 [121]</td>
<td>Dysregulation of human IL-6, inducing proliferation and preventing apoptosis of infected cells [122, 123]</td>
<td></td>
</tr>
<tr>
<td>Human papillomavirus (HPV)</td>
<td>Squamous cell carcinoma (anogenital)</td>
<td>1983 [128]</td>
<td>Production of E6 and E7 oncoproteins [112, 129]</td>
<td></td>
</tr>
</tbody>
</table>

**CagA, cytotoxin-associated gene A; CNS, central nervous system; PTLPD post-transplant lymphoproliferative disease**

EBV infection [90, 113] and *P. falciparum* malaria [137, 146, 147] are known to be involved in the pathogenesis of endemic Burkitt lymphoma. Burkitt lymphoma is a
rapidly proliferating B cell lymphoma that is characterized by a translocation involving \( c\text{-myc} \). Although the detailed mechanisms of how EBV and malaria contribute to the development of Burkitt lymphoma are not known, a scheme of carcinogenesis has been proposed. Thus, \( P. falciparum \)-infected erythrocytes attach latently EBV-infected B cells via the CIDR1\(\alpha \) domain of the \( P. falciparum \) erythrocyte membrane protein 1 (PfEMP1), which translates into expansion of EBV-infected B cells, the suppression of EBV-specific T cell immunity and the reactivation of EBV that induces genomic instability via increased expression of activation-induced cytidine deaminase (AID) in B cells [82]. AID is expressed by B cells within germinal centers and is needed for class switch recombination and somatic hypermutation of antibodies. AID has also been shown to be critically involved in forming DNA brakes in \( IgH \) as well as in \( c\text{-myc} \), allowing the well characterized Burkitt lymphoma \( c\text{-myc}/IgH \) translocation to occur [148]. Individuals with purportedly EBV/malaria-related Burkitt lymphoma show high titers of histidine-rich protein 2 (HRP-2, a marker of recent or ongoing \( P. falciparum \) infection) [146], and lower titers of serine repeat antigen 5 antibody (SE36, a marker of protection against severe \( P. falciparum \) malaria) [146, 147].

1.4 HEMATOPOIESIS AND PATHOGENESIS OF ACUTE LEUKEMIA

1.4.1 Normal hematopoiesis

The hematopoietic system is composed of cell types with specialized functions. All these cells originate from a totipotent hematopoietic stem cell (HSC) [149]. HSC has self-renewal properties and differentiates to produce the variety of hematopoietic cells in a process known as hematopoiesis. The concept of hematopoiesis was first described by Ernst Neumann in 1868 [150, 151]. Figure 6 aims to delineate the current understanding of the lineage hematopoiesis and the steps involved in each lineage to produce mature, functioning cells. Lineage hematopoiesis includes erythropoiesis (for erythrocytes), granulopoiesis (for neutrophils, eosinophils and basophils, collectively named granulocytes), lymphopoiesis (for lymphocytes), monocytopoiesis (for monocytes), and thrombopoiesis (for thrombocytes or platelets).
Figure 6. Normal hematopoiesis

The totipotent HSC divides and a progeny cell undergoes differentiation into a multipotent HSC with some self-renewal capacity. The multipotent HSC gives rise to either a common lymphoid progenitor or a common myeloid progenitor. The latter two are able to self-renew but produce only lymphoid or myeloid cells, respectively. The committed progenitor cells mature through various cell lineages to produce the mature functional forms of immune cells that are found in blood or tissues. Based on information from [152].

In humans, hematopoiesis starts early during embryogenesis to continue during fetal development and throughout life, whereby it normally takes place in the bone marrow. Most of the cells that leave the bone marrow are mature; an exception is that T lymphocytes mature within the thymus. Mature cells circulate in the bloodstream (erythrocytes, thrombocytes, granulocytes, monocytes and lymphocytes) or reside within specific tissues (lymphocytes within lymphoid organs; macrophages, dendritic cells, mast cells and plasma cells in various tissues).

HSCs are categorized into totipotent (or long-term) HSCs that are capable of unlimited self-renewal and multipotent (or short-term) HSCs with limited self-renewal capacity [152]. HSC can self-renew, differentiate, migrate and undergo apoptosis (programmed cell death) [153]. These properties are tightly regulated [154-157]. The self-renewal capacity of HSCs helps them to continually replenish the marrow tissue throughout life, while the differentiation properties allow the
HSCs to respond to the body demands as most blood cells have a limited life-span. It is estimated that in an adult human, 1.5 million blood cells are produced each second [158]. Migratory properties allow HSCs to be seeded into the respective hematopoietic organs (liver, spleen and bone marrow) during development. Apoptosis is pivotal in regulating the number of HSCs. Several of these properties are exploited in clinical and research settings, including the collection of HSCs for transplantation [159].

Growth factors and cytokines, homed within or in the vicinity of the HSC niches, govern the balance between the above-mentioned properties of HSCs [153, 158]. Thus, for example, only 8% of HSCs are allowed to enter into cell division per day [160]. A decreased rate may lead to pancytopenia, while sustained increase in the dividing HSCs and/or a decreased elimination of blood cells might lead to lympho- or myeloproliferative disorders, including leukemia.

The cell lineage and functional and maturation stages of cells may be determined using specific markers [158]. For instance, HSCs are typically CD34+/CD38−, whereas common progenitors (lymphoid and myeloid) are CD34+/CD38+ [158]. Additionally, the common lymphoid progenitor is c-kit (CD117)low/CD10 while the common myeloid progenitor is c-kit (CD117)high/CD10− [158].

### 1.4.2 Hematopoietic and lymphoid malignancies

Hematopoietic and lymphoid malignancies, sometimes referred to as blood cancers, are neoplasms that develop from abnormal hematopoietic cells. These diseases are mainly categorized into leukemias and lymphomas. In leukemias, there are malignant cells in bone marrow and frequently also in blood or other tissues [161]. Lymphomas are lymphocyte-derived solid tumors in lymph nodes or other tissues [161]. There are also uncommon types of leukemia, including myeloid sarcoma, which is a solid mass of leukemic cells that may occur in AML. Moreover, leukemia and lymphoma forms may co-occur as a single disease entity (for instance acute lymphoblastic leukemia/lymphoblastic lymphoma, and chronic lymphocytic leukemia/small lymphocytic lymphoma). Plasma cell cancer, a.k.a. multiple myeloma, comprises the accumulation of terminally differentiated B cells in bone marrow that may form osteolytic lesion throughout the blood-producing skeleton.

Leukemias are categorized into acute or chronic forms [161]. The current classification of hematopoietic and lymphoid tumors also incorporates immunophenotypes and genetic landscapes of leukemic cells [162, 163]. Markers of cell differentiation of a hematopoietic malignancy do not per se signify the maturation stage of the cell of origin, but the stage of maturation arrest. Hence, for example, all the subtypes of acute myeloid leukemia (AML) derive from the leukemic stem cell (LSC) [162-164]; the specific maturation stage at which the progeny of that LSC is arrested defines the AML phenotype. Moreover, various phenotypes of hematopoietic cancers are associated with specific cytogenetics and molecular aberrations; such genetic landscapes are incorporated into the current
classification schemes [162]. These data are useful in determining the predictive and prognostic groups of patients.

In 2015, the new cases of hematopoietic and lymphoid malignancies reported globally (in 194 countries and territories) were 1,504,000 and represented 8.6% of all cancers [165]. Among hematopoietic and lymphoid cancers, lymphomas represented 49.5%, leukemia 40.3% and multiple myeloma 10.2% [165]. In addition, hematopoietic and lymphoid cancer-related deaths in 2015 were 709,000 cases (corresponding to 8.1% of all cancer-related deaths worldwide), and almost a half of those were leukemia-related [165].

The classification of hematopoietic cancers aims to describe, define and name these diseases to guide diagnosis and therapy [162]. Table 2 provides a historical overview of the classification of hematopoietic neoplasms. Old classification systems are still in use in low-income-countries due to limited diagnostic capacities.
Table 2. History of classification of hematopoietic and lymphoid malignancies

<table>
<thead>
<tr>
<th>Classification</th>
<th>Year proposed</th>
<th>Main elements</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gall and Mallory</td>
<td>1942</td>
<td>Reticulum cell sarcoma (stem-cell lymphoma and clasmatic cell lymphoma), lymphoblastic lymphoma, lymphocytic lymphoma, Hodgkin's lymphoma, Hodgkin's sarcoma, follicular lymphoma</td>
<td>[166]</td>
</tr>
<tr>
<td>Rappaport</td>
<td>1956, revised 1976</td>
<td>Classification of non-Hodgkin's lymphomas: well-differentiated, poorly differentiated, mixed (lymphocytic-histiocytic), histiocytic and undifferentiated lymphomas.</td>
<td>[167-169]</td>
</tr>
<tr>
<td>Lukes and Collins</td>
<td>1974</td>
<td>Non-Hodgkin's lymphomas: Undefined cell type, B cell, T cell, histiocytic and unclassifiable types of lymphoma; Cell size, cleaved versus non-cleaved.</td>
<td>[170, 171]</td>
</tr>
<tr>
<td>Kiel</td>
<td>1975</td>
<td>Proposed by Karl Lennert; used in 1980-1990's; Based on cellular morphology and relationship to normal lymphoid cells: Lymphocytic (including CLL), lymphoplasmacytoid, centrocytic, centroblastic, lymphoblastic (Burkitt type, convoluted type), immunoblastic, plasmacytoma, lympho-epithelioid, unclassifiable</td>
<td>[172, 173]</td>
</tr>
<tr>
<td>WHO</td>
<td>1976</td>
<td>Histological and cytological typing of neoplastic diseases of hematopoietic and lymphoid tissues</td>
<td>[169]</td>
</tr>
<tr>
<td>FAB</td>
<td>1976, revised 1986 and 1988</td>
<td>Classification of ALL into L1-L3; Classification of AML into M0-M7. The revisions (1986 for ALL, and 1988 for AML) included morphology, immunophenotyping and cytogenetics (MIC) information</td>
<td>[174-176]</td>
</tr>
<tr>
<td>Working Formulation</td>
<td>1982</td>
<td>Used 1982-1994, essentially in the USA; Lymphomas classified as low, intermediate or high grade; nodular vs. diffuse; small, large or mixed tumor cell size.</td>
<td>[177]</td>
</tr>
<tr>
<td>REAL</td>
<td>1994</td>
<td>Integrates clinical, morphologic, immunohistochemistry and cytogenetic characteristics of lymphoid malignancies; Includes lymphocytic leukemia.</td>
<td>[178]</td>
</tr>
<tr>
<td>WHO</td>
<td>2001</td>
<td>Classification of tumors of hematopoietic and lymphoid tissues; Disease-oriented; Cell lineage: B vs T vs NK vs histiocytic; Stage of maturation of the presumed normal counterpart; Genetic subtyping, with prognostic clustering.</td>
<td>[179]</td>
</tr>
<tr>
<td>WHO</td>
<td>2008, with the current 4th revision (2016)*</td>
<td>Classification of tumors of hematopoietic and lymphoid tissues; Incorporates clinical, morphologic, immunophenotyping and genetic profiling of lymphoid and myeloid malignancies; Allows for prognostic and predictive stratification of patients.</td>
<td>[162]</td>
</tr>
</tbody>
</table>

*ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; FAB, French-American-British classification; REAL, Revised European-American classification of lymphomas
1.4.3 Acute myeloid leukemia

Acute myeloid leukemia (AML) is a heterogenous group of diseases characterized by clonal expansion of myeloid blasts in bone marrow, blood or other tissues. The initial symptoms may include anemia, thrombocytopenia, leukocytosis, and the consequences thereof such as hemorrhage and infection. The dominant current criterion is the presence of blasts with myeloid, megakaryocytic or monocytic phenotype that exceed 20% of nucleated bone marrow cells. This criterion needs not to be fulfilled when myeloid sarcoma is present or when AML-specific cytogenetic aberrations are detected such as t(8;21)(q22;q22) or RUNX1-RUNXT1 fusion, inv(16)(p13q22), t(16;16)(p13;q22), CBFB-MYH11 fusion, t(15;17)(q22;q21) or PML-RARA fusion [162].

Epidemiology of AML

AML is the most common type of acute leukemia worldwide. Its global incidence in 2015 (in 194 countries and territories) was 190,000 with 147,000 AML-associated deaths [165]. AML predominantly affects adults with a median age of approximately 70 years [180-182], and also accounts for approximately 20% of childhood leukemia. The age-standardized incidence rate (cases per 100,000 person/year) for AML is estimated at 3.5 for men and 2.2 for women [165]. In the western world, the AML incidence is relatively stable over time and between countries. For example, AML incidence in Europe in 2010 was 3.9 and 3.4 in men and women, respectively [183], and 5.0 and 3.4 in men and women, respectively, in the USA (for the 1975-2013 period) [184]. In Sweden, AML incidence (in 1973-2005) was 4.2 in men and 3.4 in women [181]. AML is more commonly diagnosed in developed than low- and middle-income countries. Accordingly, the AML incidence is 1.1 in Brazil [185], 1.0 in Egypt [186] and 0.9 in Algeria [187]. Furthermore, epidemiological data for AML show disparities in racial distribution. For example, patients of African ancestry exhibit lower incidence and poorer prognosis than those of Caucasian ancestry, even when patients are assumed to have similar access to healthcare [188-191].

Risk factors for AML

Childhood AML is known to be associated with exposure to ionizing radiation or pesticides (either to parents before conception, or in utero, or after birth), some hydrocarbons, maternal alcohol consumption during pregnancy, maternal cigarette smoking and maternal marijuana use (either before or during pregnancy) [192]. Genetic factors of relevance to the development of childhood AML include genetic syndromes such as Fanconi anemia, Bloom syndrome and Down syndrome [192]. Generic risk factors include exposure to chemical agents (benzene, pesticides, herbicides, embalming fluids), radiation and chemotherapy (such as alkylating agents, anthracyclines, taxanes, topoisomerase-II inhibitors [193]. Genetic disorders predisposing to AML (adult or childhood) include Down syndrome, Klinefelter syndrome, Patau syndrome, ataxia telangiectasia, Shwachman syndrome, Kostman syndrome, neurofibromatosis, Fanconi anemia and Li-Fraumeni syndrome [193].
Current classification

The approach in the current WHO classification of AML is to consider if the patient has a history of previous chemotherapy or prior myelodysplastic syndrome (MDS), presence of any myeloid sarcoma, or if there are recurrent genetic aberrations in leukemic cells. If none of these parameters is present, the case is considered AML not otherwise specified (NOS) [162, 194]. Immunophenotypic markers (cell-surface and cytoplasmic) in AML diagnosis comprise 5 major groups, i.e., markers for (i) precursor cells (CD34, CD117, CD33, CD13, HLA-DR), (ii) granulocytic differentiation (CD65, cytoplasmic myeloperoxidase, MPO), (iii) monocytic differentiation (CD14, CD36, CD64), (iv) megakaryocytic differentiation (CD41, CD61), and (v) erythroid differentiation (CD235a, CD36) [195]. Furthermore, mixed phenotype acute leukemia may be diagnosed as (i) MPAL-myeloid lineage (if MPO or monocytic marker in addition to at least two of the markers CD11c, or CD14, or CD64, or lysozyme), (ii) T-lineage (if AML features, with strong cytoplasmic or surface CD3), or (iii) B-lineage (if AML features, with strong CD19 plus at least one of the following: cytoplasmic CD79a, or cCD22, or CD10, or weak CD19 plus at least 2 of the following: strong CD79a, or cCD22, or CD10) [195].

The genomic landscape differs between adult AML and childhood AML. The chromosomal translocations that are common in childhood AML are, in descending order, 11q23 fusions involving KMT2A, t(8;21)(q22;q22) involving RUNX1-RUNXT1, t(15;17)(q22;q21) involving PML-RARA and inv(16)(p13q22)/t(16;16)(p13;q22) involving CBFB-MYH11, while those most common translocations in adulthood AML are (in descending order), t(15;17)(q22;q21) involving PML-RARA, t(8;21)(q22;q22) involving RUNX1-RUNXT1, inv(16)(p13q22)/t(16;16)(p13;q22) involving CBFB-MYH11, and 11q23 fusions involving KMT2A [196, 197]. The genes that are most frequently mutated in childhood AML are (by descending order of frequency), NRAS, FLT3-ITD, FLT3-N, WT1, KIT, KRAS, NPM1, PTEN11, CEBPA, and FLT3-TKD, while those frequently mutated in adulthood AML are (by descending order), NPM1, DNMT3A, FLT3-ITD, IDH1/2, FLT3-TKD, TET2, RUNX1, NRAS, TP53, and WT1 [196, 197]. The most common recurrent gene mutations are grouped into 6 major categories, based on functions of the involved genes, as shown in Table 3.

Table 3. Recurrently mutated genes in AML

<table>
<thead>
<tr>
<th>Epigenetic regulation</th>
<th>Proliferation</th>
<th>Differentiation</th>
<th>No class of function</th>
<th>Splicing</th>
<th>Cell division</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA methylation</td>
<td>Activated signaling</td>
<td>Myeloid transcription factors</td>
<td>NPM1</td>
<td>Spliceosome</td>
<td></td>
</tr>
<tr>
<td>DNMT3A/3B</td>
<td>FLT3, KIT</td>
<td>CEBPA, RUNX1</td>
<td>NPM1</td>
<td>U2AF, SRSF2</td>
<td></td>
</tr>
<tr>
<td>DNMT1</td>
<td>KRAS, NRAS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IDH1/2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TET2</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Chromatin modifiers</td>
<td>Tumor suppressors</td>
<td>Transcription factor fusions</td>
<td>TP53, WT1</td>
<td>Cohesin complex</td>
<td></td>
</tr>
<tr>
<td>AXI1</td>
<td>PHF6, TP53, WT1</td>
<td>CBFB-MYH11, PML-RARA, RUNX1-RUNXT1</td>
<td></td>
<td>SMC1/3, STAG2, RAD21</td>
<td></td>
</tr>
<tr>
<td>EZH2</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>KMT2A-fusions</td>
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<tr>
<td>KDM6A</td>
<td></td>
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<tr>
<td>NUP98-NSD1</td>
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</tbody>
</table>
The integration of clinical information, morphological, immunophenotyping and genomic data allows for the classification of AML as per the current WHO classification [162]. Table 4 outlines the current (2016) WHO classification of AML and related precursor neoplasms.

**Table 4. World Health Organization classification of AML and related precursor neoplasms**

<table>
<thead>
<tr>
<th>Classification Type</th>
<th>Subtypes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AML with recurrent genetic abnormalities</strong></td>
<td></td>
</tr>
<tr>
<td>AML with t(8;21)(q22;q22.1); RUNX1-RUNX1T1</td>
<td></td>
</tr>
<tr>
<td>AML with inv(16)(p13.1q22) or t(16;16)(p1 3.1;q22); CBFB-MYH11</td>
<td></td>
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<tr>
<td>Acute promyelocytic leukemia with PML-RARA</td>
<td></td>
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<tr>
<td>AML with t(9;11)(p21.3;q23.3); KMT2A-MLLT3</td>
<td></td>
</tr>
<tr>
<td>AML with t(6;9)(p23;q34.1); DEK-NUP214</td>
<td></td>
</tr>
<tr>
<td>AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3q26.2); GATA2, MECOM</td>
<td></td>
</tr>
<tr>
<td>AML (megakaryoblastic) with t(1;22)(p13.3;q13.1); RBM15-MKL1</td>
<td></td>
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<tr>
<td>AML with BCR-ABL1</td>
<td></td>
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<tr>
<td>AML with mutated NPM1</td>
<td></td>
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<tr>
<td>AML with biallelic mutation of CEBPA</td>
<td></td>
</tr>
<tr>
<td>AML with mutated RUNX1</td>
<td></td>
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<tr>
<td><strong>AML with myelodysplasia-related changes</strong></td>
<td></td>
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<tr>
<td>Acute myeloid leukemia, NOS</td>
<td></td>
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<tr>
<td>Acute myeloid leukemia, minimal differentiation</td>
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<tr>
<td>Acute myeloid leukemia, with maturation</td>
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<tr>
<td>Acute myelomonocytic leukemia</td>
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<tr>
<td>Acute monocytic leukemia</td>
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<tr>
<td>Acute monocytic and monoblastic leukemia</td>
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<tr>
<td>Pure erythroid leukemia</td>
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<tr>
<td>Acute megakaryoblastic leukemia</td>
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<tr>
<td>Acute basophilic leukemia</td>
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<tr>
<td>Acute panmyelosis with myelofibrosis</td>
<td></td>
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<tr>
<td><strong>Myeloid sarcoma</strong></td>
<td></td>
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<tr>
<td><strong>Myeloid proliferations related to Down syndrome</strong></td>
<td></td>
</tr>
<tr>
<td>Transient abnormal myelopoiesis</td>
<td></td>
</tr>
<tr>
<td>Myeloid leukemia associated with Down syndrome</td>
<td></td>
</tr>
<tr>
<td><strong>Blastic plasmacytoid dendritic cell neoplasm</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Acute leukemias of ambiguous lineage</strong></td>
<td></td>
</tr>
<tr>
<td>Acute undifferentiated leukemia</td>
<td></td>
</tr>
<tr>
<td>MPAL with t(9;22)(q34.1;q11.2); BCR-ABL1</td>
<td></td>
</tr>
<tr>
<td>MPAL with t(11;11)(q23.3); KMT2A rearranged</td>
<td></td>
</tr>
<tr>
<td>MPAL, B/myeloid, NOS</td>
<td></td>
</tr>
<tr>
<td>MPAL, T/myeloid, NOS</td>
<td></td>
</tr>
</tbody>
</table>

*Source: [162]. MPAL, mixed phenotype acute leukemia; NOS, not otherwise specified*
**Predictive and prognostic groups**

The European LeukemiaNet (ELN) has developed a risk stratification of AML patients by genetics [195], as shown in Table 5. Although designed for adulthood AML, the ELN protocol is suitable for risk stratification in pediatric AML [198]. Acute promyelocytic leukemia (APL, or M3-AML) is generally characterized by the t(15;17)(q24.1;q21.2) that results in fusion of PML-RARA gene product [162]. APL is associated with excellent prognosis if timely treated with all-trans-retinoic acid (ATRA) and/or arsenic trioxide [199]. Thus, APL is not included in ELN risk stratification displayed in Table 5. Additionally, presence of myeloid sarcoma without associated leukemia may herald favorable prognosis [200].

**Table 5. The 2017 ELN risk stratification of AML by genetics**

<table>
<thead>
<tr>
<th>Risk group</th>
<th>Genetic abnormality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Favorable</td>
<td>t(8;21)(q22;q22.1); RUNX1-RUNX1T1</td>
</tr>
<tr>
<td></td>
<td>inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11</td>
</tr>
<tr>
<td></td>
<td>Mutated NPM1 without FLT3-ITD or with FLT3-ITD&lt;sub&gt;low&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td>Biallelic mutated CEBPA</td>
</tr>
<tr>
<td>Intermediate</td>
<td>Mutated NPM1 and FLT3-ITD&lt;sub&gt;high&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td>Wild-type NPM1 without FLT3-ITD or with FLT3-ITD&lt;sub&gt;low&lt;/sub&gt; (without adverse-risk genetic lesions)</td>
</tr>
<tr>
<td></td>
<td>t(9;11)(p21.3;q23.3); MLLT3-KMT2A</td>
</tr>
<tr>
<td></td>
<td>Cytogenetic abnormalities not classified as favorable or adverse</td>
</tr>
<tr>
<td>Adverse</td>
<td>t(6;9)(p23;q34.1); DEK-NUP214</td>
</tr>
<tr>
<td></td>
<td>t(v;11q23.3); KMT2A rearranged</td>
</tr>
<tr>
<td></td>
<td>inv(3)(p21.3;q26.2) or t(3;3)(q21.3;q26.2); GATA2-MECOM(PL1)</td>
</tr>
<tr>
<td></td>
<td>−5 or del(5q); −7; −17/abn(17p)</td>
</tr>
<tr>
<td></td>
<td>Complex karyotype, monosomal karyotype</td>
</tr>
<tr>
<td></td>
<td>Wild-type NPM1 and FLT3-ITD&lt;sub&gt;high&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td>Mutated RUNX1</td>
</tr>
<tr>
<td></td>
<td>Mutated ASXL1</td>
</tr>
<tr>
<td></td>
<td>Mutated TP53</td>
</tr>
</tbody>
</table>

*Source: [195]. AML, acute myeloid leukemia; ELN, European LeukemiaNet; ITD, Internal tandem duplication*

**Treatment of AML**

The risk stratification of AML patients into prognostic and predictive groups provides guidance for optimal selection of treatment, including targeted therapy. The decision to treat AML (non-APL) with intensive therapy or palliation depends on the performance status and patient age. Accordingly, older patients are not always eligible for intensive therapy aiming to induce and sustain leukemia-free remission [201-204].

AML therapy with curative intent consists of two main phases, (i) induction therapy aiming at achieving complete remission, and (ii) consolidation therapy aiming at eradicating remaining leukemic cells to prevent relapse [205]. The induction therapy for children and adults consists of one or several courses of
chemotherapy based on cytarabine and anthracyclines. A patient who achieves complete remission usually proceeds to consolidation therapy. The ELN criteria for complete remission are (i) bone marrow blasts < 5%, (ii) absence of circulating blasts, (iii) absence of blasts with Auer rods, (iv) absence of extramedullary disease, (v) absolute neutrophil count ≥1.0x10⁹/l, (vi) platelet count ≥100x10⁹/l, and (vii) independence of red blood cell transfusions [195]. The WHO/Eastern Cooperative Oncology Group (ECOG) defined four performance status groups (I-IV) [201, 202] that, combined with patient age, guide the choice of therapy. The National Comprehensive Cancer Network (NCCN, USA) and ELN (Europe) have developed guidelines to follow in consolidation phase of AML therapy in older patients [195, 206]. Figure 7 aims at illustrating therapy decision-making in AML according to the ELN and NCCN guidelines [195, 206, 207].

Figure 7. Decision making in AML therapy
The diagram is based on information from [195, 206]. High risk APL denotes APL with WBC count >10,000/µL, while low-risk APL denotes APL with WBC count ≤10,000/µL [206]. AlloHCT, allogeneic hematopoietic stem cell transplantation; AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; ATRA, all-transretinoic acid; HiDAC, High Dose Ara-C (cytarabine); cytarabine + anthracycline (7 +3) refers to cytarabine continuous infusion × 7 days with anthracycline (such as daunorubicin or idarubicin) × 3 days.
1.4.4 Acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) and lymphoblastic lymphoma (LL) are considered as a single disease entity (ALL/LL); the disease manifestations may be either in leukemic (ALL) or solid mass (LL) form, or a combination of both. However, the disease is termed LL only if the disease presents as a mass lesion with no or with minimal involvement of blood or bone marrow [162]. ALL/LL results from clonal expansion of lymphoid blasts in bone marrow or blood. Initial symptoms may include unexplained weight loss, fatigue and sometimes fever (B symptoms), bleeding (due to thrombocytopenia), anemia, infections (due to impaired function of white blood cells), splenomegaly, etc.

Epidemiology of ALL

In 2015, the worldwide incidence (in 194 countries and territories) of ALL was 161,000 with approximately 110,000 ALL-related deaths/year [165]. ALL affects primarily children in both African and Caucasian populations, but a second lower peak of incidence is observed in late adulthood among Caucasians [208, 209]. The age-standardized incidence is estimated at 2.7 and 1.8 for males and females, respectively [165], and the age-standardized mortality is 1.9 (males) and 1.3 (females) [165]. There are disparities in the incidence and prognosis of ALL around the world. Small studies indicate that African populations show lower ALL incidence [189, 210] and poorer prognosis [191, 211]. The peak incidence of ALL patients of African ancestry is observed at an older age (5-9 years) than in Caucasians (0-4 years) [189, 210, 212]. Infant ALL, occurring in children <1-year old, which accounts for approximately 5% of childhood ALL in western countries, is rarely diagnosed in Africa. The quality of, and access to health care likely contribute to the patterns in overall incidence and prognosis of ALL around the world [165].

Risk factors

In addition to racial and geographical differences, environmental factors are presumed to be associated with ALL. These factors include ionizing radiation (including paternal exposure before conception), non-ionizing radiation (especially for pre-B ALL), hydrocarbons, as well as maternal use of marijuana before or during pregnancy [192]. These risk factors for ALL thus largely overlap with those identified for AML. As discussed above, the interaction of EBV and *P. falciparum* is associated with Burkitt leukemia/lymphoma [82, 213, 214].

Genetic factors are involved in the occurrence of ALL, as evidenced by a high concordance of ALL in identical twins [192, 215]. Also, there is increased risk of developing childhood ALL in children whose family have a past history of any hematopoietic/lymphoid malignancy [192]. Children with polymorphisms in genes encoding for carcinogen-metabolizing enzymes such as *CYP1A1* as well as *NQO1* have increased risk of developing ALL with relatively poor prognosis [216].


Classification of ALL

The initial FAB classification of ALL subtypes using microscopic morphological findings subdivided ALL into three groups (L1 – L3) [174]. In L1, the cells have homogeneous nuclear chromatin, a regular nuclear shape, small or no nucleoli, scanty cytoplasm, and mild to moderate basophilia. L2 is characterized by large, heterogeneous cells with variable nuclear chromatin, an irregular nuclear shape, 1 or more nucleoli, a variable amount of cytoplasm, and variable basophilia. For the L3 group, there are large, homogeneous cells with fine, stippled chromatin; regular nuclei; prominent nucleoli; and abundant, deeply basophilic cytoplasm. The most distinguishing feature is a prominent cytoplasmic vacuolation.

The initial FAB classification [174] has been superseded by the morphology, immunophenotypic and cytogenetics (MIC) classification [175]. The latter was supplemented to include clinical information and genetic profile in the current WHO classification [162]. There is no clear correlation between the FAB-ALL subtypes and the MIC subtypes, with the exception that all MIC’s (mature) B-cell ALL cases were actually classified L3 in the FAB system [175]. Immunophenotyping helps in identifying B from T or NK-cell ALL, and in determining the level of differentiation of the cell clone. Table 6 lists markers that are frequently employed in immunophenotyping of ALL.

Table 6. Antigens for acute lymphoblastic leukemia/lymphoblastic lymphoma (ALL/LL) immunophenotyping

<table>
<thead>
<tr>
<th>ALL types</th>
<th>Antigens/Antibody</th>
<th>Notes</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALL (in general)</td>
<td>Common acute lymphoblastic leukemia antigen (CALLA)</td>
<td>Also expressed by CML cells in “Blast crisis”</td>
<td>[217]</td>
</tr>
<tr>
<td></td>
<td>TdT (nuclear), CD34</td>
<td>Show less differentiated cells</td>
<td>[218-220]</td>
</tr>
<tr>
<td></td>
<td>Use of CD45 intensity expression with right-angle light scatter (RALS)</td>
<td>Show blast cells</td>
<td></td>
</tr>
<tr>
<td>B-ALL</td>
<td>BCP-CD10−, cyCD79a+, cyCD22+, CD19+, CD10+</td>
<td>Often, these 2 are put in 1 group (BCP) [218]</td>
<td>[218, 220, 221]</td>
</tr>
<tr>
<td></td>
<td>BCP-CD10+ (common ALL)</td>
<td>cyCD79a+, cyCD22+, CD19+, CD10+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pre-B</td>
<td>cyCD79a+, cyCD22+, CD19+, CD10+, cytμ+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>‘Mature’ B-ALL</td>
<td>CD19+, SmIg+, SmIg+ (gkk) or SmIg+ (λ)</td>
<td></td>
</tr>
<tr>
<td>T-ALL</td>
<td>Early</td>
<td>CD2+, CD7+, CD5+, CD1−, CD3−</td>
<td>[220]</td>
</tr>
<tr>
<td></td>
<td>Common</td>
<td>CD2+, CD7+, CD5+, CD1+, CD3−</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mature</td>
<td>CD2+, CD7+, CD5+, CD1−, CD3+</td>
<td></td>
</tr>
<tr>
<td>MPAL</td>
<td>B + myeloid</td>
<td>CD19+, CD15+</td>
<td>MPO is a myeloid marker, but cytoplasmic</td>
</tr>
<tr>
<td></td>
<td>T + myeloid</td>
<td>CD7+, CD15+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Trilineage</td>
<td>CD19+, CD7+, CD15+</td>
<td></td>
</tr>
</tbody>
</table>

ALL, acute lymphoblastic leukemia; BCP, B-cell precursor; CD, cluster of differentiation; CML, chronic myeloid leukemia; cy-, cytoplasmic; MPAL, mixed-phenotype acute leukemia; MPO, myeloperoxidase; SmIg, surface membrane immunoglobulin; TdT, terminal deoxynucleotidyltransferase

The current WHO classification includes elements that stratify patients into predictive and prognostic groups, and thus guides the management of ALL/LL cases, as summarized in Table 7.
Table 7. The World Health Organization classification of precursor lymphoid neoplasms

<table>
<thead>
<tr>
<th>B-lymphoblastic leukemia/lymphoma, NOS</th>
<th>B-lymphoblastic leukemia/lymphoma with recurrent genetic abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-lymphoblastic leukemia/lymphoma with t(9;22)(q34.1;q11.2); BCR-ABL 1</td>
<td>B-lymphoblastic leukemia/lymphoma with t(9;22)(q34.1;q11.2); BCR-ABL 1</td>
</tr>
<tr>
<td>B-lymphoblastic leukemia/lymphoma with t(v;11q23.3); KMT2A-rearranged</td>
<td>B-lymphoblastic leukemia/lymphoma with t(v;11q23.3); KMT2A-rearranged</td>
</tr>
<tr>
<td>B-lymphoblastic leukemia/lymphoma with t(12;21)(p13.2;q22.1); ETV6-RUNX1</td>
<td>B-lymphoblastic leukemia/lymphoma with t(12;21)(p13.2;q22.1); ETV6-RUNX1</td>
</tr>
<tr>
<td>B-lymphoblastic leukemia/lymphoma with hyperdiploidy</td>
<td>B-lymphoblastic leukemia/lymphoma with hyperdiploidy</td>
</tr>
<tr>
<td>B-lymphoblastic leukemia/lymphoma with hypodiploidy (hypodiploid ALL)</td>
<td>B-lymphoblastic leukemia/lymphoma with hypodiploidy (hypodiploid ALL)</td>
</tr>
<tr>
<td>B-lymphoblastic leukemia/lymphoma with t(5;14)(q31.1;q32.1); IGH/IL3</td>
<td>B-lymphoblastic leukemia/lymphoma with t(5;14)(q31.1;q32.1); IGH/IL3</td>
</tr>
<tr>
<td>B-lymphoblastic leukemia/lymphoma, BCR-ABL 1-like</td>
<td>B-lymphoblastic leukemia/lymphoma, BCR-ABL 1-like</td>
</tr>
<tr>
<td>B-lymphoblastic leukemia/lymphoma with iAMP21</td>
<td>B-lymphoblastic leukemia/lymphoma with iAMP21</td>
</tr>
</tbody>
</table>

T-lymphoblastic leukemia/lymphoma
- Early T-cell precursor lymphoblastic leukemia

NK-lymphoblastic leukemia/lymphoma

Source: [162]. NK, natural killer cell; NOS, not otherwise specified

Predictive and prognostic groups

Clinical characteristics associated with inferior prognosis are infancy, older age (≥10 years), high white blood cell counts and CNS involvement at diagnosis [162]. Moreover, slow response to therapy (evidenced by the morphology of blood and/or bone marrow examination), and positive minimal residual disease (MRD) may herald poor prognosis [162].

Concerning ALL immunophenotypes, T-ALL may imply inferior prognosis, likely because T-ALL is associated with high WBC counts, older age and increased risk of relapse than B-ALL [162]. Moreover, approximately 50% of T-ALL cases harbor somatic mutations in NOTCH1 that herald poor outcome [223]. Additionally, the prognosis of early T-cell precursor lymphoblastic leukemia is poorer than in other forms of T-ALL [224]. Prognostic and predictive information (about genetic abnormalities) provided in the current WHO classification of precursor lymphoid neoplasms [162] are summarized in Table 8.

Table 8. ALL prognostic risk groups

<table>
<thead>
<tr>
<th>Good prognosis</th>
<th>Adverse prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>- B-ALL with t(12;21)(p13.2;q22.1); ETV6-RUNX1 (or TEL/AML1)</td>
<td>- B-ALL with t(v;11q23.3); KMT2A-rearranged</td>
</tr>
<tr>
<td>- Hyperdiploid B-ALL</td>
<td>- B-ALL with BCR-ABL 1 (most common in adults)</td>
</tr>
<tr>
<td></td>
<td>- Hypodiploid B-ALL</td>
</tr>
<tr>
<td></td>
<td>- B-ALL with t(1;19)(q23;p13.3); TCF3-PBX1</td>
</tr>
<tr>
<td></td>
<td>- BCR-ABL 1-like ALL</td>
</tr>
<tr>
<td></td>
<td>- ALL with NOTCH1 mutation</td>
</tr>
<tr>
<td></td>
<td>- B-ALL with iAMP21</td>
</tr>
</tbody>
</table>

Based on information from [162]. ALL, acute lymphoblastic leukemia
Treatment of ALL

There exist several protocols for ALL treatments, including those adapted for use in developing countries. The NCCN protocols which are widely used in the western world take into account clinical, morphological, immunophenotypic and genetic landscape of individual cases in order to guide therapy in children [225], and adults [226] (Figure 8).

Figure 8. Overview of the current therapy of ALL using the NCCN protocols Based on information from [225, 226]. Interfant refers to the treatment protocol for infants younger than 1 year with acute lymphoblastic leukemia [227]. ALL, acute lymphoblastic leukemia; AlloHCT, allogeneic hematopoietic stem cell transplantation; AYA, adolescents and young adults; Ph, Philadelphia chromosome or t(9;22) resulting in ABL-BCR fusion; CR, complete remission; MRD, minimal (measurable) residual disease; NCCN, National Comprehensive Cancer Network; TKI, tyrosine kinase inhibitor.
The low-intensity treatment protocol, which are also referred to as the Hunger regimens, are commonly used in low-income countries [32] and are presented in Table 9.

**Table 9. ALL treatment in low-income countries**

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Induction (4 weeks)</th>
<th>Consolidation (4 weeks)</th>
<th>Interim maintenance (8 weeks)</th>
<th>Delayed intensification (8 weeks)</th>
<th>Maintenance (84-day cycles until 30 months from start therapy)</th>
</tr>
</thead>
</table>
| Regimen 1 | PRD prophase d 1-7  
PRD d 8-29  
VCR d 8, 15, 22, 29  
1-ASP x 3 weeks  
starting d 8  
IT MTX d 1, 8 29  
Extra IT MTX d 15, 22 if CNS3 | VCR d 1  
6-MCP d 1-28  
IT MTX d 1, 8, 15 | -  
- | DXM d 1-5, 29-33, 57-61  
VCR d 1, 29, 57  
6-MCP d 1-84  
MTX weekly start d 1  
IT MTX d 1, 29 for 1st 4 cycles then d 1 only  
(omit oral MTX when IT MTX given) | Same as in Regimen 1 for the 1st 4 molecules;  
IT MTX d 1  
CRT at start of 1st cycle  
IT MTX d 1 of each cycle  
(omit oral MTX when IT MTX given) |
| Regimen 1| Same as in Regimen 1 | Same as in Regimen 1 | Same as in Regimen 1 | Same as in Regimen 1 | Same as in Regimen 1 for the 1st 4 molecules;  
IT MTX d 1  
CRT at start of 1st cycle  
IT MTX d 1 of each cycle  
(omit oral MTX when IT MTX given) |
| Regimen 2 | PRD d 1-29  
VCR d 8, 15, 22, 29  
1-ASP x 3 weeks  
starting at d 8  
IT MTX d 1, 8 29  
Extra IT MTX d 15, 22 if CNS3 | VCR d 1  
6-MCP d 1-28  
IT MTX d 1, 8, 15 | DXM d 1-5, 29-33  
VCR d 1, 29  
6-MCP d 1-50  
MTX weekly d 1, 8, 15, 22, 29, 36, 43, 50  
IT MTX d 29 | DXM d 1-7, 15-21  
VCR d 1, 8, 15  
DOX d 1, 8, 15  
1-ASP x 2 weeks start d 3  
CPH d 29  
CTB d 29-32, 36-39  
6-Mercap d 29-43  
IT MTX d 1, 29, 36 | DXM d 1, 29, 33, 57-61  
VCR d 1, 29, 57  
6-MCP d 1-84  
MTX weekly start d 1  
IT MTX d 1, 29 for 1st 4 cycles then d 1 only  
(omit oral MTX when IT MTX given) |
| Regimen 2| Same as Regimen 2 | Same as in Regimen 2 | Same as in Regimen 2 | Same as in Regimen 2 | Same as in Regimen 1 for the 1st 4 molecules;  
IT MTX d 1 of each cycle  
CRT at start of 1st cycle  
(omit oral MTX when IT MTX given) |
| Regimen 3 | PRD d 1-29  
VCR d 8, 15, 22, 29  
1-ASP x 3 weeks  
starting at d 8  
IT MTX d 1, 8 29  
Extra IT MTX d 15, 22 if CNS3 | CPH d 1, 15  
CTB d 1-4, 8-11, 15-18, 22-25  
6-MCP d 1-28  
IT MTX d 1, 8, 15, 22  
Must have blood count recovery before start d 15 therapy | DXM d 1-5, 29-33  
VCR d 1, 29  
6-MCP d 1-50  
MTX d 1, 8, 15, 22, 29, 36, 43, 50  
IT MTX d 29 | DXM d 1-7, 15-21  
VCR d 1, 8, 15  
DOX d 1, 8, 15  
1-ASP x 2 weeks start d 3  
CPH d 29  
CTB d 29-32, 36-39  
6-Mercap d 29-43  
IT MTX d 1, 29, 36  
Must have blood count recovery before start d 29 therapy | DXM d 1, 29, 33, 57-61  
VCR d 1, 29, 57  
6-MCP d 1-84  
MTX weekly start d 1  
IT MTX d 1, 29 for 1st 4 cycles then d 1 only  
(omit oral MTX when IT MTX given) |
| Regimen 4 | PRD d 1-29  
VCR d 8, 15, 22, 29  
1-ASP x 3 weeks  
starting at d 8  
IT MTX d 1, 8 29  
Extra IT MTX d 15, 22 if CNS3 | CPH d 1, 15  
CTB d 1-4, 8-11, 15-18, 22-25  
6-MCP d 1-28  
IT MTX d 1, 8, 15, 22  
Must have blood count recovery before start d 15 therapy | VCR d 1, 11, 21, 31, 41  
IT MTX IV weekly d 1, 11, 21, 31, 41 (dose escalate)  
IT MTX d 31 | DXM d 1-7, 15-21  
VCR d 1, 8, 15  
DOX d 1, 8, 15  
L-ASP x 2 weeks start d 3  
CPH d 29  
CTB d 29-32, 36-39  
IT MTX d 1, 29, 36  
Must have blood count recovery before start d 29 therapy | DXM d 1, 29, 33, 57-61  
VCR d 1, 29, 57  
6-MCP d 1-84  
MTX weekly start d 1  
IT MTX d 1 of each cycle  
CRT at start of 1st cycle  
(omit oral MTX on d 1 of cycle #1 and when IT MTX given) |

Based on information from [32]. 6-Mercaptopurine; CNS, central nervous system (see Table 10 for the annotations 1, 2, 3); CPH, cyclophosphamide; CRT, cranial radiation; CTB, cytarabine; d, day(s); DOX, Docorubicin; DXM, Dexamethasone; IT, intrathecal; 6-MCP, L-ASP, L-asparaginase; MTX, Methotrexate; PRD, prednisone; VCR, vincristine

The Hunger regimens do not take into account risk stratification based on morphological, immunophenotypic and genetic features of leukemic cells. Instead, patients are stratified into different risk groups based on age, white blood cell (WBC) counts, marrow cellularity at day 15 and 29 of induction therapy, on CNS involvement and on whether the disease is B- or T-cell ALL [32] (Table 10). Additionally, the health facility starts with step 1 (regimen
1 or $I_{CRT}$) and when there is evidence of safety to the implemented regimens, the health facility may proceed to the next step of regimens [32]. Initially, the Hunger 1 and 2 regimens were implemented in Rwanda; a study carried out in 2015-2017 recommended to progress to Hunger 3 and 4 [228].

**Table 10. Risk stratification of ALL patients in low-income countries**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Lower risk</th>
<th>Higher risk</th>
<th>Very high risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Criteria</td>
<td>B-precursor ALL and age 1.00–9.99 years and WBC count &lt;50,000/µl and prednisone good response and CNS 1 or CNS2 and Day 15 M1/M2 marrow and Day 29 M1 marrow</td>
<td>CNS 1 or CNS2 and T-cell ALL and WBC &lt;100,000/µl or CNS1 or CNS2 and B-precursor ALL with age &lt;1 or &gt;9.99 years or WBC count &gt;50,000/µl and prednisone good response and Day 15 M1/M2 marrow and Day 29 M1 marrow</td>
<td>Prednisone poor response or CNS3 or T-cell ALL and WBC &gt;100,000/µl or Day 15 M3 marrow or Day 29 M2/M3 marrow</td>
</tr>
<tr>
<td>Therapy</td>
<td>Step 1 Regimen 1</td>
<td>Regimen 1 (or 1$_{CRT}$)</td>
<td>Regimen 1 (or 1$_{CRT}$)</td>
</tr>
<tr>
<td></td>
<td>Step 2 Regimen 1</td>
<td>Regimen 2</td>
<td>Regimen 2$_{CRT}$</td>
</tr>
<tr>
<td></td>
<td>Step 3 Regimen 2</td>
<td>Regimen 3</td>
<td>Regimen 4</td>
</tr>
</tbody>
</table>

Based on information from [32]. $I_{CRT}$ or $2_{CRT}$: cranial radiation therapy in regimen 1 or 2, respectively; ALL, acute lymphoblastic leukemia. CNS1/2/3, central nervous system status, with either no evidence of leukemia involvement (1), or with presence of leukemic cells in a cerebrospinal fluid sample that contains fewer than 5 WBCs/µl (2), or overt leukemia (nontraumatic cerebrospinal fluid sample containing ≥ 5 WBC/µl with identifiable blasts, or the presence of a cerebral mass or cranial palsy) involvement (3) [229]. CNS, central nervous system; M1, bone marrow with ≤5% blasts with normal cellularity; M2, bone marrow with 6-25% blasts; M3, bone marrow with >25% blasts (these M1, 2, 3 categories are used for ALL) [230]; WBC, white blood cell.
2. AIMS

2.1 OVERALL AIM

The overall aim of this thesis work was to determine factors associated with poor resolution of infections in African children, as well as determining the distribution, subtypes and prognosis of AML and ALL in sub-Saharan Africa, taking into account associated infections and the utilization of healthcare services. These studies used the case of Rwanda as example.

2.2 SPECIFIC AIMS

The specific aims of this thesis were to:

1) determine the role of germline IFNL4 variation for the clearance of respiratory tract pathogens in Rwandan children,
2) determine the incidence, subtypes and outcome of AML and ALL in Rwanda in comparison with the western world, and
3) assess factors associated with the current trends in the documentation and management of acute leukemia cases in Rwanda.
3. PATIENTS AND METHODS

This section provides an overview of the study population, samples and methods used in this thesis. Further details are provided in the attached papers.

3.1 PAPER I

This is a cohort study that included children ≤5 years who consulted health facilities in Rwanda for acute (≤5 days duration) respiratory infections between 2009 and 2012. Demographics and disease characteristics were recorded at baseline and at 2 weeks of follow-up. Figure 9 outlines the research methods employed in Paper I. Nasopharyngeal swabs from the children were shipped to the Department of Virology at the University of Gothenburg for analysis. Extraction of nucleic acids was performed using a MagNA Pure LC instrument (Roche Diagnostics, Mannheim, Germany) and the Total Nucleic Acid isolation kit. Detection of microbes was done by real-time polymerase-chain reaction (RT-PCR) with oligonucleotides targeting parainfluenzavirus 1–3, respiratory syncytial virus, metapneumovirus, influenza A virus, influenza B virus, coronaviruses (NL63, HKU1, OC43, 229E), enterovirus, rhinovirus, morbillivirus, bocavirus, adenovirus, B. pertussis, S. pneumonia, and H. influenzae, as previously described [231, 232].

Figure 9. Overview of case enrolment and laboratory analyses in Paper I
RT-PCR, real-time polymerase chain reaction

IFNL4 genotyping from host DNA retrieved from the nasopharyngeal swabs was performed using the 7500 Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA). In these analyses, rs12979860 (CC, CT or TT) genotyping was performed on 477 cases (99% of the study population) who had sufficient content of DNA, using a predesigned assay (Applied Biosystems, Carlsbad, CA, USA). In addition, rs368234815 (TT/TT, TT/ΔG or ΔG/ΔG) genotyping was done on 156
cases (97% of the 161 followed-up cases) using custom MGB probes (Applied Biosystems, Carlsbad, CA, USA) and primers (Sigma-Aldrich, St. Louis, MO, USA). This study was approved by the ethics committee of the National University of Rwanda and by the Regional Ethics Review Board in Gothenburg, Sweden (approval no. 052-08). Patients’ guardians provided informed signed consent prior to study enrolment.

3.2 **PAPERS II & III**

3.2.1 Case enrolment

We performed a retro- and prospective cohort study aiming to capture all cases of acute leukemia diagnosed during 2012-2017 at Rwandan centers with diagnostic and hematologic/oncologic services. For comparison, Swedish cases diagnosed in 2012-2017 were retrieved from the Swedish ALL [233] and AML [234] registries. Demographic data were obtained from national statistics centers in Rwanda [5, 235] and Sweden [236]. ASR was calculated by adjusting population demographics to the world standard population as defined in 2000 [237]. Figure 10 summarizes the enrolment of the study population.

![Figure 10. Enrolment of the study population for Papers II and III](image)

**Figure 10. Enrolment of the study population for Papers II and III**

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; OHSU, Oregon Health and Science University; TARGET, therapeutically applicable research to generate effective treatments; TCGA, The Cancer Genome Atlas
Blood samples were collected in Vacutainer Mononuclear Cell Preparation Tubes - CPT (BD, Franklin Lakes, NJ) for isolation of peripheral blood mononuclear cells (PBMCs) and dry Vacutainer serum tubes (BD, Bristol Oakville, Ontario) for serum preparation. Samples were collected from 180 ALL and 118 AML patients at diagnosis, as well as 100 hematologically healthy donors (from the Rwanda national center for blood transfusion, NCBT) in Rwanda, between October 2016 and October 2019. Serum was collected after centrifugation and PBMCs were isolated using density centrifugation. PBMCs and serum samples were aliquoted in four cryovials each. The samples were kept at -80°C until shipment on dry ice to the University of Gothenburg for analyses including subtyping of the disease and identification of infectious agents as outlined in the following subsection. To compare the subtypes of ALL and AML in Rwanda versus the western world, we retrieved genomic and immunophenotypic data for ALL patients from the Therapeutically Applicable Research to Generate Effective Treatments (TARGET) database [238]. Likewise, genomic profiling data for AML patients were retrieved from The Cancer Genome Atlas (TCGA) [239], TARGET [240], and the Oregon Health and Science University (OHSU) [241] database.

The survival of ALL and AML patients was recorded for Rwandan patients diagnosed between 2012 and 2017. The follow-up was at least 2 years (up to December 2019). Cases diagnosed before October 2016 were followed-up retrospectively by consulting their hospital files and by contacting their families. For comparison, survival data for AML patients were retrieved from the western world databases, i.e. TCGA for adult AML patients [239] and the TARGET for pediatric AML [240] and ALL [238] patients.

Ethical approval was obtained from the University of Rwanda College of Medicine and Health Sciences (CMHS)’s institutional review Board (IRB) (ethical clearance no. 158/CMHS IRB/2016). The participating institutions provided permission to access the data. Informed signed consent or assent was obtained (for cases enrolled prospectively) from each participant or guardian, respectively. Material transfer agreement was signed between UR-CMHS (Rwanda) and Sahlgrenska Academy (Sweden) for the shipment of samples.

### 3.2.2 Laboratory methods

Analyses were performed on serum and PBMC as summarized in Figure 11. Not all tests were performed for each case, as poor cell viability, insufficient DNA yield and/or low serum volume precluded some cases from undergoing flow cytometry, whole exome sequencing and/or serological assays, respectively.

**Serological tests**

Upon arrival of samples at the University of Gothenburg, serum samples were analyzed for presence of anti-human immunodeficiency virus (HIV) antibodies; hepatitis B surface antigen for active hepatitis B virus (HBV) infection; anti-hepatitis B core immunoglobulin (Ig) G (IgG) antibodies for past or ongoing HBV infection; anti-hepatitis C IgG for past or ongoing hepatitis C virus (HCV)
infection; anti-cytomegalovirus (CMV) IgG for previous CMV infection; IgG antibodies against viral capsular antigen (VCA) for previous or ongoing Epstein-Barr virus (EBV) infection and IgM antibody against EBV VCA for recent or ongoing EBV infection. Samples reactive to either HIV Ab, HBsAg or HCV Ab were excluded from further analyses.

**Enzyme-linked immunosorbent assay (ELISA)**

We used the indirect enzyme-linked immunosorbent assay (ELISA) on serum samples from ALL patients, AML patients and healthy donors to quantify levels of *P. falciparum* histidine-rich protein-2 antigen (HRP-2) and an antibody to *P. falciparum* serine-repeat antigen protein (SE36). ELISA was performed using recombinant antigens for HRP-2 and SE36 (East Coast Biologics, North Berwick, ME) as previously described [146], with modification of the substrate used for reaction detection using the substrate 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma-Aldrich) mixture. The plates were read immediately for optical density at 450 nm using the FLUOstar Omega Microplate Reader (BMG Labtech).

**Flow cytometry**

PBMCs were stained with live-dead markers to identify live cells. Samples from AML and ALL patients were stained with cocktails of fluorochrome-labelled antibodies to define the respective leukemic immunophenotype. Samples were run...
on a BD LSRFortessa™ flow cytometer (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo 10.0 software (Flowjo, LLC). Figure 12 outlines the strategy employed to determine the immunophenotype of ALL cases and acute leukemia cases with ambiguous phenotypes. Immunophenotyping of AML cases utilized the pattern of markers expressed by cells according to AML-FAB subtypes, as described in the current WHO classification of hematopoietic and lymphoid tumors [162] and by the European LeukemiaNet (ELN) [195]. Table 11 outlines the approach employed to determine the AML immunophenotype.

**Figure 12. Strategy for immunophenotyping of ALL and MPAL**

Myeloid markers included CD33, HLA-DR, CD14, CD15 and CD11b. ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; BCP, B cell precursor; CD, cluster of differentiation; HLA-DR, human leucocyte antigen DR; int, intensity; MPAL, mixed-phenotype acute leukemia; smIg, surface membrane immunoglobulin; SSC, side light scatter. A marker was considered positive if at least 20% of the stained cells were positive.
Table 11. Strategy for immunophenotyping of AML cases

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Markers</th>
</tr>
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<tbody>
<tr>
<td>Blast gating</td>
<td>CD45^low/SSC^low-int</td>
</tr>
<tr>
<td>M0</td>
<td>CD34^+, HLA-DR^++, CD33^+, CD15^+, CD11b^−, CD14^−</td>
</tr>
<tr>
<td>M1</td>
<td>CD34^+, HLA-DR^+, CD33^+, CD15^+, CD11b^+, CD14^−</td>
</tr>
<tr>
<td>M2</td>
<td>CD34^+, HLA-DR^+, CD33^+, CD15^+, CD11b^+, CD14^−</td>
</tr>
<tr>
<td>M3</td>
<td>CD34, HLA-DR, CD33^++, morphological description</td>
</tr>
<tr>
<td>M4</td>
<td>CD34^+, HLA-DR^+, CD33^+, CD15^+, CD11b^+, CD14^−</td>
</tr>
<tr>
<td>M5</td>
<td>CD34^+, HLA-DR^++, CD33^+, CD15^+, CD11b^+, CD14^+</td>
</tr>
<tr>
<td>M6</td>
<td>CD34, HLA-DR, CD33, E-Cadherin^++ §</td>
</tr>
<tr>
<td>M7</td>
<td>CD34, HLA-DR, CD33^+, CD15^+, CD11b^+, CD14^+, CD123^++§</td>
</tr>
<tr>
<td>Acute basophilic leukemia</td>
<td>CD34, HLA-DR, CD33^++, CD15^+, CD11b^+, CD14^+, CD123^++§</td>
</tr>
<tr>
<td>Acute panmyelosis with myelofibrosis</td>
<td>(requires bone marrow biopsy specimen)</td>
</tr>
</tbody>
</table>

Based on information from [162]. These markers were planned to be run after the initial flow cytometry on cases eventually suspected to be either M6, M7 or acute basophilic leukemia types of AML. Key interpretation: ++ brightly positive in most (>70%) cells; + positive in a moderate proportion (40-70%) of cells; ± positive in few (20-40%) cells, negative <20% positive cells. AML, acute myeloid leukemia; CD, cluster of differentiation; HLA-DR, human leucocyte antigen DR; int, intensity; surface membrane immunoglobulin; SSC, side light scatter.

Genomic profiling

DNA extraction from PBMCs was done using QIAamp DNA Blood Mini kit (QIAGEN), according to the manufacturer’s guidelines. DNA samples with sufficient yield and quality were sent to BGI Global Genomics Services (Copenhagen, Denmark) for library preparation and whole exome sequencing (WES). Library preparation used the Agilent V6 exome promoted kit. Sequencing data were transferred back to Sahlgrenska Academy for quality check and analysis at Bioinformatics core facility.

FastQC 0.11.2 software was used to assess the quality of sequencing data and Samtools 1.3.1 was used for statistics mapping. The reads alignment to reference genome (hg19) used the Burrows-Wheeler Aligner BWA_0.7.13 [242]. Genome Analysis ToolKit (GATK) 3.1-1 was used for reads realignment [243]. HaplotypeCaller was utilized for variant calling. Variant filtration was done using QD < 2.0, MQ < 40.0, FS > 60.0, ReadPosRankSum < -8.0, MQRankSum < -12.5 for small nucleotide variants (SNVs) and QD < 2.0, FS > 200.0, ReadPosRankSum < -20.0 for small insertions/deletions (INDELs). Obtained variants were further filtered against 1000 Genomes [244], whereby variants with a minor allele frequency (MAF) >0.01 were removed. ANNOVAR [245] was then used to annotate the variants and to determine exonic functional predictions using the following tools: whole-exome SIFT, PolyPhen2 H DIV, PolyPhen2 HVAR, LRT, MutationTaster, MutationAssessor, FATHMM, MetaSVM, MetaLR, VEST, CADD, GERP++, ClinVar, PhyloP and SiPhy scores from dbNSFP version 2.6, and with variants present in the COSMIC70 database. The calling of large structural variants (SVs) was done using MANTA version 1.6.0 [246] and Control_FREEC version 9.1 [247]. Annotation of SVs was done by the structural
variant database software (SVDB).2.3.0 against allele frequencies retrieved form Gnomad SV database [248]. Additional SVs annotation was done with known genes from UCSC using ANNOVAR software [245]. A hematologically healthy donor from the same Rwandan population was used as control in these analyses.

### 3.3 PAPER IV

We performed a qualitative study, adopting a phenomenological approach, to document factors associated with low detection of acute leukemia at individual/family (or community), healthcare facility or healthcare system management levels. The study involved 41 participants including patients, patients’ guardians and key informants (physicians from district hospitals and specialists in different acute leukemia-related fields from referral hospitals), as well as directors from Rwanda Biomedical Center (RBC). Procedures in this study are summarized in Figure 13. In-depth interviews were conducted, and we used thematic analysis to interpret the data. This study was approved by UR-CMHS’s IRB (approval N° 315/CMHS IRB/2019). Participating institutions also offered permissions to collect data. Research participants provided signed informed consents before conducting the interviews.

*Figure 13. Overview of research methods for Paper IV*
4. RESULTS AND DISCUSSION

4.1 PAPER I

The study investigated the impact of genetic variation at IFNL4 for the outcome of upper respiratory tract infections in Rwandan children (n=480). Fifty-one % of the patients were 1-3 years old, 27 % were <1 year, and 22% were 4-5 years old. Symptoms were dominated by cough (98%) and fever (92%). Most patients (85%) carried multiple respiratory pathogens. The most common pathogens were Streptococcus pneumoniae (in 82%), Hemophilus influenzae (73%), rhinovirus (38%), enterovirus (21%), and respiratory syncytial virus (18%).

In this cohort, IFNL4 rs12979860 and rs368234815 were in strong linkage disequilibrium (D' = 0.998; r^2 = 0.89), implying that the demographic distribution and impact of the rs12979860-C allele mirror those of rs368234815-TT, whereas rs12979860-T allele distribution and impact mirror those of rs368234815-ΔG. The rs12979860 CC genotype was detected in 18% of the study population, which is similar to results obtained in other cohorts of African descent [65]. The distribution of IFNL4 genotypes is shown in Table 12, which includes genotypes in racial groups as reported in previous studies.

### Table 12. Distribution of IFNL4 genotypes among world populations

<table>
<thead>
<tr>
<th>IFNL4 rs12979860 genotypes</th>
<th>Proportions (%) of IFNL4 genotypes in population (country of residence)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Africans (Rwanda), n=477 (Paper I)</td>
</tr>
<tr>
<td></td>
<td>Africans (USA, UK), n=290 [65]</td>
</tr>
<tr>
<td></td>
<td>Caucasians (USA, UK), n=642 [65]</td>
</tr>
<tr>
<td></td>
<td>Caucasians (Italy), n=177 [75]</td>
</tr>
<tr>
<td></td>
<td>Middle-East (Iran), n=158 [249]</td>
</tr>
<tr>
<td></td>
<td>East-Asians (China), n=1,012 [250]</td>
</tr>
<tr>
<td>CC</td>
<td>18</td>
</tr>
<tr>
<td>CT</td>
<td>49</td>
</tr>
<tr>
<td>TT</td>
<td>33</td>
</tr>
</tbody>
</table>

UK, United Kingdom; USA, United States of America. Sources: [65, 75, 249, 250]

The results implied that carriers of the IFNL4 rs12979860-CC genotype clear respiratory RNA viruses more efficiently than do carriers of CT and TT genotypes (Chi-square for trend P=0.006, Figure 14A). A similar trend was observed for the rs368234815-TT/TT genotype. The benefit of the rs12979860-CC genotype was observed for the clearance of ss(+)RNA viruses (P=0.03, Figure 14B) but not for clearance of ss(-)RNA viruses (P=0.5) or DNA viruses (P=0.7).
Figure 14. Respiratory RNA virus clearance versus host rs12979860 genotypes

Earlier studies show that carriers of rs12979860-CC and rs368234815-TT/TG IFNL4 genotypes are more likely to clear infection with HCV (a ss(+)RNA virus) [49, 62-66, 75, 251, 252]. To our knowledge, the results presented in Paper I are the first to indicate that the course of infection by RNA viruses other than HCV is influenced by IFNL4 genotypes, although further studies are required to validate and extend this association. On a similar note, a recent study showed that the rs12979860-T allele is associated with severe dengue virus (also a ss(+)RNA virus) infection [253]. In addition, a recent study from Mali showed that young children carrying the rs368234815-TT allele were relatively protected against early episodes of gastrointestinal, malaria and respiratory infections [254]. Furthermore, in a recent study of Rwandan women who participated in a cervical cancer screening program, carriers of rs12979860-CC tended to be protected from contracting human papilloma virus (HPV) infection (a DNA virus) compared with those carrying CT or TT rs12979860 genotypes (Paper SI). Overall, these results should inspire additional studies to explore the role of IFNL4 gene variation for the course of infectious and non-communicable diseases.

4.2 PAPER II

This study aimed to define the incidence and and outcome of AML in Rwanda. In 2012-2017, we identified 180 new AML cases among Rwandans versus 3,043 new AML cases diagnosed in Sweden. In-country incidence crude ratio (per 100,000 person-years) for AML was 0.27 in Rwanda and 5.17 in Sweden. After adjusting the incidence for age according to the world standard population, the age-standardized rate (ASR) was 0.30 in Rwanda and 2.62 in Sweden. Figure 15 is a diagram that compares age-specific rates of AML in Rwanda and Sweden. In both cohorts, there was a slightly higher male to female ratio, in accordance with previous reports [165]. AML affected younger patients in Rwanda than in Sweden. AML patients aged <30 years hence represented 61.4% in Rwanda and 5.6% in Sweden. The low incidence
and earlier onset on AML in Africans compared with Caucasians have been previously reported [208, 209, 255, 256], even in settings of socioeconomic and health equity. The rise in the number of detected AML cases in Rwanda since 2014 may be due to the increased number of diagnostic facilities as well as an increment of trained medical specialists in cancer diagnosis fields [31].

**Figure 15. Diagram of the ASR of AML in Rwanda and Sweden, 2012-2017**
The ASRs for AML are plotted on the left side for Sweden and the right side for Rwanda. AML, acute myeloid leukemia; ASR, age-standardized incidence rate

The characterization of AML in Rwanda is currently limited to the FAB classification using morphology and, in some cases, immunohistochemistry. Immunophenotyping using flow cytometry performed at Sahlgrenska Cancer Center showed that AML-M1 (AML without maturation) was the most frequent phenotype (43%) followed by AML-M2 (AML with maturation) (36%). One case of AML with associated myeloid sarcoma was recorded. Figure 16 shows the morphology by light microscopy of a peripheral blood film of an AML case (Figure 16A), histology of a myeloid sarcoma biopsy (Figure 16B) and an example of AML immunophenotyping using flow cytometry (Figure 16C). In studies of AML in western countries, M1- and M2-AML phenotypes were reported each to represent up to 10% of cases [162], which suggests that these FAB classes are overrepresented in the Rwandan AML cohort.
Figure 16. Morphology and immunophenotyping (flow cytometry) of AML

A. Peripheral blood film (PBF) of a patient with AML; most cells are myeloid blasts, with moderate cytoplasm and large hyperchromatic nuclei with some nuclear indentations (head-arrow); only few erythrocytes (arrow) are present (Giemsa stain, 1000X). B. Myeloid sarcoma mass biopsy, showing medium-sized cells with round nuclei. Nuclear indentation (head-arrow) is seen in some cells (Hematoxylin & Eosin stain, 400X). C. Flow cytometry immunophenotyping of an AML case. From upper left to lower right: gating around preserved PBMCs was followed by live cell gate (plot not shown), which in turn was followed by gating around CD45low cells. The following plots all show gated CD45low cells. The case displayed in C was positive for CD34, CD33, CD15 and HLA-DR, therefore subtyped as AML with maturation (M2). Microphotography (A and B) courtesy of the Butaro Hospital Pathology Laboratory – modified by author.

Initial analysis of the genomic landscape of AML in Rwanda suggested differences in the frequency of specific small nucleotide variations (SNVs) and insertions/deletions (INDELs) compared with AML cases in the TARGET-OHSU
databases comprising patients in the western world. Accordingly, mutations in **NOTCH1** were frequent in pediatric AML in Rwanda, **versus NRAS** and **KRAS** in TARGET cohort Africans and **NRAS** and **FLT3** in Caucasians in the western world (Figure 17). In adult AML, the most frequently mutated genes were **TET2** and **NRAS** in Rwanda, **FLT3** and **TP53** in Africans and **FLT3** and **DNMT3A** in Caucasians in the western world. For chromosomal aberrations, **BCR-ABL1**, which was most frequently detected in Rwanda (2/9 cases), was only encountered in 0.6% of patients in the TARGET-TCGA-OHSU database. The most frequent chromosomal aberration in the TARGET-TCGA-OHSU database was **KMT2A** amplification (8.1%), which was not detected in Rwandan cases. Earlier studies show that the most frequent genetic abnormalities associated with favorable outcome are **RUNX1-RUNX1T1** fusion (11% **versus** 2% for Rwandan and TARGET-TCGA-OHSU cohort) and **NPM1** mutation (23% and 11% in western world Caucasians and Africans, respectively, **versus** 0% in the Rwandan cohort) [195]. In addition, the most frequent adverse genetic abnormalities were **TP53** and **RUNX1** mutations (25% each in Rwandan pediatric cohort **versus** 0% in TARGET cohort, and 20% each in Rwandan adult cohort **versus** approximately 11% for **RUNX1** in both Africans and Caucasians, and 22% and 9% for **TP53** in Africans and Caucasians, respectively), and **BCR-ABL1** fusion (22% in Rwandan **versus** 0.6% in TARGET-TCGA-OHSU cohort). We thus propose, with the precaution that AML genet were available in a limited number of Rwandan AML cases, that genetic abnormalities associated with adverse prognosis are more frequently noted in Rwanda than in the western world.

**Figure 17. Frequency of mutations (A, B) and chromosomal aberrations (C) in AML**

The figures display the most frequent variations in the Rwandan cohort, and the most frequent variations from the comparative cohorts. Cases are from Rwanda (n=9) and the TARGET-TCGA-OHSU cohorts (n=1,505 cases for gene fusions, and n=813 cases for copy number abnormalities). For gene mutations, we show only cancer genes according to the TARGET (Caucasians n=114, Africans n=16) and OHSU (Caucasians n=514, Africans n=27) database information. AML, acute myeloid leukemia; TARGET, Therapeutically Applicable Research to Generate Effective Treatments; TCGA, The Cancer Genome Atlas; OHSU, Oregon Health and Science University.

The overall survival of Rwandan AML patients was poor with 23% 2-year survival. Of 128 AML patients with documented follow-up, 54 had received treatment from abroad or at local private clinics, while the remaining patients received palliation. As expected, the survival of patients who received any form of chemotherapy was better than patients receiving palliative treatment (log-rank test P<0.0001, hazard
ratio 0.1 with 95% CI of 0.05 to 0.26). Furthermore, the survival of the treated Rwandan pediatric patients was inferior to that of patients of African descent in western countries (log-rank test $P=0.012$, hazard ratio 0.33 with 95% CI of 0.14 to 0.79, Figure 18A). This difference was not observed in adult patients (log-rank test $P=0.65$, hazard ratio 0.86 with 95% CI of 0.45 to 1.65, Figure 18B). In addition, the survival of treated AML Rwandan patients was inferior to that of western Caucasian patients for pediatric (log-rank test $P<0.0001$, hazard ratio 0.10 with 95% CI of 0.03 to 0.28, Figure 18A) but not adult (log-rank test $P=0.13$, hazard ratio 0.70 with 95% CI of 0.44 to 1.11, Figure 18B) patients. Whilst there was no significant difference in overall survival between Rwandan adult and pediatric patients, it was observed that in western countries, pediatric AML patients (TARGET cohort) show considerably better survival than adult patients (TCGA cohort).

Earlier studies have established that AML patients of African descent show poorer survival than those of Caucasian descent [208, 209, 255, 256]. We observed that patients in the Rwandan AML cohort exhibited a high frequency of genetic abnormalities associated with adverse prognosis and a low frequency of abnormalities associated with favorable prognosis. Nevertheless, the shortage of therapy with curative intent in Rwanda is likely the main contributor to the poor survival noted in the Rwandan AML cohort. The introduction of curative therapy is likely to markedly improve AML survival, in particular in pediatric patients. Curative-intent therapy should be implemented along with the determination of predictive and prognostic groups using immunophenotyping and genomic profiling of AML cases.

In conclusion, improved detection of AML, treatment with curative intent and improved subtyping, including genetic features of leukemic cells, are direly needed in Rwanda.
4.3 PAPER III

This study assessed the incidence and outcome of ALL in Rwanda using Sweden as the comparator for incidence and a western world database (TARGET) as a comparator for outcome. In 2012-17, we observed 318 cases of ALL in Rwandans versus 871 cases in Sweden. Approximately 68% of patients in Rwanda were <15 years at diagnosis, versus 44% of patients in Sweden. There was a striking early peak age (0-4 years) of incidence in Sweden, while ALL tended to peak at 5-9 years in Rwanda (Figure 19). There was a second peak (60-65 years) of ALL incidence in Sweden that was less evident in Rwanda (Figure 19). These findings are coherent with reports of earlier age at ALL diagnosis in developed countries [189, 210, 212, 257]. The mechanisms explaining the higher incidence of infant ALL in developed countries are not known but may include delayed exposure to common infectious agents in developed countries [258-260].

![ASR of ALL in Rwanda and Sweden](image)

**Figure 19. Age-standardized incidence rates of ALL in Rwanda and Sweden**
The ASRs are presented on left for Sweden and on right for Rwanda. ALL, acute lymphoblastic leukemia; ASR, age-standardized incidence rate

The crude incidence rate (cases per 100,000 person-years) was 0.48 and 1.48 for Rwanda and Sweden, respectively. The age-standardized (adjusted to the world standard population) incidence rate (ASR) was 0.38 in Rwanda versus 1.61 in Sweden. Age-specific rates standardized to the world population are shown in Figure 19. Previous studies show low ALL incidence among Africans and lower incidence in developing versus developed countries [189, 210, 256], which may be explained by racial or environmental factors; however, insufficient detection of ALL likely plays a role [7, 261], (see also Paper IV).

The diagnosis of ALL in Rwanda was solely based on morphology, while subtyping used the French-American-British (FAB) classification into L1-L3 classes, and occasionally supplemented by immunohistochemistry to classify cases as B-ALL or T-ALL. Figure 20 provides examples of ALL cases morphology of a peripheral blood film (Figure 20A) and a bone marrow biopsy (Figure 20B).
Figure 20. Morphology and immunophenotyping (flow cytometry) of ALL

A, Peripheral blood film of a patient with ALL; most cells are lymphoblasts, with scanty cytoplasm and large round hyperchromatic nuclei; erythrocytes (arrow) are seen in background (Giemsa stain, 1000X).

B, Bone marrow biopsy, showing diffuse proliferation of medium-sized round cells with round hyperchromatic nuclei and scanty cytoplasm (Hematoxylin & Eosin stain, 400X).

C, Flow cytometry immunophenotyping of an ALL case where almost all CD45<sup>low</sup> cells were CD19<sup>+</sup> and CD10<sup>+</sup> but negative to smIg. The case was therefore classified as CD10<sup>+</sup> B-cell precursor (or common) ALL.

D, Flow cytometry immunophenotyping of another ALL case where CD45<sup>low</sup> cells were CD19<sup>-</sup> but CD7<sup>+</sup>, CD3<sup>+</sup>, and CD1a<sup>-</sup>, therefore corresponding to mature T-ALL. Microphotography (A and B) courtesy of the Butaro Hospital Pathology Laboratory – modified by author.

L1-L3 subtyping of ALL cases in Rwanda was done in 25% of cases. Further subtyping in Rwanda was, if performed, limited to determining B or T cell origin of disease (reported for 24% of the cases in our cohort). We performed
immunophenotyping using flow cytometry on 33% of the cases with available blood samples, as illustrated in Figures 20 C-D. Pro-B ALL and common B-ALL (CD10+ B cell precursor ALL) were the most common ALL immunophenotypes (46%) in the Rwandan cohort; a similar frequency of precursor B-ALL (48%) was observed in the TARGET cohort. Additionally, T-ALL was apparently overrepresented in the Rwandan cohort (27% according to flow cytometry and 37% of cases immunophenotyped using immunohistochemistry) compared to the TARGET cohort (15%).

The genomic profile of ALL cases in Rwanda was compared with that of ALL cases in the TARGET cohort. The TARGET database includes only children (<18 years old), and the vast majority of the Rwandan ALL cases were <18 years old (73%). We observed differences in the frequency of small and large variations between these cohorts. The most frequent variations in the Rwandan ALL cohort were rarely seen in the TARGET cohort [238, 262], and vice versa (Figure 21). For example, NOTCH1 and RUNX1 mutations were frequent in the Rwandan cohort but not observed in the pediatric TARGET cohort. This may partly be explained by these mutations being more common in T-ALL than in B-ALL. There were no T-ALL cases with available genomic analysis in the TARGET database. However, NOTCH1 and RUNX1 mutations were present also in Rwandan patients with B-ALL (50% of B-ALL versus 80% of T-ALL with NOTCH1 mutations, and 33% of B-ALL versus 20% of T-ALL with RUNX1 mutations). Furthermore, none of the 10 most common chromosomal aberrations in the TARGET database was found in Rwandan patients.

The overall survival of Rwandan ALL patients at 2 years of diagnosis was 39% versus >90% in the TARGET cohort. In the Rwandan cohort, young and adult ALL patients showed no difference in survival (log-rank test P=0.4, Figure 22A). Young

Figure 21. Frequency of mutations (A) and chromosomal aberrations (B) in ALL
The figures display the most frequent variations in the Rwandan cohort, and the most frequent variations from the comparative cohort. Cases are from Rwanda (n=13 cases) and the TARGET cohort for gene mutations (n=22 Africans and 103 Caucasians) [262] and for large structural aberrations (n=764 cases for copy number abnormalities and n=306 for fusion genes) [238]. For small variations, we show only cancer genes according to the TARGET database information. ALL, acute lymphoblastic leukemia; TARGET, Therapeutically Applicable Research to Generate Effective Treatments
Rwandan patients showed inferior survival compared with Africans (log-rank test \( P<0.0001 \), hazard ratio 0.23 with 95% CI of 0.17 to 0.33, Figure 22B) and Caucasians (log-rank test \( P<0.0001 \), hazard ratio 0.005 with 95% CI of 0.003 to 0.007, Figure 22B) in the TARGET cohort.

**Figure 22. Survival of ALL patients in Rwanda and TARGET (children) cohorts**

*Panel A.* Survival of ALL patients in Rwanda by age at diagnosis. *Panel B.* Survival of pediatric ALL patients (<18 years) in Rwanda and the TARGET (Africans and Caucasians) database. Censored cases are those alive at the last follow-up date and those lost-to-follow-up. Afr, Africans; ALL, acute lymphoblastic leukemia; Cauc, Caucasians; Rwa, Rwanda; TARGET, Therapeutically Applicable Research to Generate Effective Treatments

We analyzed malarial HRP-2 antigen (a marker for a recent *P. falciparum* infection) and SE36 antibody (a marker of protection against severe *P. falciparum* malaria) in serum from 168 ALL patients at diagnosis, using adult healthy donors (n=95) and AML patients (children and adults; n=110) as controls. We observed higher serum levels of HRP-2 (Mann-Whitney test \( P<0.05 \), Figure 23A) in ALL patients versus AML patients. A significant proportion of ALL patients showed very high levels of HRP-2 (circled in Figure 23A; Figure 23B-C). ALL patients with high HRP-2 levels frequently tended to have detectable EBV IgM in serum (Figure 23D). On the other hand, ALL patients showed lower titers of SE36 than AML patients (Mann-Whitney test \( P=0.023 \)) and healthy donors (Mann-Whitney test \( P<0.0001 \)) (Figure 23F).

The combination of high HRP-2 and low SE36 levels has been reported for African patients with endemic Burkitt leukemia/lymphoma, in whom EBV likely promotes the development of leukemia of B cell origin [146]. Our study detected a group of ALL patients with high HRP-2 levels (circled in a Figure 23A) that also showed a high frequency of seropositivity for EBV-IgM (44%). Unexpectedly, these cases of ALL did not show genetic or morphological features of Burkitt leukemia. Hence, all analyzed samples with high HRP-2 and EBV-IgM seropositivity showed L1 morphology, while Burkitt leukemia/lymphomas are of L3 phenotype [263]. Furthermore, out of the analyzed samples the t(8;14)(q24;q32) involving the MYC gene, a hallmark of Burkitt leukemia/lymphoma [162], was seen in only 1 of 13 (7.7%) Rwandan ALL patients and, unexpectedly, this was not one of the patients showing high HRP-2 levels. Our findings may thus reflect a previously unknown form of ALL, distinct from Burkitt leukemia, which is related to malaria and EBV
infection and thus likely only occurring in regions where malaria is holo-endemic. We cannot exclude, however, that ALL may have been misdiagnosed in these cases as the expansion of B cells, resulting from primary or reactivated EBV infection, may have been reinforced by concomitant malaria. Further studies are needed to clarify these issues.

**Figure 23. Malarial HRP-2 and SE36 and EBV-IgM in serum**

A, HRP-2 titers among ALL patients versus controls (AML patients and healthy donors). In addition, we compare the proportions of cases with a high HRP-2 (≥4000 units/µl) in ALL patients versus healthy donors (B) and ALL versus AML patients (C) using Fisher’s exact test. D, HRP-2 levels versus EBV-IgM among ALL patients. E, HRP-2 levels versus ALL-FAB types. F, SE36 titers among ALL patients and healthy donors. ALL; acute lymphoblastic leukemia; AML, acute myeloid leukemia; FAB, French-American-British classification; HD, healthy donors

In summary, the results presented in Paper III are in line with previous reports of poor survival after ALL diagnosis among Africans [189, 191, 210, 211, 228, 264, 265], and improved therapy, in particular in pediatric ALL, is needed. Our results also imply that genetic features of ALL cells, including NOTCH1 mutations, may be overrepresented in sub-Saharan Africa. The association between ongoing malaria and EBV for non-Burkitt leukemia/lymphoma merits further investigation.
4.4  PAPER IV

This study aimed to give an overview of the current health system utilization for acute leukemia in Rwanda by interviewing patients and health professionals. The analysis of information provided by the research participants shows that the low detection and poor outcome of acute leukemia in Rwanda is likely linked to multiple factors. These factors can be categorized into individual/societal barriers, healthcare system factors and health system administration factors. Nevertheless, to some extent, these categories overlap or are cross-cutting. For example, the tediousness of the referral system is in part due to poor knowledge of some healthcare professionals but also to the health system management. Table 13 summarizes the observations of the study respondents on factors affecting early detection and outcome of acute leukemia in Rwanda. Most of these factors were reiterated by all the categories of respondents (patients/guardians, healthcare professionals and health system administrators).

<table>
<thead>
<tr>
<th>Process</th>
<th>Category</th>
<th>Barriers</th>
<th>Examples</th>
<th>Comparative references</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decision to seek for healthcare</td>
<td>Patient/family/social factors</td>
<td>Poor knowledge/ cultural factors</td>
<td>Underestimation of the disease</td>
<td>[266-268]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Consultation of traditional healers</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fearing a bad diagnosis, or stigma</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Financial accessibility</td>
<td>No health insurance or no copayment fee</td>
<td>[268]</td>
</tr>
<tr>
<td>Healthcare facilities</td>
<td>Quality of healthcare services at a health facility (including private facilities)</td>
<td>Poor knowledge</td>
<td>Misdiagnosis</td>
<td>[269]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Scarcity of specialized professionals</td>
<td>Delay to diagnosis</td>
<td>[18, 270, 271]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Infrastructure shortage</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Procurement issues</td>
<td>Recurrent stock-out</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Financial (and geographical) accessibility</td>
<td>Lack of subsistence means (health facility located far; payment of caution fee)</td>
<td>[18, 268, 272]</td>
</tr>
<tr>
<td>Referral system</td>
<td>Poor knowledge, lack of collaboration, poor customer care</td>
<td>Poor knowledge</td>
<td>Health professionals unwilling or delaying to refer patients</td>
<td>[272-274]</td>
</tr>
<tr>
<td>Health system management</td>
<td>Tedious hierarchical referral system</td>
<td>Tiedious hierarchical referral system</td>
<td>Going through health facilities, even if it is known they can’t handle the case, until getting to the needed health service.</td>
<td></td>
</tr>
<tr>
<td>Clinical practice guidelines</td>
<td>Scarcity of protocols</td>
<td>No protocols for early detection and management of acute leukemia from basic health facilities</td>
<td>[275, 276]</td>
<td></td>
</tr>
<tr>
<td>Training issues</td>
<td>Lack of training for health professionals regarding acute leukemia detection and management</td>
<td>Lack of training for health professionals regarding acute leukemia detection and management</td>
<td>[269]</td>
<td></td>
</tr>
<tr>
<td>Prioritization</td>
<td>Acute leukemia not included in the priority cancers [11]</td>
<td>Therapy is available (low-intensity chemotherapy) for ALL, although ALL not listed among priorities</td>
<td>[277, 278]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lack of infrastructures for acute leukemia optimal subtyping [162]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AML has no curative-intent therapy in public health facilities</td>
<td></td>
</tr>
</tbody>
</table>

*ALL: acute lymphoblastic leukemia; AML, acute myeloid leukemia*
Similar factors have been reported as playing role in the utilization of health services and improvement of healthcare services in various diseases in other developing countries (references in Table 13). Problem-solving strategies to improve detection and outcome of acute leukemia in Rwanda, and other developing countries, could include education of the population to improve care-seeking behaviors [268], subsidization in care of patients diagnosed with cancer [268], developing cancer detection and management guidelines adapted to each level of healthcare facility [275, 276], design and implement a special referral system for cancer patients, continuous professional education for healthcare professionals and training of specialists in cancer fields, as well as including acute leukemia among the cancer disease priorities to be addressed.
5. CONCLUSIONS AND FUTURE PERSPECTIVES

Paper I

IFNL4 genotypes are known to predict the spontaneous clearance of hepatitis C virus (HCV) [63, 64, 66, 71], and IFNL4 genotypes associated with insufficient clearance of HCV are common in subjects of African descent [65], (Paper I). Our results suggest that the clearance of RNA viruses from the respiratory tract was impaired in Rwandan children with IFNL4 genotypes that are common in Africa. These findings may, in part, explain the morbidity of respiratory tract infections in sub-Saharan African children, although confirmatory studies are warranted. Further studies should also define the potential impact of e.g. IFNL4 variation on the clinical course of infections of the lower respiratory tract (including coronavirus infections), digestive system and urogenital tract, in children and adults.

Papers II, III and IV

We found a low incidence of ALL and AML compared with western countries. We assume that this trend may result, in part, from an insufficient detection rate in developing countries. Our preliminary results suggest that the genomic landscape of ALL and AML in Rwanda may differ from that observed in Africans or Caucasians in western countries. ALL and AML in Rwanda thus frequently entailed mutations and chromosomal aberrations conferring adverse prognosis. We propose that treatment programs for acute leukemia be further implemented in Rwanda, and that the need for such treatment is particularly imminent in pediatric leukemia. The potential role of ongoing P. falciparum and EBV infection for the occurrence of B cell neoplasms was also investigated, aiming to define the occurrence of Burkitt leukemia/lymphoma. We observed that >15% of newly diagnosed ALL cases in Rwanda had very high malaria antigens in blood, and that a significant proportion of these patients showed signs of ongoing EBV infection, despite that these cases did not show features of Burkitt leukemia. These findings merit additional study.
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“I will meditate also of all thy work, and talk of thy doings” Asaph the Psalmist, 1000 B.C.

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