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Determination of haematological and biochemical reference intervals for infants and children in Gabon

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1 Table of Contents

Table of contents

2 Introduction

2.1 The need for reference values

Reference values serve as the basis of laboratory testing. As one of the most used medical decision-making tools, they aid physicians in differentiating between healthy and non-healthy patients and guide them in taking appropriate clinical actions. Defined reference ranges are useful not only in clinical diagnostics but also in medical research studies where they are used to achieve high standards and adequate results for a population. Through the use of reference values, physicians are pointed toward a signifier of normality, which then leads them to a medical decision.

But how do we define “normality”? What is “normal” in medicine?

In the beginning of medicine, ancient physicians based their medical decisions on Galen’s theory of humoralism, the mix ratio of humours [55]. They used categorical thinking as a basis to describe abnormal states and thus, early on used “normality” and “abnormality” as limiting factors for health and disease. Seventeenth century physicians were observers who specified a homogeneous and continuous physiological field on the basis of categories. For example, Daniel Sennert (1572 – 1637) is notable for basing medicine on systematically aquired empirical knowledge [55]. He classified the state of a patient based on universal prevailing categories that were obtained by experience or observation, [55] and thus became one of the first physicians to categorize observations. His experience was gained by observing the human body’s physical structures and features. These factors, like other biological systems, result from evolution and therefore are part of a lengthy progression towards materialized equipoise or harmony. The term “normal“ serves as a crude term to describe the spectrum of “healthy” states while conversely, one uses the term “pathologic” to describe states not within the domain of normality. As a result, therapy was, and still is, universally seen as an intervention to normalize. However, this categorical

thinking is limited to only those health and disease phenomena which are easily captured within certain parameters.
In his writings, the French philosopher and physician Georges Canguilhem [102] takes a step forward in distinguishing between the virtual “pathologic abnormality“ of the disease from “evolutionary abnormality“. Canguilhem declares the “evolutionary abnormality” as empathic “normality“, which is overlapping with “normativity“, meaning “potency of life to create new life-forms“. However, confusing "pathologic abnormality" with "evolutionary abnormality" could lead to fatal mistakes because part of biological evolution, “the normativity of life”, involves a constant yet slight fluctuation in so-called “normal” biological parameters.

Since the advent of haematological measurement and analysis it has been shown that the cellular constituents of blood vary in number and are not stable constants. In fact, these elements are determined and influenced by several factors. Age and sex, for example, are very clear, definable, influencing factors [182, 185]. Neonates, who have an initial elevation of hemoglobin, then consistently demonstrate a decrease of hemoglobin concentration to 10 g/dl starting six to twelve hours postpartum and continuing until the first 3-6 months of life. Fetal and neonatal erythrocytes have a briefer life span (70 – 90 days) and a greater mean corpuscular volume (MCV 110-120 fL) than adults [157]. Erythrocytes enlarge as people age [78] and one can begin to see measurable sex differences in red cell values starting around 11 to 15 years of age. Mean haemoglobin levels for men over age 30 decline gradually, while in women these levels rise. After age 60, these sex differences diminish. Sex-dependent differences are also seen in black populations, as black males, for example, are found to have higher red cell values than black females. Additional sex differences are seen with leukocyte counts, as young children and women have higher values compared to men between the ages of 21 and 50 years, but then similar values after age 60 [36].
Furthermore, some behavioural patterns have been shown to influence the number of haematological cells. The mean leukocyte count differs significantly

across lifestyle factors (overall obesity, alcohol consumption, cigarette smoking, eating breakfast, nutritional balance, physical exercise and hours of work) [122]. It is higher in smokers, and increases in proportion to the intensity of smoking [78, 123, 146]. In addition, smokers have significantly decreased red blood cell (RBC) counts and increased mean cellular volumes (MCV) when they regularly consume alcohol and generally have larger erythrocytes than non-smokers [78, 172, 173]. Non-smokers, who regularly consume alcohol, have significantly increased WBC counts [59]. However, it is not only “unhealthy” life styles such as smoking and alcohol consumption that influence blood cells, but also “health” life styles involving intense physical exercise [25]. The “sports anaemia”, for example, that occurs in highly trained people (e.g. more than one year high intensity sports training twice a day/five days a week) is exhibited by lower red blood cell counts, lower packed cell volume, and lower haemoglobin concentrations than in untrained control groups [25, 35]. Eating habits also impact the blood count. Vegans, in comparison to lacto- or lacto-ovo-vegetarians, have lower lymphocyte counts and higher mean corpuscular volumes (MCV), as determined by iron and vitamin B12 status [127].

Blood parameters are determined not only by behavioural patterns, such as smoking and nutrition, or biological influences, such as age and sex, but additionally by environmental factors. Altitude is one of these well known influences. Exposing humans to increased altitude leads to a reduction in the partial pressure of oxygen due to the drop in barometric pressure. A rapid increase in the concentration of haemoglobin helps mountaineers to compensate for the fall in arterial oxygen saturation [183]. Residents of high altitudes, like Tibetans or Bolivians, who have adapted to their environment over thousands of years, were shown to have higher haemoglobin values in contrast to people living at sea level [18, 19].

Clearly, explication is only possible through comparison of different findings in the context of accurate science-based interpretation.

Contrary to easily detectable influences like altitude, age and sex, the exact factors which influence blood cells are not always definable. For example, why do Yemenite and Falashah Jews have lower values of neutrophil cells in their

blood than Caucasians [152]? Or why do haemoglobin and erythrocyte indices of boys and girls aged 7 to 10 years show a tendency to increase in autumn and winter, but decrease in spring [134]?

By the early thirties, the existence of specific population-based differences in haematological reference values was well known [23, 65]. Following Forbes, who in 1941 demonstrated leucopenic conditions among Negro workmen around the area of the Mississippi River [65], several subsequent studies reported differences on reference intervals for haematological and biochemical parameters according to sex, age and ethnic origin [4, 13-15, 21, 27, 32, 36-38, 45, 48, 57, 58, 61, 63, 67-69, 83, 104, 106, 107, 125, 128, 136, 138, 140, 143, 148, 149, 159, 186]. For instance, multiple data showed lower red blood cell parameters or modified white blood cell counts for African populations when compared to Caucasians. But why do African blood values differ so much from European or American values? Is it due to (mal-) nutrition? Even if Africans are found to be iron or Vitamin A deficient [121, 155, 158, 163, 168, 187], why then are African Americans, who are adapted to the “western” lifestyle, found to have lower red blood cell values than their Caucasian counterparts [21, 138, 140, 186]? Is it due to chronic or re-occurring infections, such as with helminths or plasmodia, that have an evolutionary impact on the structure and the function of red blood cells? And why are African blacks found to have lower haemoglobin values than Caucasians whereas the traditional “San” bushmen from Namibia and South Africa have haemoglobin values comparable to Caucasians [45]? Or why are black populations often neutropenic [61, 63, 79]? Assuming environmental factors – why are such variables in hematological factors found in expatriates [14, 15]?

Consequently, it is frequently difficult to interpret differences amidst varying ethnicities and it is therefore questionable that concepts of medical textbooks are not designed for such grievances [87, 157, 184]. Reference values printed in these books are derived from a Caucasian population, which represents only about 25 % (Europe and America) [1] of the world population, and thus do not account for other ethnic groups. Dallmann et al. [48], who found lower red cell parameters in a black population, assumed that 10% of the black population

would be deemed falsely anaemic if such ethnic differences were not taken into account. Because certain diagnoses are highly dependent upon laboratory values, one may reach the wrong diagnosis by using laboratory values representative of a different population. The danger in this is that a wrong diagnosis can then lead to a wrong therapeutic decision. As HIV therapy is based upon lymphocyte counts, so too is cancer chemotherapy based on hematological values. The difference, however, is that the “normal” range for lymphocyte values varies less than the cells whose quantity one uses as a threshold with which to provide toxic chemotherapeutic agents. Hershman et al. highlighted such risks in a study comparing African-American and white women with breast cancer. In this study it appears that one of the reasons why African-Americans might have had a less beneficial outcome, is that their continuation of chemotherapy was dictated by their having lower leukocyte counts, when in fact the lower limit of their leukocyte count might naturally be lower [79]. Due to neutropenia and thrombocytopenia among blacks Shija et al. [151] point out the necessity of using special defined counts to accurately provide tumour chemotherapy.

Put simply, a reference value for a particular laboratory parameter provides a range of acceptable values based on measured data of a defined cohort, that is characterised by equality of, for example, health status, race, gender or age.

The aforementioned conclusions question the validity of using reference values derived from data collected from non-local populations and limit the degree to which reference values can be generalised. To improve quality in health care and clinical research, it is therefore vital to develop reference values for local populations. The dangerous assignment of reference ranges derived from one population to a second, nonrepresented population may ultimately harm those subjects of the second population.

Many factors influence haematological parameters. To investigate these influences, to demonstrate the impact of diseases, or furthermore, to prove the tolerability, safety, and efficacy of therapeutic regimens, clinical research studies need to be done. Important physiological and biochemical parameters of study participants can be examined through the haemogramme with differential, and

liver or renal function tests. It is crucial to have reference values based on and oriented to special defined populations in order to avoid misinterpretion of findings and misconclusion in medical research. These reference values must derive from a part of the population which represents the study participants.

On these accounts, the focus of this thesis is the establishment of haematological and biochemical reference values for children aged 4 weeks to 9 weeks and 18 months to 5 years from a population in Gabon.

Regarding terminology, this thesis preferentially uses the terms “reference values” or “reference intervals” over the term “normal values”. The term “normal values” is not used because this phrase assumes that physiological variability is well understood and based simply on ethnic differences, but, as the abovementioned studies demonstrate, the observed differences between Caucasians and Africans actually are not fully understood.

The current determination of reference values is based on the methods of the National Committee for Clinical Laboratory Standards (NCCLS) of the Clinical and Laboratory Standards Institute (CLSI). The Clinical and Laboratory Standards Institute (CLSI) is “a global, nonprofit, standards-developing organization that promotes the development and use of voluntary consensus standards and guidelines within the health care community” [44] and was first accredited by the American National Standards Institution (ANSI) in 1977 [43]. As a World Health Organization Collaborating Center, the committee attempts “to improve the quality of clinical and laboratory services worldwide”. To promote its practice standards globally, the CLSI consists of over 2,000 member organisations and works with institutions like the International Organization for Standardization (ISO), the International Federation of Clinical Chemistry (IFCC) and the International Federation of Biomedical Laboratory Sciences (IFBLS) [42]. Other Institutions and companies such as the National Institute of Standards and Technology (NIST), American Association for Clinical Chemistry, Siemens Healthcare Diagnostics Inc., Bayer Corporation, GlaxoSmithKline and Roche Diagnostics, Inc. support the CLSI “to maintain its position of leadership in developing needed documents […]” [41].

The conclusions of this thesis are based on the raw data derived from haematological and biochemical analytes of healthy Gabonese children who were seen during the screening visits of two anti-malarial vaccine trials in the Medical Research Unit at the Albert Schweitzer Hospital in Lambaréné. One cardinal difference between these trials concerns the participants' ages. The first trial was conducted in the spring of 2006 and concentrated on children aged 18 months to 5 years (60 months). In the second anti-malarial vaccine study, which is ongoing (as of October 2008), venuous blood from children aged 4 weeks to 9 weeks was drawn between May 2007 and January 2008. These two studies were chosen for calculation of reference values for the following reasons: Firstly, because blood collection was an obligatory component for the participation in each trial, the data could be used for value estimation without taking additional blood samples or performing additional informed consent. Secondly, because a thorough history and medical examination were performed at the start of study participation and because patients were followed closely over time, the probability that the blood samples herein analysed were from healthy participants is quite high. A further advantage of using the analytes from the vaccine trials conducted in the Medical Research Unit (MRU) concerns data quality. The MRU has conducted numerous clinical trials in accordance with ICH/GCP standards, and because the data is derived using written standard operating procedures (SOPs), regular internal and external controls of technical equipment and qualified medical personnel, the quality is assuredly high. Both trials were monitored by external study monitors to ensure accuracy and consistency of the data.

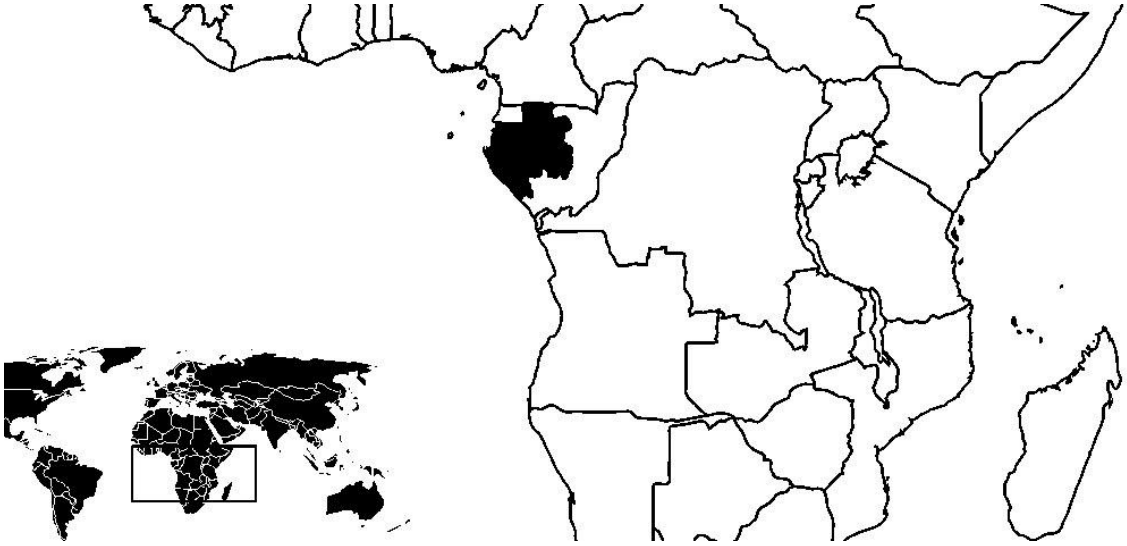
The major parameters of whole blood count with differential count were measured, as well as three biochemical parameters commonly used in paediatrics.

3 Materials and Methods

3.1 Geographical data of study site

3.1.1 Geography and climate of Gabon (République du Gabon)

Fig. 1. Location of Gabon in Central Africa [176]



Gabon is located in Central Africa, bordered by the Atlantic Ocean in the west, which creates a coastline of 885 km. The Republic of the Congo lies to the east and south of Gabon. Equatorial Guinea and Cameroon border Gabon in the north. The country's territory comprises 257,667 sq km [39].

Located on the equator, Gabon has a tropical climate with an extensive system of rainforests which cover 85% of the country. Gabon's largest river is the Ogooué which is 1200 km long [178]. There is a difference of 1575 m from Gabon's lowest point, the Atlantic Ocean to its highest point, Mont Iboundji [39].

The climate is hot and humid with high temperatures all year round. The average temperature is 22° C – 32° C with a humidity of 85% and an average rainfall between 2000 and 3800 mm^3. There are four seasons throughout the year. There is a dry season during December and January, followed by a wet season between January and May, followed by another dry season from June until August, and then there is a second wet season from September through December [3].

3.1.2 Population of Gabon

Gabon has a population of around 1 500 000 inhabitants. Children, aged 0-14 years, account for a majority of the population with 42 %. People aged 15 to 64 years comprise 54 % of the Gabonese population. A minority of the population is over the age of 65, comprising only 4% of Gabon's population. The median age is 18.6 years. 63 % of the total population are able to read and write (male: 74%, female: 53%).

Gabon enjoys a per capita income four times that of most of sub-Saharan African nations. This has supported a sharp decline in extreme poverty; yet, because of high income inequality, a large proportion of the population remains poor. A small population with oil and mineral reserves has helped Gabon become one of Africa's wealthier countries with an actual human development index (HDI) ranking of 119 (South Africa 121, Sierra Leone 177, Norway 2, Germany 22) [165].

The local population consists of different ethnic groups, the majority of which are Bantu tribes, including four major tribal groupings (Fang, Bapounou, Nzebi, Obamba). The different tribes merged over time and the people of Gabon became a homogenous population [39, 147, 175, 178]. Furthermore, as part of the Central African region, some commonalities such as malaria endemicity, language origin (Bantu) and vegetation exist between Gabon and neighbouring countries. The following maps (figure 2 – 4) demonstrate these commonalities of the Central African region. The tropical rainforest, widespread in Central Africa, determines the daily life in this region. The rainforest serves not only as a resource for several industrial sectors, but is an important source of food and income for the people living within and near it. Hunters and farmers use it to feed their families or to sell the products and animals at local markets. One further characteristic of the Central African region is the high to very high endemic malaria transmission [174] with an estimated incidence of around 0.2 to 1.5 *P. falciparum* episodes per person per year [101, 111, 174] (see 5.1.1.1).

It is difficult to distinguish or compare several tribes and families in order to derive similarities or differences between them. A frequently used and well known ethnological method is the comparison of languages and dialects from

several areas. The people of Central Africa are comprised of several subpopulations, each with their own languages or dialects, and these languages all belong to one larger family of languages, the Bantu language (Niger-Congo B) [177]. Ethnologists assume an expansion of the Bantu people over several thousands of years [177] and, although the ethnologists do not agree on how this occurred, they all state a common origin of Central Africa's people [177].

Fig. 2. Endemic distribution Of Malaria in Africa [109]

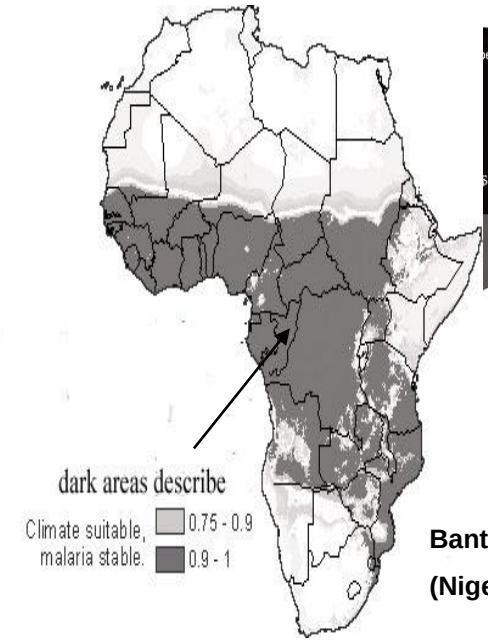


Fig. 3. African language families [52]

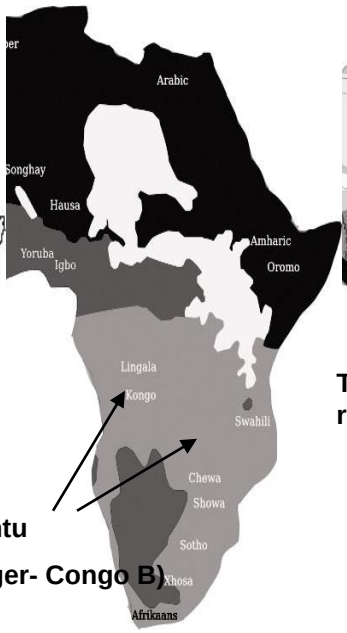
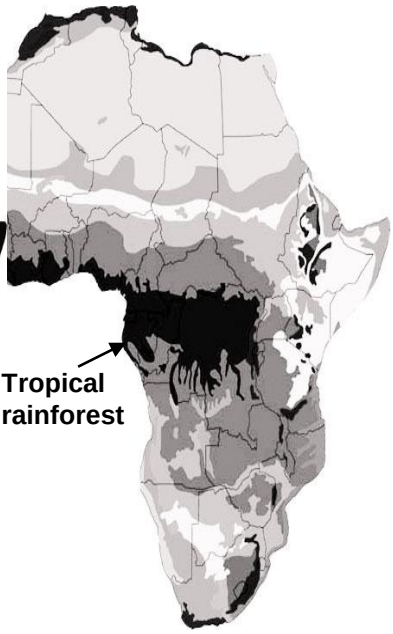


Fig. 4. Vegetation in Africa [6]



3.1.2.1 Health

The infant mortality rate is 54 deaths per 1000 live births. At birth a child has a life expectancy of 54 years [39] in contrast to the average life of 48 years in the African region (WHO) [175]. The data from 2000-2001 show that 21 % of children under 5 years were stunted for age. 12 % were determined to be underweight for their age. 4 % were diagnosed as overweight [147, 175].

In 2000-2003, malaria, as cause of death in children under 5 years of age, was only surpassed by miscellaneous neonatal diseases like diarrhoea (in 35% of all deaths). 28% of the children under 5 years of age died from malaria, 11% from pneumonia and 10% from HIV/AIDS [175].

The HIV/AIDS prevalence rate is decreasing. The estimated adult HIV/AIDS prevalence rate in 2003 was 8 % with 3 000 (2003) deaths caused to HIV/AIDS [39] and in January 2008 the estimated HIV/AIDS prevalence rate was 6 % [139]. The prevalence of tuberculosis in 2004 was at 339 cases per 100 000 for both sexes, compared to 518 cases per 100,000 in the WHO African region [175]. In 2004 the immunization coverage among 1-year-olds was 55% against measles and 38% for DTP3 immunization in cases surveyed in Gabon. This is in contrast to the 66% immunization coverage against measles and DTP3 in the WHO-defined “African region”.

3.1.2.2 Lambaréné, the Albert Schweitzer Hospital and the Medical Research Unit

Lambaréné is the capital of the political district Moyen-Ogooué. Located about 50 km south of the equator and 180 km southwest of the national capital of Libreville, Lambaréné with about 20,000 inhabitants is situated in the Central African rainforest at the Ogooué river.

Today, the Albert Schweitzer Hospital consists of three major departments (internal medicine, paediatrics, surgery) and additionally has a maternity clinic, a dental clinic and a psychiatric ward. Public health matters are addressed by the PMI (Protection Maternelle et Infantile) through their vaccination services and through their HIV/ AIDS counselling and testing. The hospital has 150 beds and a staff of approximately 180 people. In 2007, there were 21,420 consultations performed and roughly 5,500 patients hospitalised. On the paediatric ward children with respiratory illness were seen most commonly followed by malnourished children [2].

Since 1981 a Medical Research Unit has been in place, currently under the direction of Prof. Dr. Kremsner, and with a focus mostly on malaria research. The main scientific areas of interest are parasite biology, pathophysiology, pathogen-host interaction, host immune response as well as clinical aspects and chemotherapy of malaria. Other areas of research include schistosomiasis, filariases and mechanisms of allergy development.

3.1.3 Haemic and parasitic diseases in Lambaréné

Malaria transmission in the study area is high and there is little seasonal variability in transmission rates or parasite prevalence [161, 179]. *Plasmodium falciparum* is the predominant species and responsible for 95% of all infections. The main vectors are *Anopheles gambiae* and *An*. *moucheti*. The entomological inoculation rate lies around 50 infective bites per person per year [160]. The level of chloroquine resistance is high, reaching 100% both in vitro and in vivo[24]. Resistance of local parasite strains to sulfadoxine/pyrimethamine is moderate and rising steadily. First line treatment of Plasmodium falciparum malaria in Gabon is Artemisinin-based Combination Therapy (ACT).

In Lambaréné bednet use is highest among the poorest families, but the condition of these nets is the worst [71].

Depending on area of residence, the prevalence of schistomiasis infection varies and increases with age [166].

Intestinal helmints are highly prevalent among children living in Lambaréné. In 1998, 46% of the children, who participated in one study, were estimated to be infected with *Ascaris lumbricoides* and 71% with *Trichuris trichiura*. Median egg loads for *Ascaris* were found of 25,560 eggs/g and for *Trichuris* of 1240 eggs/g [167].

3.2 Material

3.2.1 Laboratory standards

All laboratory standards are recorded in the Standard Operating Procedures (SOPs) of the MRU Lambaréné. The following procedures are part of the SOPs.

3.2.1.1 Material for blood sampling

For blood sampling, tubes from Sarstedt (S-Monovette®) were used. For haematological analysis, EDTA served as anticoagulant in a concentration of 1.2 – 2 mg EDTA per one millilitre blood. For biochemical serum analysis S-Monovette® were used with a propriety coagulator. For coagulation, blood had to stay in the tubes and coagulate for 20 to 30 minutes. After centrifugation biochemical analysis of serum was undertaken.

In the elder group of children, aged 18 months to 60 months, blood was taken in a closed system with a “Butterfly®” needle produced by Hospira, Inc. The infants were stuck with a sterile cannula. In this case, blood was taken in an open system and dropped from the needle into an opened S-Monovette®.

3.2.1.2 Collection, handling and storage of blood samples

All blood samples were taken in the morning following medical observation and examination.

Children aged 4 to 9 weeks were punctured on the back of the hand using a sterile cannula. EDTA tubes were opened and the correct amount of blood between 0.5 ml and 2.7 ml was dropped from the cannula into the tube. The children aged 18 months to 5 years were venipunctured in the antecubital vein with a “Butterfly®” needle and EDTA tubes were used for collection and anticoagulation. For biochemical analysis, coagulant tubes were used in the same way.

The labelled tube was mixed immediately by gently inverting the tubes several times. It was stored in the examination room which is cooled down to 25°C. Haematological samples were analysed within 30 to 60 minutes after blood collection. Biochemical analysis was done after 30 minutes of coagulation in the

tube. Within one hour after sampling the serum was analysed. Sampling and analysing time for each tube was documented in logs.

3.2.2 Analysis instruments

3.2.2.1 Haematology analyser

For haematology, an ABX Pentra 60 machine was used. Davis et al. [50] showed the performance of the ABX Pentra 60 to be comparable to other haematology analyzers for all complete blood counts (CBC) and differential parameters.

3.2.2.2 Description

The ABX *PENTRA 60* is a fully automated haematology analyzer used for in vitro diagnostic testing of whole blood specimens. It is able to measure WBC, LYM % and #, MON % and #, NEU % and #, EOS % and #, BAS % and #, LIC % and #, ALY % and #, RBC, HGB, HCT, MCV, MCH, MCHC, RDW, PLT, PDW*, MPV, PCT*. PDW and PCT have not been established as indications for this product in the United States. The use of PCT and PDW should be restricted to research and investigational measurements only [84].

3.2.2.3 Installation of ABX PENTRA 60

The PENTRA 60 is located on a clean and level workbench in a special room without windows, thus avoiding exposure to sunlight. The instrument is not exposed to water or vapour and is free from vibration or shock. The ABX PENTRA 60 operates in a dry room cooled to between 20°C and 24°C. Controls of room temperature are performed three times per day.

3.2.2.4 Haematological quality control

When carrying out the daily start-up, three levels of controls (low, normal and high) are run. The results are printed regularly, filed and viewed daily on Levy-Jennings charts. Regular inter-laboratory comparisons (10 samples every 3 months) are run between the laboratory of the MRU and the laboratory of the Albert Schweitzer Hospital. The results of these comparisons are documented by a technician.

External quality control is performed by UKNEQAS. All schemes are accredited by Clinical Pathology Accreditation (UK) Ltd. Two whole blood specimens are issued per distribution for the Full Blood Count, with one distribution per month, for a total of 12 distributions per year, thus 24 specimens are examined per year. The specimens are prepared from pooled human donations, partially fixed and treated with antibiotics. The analytes to be measured are WBC, HGB, RBC, HCT, MCV, MCH, MCHC and PLT.

3.2.2.5 Known interfering substances

Due to clinical examination and standardized history taking, many confounders such as multiple myeloma, cytotoxic and immunosuppressive drugs (which may increase the fragility of the leukocytes, cause low WBC counts and cause low PLT counts), cold agglutinizing disease (which could cause lower RBC, PLT counts and increased MCV), nutritional deficiency and blood transfusion (which may cause high RDW results due to iron and/or cobalamin and/or folate deficiency) were excluded.

3.2.2.6 Biochemical analyser

Biochemical analysis was performed with the ABX MIRA PLUS. It is located in a dry, cooled room (20°C-25°C). Daily controls of room temperature are carried out.

The results are derived from calculations that are performed on the absorbance values of the photometric measurement. All absorbance values used for the calculations are checked to make sure they are less than 3.5 absorbance units. A sample may fail this check, if it is lipemic, icteric or haemolytic. Visual inspection by the operator excluded conspicuous samples and therefore avoided invalid results [5].

3.2.2.7 Biochemical quality control

Normal and abnormal controls are run daily after calibration and are viewed daily on Levy-Jennings charts. The results are regularly printed and filed. A quality report is printed at the first of every month before the daily routine work starts. The report contains all the first values generated for each test. Regular inter-laboratory comparisons are run every 3 months between the laboratory of the

MRU and the laboratory of the Albert Schweitzer Hospital. Depending on the specimens' availability at the hospital laboratory, 2 or 3 biochemical parameters of 10 blood samples are analysed. All results are documented by a technician.

External quality control is again performed by UKNEQAS. The scheme is dispatched every two weeks.

3.3 Methods

3.3.1 The estimation of reference values for Gabonese children on the basis of the NCCLS approved guideline C28-A2 for the definition and determination of reference intervals in the clinical laboratory

In this thesis the calculation of the reference intervals is based on the approved Guideline (2nd Edition) of the National Committee for Clinical Laboratory Standards (NCCLS) “How to Define and Determine Reference Intervals in the Clinical Laboratory” (NCCLS document C28-A2) [124]. Methodological approaches and recommended procedures for establishing reliable reference intervals for use in clinical laboratory medicine are described.

3.3.1.1 Definitions

The following definitions are excerpts from the NCCLS document C28-A2 [124] pp. 3-4.

Following the propositions by the Expert Panel on Theory of Reference Values (EPTRV) of the International Federation of Clinical Chemistry (IFCC) and the International Council for Standardization in Haematology (ICSH) the presented definitions present a universal terminology to achieve a relatively unambiguous description of the subject of reference values.

Reference individual: “A person selected for testing on the basis of well-defined criteria. It is usually important to define the person’s state of health.”

Reference population: “A group consisting of all the reference individuals. The reference population usually has an unknown number of members and, therefore, is a hypothetical entity.”

Reference sample group: “An adequate number of persons selected to represent the reference population.”

Reference value: “The value (test result) obtained by the observation or measurement of a particular type of quantity on a reference individual. [These] reference values are obtained from a reference sample group.”

Reference distribution: “The distribution of reference values”. The parameters of the hypothetical distribution of the reference population may be estimated using the reference distribution of the reference sample group and adequate statistical methods. (p. 3, [124])

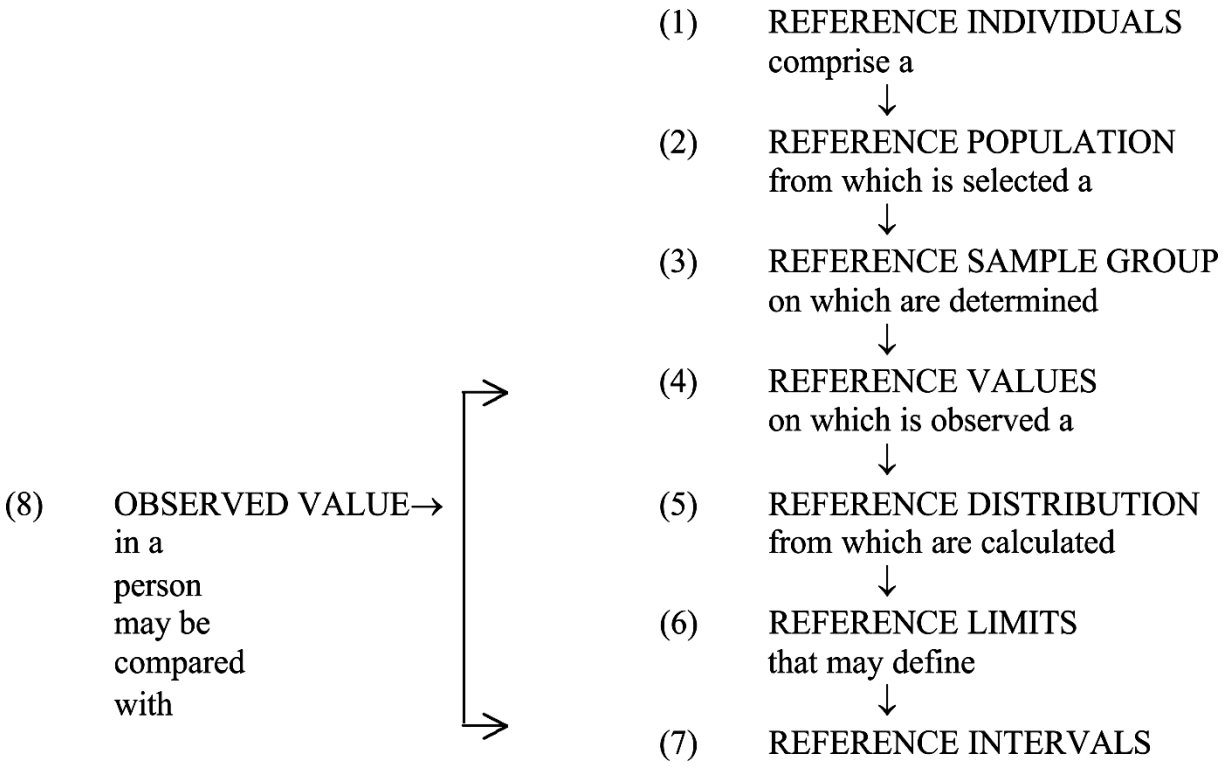
*Reference limit: “*A value derived from the reference distribution and used for descriptive purposes.”

Reference interval: “The interval between, and including, two reference limits.”

Observed value: “The value of a particular type of quantity, obtained by observation or measurement of a test subject (i.e., patient), to be compared with reference values, reference distributions, reference limits, or reference intervals.”

The following scheme demonstrates the relationship between the terms defined.

Fig. 5. Scheme of relationship between the defined terms (taken from the NCCLS document C28-A2 [124])



3.3.1.2 Analysis of reference values

The NCCLS proposes a nonparametric method for the estimation of reference values. The following descriptions are adopted from page 13 of the NCCLS document [124].

Basically there are two general statistical methods for determining reference limits – the parametric method and the nonparametric method. Corresponding to Solberg et al. [156] the “nonparametric method of estimation makes no specific assumption about the mathematical form of the probability distribution represented by the observed reference values.” Contrary to the nonparametric estimation method “the parametric method assumes that the observed values follow a Gaussian probability curve.” “Because the reference values of many analytes do not follow the Gaussian form, use of the parametric method requires that they be transformed to some other measurement scale, which will "normalize" them. This requires selecting the most suitable transformation (e.g., log, power, or some other function of the original scale) and then testing whether, on this new scale, the reference values do indeed appear to conform to a Gaussian distribution. This involves some moderately complex statistical theory and corresponding computer programs.”

The NCCLS states (p.13, [124]) that the simpler nonparametric method for estimation of reference values depends only on the ranks of the reference data. These data are arrayed in order of increasing size. The reliability of the achieved reference values depends, according to the NCCLS, more on a “proper selection of reference subjects, testing an adequate number of subjects and avoidance of preanalytical sources of error” than on the statistical method used. Therefore, the NCCLS recommends the nonparametric method of computing the lower reference limit (the 2.5th percentile) and the upper reference limit (the 97.5th percentile) (p.13, [124]).

3.3.1.2.1 Minimum number of reference values

“The nonparametric method is based solely on the ranks of the observations (in order of magnitude) and ignores their measured values” (p.13, [124]). Consequently, “it is impossible to distinguish between two percentiles of a

distribution that are P% apart unless at least $n = (100/P) - 1$ observations have been obtained". For example, "to estimate the 2.5th percentile distinct from the 5th percentile, or the 95th percentile distinct from the 97.5th percentile, a minimum of 39 samples are required." The 2.5th percentile of the population is represented by the smallest observation in the sample, while the largest observation of the sample estimates the 97.5th percentile. In this case P equals 2.5 and using the term for estimation of the population size ($n = (100/P) - 1$) yields an $n = 39$, and thus 39 samples are required (p.13, [124]).

In reference to Reed et al. [137], the subcommittee of the NCCLS Guideline recommends a minimum size of 120 observations, one from each reference subject. By obtaining a sufficiently large number of samples it would be possible to reduce vulnerability to outliers. One may calculate the two-sided confidence intervals from the data. In that case the experimenter has an interval derived from the sample values that includes the true 2.5th percentile within the population. By using the following term

$$(1) \qquad \sum_{j=a}^{b-1} \binom{n}{j} (0{,}025)^j (0{,}975)^{n-j} \geq 0.90 \quad [137]$$

with the ath lowest sample value describing the lower limit of 90% confidence interval for the 2.5th percentile in the target population and the bth lowest sample value representing the upper limit of 90% confidence interval for the 2.5th percentile, Reed et al. [137] showed that the smallest sample size which permits 90% confidence intervals for the reference limits is n = 120 (p.280-281, [137]). To estimate reference limits for the 2.5th and the 97.5th percentile with 95% confidence, 153 observations are required. To reach 99% confidence, 198 examples are to be obtained [137].

The threshold value of $n = 120$ should be strictly adhered to. In an observation set of $n = 120$ examples, any deleted aberrant or outlying observations should be substituted with additional subjects "until at least 120 acceptable reference values are obtained for each determination of a reference interval" (p.14, [124]). For obtaining separate intervals "for different subclasses (by sex or age-class),

each such interval should be based on the recommended number of 120 reference observations" (p.14, [124]).

3.3.1.2.2 Treatment of outlying observations

"Unless the number of observations is extremely large, reference range estimation by nonparametric methods almost entirely depends on the extremely lowest and highest values" (p.282, [137]). Thus, one or two persons who are subclinically ill, but were included into the sample "could have a considerable influence on the final reference range estimate" (p.282, [137]). Technical or clerical errors could be a risk and also influence the estimate. When such values are included with the others, they are practically unidentifiable, unless the person performing the haematological and biochemical analysis happens to know that these observations represent atypical analytical conditions or are the result of some arithmetic or procedural mistake. Such "aberrant" values often lie outside the range of the remaining measurements and are easily identified as "outliers" requiring special mathematical attention.

To detect such outliers mathematically, different techniques are described [10, 17, 73]. According to the NCCLS, the majority of these techniques assume that the observed reference values are homogenous random samples from a Gaussian distribution. The problem of masking less extreme outliers is always possible when any test for outliers is performed on extreme values individually (p.15, [124]).

The NCCLS subcommittee, based on the work of Reed et al. [137], proposes use of the Dixon test for reference value estimation [53]. The goal of this test is to reject an extreme observation (large or small) by assessing the ratio *D/R*, where D is the absolute difference between an extreme value $x_{(n)}$ and the next value $x_{(n-1)}$. *R* represents the range of all observed values, including the extreme observations. Reed et al. [137] recommend a cut-off value of 1/3 for the ratio *D/R*. Extreme values who are equal to or greater than one-third of the range *R* have to be deleted. This might produce some problems. If two or three outliers exist in the same example on the same side of the distribution, the *D/R* rule may fail to detect the most extreme value as statistically significant. Nearly similar

observed extreme values that could also be outliers are masked and thereby not detected. The NCCLS subcommittee suggests treating such observations as either one group or as only one outlier. If the Dixon rule detects such values as outliers, then the whole group should be deleted from the sample. Proper medical examination for the inclusion of healthy and exclusion of unhealthy participants should always precede mathematical methods such as the Dixon test. Furthermore, Dixon et al. [53] highlighted the importance of obtaining a sufficient number of values to reduce the risk of obtaining samples from subjects in early stages of disease, as this might induce outliers that cannot be resolved by a mathematical formula. Careful selection of individuals at the beginning of the study is as important as reaching a sufficient sample size. For this, and on the basis of Dixon et al. [53], the NCCLS recommends a sample size of 120 observations (p.15, [124]).

3.3.1.2.3 Partitioning of reference values

For clinical usefulness, reference values sometimes must be separated into subclasses. The NCCLS subcommittee advises to consider subclass reference intervals at an early stage in the analysis. Biological factors, such as age or sex, may bias the reference values and thus the sample may need to be divided into subclasses (p.15, [124]).

If subclass distinction appears clinically significant, 60 subjects should be sampled in each subclass at the beginning. If further analysis of these 60 observations show significance, each subclass should be comprised of at least 120 values (p.16, [124]).

To decide whether reference values must be divided into subclasses, approximately 60 samples for each subclass are needed. To measure statistical significance of the difference between two subclasses, the NCCLS subcommittee references Harris et al. [76] and recommends calculating the statistical significance of the difference between subclass means by the standard normal deviate test. Let $\bar{x}_1$ and $\bar{x}_2$ be “the observed means of the two subgroups, s_1^2 and s_2^2 the observed variances, and n_1 and n_2 the number of reference values in each subclass” (p.16, [124]). Then is z

(2) $$z = \frac{\bar{x}_1 - \bar{x}_2}{\left[\left(\frac{s_1^2}{n_1}\right) + \left(\frac{s_2^2}{n_2}\right)\right]^{1/2}}$$ (p.16, [124])

the calculated statistic which has to be compared with a “critical” value [76].

To calculate the “critical” value z^*, the following term should be used:

(3) $$z^* = 3\left[\left(n_1 + n_2\right)/240\right]^{1/2}$$ (p.16, [124])

“In addition, the larger standard deviation, for example s_2, should be checked to see whether it exceeds 1.5 s_1, or if $s_2/\left(s_2 - s_1\right) < 3$ [76]” (p.16, [124]).

Partitioning into subclasses should be based on these formulas. If the calculated z value is greater than z^*, or if the larger standard deviation exceeds 1.5 times the smaller standard deviation (irrespective of the calculated z-value), then the reference group should be divided into subclasses and reference intervals should be established for each subclass, supposing that the two different reference intervals are of equal importance in medical practice and relate to true medical variables (p.16, [124]).

Whether or not the values conform to a Gaussian distribution, the “z-test is essentially a nonparametric test and may be applied to the original data” (p.16, [124]). If the original data are highly skewed and a transformation nearly reaches a Gaussian distribution, use of these transformed values for the z-test is preferable (p.16, [124]).

To further understand the tables in the “Results” section, the following definitions are given.

i) $$z = \frac{\bar{x}_1 - \bar{x}_2}{\left[\left(\frac{s_1^2}{n_1}\right) + \left(\frac{s_2^2}{n_2}\right)\right]^{1/2}}$$

ii) $$z^* = 3\left[\left(n_1 + n_2\right)/240\right]^{1/2}$$

iii) Test A: $StdDev(great)/StdDev(small)$, (use subclasses if Test A> 1.5)

iv) Test B: $StdDev(great)/(StdDev[great]-StdDev[small])$ (use subclasses if Test B < 3)

3.3.1.2.4 Confidence intervals for reference limits

The NCCLS subcommittee describes the importance and the use of confidence intervals as follows (p.21-22, [124]). “The reference limits computed from a sample of selected subjects are estimates of the corresponding percentiles in the population of persons studied. Another sample of persons from the same population would probably yield somewhat different reference limits. A useful way of recognizing and assessing the variability in sample estimates is by computing a confidence interval for the population percentile being estimated, using information provided by the sample. In the present case, a confidence interval is a range of values that includes the true percentile (e.g., the 2.5th percentile of the population) with a specified probability, usually 90 or 95%. This probability is called the “confidence level” of the interval.

The concept of confidence intervals rests on the presumption that a representative sample of observations (in the case of the subject of this document, reference individuals) has been drawn from some defined population. This implies that each member of the population is equally likely to be selected. This ideal is often not attained in practice. The most that can be expected is that the sample of reference individuals selected will be, in fact, healthy persons from whom reference specimens will be secured under the recommended preanalytical conditions. The reference individuals are at least randomly obtained from a described pool, e.g., laboratory employees. Therefore, the basic assumptions for the validity of confidence intervals are that the observations are obtained independently of each other and that the reference sample is representative of the population even if not drawn strictly at random.

Nevertheless, confidence intervals are useful for two reasons. First, they remind the investigator of the variability of estimates and provide a quantitative measure of this variability. Second, confidence intervals narrow as the size of the sampling increases. Therefore, an investigator may choose a larger sampling of reference

individuals in order to obtain improved precision in the estimated reference interval.

Nonparametric confidence intervals are given by the observed values corresponding to certain rank numbers. Table 1 [137, 156] shows the rank number defining the 90% confidence interval (CI) for the 2.5th percentile based on a given sample size.

Table 1. Rank Number Defining the 90% Confidence Interval for the 2.5th Percentile [137]

No. of Sample, *n*		**Rank***	
From	To	a	b
120	131	1	7
132	159	1	8
160	187	1	9
188	189	1	10
190	216	2	10
217	246	2	11
247	251	2	12
252	276	3	12
277	307	3	13
308	310	3	14
311	338	4	14
339	366	4	15
367	369	5	15

**a^{th} lowest sample value = lower limit of 90% confidence interval for 2.5 percentile in target population; b^{th} lowest sample value = upper limit of 90% confidence interval for 2.5 percentile in target population. To obtain ranks corresponding to a 90% confidence interval for the 97.5 percentile, substract the values given for a and b from n + 1 [137].*

For example, when the reference sample consists of 120 persons, the observations corresponding to rank numbers 1 and 7 define the 90% confidence interval for the lower reference limits. To obtain the comparable rank numbers defining the 90% confidence interval for the upper reference limit, these rank

numbers are subtracted from 121 (in general, *n* + 1), giving 114 and 120. Thus, the smallest observation serves as the lower limit of the 90% confidence interval for the lower reference limit, while the largest observation is the upper limit of the 90% confidence interval for the upper reference limit" (p.21-22, [124]).

3.3.1.2.5 "Critical values"/Medical Decision Limits

The subcommittee of the NCCLS points out that the NCCLS guidelines are not intended to define "critical values" or other medical decision limits. The discrepancy between decision limits and reference limits is that scientific and medical knowledge serve as the basis for decision limits and may be related to a specific medical condition. For this, the medical decision limits, rather than the "healthy reference intervals", should accompany the patient's results on the laboratory report (p.28, [124]).

3.3.2 Selection of Reference Individuals

3.3.2.1 Reference sample group

3.3.2.1.1 Recruitment

The data for establishing the reference values derive from the healthy study participants of two vaccine trials carried out at the Medical Research Unit in Lambaréné, Gabon. Both trials, performed in two different age groups, examined the safety and immunogenicity of GlaxoSmithKline Biologicals' candidate Plasmodium falciparum malaria vaccine RTS,S.

For the first study, children aged 18 months to 60 months were screened. The study population will be referred to as "CHILDREN COHORT".

The study is entitled:

"A Phase II randomized, double-blind bridging study of the safety and immunogenicity of GlaxoSmithKline Biologicals candidate Plasmodium falciparum malaria vaccine RTS,S/AS01E (0.5 mL dose) to RTS,S/AS02D (0.5 mL dose) administered IM according to a 0, 1, 2- month vaccination schedule in children aged 18 months to 4 years living in Gabon".

For the second study, infants aged 4 weeks to 9 weeks were screened. The study population will be referred to as “INFANT COHORT”.
The study is entitled:
“A Phase II randomized, open, controlled study of the safety and immunogenicity of GlaxoSmithKline Biologicals. Candidate Plasmodium falciparum malaria vaccine RTS,S/AS01E, when incorporated into an Expanded Program on Immunization (EPI) regimen that includes DTPwHepB/Hib, OPV, measles and yellow fever vaccination in infants living in malaria-endemic regions”.

The present anlysis is based on values from blood sampling performed at screening. At this time point, medical history was taken and a medical exam was performed to ensure that the study subjects were healthy.

3.3.2.1.2 Ethics

The “CHILDREN COHORT” trial was approved by the “Ethics Committee of the International Foundation of the Albert Schweitzer Hospital” and the “INFANT COHORT” study by the ”Comité d’Ethique Regionale Independent de Lambaréné (CERIL)”.

Obtained informed consents complied with the applicable regulatory requirement(s), and adhered to GCP and to the ethical principles that originate from the appended Declaration of Helsinki. Information was given in both oral and written form. An investigator or designate described the protocol to parents/guardians face to face including workflow of blood sampling.

3.3.2.1.3 Inclusion/exclusion criteria

3.3.2.1.3.1 Inclusion criteria of the CHILDREN COHORT

Male or female children between 18 months and 4 years of age (up to, but not including 5th birthday) at the time of first vaccination were eligible for participation.

3.3.2.1.3.2 Inclusion criteria of the INFANT COHORT

Male or female infants between 6 and 10 weeks of age at the time of their first vaccination were eligible for trial participation. Subjects who had received one previous dose of OPV (oral polio vaccine) and BCG (Bacillus Calmette-Guérin

vaccine) and who were born after a normal gestation period (between 36 and 42 weeks) were eligible.

3.3.2.1.3.3 Exclusion criteria of the CHILDREN COHORT

Exclusion criteria were:

- acute disease at the time of enrolment (acute disease defined as the presence of a moderate or severe illness with or without fever, i.e. axillary temperature < 37.5°C)
- Serious acute or chronic illness determined by clinical or physical examination and laboratory screening tests including, but not limited to:
    - Any confirmed or suspected immunosuppressive or immunodeficient condition, based on medical history and physical examination (no laboratory testing required)
    - A family history of congenital or hereditary immunodeficiency
    - History of splenectomy
    - Major congenital defects
    - History of any neurologic disorders or seizures
    - Moderate malnutrition at screening defined as weight-for-age Z-score less than -2
- Planned administration of a vaccine not foreseen by the study protocol within 30 days of the first dose of vaccine(s) with the exception of tetanus toxoid
- Use of any investigational or non-registered drug or vaccine within 30 days preceding the first dose of study vaccine, or planned use during the study period
- Administration of immunoglobulins, blood transfusions or other blood products within the three months preceding the first dose of study vaccine or planned administration during the study period
- Chronic administration (defined as more than 14 days) of immunosuppressants or other immune-modifying drugs within six months prior to the first vaccine dose (for corticosteroids, this will mean

prednisone, or equivalent, ≥ 0.5 mg/kg/day Inhaled and topical steroids are allowed)

- Previous participation in any other malaria vaccine trial
- Simultaneous participation in any other clinical trial
- Same sex twin
- History of allergic reactions (significant IgE-mediated events) or anaphylaxis to previous immunizations
- History of allergic disease or reactions likely to be exacerbated by any component of the vaccine
- Any other findings that the investigator feels would increase the risk of having an adverse outcome from participation in the trial

3.3.2.1.3.4 Exclusion criteria of the "INFANT COHORT"

The criteria for exclusion from this study are similar to the exclusion criteria from the "CHILDREN COHORT". Additional exclusion criteria were: previous vaccination with diphtheria, tetanus, pertussis (whole-cell or acellular), Hemophilus influenzae type b or hepatitis B; BCG administration within one week or an OPV administration within four weeks of the proposed administration of a study vaccine.

3.3.2.2 Questionnaire and examination of study participants

Patient record form (PRF) and physical examination as well as all laboratory procedures were similar for both trials. The following descriptions apply to both trials.

The questionnaire was used in conjunction with medical measurements, such as blood pressure, height and weight, axillary temperature and pulse alongside an interview to ask interviewees, if they considered themselves or their children to be in good health. Furthermore, the investigator had to check several medical conditions concerning the cardiovascular system, respiratory system, gastrointestinal system including liver, central and peripheral nervous system, spleen, lymph nodes, skin and the uro-genital system. Distinctive features had to be noted in a designated field.

To protect the reference individuals, the questionnaire information and the testing results were maintained in a confidential manner. Name, address, and phone number were included to facilitate contacting the reference individual in the event that the analysis detected any abnormalities.

3.3.3 Exclusion of subjects prior to analysis

Participants who did not fulfil the inclusion criteria of both trials were not considered for the reference sample group. Because the criteria for being considered healthy was less strict for the trials than for reference subjects, the clinical records of each study subject was examined to acertain good health of reference value population. By searching the study database, children with systemic infections, diarrhoea, otitis media, antibiotic treatment up to eight days before blood sampling, burns and large abscesses were not included in the reference sample group.

3.3.4 Data management and analysis

All patient record forms (PRFs) of both trials were reviewed for age, sex, date of blood sampling, any acute or histories of disease, prior medications and criteria for eventual exclusions. Subsequently occurring diseases were detected by monitoring follow-up patient record forms. All blood values on the lab-printouts were entered into an electronic data base (Microsoft® Excel 2002). Database printouts were subsequently checked against the lab-printouts. Calculations were performed with SAS Statistical Software JMP 7.0 [164]. Firstly, the individual data were ranked and listed in order of increasing size. The Dixon test (the ratio D/R) was carried out until no outlier could be detected. Subclasses were assessed by the z-test. All parameters were plotted and inspected in frequency plots, CDF plots and box plots. If parameters appeared Gaussian distributed, the median, mean, skewness and kurtosis were calculated and compared. Quantiles were computed with JMP 7.0, and the 90% confidence intervals were calculated as described in the NCCLS Protocol.

3.3.5 Comparison of estimated reference values with reference values from other populations

The parameters estimated for the population of Lambaréné were compared to those deriving from their Caucasian and African counterparts. For Caucasian populations, values were taken from the textbooks “Pädiatrie [157]” and “Klinkleitfaden Pädiatrie: Untersuchung, Diagnostik, Therapie, Notfall [87]”. For comparison to African values, the data of Lugada et al. [104] from Uganda were used. In addition, for the CHILDREN COHORT, the data of Quinto et al. [136] from Mozambique were used. These sources are described in more detail in the following paragraphs.

3.3.5.1 Pädiatrie

Published in 2005 as the second edition by Christian P. Speer and Manfred Gahr, this German textbook is one of the most used reference sources in paediatrics. The authors do not precisely explain where the cited parameters are taken from. The age groups for reference ranges in this textbook are divided into newborns first week of life, newborns second week of life, infants two to 12 months and children aged 2 – 6 years. For simplification, this book will be referred to as “Speer” in this thesis.

3.3.5.2 Klinkleitfaden Pädiatrie: Untersuchung, Diagnostik, Therapie, Notfall

This German “pocket book“ was published in 1994 by Illing et al. [87] and is a standard pocket book used by German paediatricians. Unfortunately, Illing et al. do not give any references of the source for their printed ranges. Therefore these values should only guide the clinican in interpreting laboratory values and are not designed to give strict cut-off values. For this analysis the values of Illing et al. are sufficient for demonstrating trends between the different groups. For simplification, this book will be referred to as “Illing” in this thesis.

3.3.5.3 Uganda

“Population-Based Hematologic and Immunologic Reference Values for a Healthy Ugandan Population” is the title of a publication by Lugada et al. [104] in

2004. The investigators estimated the 90 % interval for Ugandan infants less than 1 year of age on the basis of 373 samples. 518 samples were used for children aged 1 – 5 years. For statistical determination Lugada et al. used the 5% and 95% quantiles and therefore caution needs to be exercised when directly comparing these reference values with those of other populations.

3.3.5.4 Mozambique

“Haematological and biochemical indices in young African children: in search of reference intervals” was published in 2006 by Quinto et al. [136]. The children aged 1 – 5 years were divided into four “one year” cohorts and reference values were estimated for children aged 1 – 2 years, 2 - 3 years, 3 – 4 years and 4 – 5 years following the same NCCLS methods as in this present document. It is only known that the data derive from 2226 blood samples and nothing is said about the numbers of participants in each age group. One disadvantage of using the data for comparison is the selection of age. To compare the reference ranges, the lower limit of the one-year group and the upper limit of the 5-year group were used. Therefore, similarly to the Ugandan cohort, caution needs to be exercised when directly comparing these reference values with those of other populations.

4 Results

4.1 Subjects

4.1.1 Analysed parameters

For statistical analysis the following parameters were available.

Red blood cells

- red blood cell count (RBC [10^6/µl])
- haemoglobin (HGB [g/dl])
- haematocrit (HCT [%])
- mean corpuscular volume (MCV [µm³])
- mean corpuscular haemoglobin (MCH [pg])
- mean corpuscular haemoglobin concentration (MCHC [g/dl])
- red cell distribution width (RDW [%])

Platelets

- platelet count (PLT [10³/µl])
- mean platelet volume (MPV [µm³])
- plateletcrit (PCT [%])
- platelet distribution width (PDW [%])

Leukocytes

- white blood cell count (WBC [10^3/µl])
- lymphocytes (LYM [%] and LYM [10³/µl])
- monocytes (MON [%] and MON [10³/µl])
- neutrophils (NEU [%] and NEU [10³/µl])
- eosinophils (EOS [%] and EOS [10³/µl])

- basophils (BAS [%] and BAS [10^3/µl])
- atypical lymphocytes (ALY [%] and ALY [10^3/µl])
- large immature cell (LIC [%] and LIC [10^3/µl])

Biochemistry

- glutamic-pyruvic transaminase (GPT [U/l])
- creatinine (CREA [µmol/l])
- total bilirubin (BILI [µmol/l]) (only assessed in the “CHILDREN COHORT” trial)

4.1.2 Age of included children

4.1.2.1 The CHILDREN COHORT

On the day of blood sampling, the mean age of the 185 participants included for analysis was 39 months. The age range was 42 months with the youngest participants being 18 months and the oldest being 60 months. The age distribution over the participants’ sample was very balanced, although there was an overrepresentation of 30-35 month olds, corresponding to 19% of the population.

4.1.2.2 The INFANT COHORT

On the day of medical screening and blood sampling, the mean age of the 226 participants was 5.8 weeks. The age range was 6 weeks with the youngest participant being 3 weeks and the oldest being 9 weeks. There was an overrepresentation of children aged 5 to 6 weeks, corresponding to 82 % of the population. 10 % of the children had an age of 7 weeks.

4.1.3 Inclusions and exclusions

4.1.3.1 The CHILDREN COHORT

277 children were initially screened for the RTS,S trial. 48 children were not available for blood sampling due to illness, impossibility of taking blood, withdrawal of consent or had other characteristics which did not fit the inclusion criteria. 13 blood result printouts were printed out incorrectly and 19 blood

analyses were performed with a different blood counter. Based on our definition of health and to avoid data being biased by systemic illness, 6 children were excluded from analysis after searching through the data base. They suffered from otitis media and worm infestation, otitis media and skin postule, common cold with diarrhoea, cervical lymphadenitis, st.p burn or scalp abscess.

The data from children with tinea capitis, scarification on the abdomen after skin transplantation, common cold, upper respiratory tract infection, skin infection and furuncolosis on the scalp, mild conjunctivitis and staphyloderma were left in the analysis data set. These conditions were deemed as both prevalent enough to be considered representative of the local population and as having little influence on the laboratory parameters being analysed.

Table 2. Disease or history of disease of included children at blood sampling in the CHILDREN COHORT trial

Medical conditon	N
Tinea capitis	6
Scarification on the abdomen after skin transplantation	1
Common cold	1
Upper respiratory tract infection	1
Skin infection	1
Furuncolosis on the scalp	1
Mild conjunctivitis	1
Staphyloderma	1

N: number of children with disease or history of disease

4.1.3.2 The INFANT COHORT

For this study 267 children were screened. Due to HIV, febrile infections, weight-for-age $z-$score out of range, withdrawal of consent or other medical reasons, 33 children were not included into the study. Seven study children were excluded from the reference value analysis cohort due to: blister with pus, common cold with bronchiolitis and antibiotic treatment 7 days prior, diarrhoea, runny nose and bilateral lymphadenopathy, cough with fever and proteinuria and common cold with diarrhoea. One child was excluded as the description of illness was illegible.
The following diseases were considered as not influencing the haematological or biochemical values and describing the common reference population and were

therefore kept in the analysis cohort: rhinitis, conjunctivitis, mild skin disease and other mild medical conditions. Table 3 shows the amount and distribution of the diseases or history of diseases during blood sampling of the included children.

Table 3. Disease or history of disease of included children at blood sampling in the INFANT COHORT trial

Medical conditon	N
At least one of the following symptoms: cough, rhinitis, common cold, conjunctivitis	37
At least one of the following symptoms: dermatitis, sore, mycosis, mild staphyloderma, cutaneous eruption	10
Albinism	1
Fever three weeks before inclusion with rocephin treatment as outpatient	1
Rocephin treatment ten days before inclusion for unknown reasons and Depigmented macules on skin	1
Rhinitis and nappy dermatitis	1

N: number of children with disease or history of disease

4.1.3.3 Dixon test for outliers

4.1.3.3.1 The CHILDREN COHORT

The Dixon test was performed three times.

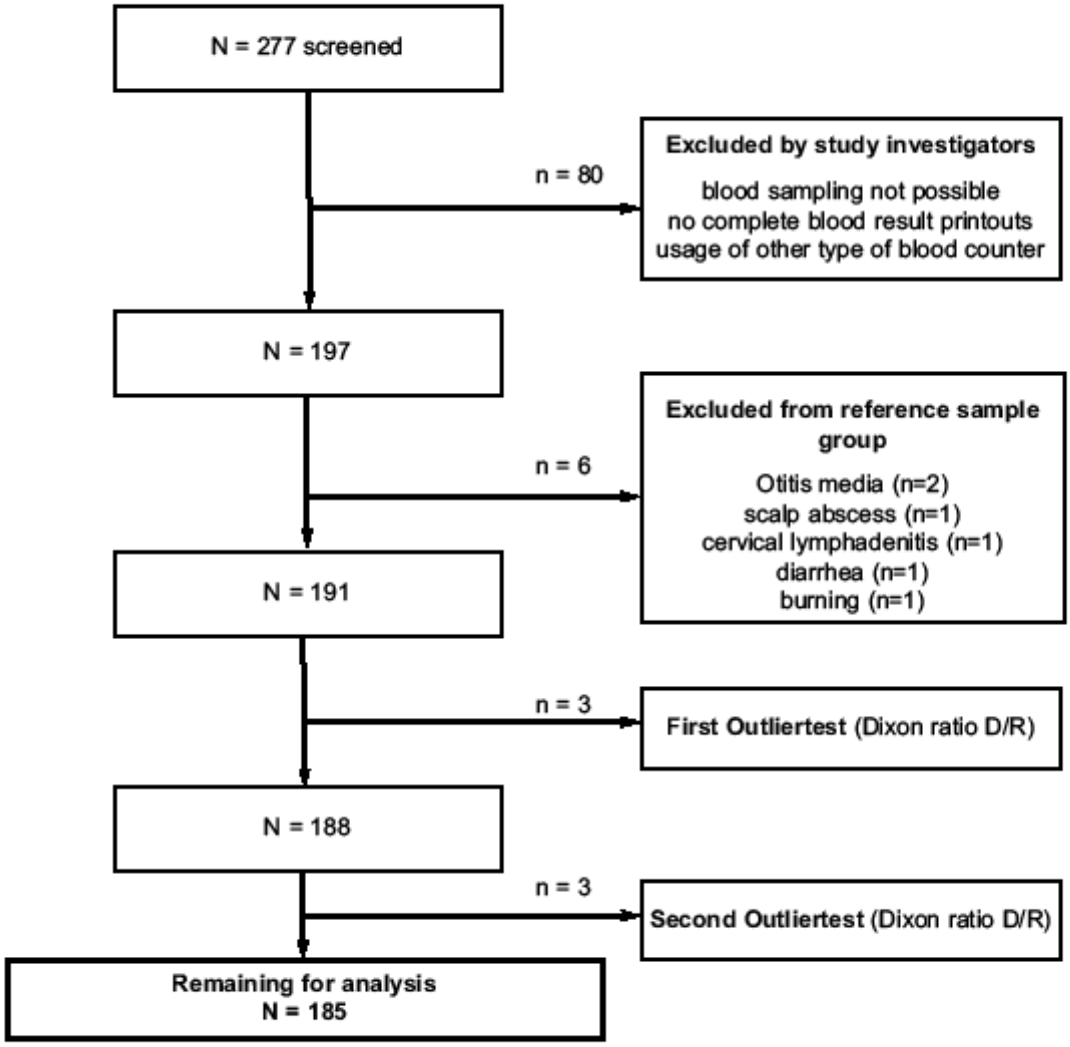
The first test detected three data sets to be excluded from the database. One blood sample showed a high bilirubin value of 158.5 µmol/l, one creatinine value was more than one third higher than the following value and one patient had a high amount of atypical lymphocytes.

At the second cycle of testing, three further data samples had to be eliminated. Two samples showed high basophil values and one sample detected a high number of large immature cells.

The third Dixon test did not detect any outliers.

In summary, for analysis of the „CHILDREN COHORT“ data, 185 blood samples were of sound quality. The participants passed the screening and were healthy at the time of blood sampling. The flowchart is summarized in figure 5.

Figure 5. Exclusions for CHILDREN COHORT

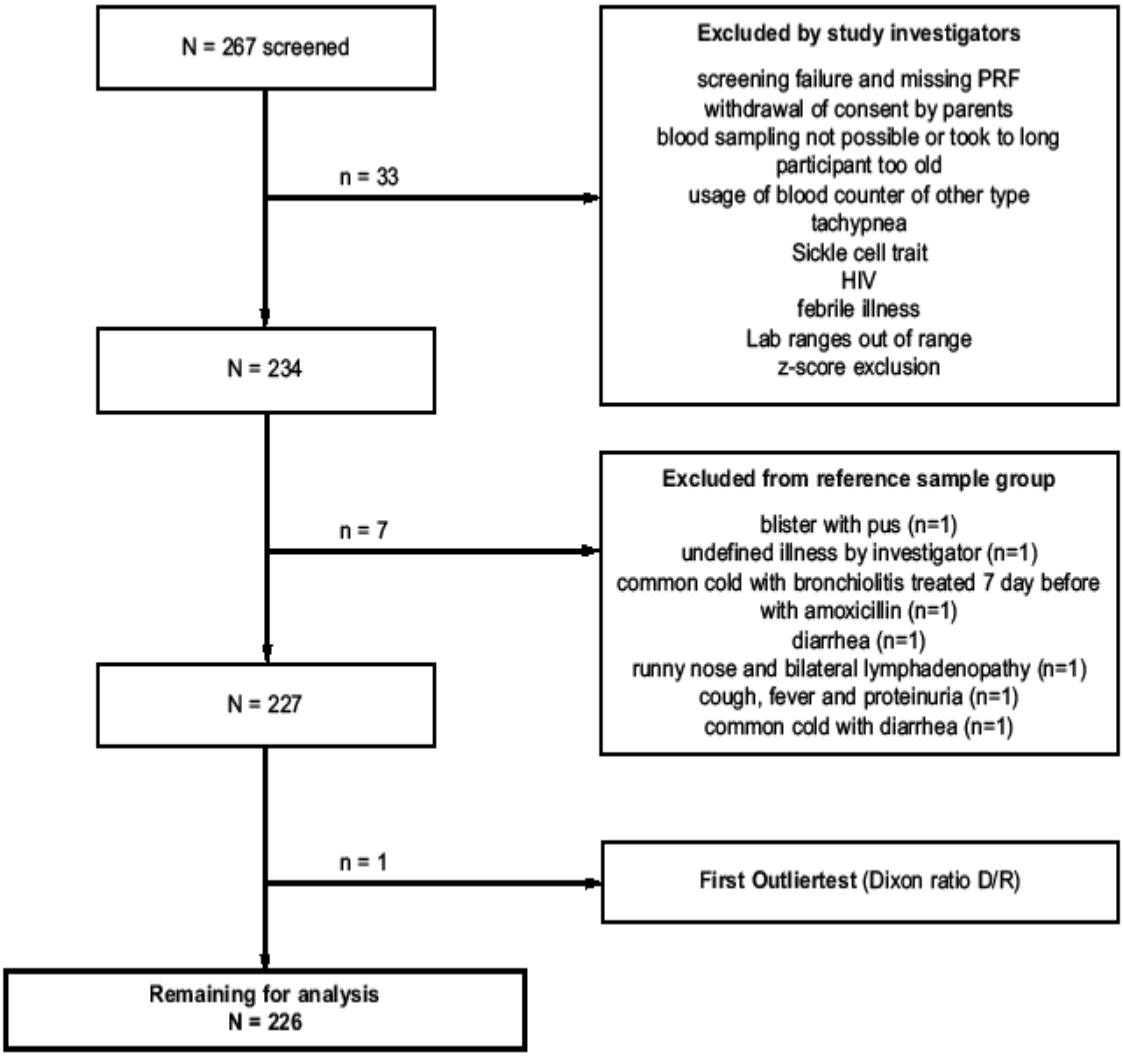


4.1.3.3.2 The INFANT COHORT

The platelet count of one participant failed the Dixon test. The second cycle did not show any outliers.

In summary, for analysis of the „INFANT COHORT“ data, 226 blood samples were of sound quality. The participants passed the screening and were healthy at the time of blood sampling. The flowchart is summarized in figure 6.

Figure 6. Exclusions for INFANT COHORT



4.1.4 Reject flag on lab printouts

Some analysis values were marked with a “reject flag” (shown by *) on the result printout. A reject flag occurs when two counts on a parameter differ more than the pre-defined limits. It indicates that the result is not coherent and the sample has to be rerun. In a few cases, despite several reruns of the analysis, some values remained flagged. Because of the uncertainty of a correct reading for these measurements, they were not included in the analysis dataset. The following tables show the amount of reject flags for each parameter.

Table 4. Amount of reject flags on the laboratory printouts of the CHILDREN COHORT

Parameter	Number of reject flags	Total left for analysis
Platelets		
PLT	9	176
MPV	37	148
PCT%	37	148
PDW%	38	147
White blood cells		
WBC	9	176
LYM	8	177
MON	8	177
NEU	22	163
EOS	21	164
BAS	12	173
ALY	12	173
LIC	10	175
Biochemistry		
GPT	5	180
BILI	3	182
CREA	4	181

Table 5. Amount of reject flags on the laboratory printouts of the INFANT COHORT

Parameter	Amount of reject flags	Total left for analysis
Platelets		
PLT	6	220
MPV	6	220
PCT%	6	220
PDW%	6	220
White blood cells		
WBC	9	217
LYM %	10	216
LYM	11	215

MON %	9	217
MON	10	216
NEU %	11	215
NEU	12	214
EOS	12	214
BAS	11	215
ALY %	12	214
ALY	13	213
LIC	11	215
Biochemistry		
GPT	3	223
CREA	3	223

4.2 Data analysis

4.2.1 Subgroups

The z-test was performed to test the disparity between the subgroups “female” and “male”. $z*$ was larger than z in all cases. In addtion, Test A ($StdDev(great)/StdDev(small)$) was lower than 1.5 for all parameters. Furthermore, all values of Test B ($StdDev(great)/(StdDev[great]-StdDev[small])$) were above the threshold of 3, with the exception of bilirubin in the CHILDREN COHORT. The results are summarised in table 6 and 7.

Table 6. Tests for subclassing reference values of CHILDREN COHORT data

Parameter	N		Mean (SD)		z	z*	Test A *	Test B **
	Male	Female	Male	Female				
Red blood cells								
RBC	99	86	4,6 (0.51)	4.58 (0.5)	0.27	2.63	1.02	97.28
HGB	99	86	10.45 (0.89)	10.72 (0.94)	2.04	2.63	1.06	22.36
HCT	99	86	31.55 (2.46)	32.24 (2.66)	1.8	2.63	1.08	13
MCV	99	86	68.95 (6.49)	70.84 (6.3)	-2	2.63	1.03	32.87
MCH	99	86	22.9 (2.73)	23.6 (2.54)	-1.8	2.63	1.07	14.43
MCHC	99	86	33.12 (1.11)	33.25 (0.99)	-0.81	2.63	1.21	9.2
RDW	99	86	15.65 (2.31)	15.32 (2.43)	-0.94	2.63	1.05	19.9
Platelets								
PLT	94	83	392.66 (109.65)	381.33 (119.63)	-0.65	2.58	1.09	11.99
MPV	78	71	8.11 (0.8)	8.06 (0.75)	0.37	2.36	1.06	16.75
PCT	78	71	0.31 (0.08)	0.3 (0.09)	-0.66	2.36	1.13	8.97
PDW	77	71	13.4 (2.95)	13.25 (2.89)	0.3	2.36	1.02	45.48
Leukocytes								
WBC	96	81	9.12 (2.5)	9 (2.35)	0.34	2.58	1.06	16.56

LYM	96	81	44.98 (9.83)	49.01 (9.18)	-2.82	2.58	1.07	15.08
LYM	96	81	3.99 (1.08)	4.38 (1.48)	1.97	2.58	1.37	3.7
MON	96	81	8.2 (2.55)	7.89 (1.82)	0.95	2.58	1.4	3.52
MON	96	81	0.74 (0.31)	0.7 (0.23)	0.99	2.58	1.35	3.99
NEU	88	76	35.74 (9.84)	33.95 (8.98)	1.22	2.48	1.10	11.51
NEU	88	76	3.22 (1.38)	3.03 (1.16)	0.98	2.48	1.19	6.34
EO	88	77	9.38 (6.6)	7.92 (5.21)	1.58	2.49	1.27	4.75
EOS	88	77	0.9 (0.82)	0.73 (0.57)	1.57	2.49	1.44	3.32
BAS	93	80	1.04 (0.31)	1.1 (0.29)	-1.14	2.55	1.07	11.27
BAS	93	80	0.1 (0.05)	0.1 (0.05)	-0.44	2.55	1	13.91
ALY	93	81	1.51 (0.52)	1.46 (0.45)	0.6	2.55	1.16	7.04
ALY	93	80	0.13 (0.06)	0.13 (0.05)	0.46	2.55	1.2	6.2
LIC	94	81	1.08 (0.55)	1.03 (0.4)	0.73	2.56		3.87
LIC	94	81	0.1 (0.06)	0.09 (0.05)	1.12	2.56	1.38	3.29
Biochemistry								
GPT	97	85	15.09 (6.97)	15.59 (6.5)	-0.5	2.61	1.07	14.87
BILI	98	85	9.31 (6.08)	9.77 (5.35)	0.54	2.62	1.14	-7.28
CREA	97	85	25.21 (7.97)	22.41 (6.78)	2.56	2.61	1.18	6.72

* Test A: $StdDev(great)/StdDev(small)$

** Test B: $StdDev(great)/(StdDev[great]-StdDev[small])$

Table 7. Tests for subclassing reference values of INFANT COHORT data

Parameter	N		Mean (SD)		z	z*	Test A*	Test B**
	Male	Female	Male	Female				
Red blood cells								
RBC	112	114	3.63 (0.49)	3.79 (0.44)	-2.61	2.91	1.11	11.28
HGB	112	114	10.4 (1.17)	10.92 (1.04)	-3.5	2.91	1.13	8.76
HCT	112	114	30.83 (3.53)	32.46 (3.14)	-3.67	2.91	1.12	9.01
MCV	112	114	85.42 (7.1)	85.86 (6.56)	-0.48	2.91	1.07	12.99
MCH	112	114	28.84 (2.73)	28.96 (2.53)	-0.33	2.91	1.08	13.79
MCHC	112	114	33.75 (0.77)	33.65 (0.88)	-0.89	2.91	1.14	8.13
RDW %	112	114	12.79 (1.15)	12.64 (0.97)	1.06	2.91	1.19	6.41
Platelets								
PLT	108	112	388.5 (129.58)	404.14 (127)	-0.9	2.87	1.02	47.5
MPV	108	112	8.38 (0.86)	8.43 (0.84)	-0.4	2.87	1.02	43.95
PCT	108	112	0.32 (0.09)	0.34 (0.09)	1.32	2.87	1	36.64
PDW	108	112	14.96 (3.3)	14.84 (3.15)	0.28	2.87	1.05	22.16
Leukocytes								
WBC	106	111	8.71 (1.93)	9.47 (2.11)	2.76	2.85	1.09	11.85
LYM	106	110	63.63 (7.75)	62.93 (7.29)	0.68	2.85	1.06	16.72
LYM	105	110	5.54 (1.33)	5.95 (1.47)	2.16	2.84	1.11	10.78
MON	107	110	14.37 (4.25)	13.89 (3.66)	0.9	2.85	1.16	7.23
MON	106	110	1.26 (0.53)	1.31 (0.49)	-0.8	2.85	1.08	12.3
NEU	106	109	17.35 (6.27)	18.65 (6.34)	1.51	2.84	1.01	87.06
NEU	105	109	1.52 (0.67)	1.78 (0.77)	2.6	2.83	1.15	7.52
EOS	105	109	2.78 (1.13)	2.93 (1.51)	0.83	2.83	1.34	4

EOS	105	108	0.24 (0.1)	0.27 (0.14)	2.15	2.83	1.4	3.4
BAS	105	110	1.54 (0.45)	1.69 (0.62)	2.03	2.84	1.38	3.78
BAS	105	110	0.14 (0.06)	0.16 (0.08)	2.66	2.84	1.33	5.21
ALY	105	108	5.88 (1.99)	5.46 (1.92)	1.55	2.83	1.04	27.01
ALY	105	109	0.52 (0.24)	0.52 (0.23)	-0.14	2.83	1.04	40.19
LIC	106	109	1.4 (0.76)	1.49 (0.76)	0.83	2.84	1	502.4
LIC	106	109	0.14 (0.12)	0.14 (0.08)	-0.32	2.84	1.5	3.1
Biochemistry								
GPT	110	113	20.9 (7.92)	21.8 (7.61)	-0.86	2.89	1.04	25.6
CREA	110	113	24.22 (7.05)	25.33 (6.95)	-1.18	2.89	1.01	72.27

* Test A: $StdDev(great)/StdDev(small)$

** Test B: $StdDev(great)/(StdDev[great]-StdDev[small])$

4.2.2 Distribution of observed values

For each parameter, the assessment of the frequency distribution and the classification into Gaussian vs. non-Gaussian distribution was done by visual inspection of frequency plots and CDF plots as well as by inspection of mean, median, skewness and kurtosis. For both cohorts, the major red cell parameters, platelets and biochemical parameters were normally distributed, whereas white blood cell parameters showed a skewed distribution. The results are summarised in tables 8-11.

Table 8. Mean, median, skewness and kurtosis of Gaussian distributed parameters of the CHILDREN COHORT

Blood parameter	Mean	Median	Skewness	Kurtosis
Red blood cells				
RBC [10^6/µl]	4.6	4.5	0.5	0.7
HGB [g/dl]	10.6	10.6	-0.5	-0.1
HCT [%]	31.9	31.9	-0.2	0.5
Platelets				
PLT [10^3/µl]	387	378	0.38	0.5
MPV [µm³]	8	8	0.6	0.3
PCT [%]	0.3	0.3	0.3	1.1
Biochemistry				
BILI [µmol/]	9.5	9.7	0.3	-0.4
CREA [µmol/l]	23.9	23.6	0.0	0.9

Table 9. Mean, median, skewness and kurtosis of non-Gaussian distributed parameters of the CHILDREN COHORT

Blood parameter	Mean	Median	Skewness	Kurtosis
Red blood parameters				
MCV [µm³]	69.8	70	-0.7	0.0
MCH [pg]	23.2	23.6	-0.6	-0.2
MCHC [g/dl]	33.2	33.4	-0.7	0.2
RDW [%]	15.5	15.1	0.9	1.2
Platelets				
PDW [%]	13.3	12.8	1.1	1.8
White blood cells				
WBC [10^3/µl]	9.1	8.9	0.6	0.0
LYM [%]	46.8	47.5	-0.2	-0.5
LYM [10³/µl]	4.2	3.9	1.3	3.0
MON [%]	8.1	7.8	1.7	6.1
MON [10³/µl]	0.7	0.7	1.6	4.2
NEU [%]	34.9	33.6	0.5	0.3
NEU [10³/µl]	3.1	2.9	1.2	1.7
EOS [%]	8.7	7.5	1.6	3.8
EOS [10³/µl]	0.8	0.6	2.3	7.9
BAS [%]	1.1	1	1.2	3.0
BAS [10³/µl]	0.1	0.1	2.1	8.9
ALY [%]	1.5	1.4	1.1	2.2
ALY [10³/µl]	0.1	0.1	2.1	7.3
LIC [%]	1.1	1	2.5	12.2
LIC [10³/µl]	0.1	0.1	1.3	2.0
Biochemistry				
GPT [U/l]	15.3	14	1.7	5.2

Tabelle 10. Mean, median, skewness and kurtosis of Gaussian distributed parameters of the INFANT COHORT

Blood parameter	Mean	Median	Skewness	Kurtosis
Red blood cells				
RBC [10^6/µl]	3.7	3.7	0.3	0.5
HGB [g/dl]	10.7	10.7	-0.1	-0.1
HCT [%]	31.7	31.4	-0.1	-0.1
MCH [pg]	28.9	29.4	-0.7	0.1
MCHC [g/dl]	33.7	33.7	-0.2	-0.1
Platelets				
PLT [10³/µl]	396	401	-0.2	-0.2
Biochemistry				
CREA [µmol/l]	24.8	24.2	0.2	0.1

Tabelle 3. Mean, median, skewness and kurtosis of non-Gaussian distributed parameters of the INFANT COHORT

Blood parameter	Mean	Median	Skewness	Kurtosis
Red blood cells				
MCV	85.6	87	-0.6	0.1
RDW	12.7	12.6	1.0	2.0
Platelets				
MPV	8.4	8.4	0.9	-0.3
PCT	0.3	0.3	-0.2	-0.4
PDW	14.9	14.5	0.5	-0.3
White blood cells				
WBC	9.1	9.2	0.5	0.3
LYM %	63.3	64.4	-1.0	2.0
LYM	5.8	5.6	0.4	-0.5
MON %	14.1	13.4	1.2	2.9
MON	1.3	1.2	2.3	10.3
NEU %	18.0	17.3	1.0	1.7
NEU	1.7	1.5	1.6	5.3
EOS %	2.9	2.6	0.9	0.5
EOS	0.3	0.2	1	49.4
BAS %	1.6	1.5	2.0	6.4
BAS	0.2	0.1	1.6	3.3
ALY %	5.7	5.2	1.6	4.2
ALY	0.5	0.5	2.3	9.7
LIC %	1.5	1.3	2.4	8.0
LIC	0.1	0.1	3.7	19.5
Biochemistry				
GPT	21.4	20	0.6	0.2

4.2.3 Quantiles of blood parameters

The following tables summarize the 2.5th and 97.5th quantiles on which the reference intervals are based as well as median, minimum and maximum values of each parameter (table 12 and 13).

Table 4. Quantiles of „CHILDREN COHORT“ for children aged 18 to 60 months

Parameter	Minimum	2.5% Quantile	Median	97.5% Quantile	Maximum
Red blood cells					
RBC [10^6/µl]	3.3	3.66	4.52	5.81	6.13
HGB [g/dl]	8	8.5	10.6	12	13
HCT [%]	23	26.5	31.9	37.2	38.4
MCV [µm³]	52	54	70	80	81
MCH [pg]	16	17	23.6	27.1	27.8
MCHC [g/dl]	29.8	30.7	33.4	34.8	35.4
RDW [%]	10.3	11.7	15.1	20.9	25.2

Platelets					
PLT [10³/µl]	61	192	378	646	724
MPV [µm³]	6.5	6.9	8	10	10.5
PCT [%]	0.064	0.16	0.3	0.488	0.593
PDW [%]	8.5	9	12.8	20.6	24.8
WBC [10^3/µl]	4.7	5.4	8.9	14.8	16.2
Leukocytes					
LYM [%]	20.8	27.4	47.5	64.2	70.1
LYM [10³/µl]	1.82	2.34	3.88	7.11	10.6
MON [%]	3.7	4.7	7.8	13	20.2
MON [10³/µl]	0.29	0.36	0.68	1.62	2.01
NEU [%]	15.9	18	33.6	54.2	68.4
NEU [10³/µl]	1.07	1.27	2.9	6.85	7.61
EOS [%]	0.8	1.3	7.5	24.4	38.7
EOS [10³/µl]	0.07	0.12	0.61	3.05	4.93
BAS [%]	0.5	0.6	1	1.9	2.5
BAS [10³/µl]	0.02	0.04	0.09	0.22	0.4
ALY [%]	0.7	0.8	1.4	2.4	3.7
ALY [10³/µl]	0.06	0.06	0.12	0.28	0.45
LIC [%]	0.4	0.5	1	2.2	4.3
LIC [10³/µl]	0.02	0.03	0.09	0.27	0.33
Biochemistry					
GPT [U/L]	2	6	14	32	51
BILI [µmol/l]	0	0.44	9.67	22.12	27.09
CREA [µmol/l]	1.9	7.3	23.6	40.5	46.8

Table 5. Quantiles of „INFANT COHORT“ for children aged 4 to 9 weeks

Parameter	Minimum	2.5% Quantile	Median	97.5% Quantile	Maximum
Red blood cells					
RBC [10^6/µl]	2.19	2.89	3.69	4.77	5.19
HGB [g/dl]	6.9	8.4	10.7	12.8	13.5
HCT [%]	19.7	25	31.4	38.3	39.8
MCV [µm³]	67	70	87	96	105
MCH [pg]	21.7	23	29.4	33	35.3
MCHC [g/dl]	31.3	32	33.7	35.3	35.7
RDW [%]	10.8	11	12.6	15.2	17.2
Platelets					
PLT [10³/µl]	39	143	401	638	669
MPV [µm³]	6.7	6.8	8.4	10.1	11
PCT [%]	0.07	0.145	0.338	0.498	0.516
PDW [%]	9	9.9	14.5	22.1	24
Leukocytes					
WBC [10^3/µl]	5.4	5.8	9.2	13.5	17.4
LYM [%]	33	42.9	64.4	74.8	83.9
LYM [10³/µl]	2.76	3.42	5.55	8.69	9.42

MON [%]	6.6	7.7	13.4	23.4	32.4
MON [10³/µl]	0.59	0.65	1.2	2.51	4.42
NEU [%]	5.5	8.1	17.3	34.9	43.3
NEU [10³/µl]	0.35	0.54	1.53	3.57	5.62
EOS [%]	0.6	0.8	2.6	6.1	7.4
EOS [10³/µl]	0.05	0.07	0.23	0.58	0.71
BAS [%]	0.8	1	1.5	3.1	4.6
BAS [10³/µl]	0.05	0.07	0.14	0.37	0.43
ALY [%]	2.3	3.1	5.2	11	15.1
ALY [10³/µl]	0.2	0.22	0.47	1.09	2.06
LIC [%]	0.4	0.7	1.3	3.9	5.8
LIC [10³/µl]	0.02	0.04	0.11	0.42	0.9
Biochemistry					
GPT [U/L]	5	8	20	41	44
CREA [µmol/l]	4.9	11.7	24.2	39.6	46

4.2.4 Reference Limits and corresponding Confidence Intervals

The following tables summarize the 95% reference ranges and the corresponding 90% confidence intervals.

Table 14. 95% Reference values for children aged 18 months to 5 years in Lambaréné and 90% Confidence Intervals for Lower and Upper 95% Reference Limits

Analyte	95 % Reference Range	90 % Confidence Intervals	
		Lower Reference Limit	Upper Reference Limit
Red blood cells			
RBC [10^6/µl]	3.66 - 5.81	3.3 - 3.78	5.59 - 6.13
HGB [g/dl]	8.5 - 12	8 - 8.8	11.9 - 13
HCT [%]	26.5 - 37.2	23 - 27.6	36.2 - 38.4
MCV [µm³]	54 - 80	52 - 57	79 - 81
MCH [pg]	17 - 27.1	16 - 18.2	27 - 27.8
MCHC [g/dl]	30.7 - 34.8	29.8 - 31.2	34.8 - 35.4
RDW [%]	11.7 - 20.9	10.3 - 12.4	20.2 - 25.2
Platelets			
PLT [10³/µl]	192 - 646	61 - 211	613 - 724
MPV [µm³]	6.9 - 10	6.5 - 6.9	9.5 - 10.5
PCT [%]	0.16 - 0.488	0.064 - 0.176	0.439 - 0.593
PDW [%]	9 - 20.6	8.5 - 9.5	18.8 - 24.8
Leukocytes			
WBC [10^3/µl]	5.4 - 14.8	4.7 - 5.6	13.9 - 16.2
LYM [%]	27.4 - 64.2	20.8 - 29.3	62.5 - 70.1
LYM [10³/µl]	2.34 - 7.11	1.82 - 2.63	6.72 - 10.6
MON [%]	4.7 - 13	3.7 - 5.1	12 - 20.2
MON [10³/µl]	0.36 - 1.62	0.29 - 0.38	1.22 - 2.01

NEU [%]	18 - 54.2	15.9 - 21.7	51.6 - 68.4
NEU [10³/µl]	1.27 - 6.85	1.07 - 1.54	6.36 - 7.61
EOS [%]	1.3 - 24.4	0.8 - 2	20.2 - 38.7
EOS [10³/µl]	0.12 - 3.05	0.07 - 0.15	2.33 - 4.93
BAS [%]	0.6 - 1.9	0.5 - 0.7	1.7 - 2.5
BAS [10³/µl]	0.04 - 0.22	0.02 - 0.04	0.18 - 0.4
ALY [%]	0.8 - 2.4	0.7 - 0.9	2.3 - 3.7
ALY [10³/µl]	0.06 - 0.28	0.06 - 0.07	0.23 - 0.45
LIC [%]	0.5 - 2.2	0.4 - 0.5	1.8 - 4.3
LIC [10³/µl]	0.03 - 0.27	0.02 - 0.03	0.21 - 0.33
Biochemistry			
GPT [U/L]	6 - 32	2 - 6	23 - 51
CREA [µmol/l]	7.3 - 40.5	1.9 - 12.3	37.9 - 46.8
BILI [µmol/l]	0.44 - 22.12	0 - 0.82	20.01 - 27.05

Table 15. 95% Reference values for children aged 4 weeks to 9 weeks in Lambaréné and 90% Confidence Intervals for Lower and Upper 95% Reference Limits

Analyte	95 % Reference Range	90 % Confidence Intervals	
		Lower Reference Limit	Upper Reference Limit
Red blood cells			
RBC [10^6/µl]	2.89 - 4.77	2.65 - 2.99	4.45 - 5.16
HGB [g/dl]	8.4 - 12.8	8.0 - 8.6	12.4 - 13.52
HCT [%]	25 - 38.3	23.9 - 25.8	37.4 - 38.7
MCV [µm³]	70 - 96	67 - 72	96 - 98
MCH [pg]	23 - 33	22.2 - 23.2	32.6 - 33.9
MCHC [g/dl]	32 - 35.3	31.6 - 32.2	35 - 35.6
RDW [%]	11 - 15.2	10.8 - 11.3	14.5 - 16.7
Platelets			
PLT [10³/µl]	143 - 638	49 - 174	606 - 664
MPV [µm³]	6.8 - 10.1	6.7 - 7	9.7 - 10.5
PCT [%]	0.145 - 0.498	0.124 - 0.167	0.484 - 0.51
PDW [%]	9.9 - 22.1	9.5 - 10.3	21 - 23.5
Leucocytes			
WBC [10^3/µl]	5.8 - 13.5	5.5 - 6.1	12.9 - 14
LYM [%]	42.9 - 74.8	37.2 - 49.3	73.4 - 79
LYM [10³/µl]	3.42 - 8.69	3.13 - 3.74	8.41 - 9.39
MON [%]	7.7 - 23.4	6.9 - 8.6	21 - 31
MON [10³/µl]	0.65 - 2.51	0.6 - 0.71	2.1 - 4.12
NEU [%]	8.1 - 34.9	5.7 - 8.7	30.9 - 39.1
NEU [10³/µl]	0.54 - 3.54	0.52 - 0.7	2.87 - 4.63
EOS [%]	0.8 - 6.1	0.6 - 1.1	5.4 - 7.1
EOS [10³/µl]	0.07 - 0.58	0.05 - 0.09	0.52 - 0.68
BAS [%]	1 - 3.1	0.9 - 1	2.9 - 4
BAS [10³/µl]	0.07 - 0.37	0.06 - 0.07	0.27 - 0.42
ALY [%]	3.1 - 11	2.7 - 3.4	9.3 - 14.2
ALY [10³/µl]	0.22 - 1.09	0.21 - 0.26	0.97 - 1.49
LIC [%]	0.7 - 3.9	0.5 - 0.7	3.1 - 4.9

LIC [10³/µl]	0.04 - 0.42	0.03 - 0.05	0.33 - 0.77
Biochemistry			
GPT [U/L]	8 - 41	5 - 11	38 - 41
CREA [µmol/l]	11.7 - 39.6	7.7 -13.2	37.1 - 44.1

4.2.5 Reference values for children in Lambaréné

In order to compare the reference limits of the present analysis, values deriving from two textbooks refering to Caucasian subjects [87, 157] and one study performed in Uganda [104] are shown in table 16 and 17. For major blood parameters, a graphical presentation of the comparison is shown in figure 7 and 8.

Table 16. Reference Values for children aged 18 months to 5 years in Lambaréné compared to other populations

Parameter	Reference Range			
	18 months to 5 years	**Caucasian 2 to 6 years* Caucasian 2 to 6 years****	**Mozambique 1 – 5 years*****	**Uganda 1 – 5 years********
Red blood cells				
RBC [10^6/µl]	3.66 - 5.81	4.3 – 5.5 4.0 – 5.4	not available	3.5–5.2
HGB [g/dl]	8.5 - 12	12 – 15 11 – 16	6.8 – 13.5	8.8–12.5
HCT [%]	26.5 - 37.2	34 – 41 31 – 48	23.4 – 40.4	25.9–36.3
MCV [µm³]	54 - 80	76 ± 8 72 – 100	not available	60.7–82.8
MCH [pg]	17 - 27.1	not available 22 - 35	not available	not available
MCHC [g/dl]	30.7 - 34.8	not available 26 - 36	not available	not available
RDW [%]	11.7 - 20.9	not available not available	not available	not available
Platelets				
PLT [10³/µl]	192 - 646	not available 220 - 500	133 – 533.8	126–376

MPV [µm³]	6.9 - 10	not available not available	not available	not available
PCT [%]	0.16 - 0.488	not available not available	not available	not available
PDW [%]	9 - 20.6	not available not available	not available	not available
Leukocytes				
WBC [10^3/µl]	5.4 - 14.8	8 – 12 5 – 18	6.2 – 14.4	4.9–13.6
LYM [%]	27.4 - 64.2	25 – 50 20 – 60	not available	not available
LYM [10³/µl]	2.34 - 7.11	2 – 6 not available	not available	2.4–8.4
MON [%]	4.7 - 13	1 – 6 1 – 6	not available	not available
MON [10³/µl]	0.36 - 1.62	0.08 – 0.73 not available	not available	0.26–1.04
NEU [%]	18 - 54.2	35 – 70 20 – 65	not available	not available
NEU [10³/µl]	1.27 - 6.85	2.8 – 8.4 not available	not available	1.0–3.9
EOS [%]	1.3 - 24.4	1 – 5 1 – 5	not available	not available
EOS [10³/µl]	0.12 – 3.05	0.08 – 0.6 not available	not available	0.14–2.03
BAS [%]	0.6 - 1.9	0 – 1 0 – 1	not available	not available
BAS [10³/µl]	0.04 - 0.22	0 - 0.12 not available	not available	0.03–0.17
ALY [%]	0.8 - 2.4	not available not available	not available	not available
ALY [10³/µl]	0.06 - 0.28	not available not available	not available	not available
LIC [%]	0.5 - 2.2	not available not available	not available	not available
LIC [10³/µl]	0.03 - 0.27	not available not available	not available	not available
Biochemistry				
GPT [U/L]	6 - 32	8 – 20 < 40	11 – 56.3	not available
CREA [µmol/l]	7.3 - 40.5	0 – 88 25 – 64	24 – 49*****	not available
BILI [µmol/l]	0.44 - 22.12	0 – 17 1.7 – 22	1.7 – 18.9	not available

* according to Illing et al., “Klinikleitfaden Pädiatrie” [87]

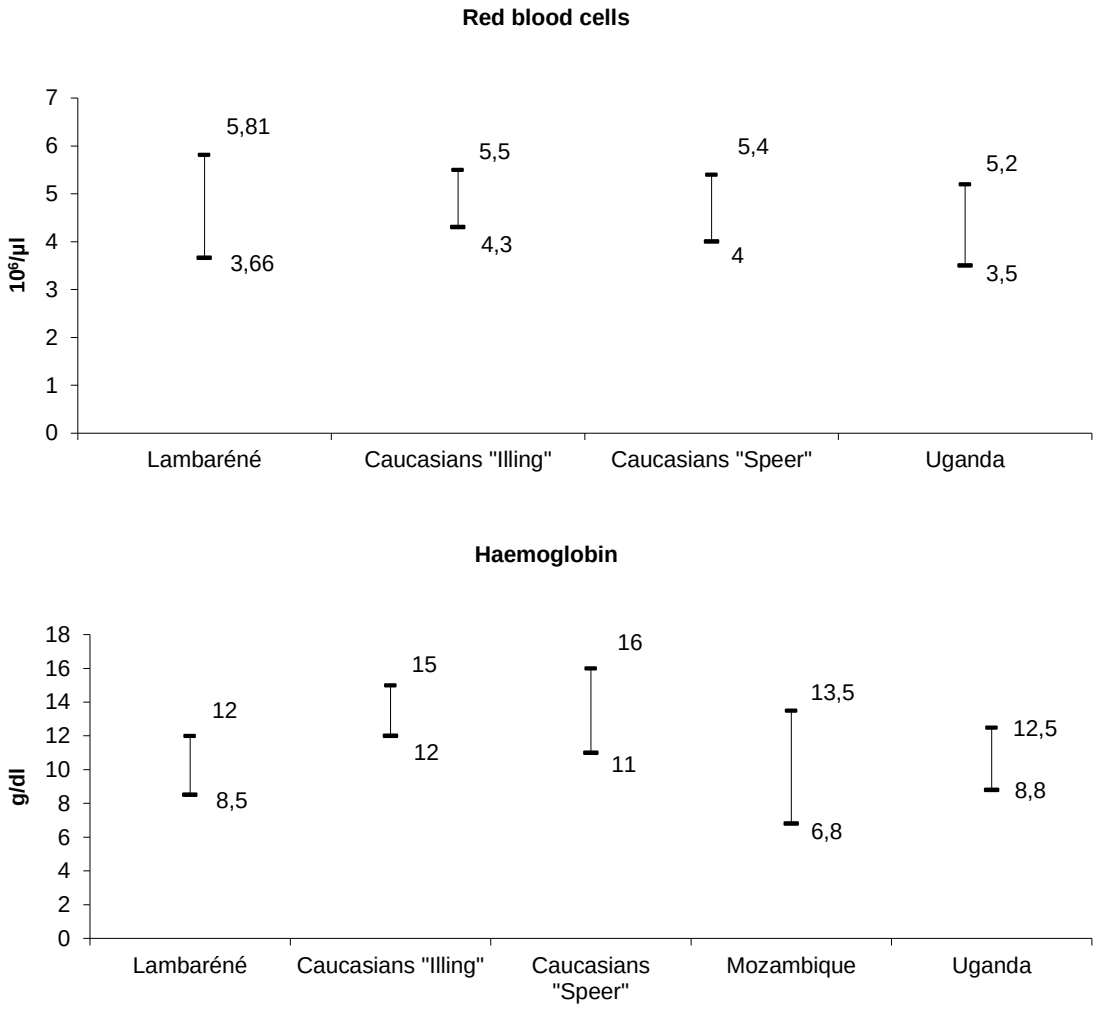
** according to Speer et al., “Pädiatrie” [157]

*** data are taken from Quinto et al. [136]. Due to the segregation of the 1 - 5 years group into 4 age groups (1-2 years; 2-3 years; 3-4 years; 4-5 years) in the data from Mozambique, this table presents the lower reference value of the one year group and the upper reference limit of the 5 year group

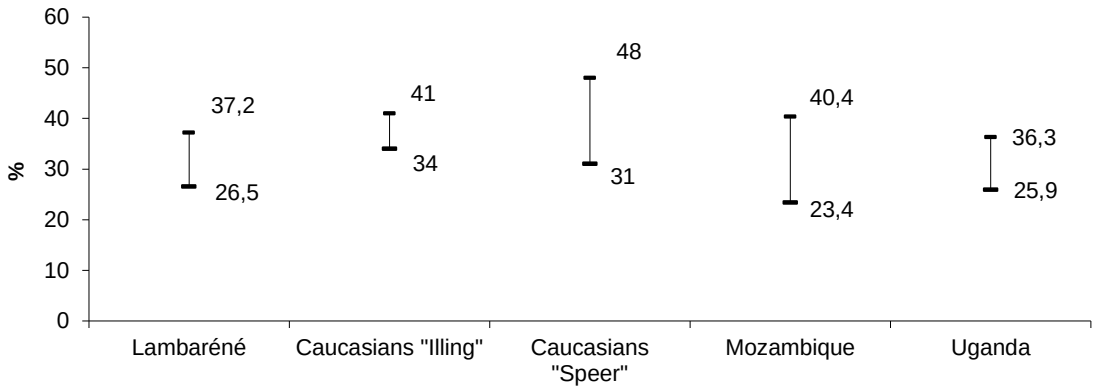
**** Lugada et al. [104] estimated the 5% and 95% percentile of their data set from Uganda

***** Due to the subdivision into gender, this reference range derives from the lower one-year old girls' value; the upper reference limit is taken from the 5-year old boys' value

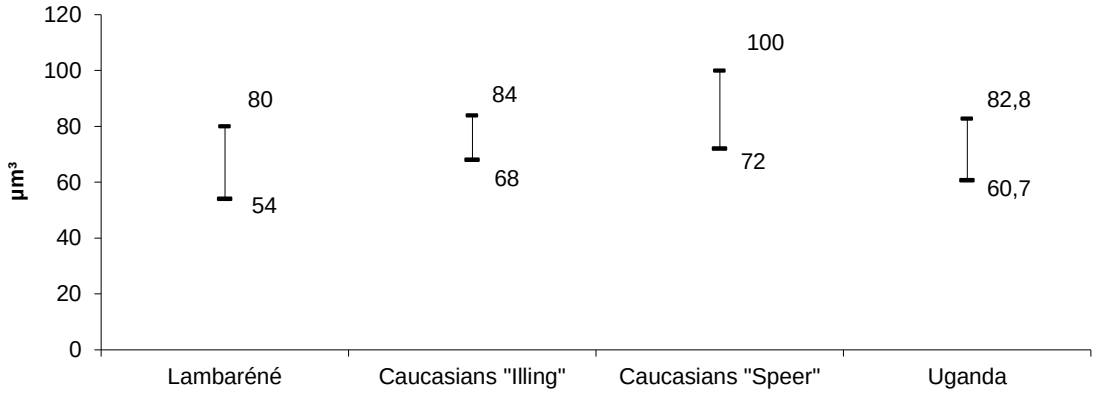
Fig. 7. Reference limits of major parameters for Lambaréné in comparison to other populations (CHILDREN COHORT).



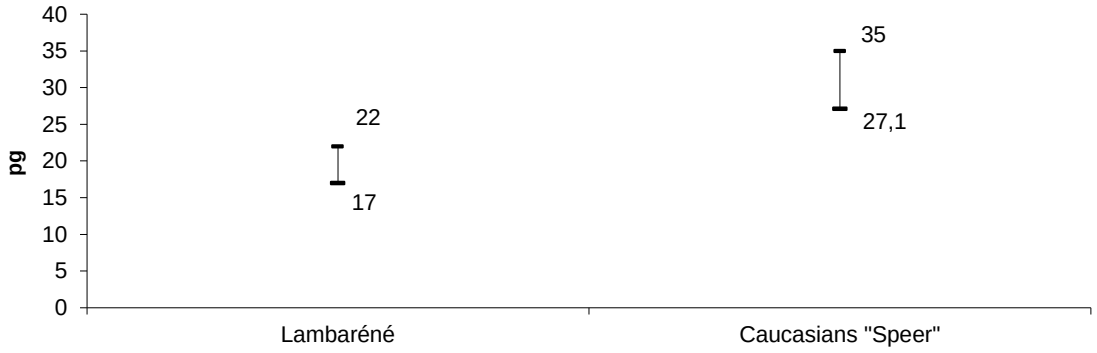
Haematocrit

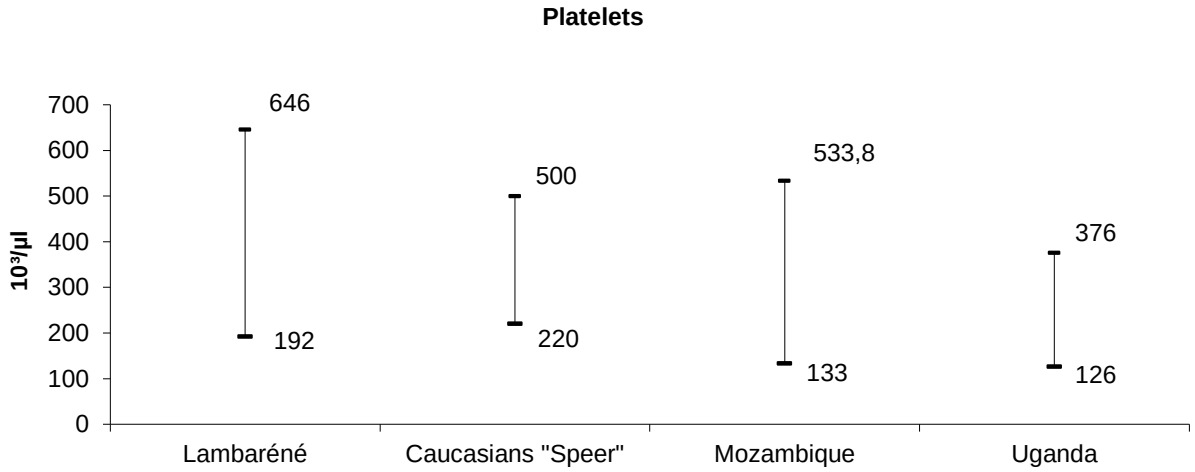


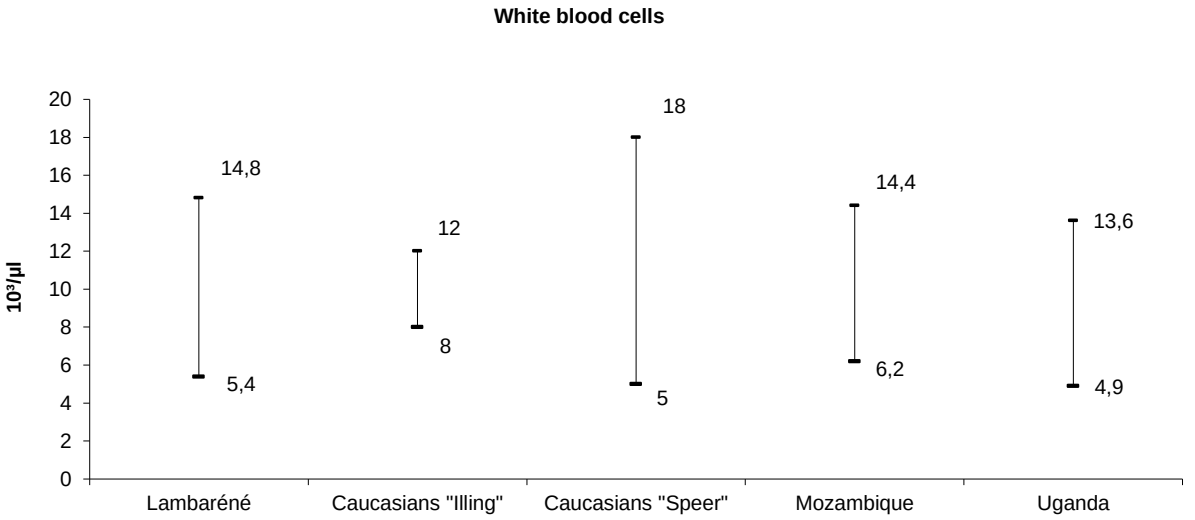
Mean cell volume

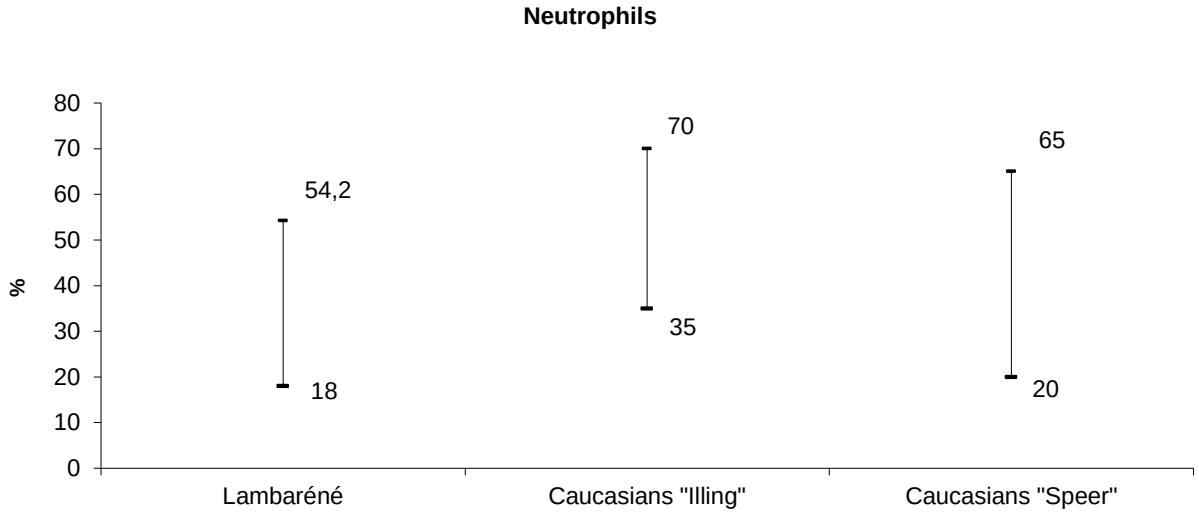


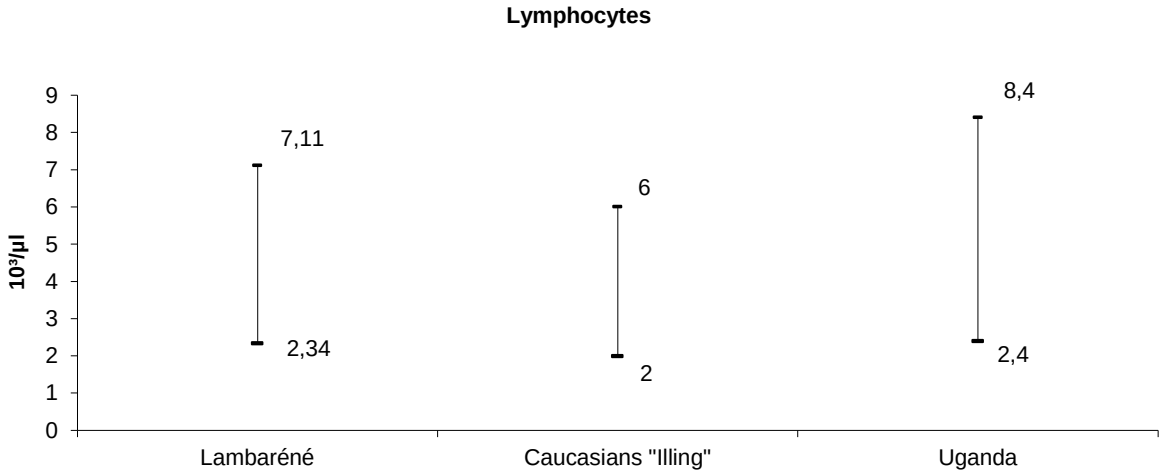
Mean cell haemoglobin

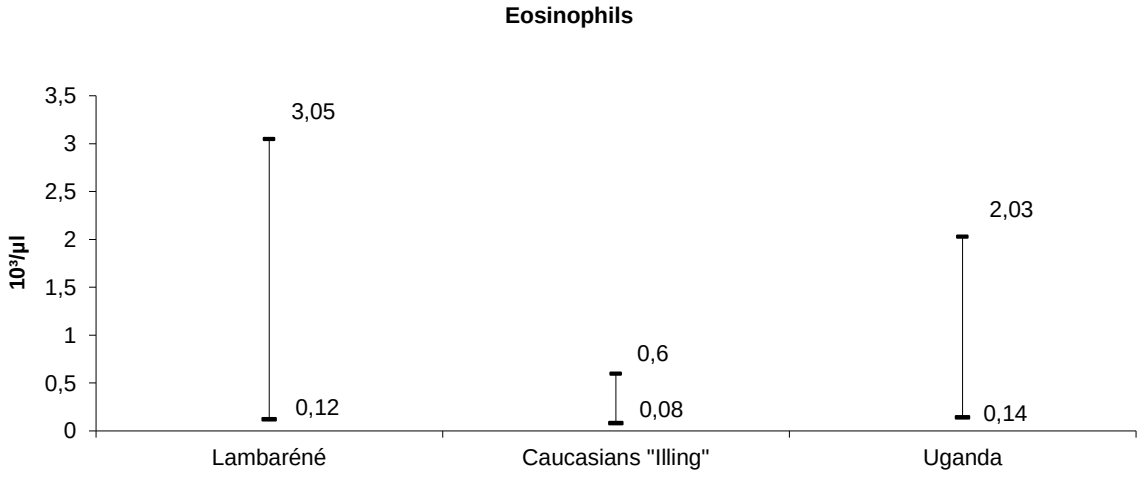


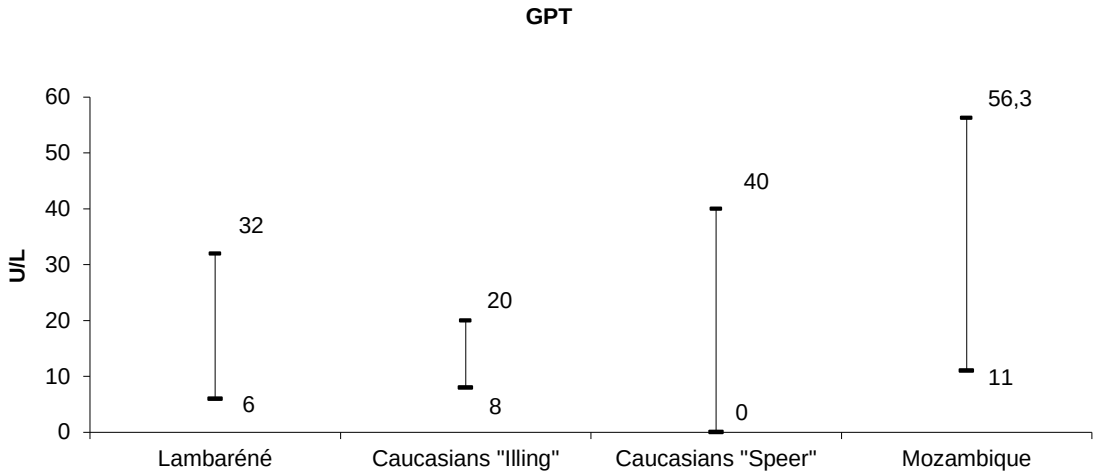

Platelets
10³/µl
700
600
500
400
300
200
100
0
646
192
500
220
533,8
133
376
126
Lambaréné
Caucasians "Speer"
Mozambique
Uganda

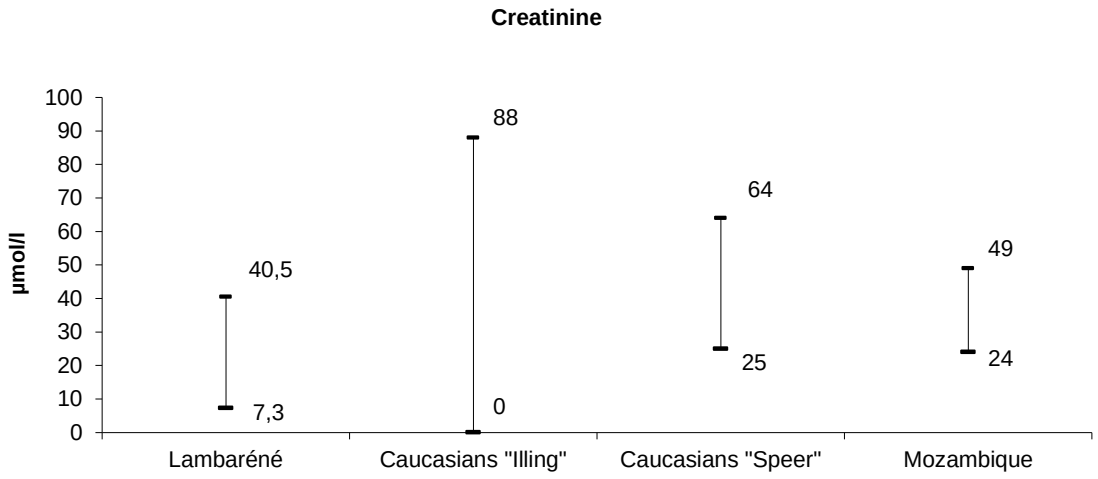

White blood cells
10³/µl
20
18
16
14
12
10
8
6
4
2
0
14,8
5,4
12
8
18
5
14,4
6,2
13,6
4,9
Lambaréné
Caucasians "Illing"
Caucasians "Speer"
Mozambique
Uganda


Neutrophils
%
80
70
60
50
40
30
20
10
0
54,2
18
70
35
65
20
Lambaréné
Caucasians "Illing"
Caucasians "Speer"


Lymphocytes
10³/µl
9
8
7
6
5
4
3
2
1
0
7,11
2,34
6
2
8,4
2,4
Lambaréné
Caucasians "Illing"
Uganda


Eosinophils
10³/µl
3,5
3
2,5
2
1,5
1
0,5
0
3,05
0,12
0,6
0,08
2,03
0,14
Lambaréné
Caucasians "Illing"
Uganda


GPT
U/L
60
50
40
30
20
10
0
32
6
20
8
40
0
56,3
11
Lambaréné
Caucasians "Illing"
Caucasians "Speer"
Mozambique


Creatinine
µmol/l
100
90
80
70
60
50
40
30
20
10
0
40,5
7,3
88
0
64
25
49
24
Lambaréné
Caucasians "Illing"
Caucasians "Speer"
Mozambique

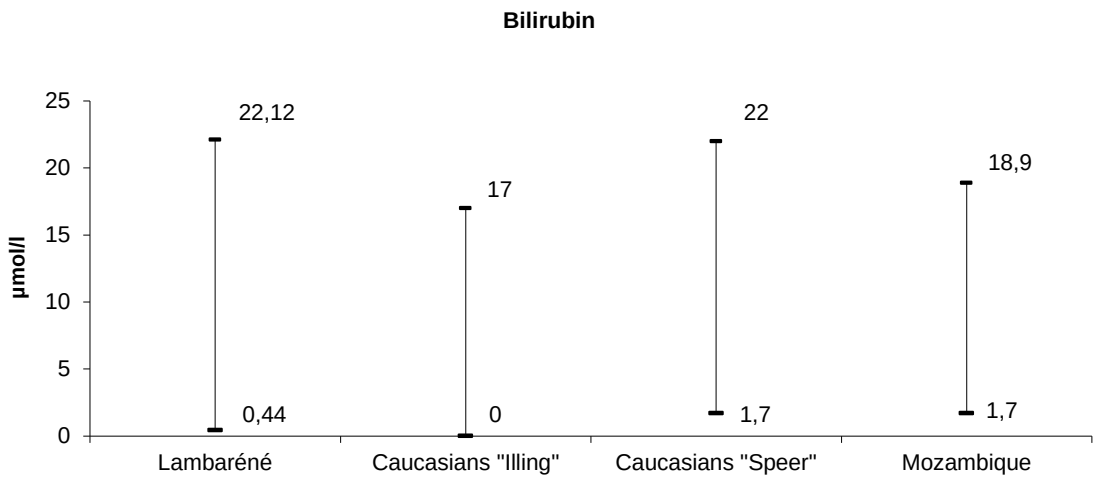

Bilirubin
µmol/l
25
20
15
10
5
0
22,12
0,44
17
0
22
1,7
18,9
1,7
Lambaréné
Caucasians "Illing"
Caucasians "Speer"
Mozambique

Table 17. Reference Values for children aged 4 to 9 weeks in Lambaréné compared to Caucasian values

Parameter	Reference Range		
	4 to 9 weeks	**Caucasian Infants * Caucasian Infants 2 – 12 months ****	**Uganda (<1 year) *****
Red blood cells			
RBC [10^6/µl]	2.89 - 4.77	3.9 – 5.3 3.0 – 5.4	3.0 – 5.4
HGB [g/dl]	8.4 – 12.8	11 – 17 9.5 – 16	6.8 – 14.7
HCT [%]	25 - 38.3	41 – 48 30 – 55	20.4 – 42.6
MCV [µm³]	70 - 96	100 ± 6 74 – 125	54.9 – 88.3
MCH [pg]	23 – 33	not available 21 – 38	not available
MCHC [g/dl]	32 - 35.3	not available 25 – 37	not available
RDW [%]	11 - 15.2	not available not available	not available
Platelets			
PLT [10³/µl]	143 - 638	100 - 250 100 – 250	123 – 487
MPV [µm³]	6.8 – 10.1	not available not available	not available
PCT [%]	0.145 - 0.498	not available not available	not available
PDW [%]	9.9 – 22.1	not available not available	not available
Leukocytes			
WBC [10^3/µl]	5.8 – 13.5	9 – 15 5 – 18	4.1–15.8
LYM [%]	42.9 - 74.8	20 – 70 41 – 56	not available
LYM [10³/µl]	3.42 - 8.69	1.8 – 10.5 not available	1.9 – 10.3
MON [%]	7.7 – 23.4	7 – 20 1 – 11	not available
MON [10³/µl]	0.65 - 2.51	0.63 – 3 not available	0.22 – 1.83
NEU [%]	8.1 – 34.9	25 – 65 20 – 65	not available

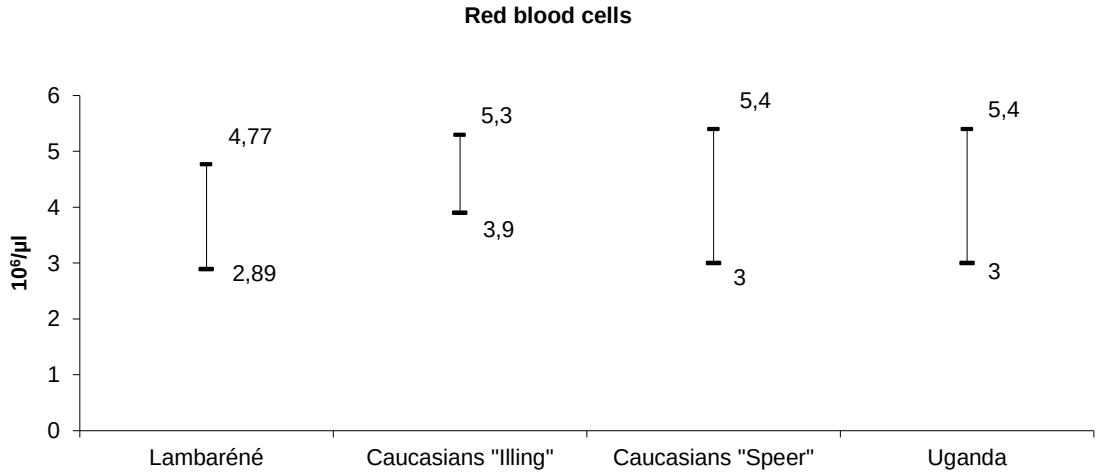
NEU [10³/µl]	0.54 - 3.54	2.25 – 9.75 not available	0.9 – 4.4
EOS [%]	0.8 - 6.1	1 – 7 1 – 6	not available
EOS [10³/µl]	0.07 - 0.58	0.09 – 1.05 not available	0.07 – 1.85
BAS [%]	1 - 3.1	0 – 2 0 – 1	not available
BAS [10³/µl]	0.07 - 0.37	0 – 0.3 not available	0.02 – 0.30
ALY [%]	3.1 - 11	not available not available	not available
ALY [10³/µl]	0.22 - 1.09	not available not available	not available
LIC [%]	0.7 - 3.9	not available not available	not available
LIC [10³/µl]	0.04 - 0.42	not available not available	not available
Biochemistry			
GPT [U/L]	8 – 41	5 – 25 <60	not available
CREA [µmol/l]	11.7 - 39.6	0 – 44 22 – 55	not available

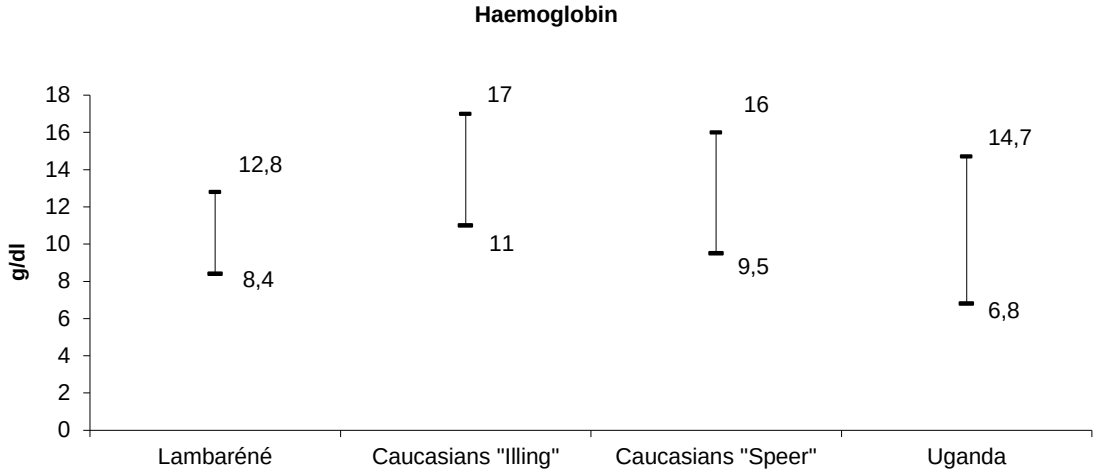
* according to Illing et al., “Klinikleitfaden Pädiatrie” [87]

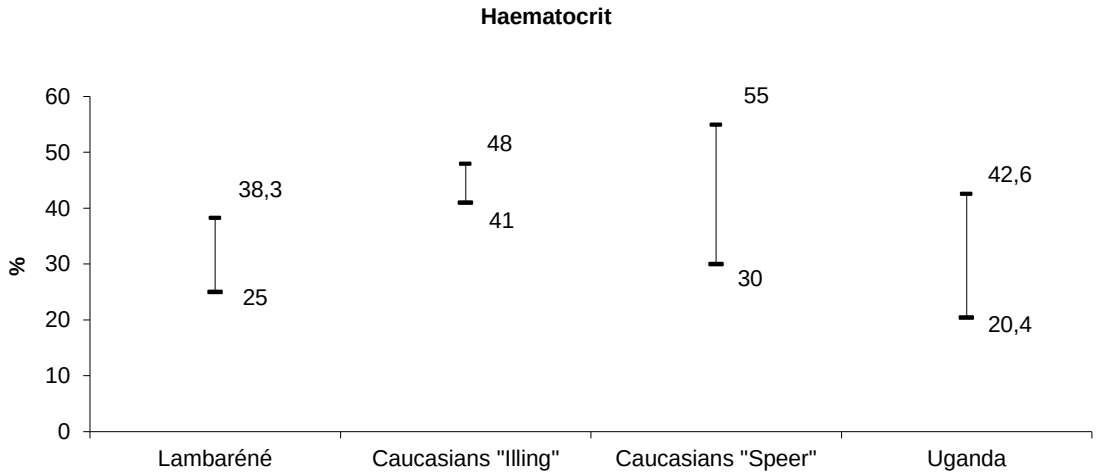
** according to Speer et al., “Pädiatrie” [157]

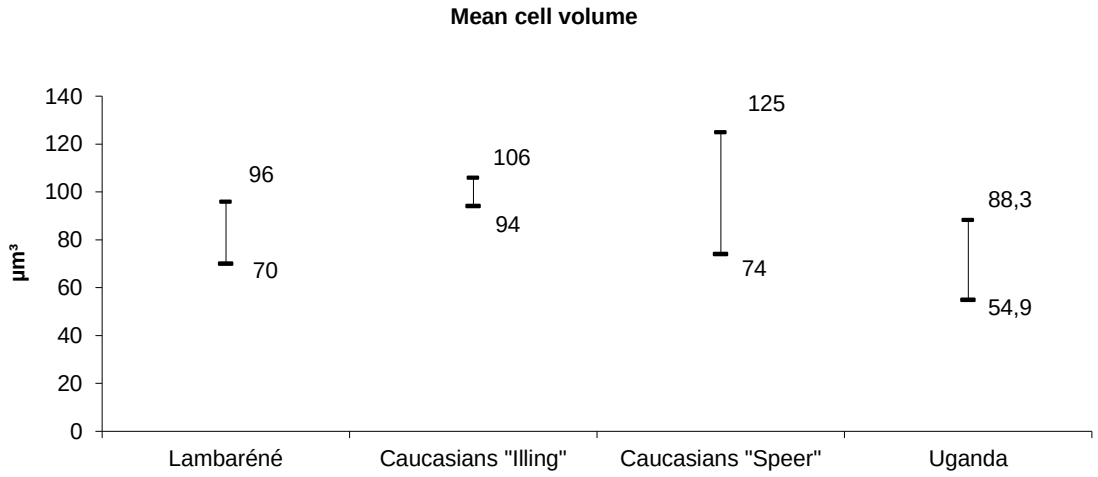
*** Lugada et al. [104] estimated the 5% and 95% percentile of their data set from Uganda

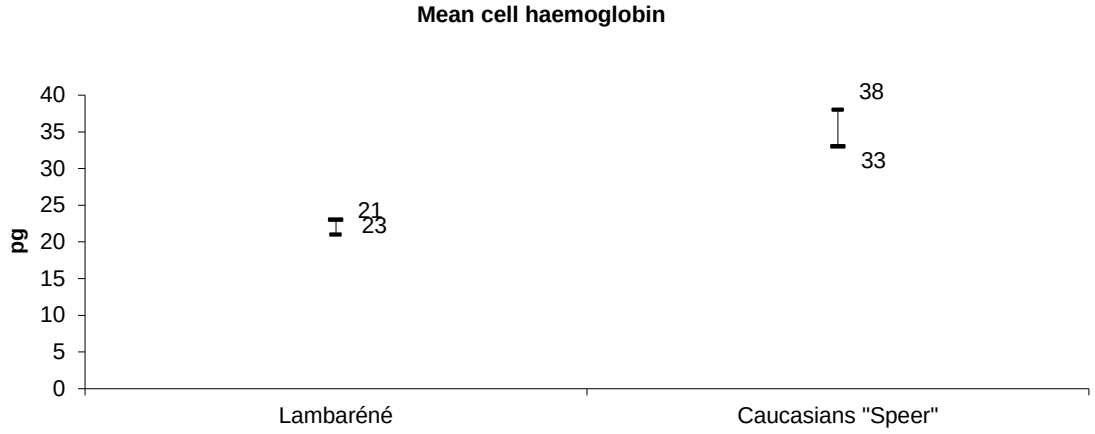
Fig. 8. Reference limits of major parameters for Lambaréné in comparison to other populations (INFANT COHORT).

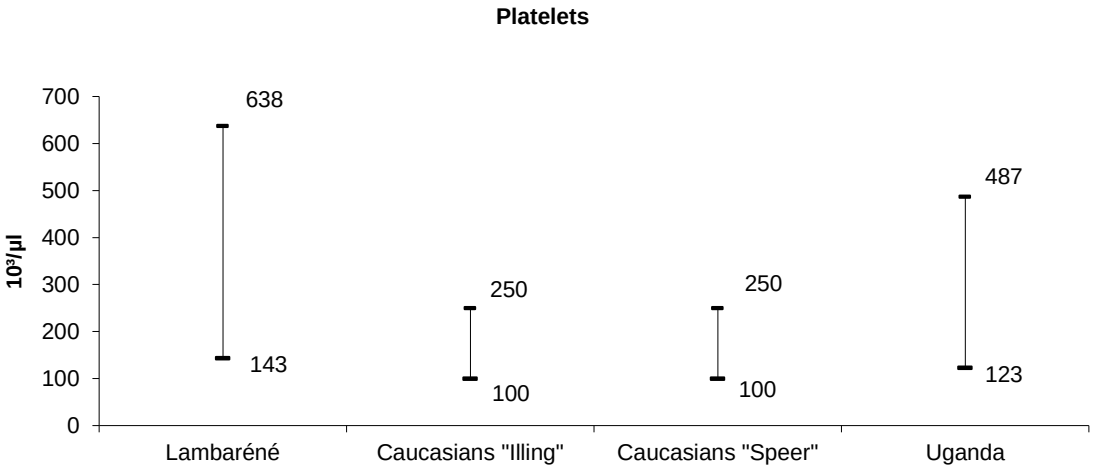


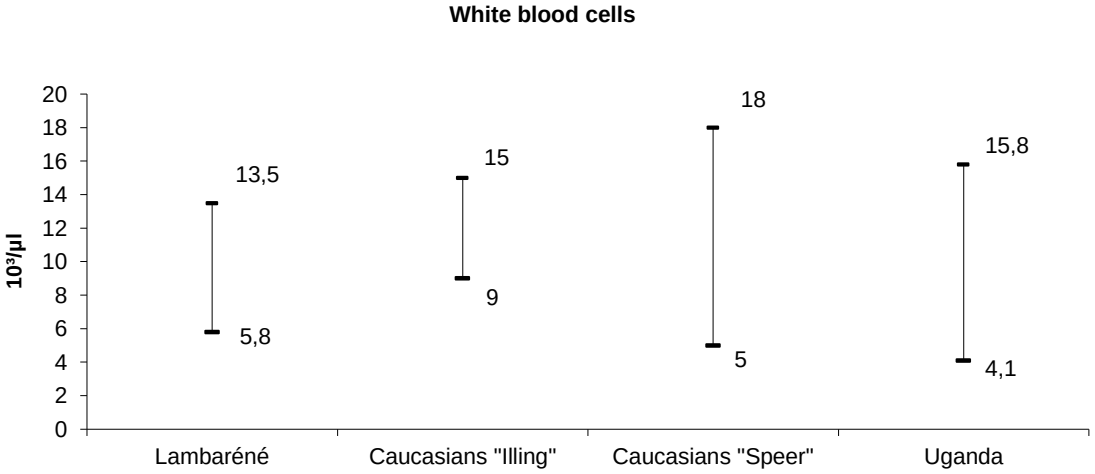

Haemoglobin
g/dl
18
16
14
12
10
8
6
4
2
0
12,8
8,4
17
11
16
9,5
14,7
6,8
Lambaréné
Caucasians "Illing"
Caucasians "Speer"
Uganda

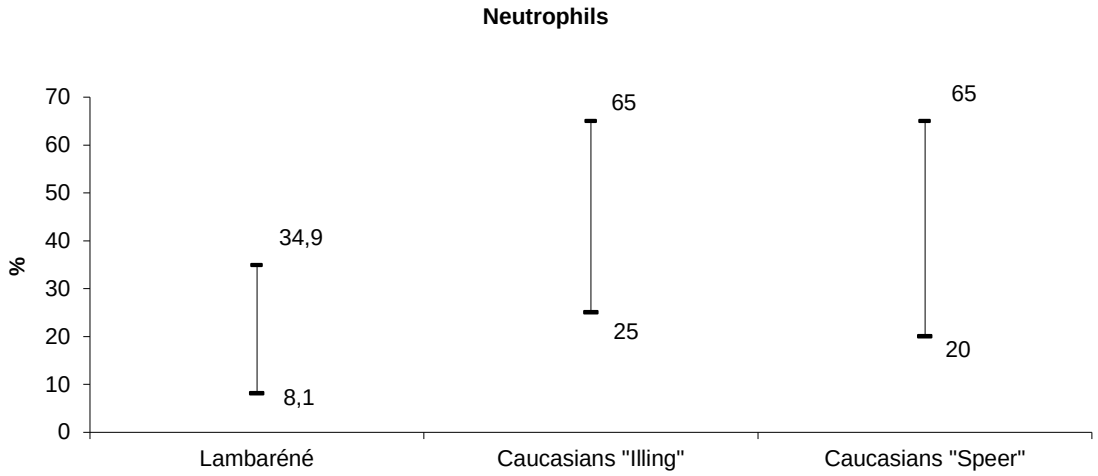

Haematocrit
%
60
50
40
30
20
10
0
38,3
25
48
41
55
30
42,6
20,4
Lambaréné
Caucasians "Illing"
Caucasians "Speer"
Uganda

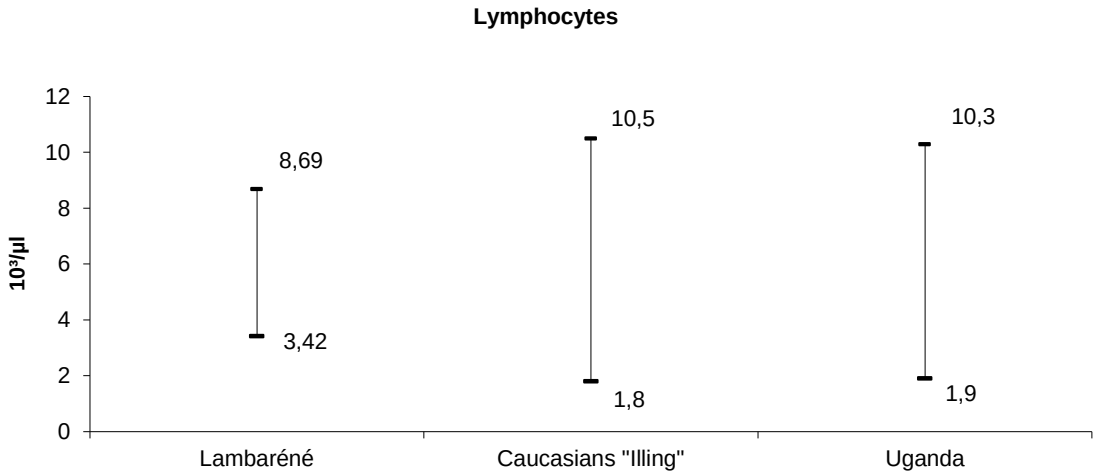

Mean cell volume
µm³
140
120
100
80
60
40
20
0
96
70
106
94
125
74
88,3
54,9
Lambaréné
Caucasians "Illing"
Caucasians "Speer"
Uganda

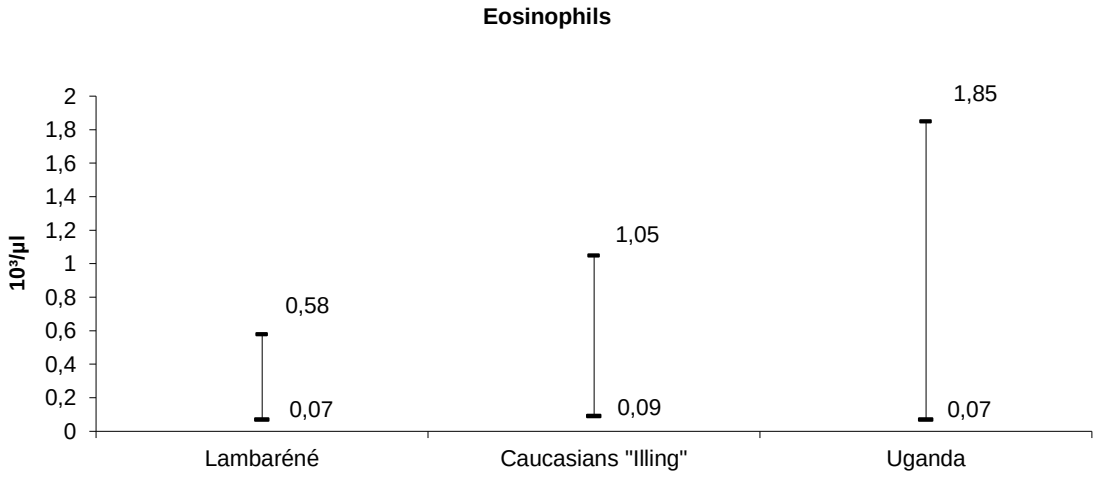

Mean cell haemoglobin
pg
40
35
30
25
20
15
10
5
0
38
33
21
23
Lambaréné
Caucasians "Speer"

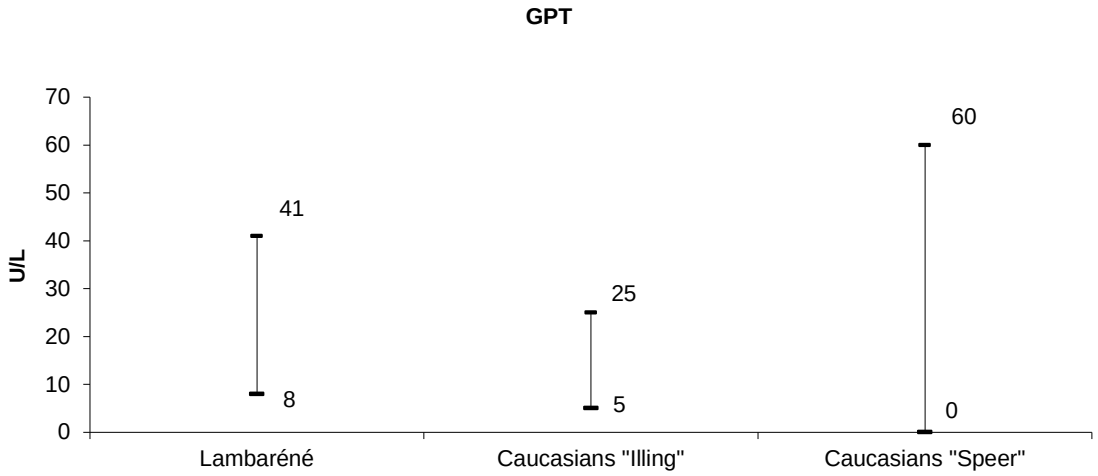

Platelets
10³/µl
700
600
500
400
300
200
100
0
638
143
250
100
250
100
487
123
Lambaréné
Caucasians "Illing"
Caucasians "Speer"
Uganda

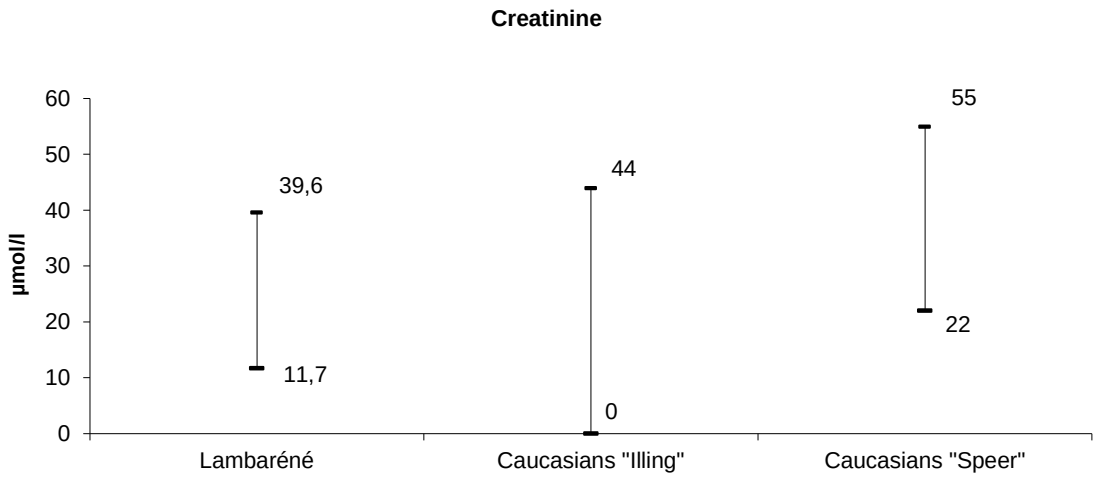

White blood cells
10³/µl
20
18
16
14
12
10
8
6
4
2
0
13,5
5,8
15
9
18
5
15,8
4,1
Lambaréné
Caucasians "Illing"
Caucasians "Speer"
Uganda


Neutrophils
%
70
60
50
40
30
20
10
0
34,9
8,1
65
25
65
20
Lambaréné
Caucasians "Illing"
Caucasians "Speer"


Lymphocytes
10³/µl
12
10
8
6
4
2
0
8,69
3,42
10,5
1,8
10,3
1,9
Lambaréné
Caucasians "Illing"
Uganda


Eosinophils
10³/µl
2
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
0,58
0,07
1,05
0,09
1,85
0,07
Lambaréné
Caucasians "Illing"
Uganda


GPT
U/L
70
60
50
40
30
20
10
0
41
8
25
5
60
0
Lambaréné
Caucasians "Illing"
Caucasians "Speer"


Creatinine
µmol/l
60
50
40
30
20
10
0
39,6
11,7
44
0
55
22
Lambaréné
Caucasians "Illing"
Caucasians "Speer"

5 Discussion

Previous studies have clearly shown that ethnic differences in haematologic and biochemical analytes exist. Therefore, it is necessary to establish haematologic and biochemical reference values specifically for the populations in which they will be used. In this thesis, the main objective is to determine reference ranges for haematologic and biochemical parameters in healthy children from rural Gabon.

In the Medical Research Unit (MRU) at the Albert Schweitzer Hospital in Lambaréné a large number of clinical studies are conducted, the majority in a paediatric population and with strict adherance to ICH/GCP guidelines. These guidelines list normal values as essential document Nr. 8.2.11. [86]

These presented reference values provide a basis for better and safer clinical research and closer adherence to ICH/GCP guidelines. Furthermore, the MRU serves as a medical facility for the health care of children, who participate in several long-term studies (e.g. vaccine studies). To improve detection of Adverse Events and for medical decisions in clinical practice using properly established reference values is essential.

Selection of reference sample group. The establishment of a suitable reference sample group is essential but difficult. Inclusion/exclusion criteria for the present reference sample group were based on an appropriate medical examination and questioning. Nevertheless, a number of diseases may not have been picked up. For example, one child in the “INFANT COHORT“ trial was determined to be positive for sickle cell anemia a few months after blood sampling and therefore had to be excluded a posteriori. Other diseases that are difficult to detect clinically, such as HIV, are present in only low prevalences in the local population or would not have a major influence on laboratory values. Lastly, because of the difficulty in interviewing children, data was obtained through the questioning of parents and as such, there is the potential that information was missed due to observational oversights and that recall bias on the behalf of parents occurred.

Data and statistical analysis. For the calculation of reference values, 185 sets of lab results for the CHILDREN COHORT and 266 sets of lab results for the INFANTS COHORT were used. Even though some readings were rejected by the analyser, there was enough data for a rigorous statistical analysis.

Expectedly, the z- test showed that none of the values differed by gender. Previous studies show that differences by gender are dependent on age and become evident at about 12 years [36, 64, 185]. Only the value for bilirubin in Test B fell below the threshold of 3, but all other tests did not show any evidence for needed subclass dividing. Furthermore, no physiological explanation exists which could give evidence for possible differences between males and females concerning the levels of bilirubin. Therefore, all reference values are valid for both sexes.

The major parameters followed a Gaussian distribution with RBC, HGB, HCT, PLT and CREA being normally distributed in both cohorts. Reed et al. [137] have shown that if data are in fact gaussian or log-gaussian distributed, “nonparametric estimates are practically indistinguishable from the best gaussian or log-gaussian estimates”. Furthermore, they have shown that when neither assumption is true, nonparametric estimates were much better. Therefore, the present analysis is based entirely on the nonparametric methods recommended by the NCCLS.

Different methods by which to calculate reference values have been developed. According to Horn et al. [83], the method used in the present analysis shows some advantages over the other methods and is deemed best for parameters derived from a non-gaussian distributed data set. Concerning detection of outliers, Horn et al. [82] caution that there might be some sources of error using the Dixon test. On balance, however, NCCLS recommends this method as appropriate for non-parametric analysis.

5.1 Comparison of reference values to other populations

5.1.1 Environmental and genetic factors acting upon the Lambaréné population

5.1.1.1 Malaria

To understand the impact of malaria as an environmental influence, it is important to know that the population of Lambaréné, like other Central African populations, lives in a malaria endemic setting, with year long exposure to these parasites. The disease can influence blood values through two distinct mechanisms: on a population level, the exposure to malaria over thousands of years has led to erythrocyte polymorphisms which effect red cell count values. On an individual level, infection with *P. falciparum* is common and often long-lasting and untreated, leading to a change for certain red cell parameters.

The protozoan parasites of the genus *Plasmodium* have persisted for thousands of years, and so it should come as no surprise that malaria parasites have had a profound impact on recent human evolution. The so called “malaria protective hypothesis” posits that certain human genetic polymorphisms, especially those affecting red blood cell structure or function, have been selected for, and thus exist in high frequency, because they have protected against the effects of malarial infection.

After extensive clinical and epidemiologic investigation, it is has been shown that a single point mutation in the gene for the beta chain of haemoglobin causes an altered form of haemoglobin, called haemoglobin S [22, 40, 88, 181]. One study illustrated that West African children who are heterozygous for the sickle cell gene have approximately 1/10 of the risk of death from *P. falciparum* malaria as do those children who are homozygous for the normal gene [70, 81]. However, there are vast disadvantages that accompany this genetic malaria resistance. The result of the haemoglobin S in homozygote carriers is a hypoxia-induced irreversibly sickled cell due to membrane changes which causes infarction and haemolytic anaemia. These patients suffer from increased susceptibility to

infection, disturbance of growth and development and chronic organ damage [133].

The thalassaemias are a heterogeneous group of disorders that result from the reduced or absent synthesis of the α- or β-globin chains. Carrier for the α-thalassaemia gen usually have mild anaemia [8]. The extent and mechanism of protection of thalassaemia against malaria is unclear [181]. Alpha – thalassaemia is very common in Lambaréné, with a gene frequency of around 0.3 [101].

Other haemoglobinopathies, like HbC and HbE, have only mild clinical effects, have less malaria-protective effects and are rare in the local population [34, 119, 181].

Another red blood cell defect present in the study population is the group of mutations in the sex-linked gene for glucose-6-phosphate dehydrogenase (G6PD), which manifest clinically as neonatal jaundice, acute haemolytic anaemia, favism or chronic non-spherocytic haemolytic anaemia [30]. The areas, in which *Plasmodium falciparum* is prevalent overlap greatly with where G6PD deficiency is found, providing further evidence for the “malaria hypothesis”. Present in more than 400 million people worldwide, the highest prevalence of G6PD deficiency is found in Africa [30]. *In vivo* studies have shown a reduction in the risk of severe *P. falciparum* malaria in those with G6PD deficiency and several *in vitro* studies showed a slower growth of parasites in G6PD deficient red blood cells [115, 141], thus demonstrating the protective nature of this genetic polypmorphism. In Lambaréné, the G6DPH deficiency is common, with gene frequencies of the B, A, and A^- alleles of around 0.6, 0.2 and 0.2, respectively [101].

Haemoglobin E, Ovalocytosis, Duffy negativity, Pyruvate kinase deficiency and other genetic mutations all serve as variations which are protective against a severe course of malaria and even infection with this potentially deadly parasite [12, 34, 54, 66, 70, 81, 89, 100, 101, 114, 116, 117, 119, 162, 171, 180, 181].

Malaria, as an infectious disease, does not only have a genetical and evolutionary impact on red blood cells, but also acts on the individual, as an “environmental factor” responsible for anaemia. Approximately more than half of

all deaths in African children are related to severe anaemia caused by *Plasmodium falciparum* infection [46] and estimates of prevalence of anaemia in malaria-endemic areas vary from 31% to 90% in children [113]. Furthermore, mild-to-moderate degrees of anaemia due to a cumulative effect by continous asymptomatic parasitemia often remain undetected [46]. One study reports that about one quarter of infants was detected anaemic through cross-sectional surveys [113] and children with mean asymptomatic parasitemia ≥ 400/µl had lower median Hb levels than those with a mean density of ≤ 400/µl [56]. This implicates severe public health problems and “may impair cognitive and motor development, growth, immune function, and physical work capacity” in children with asymptomatic *P. falciparum* infection [56].

Though the fundamental pathophysiology of malarial anaemia remains unexplained, multiple factors involving both destruction and decreased production of red blood cells are known. Besides phagocytosis of parasitized and unparasitized red blood cells, hypersplenism and autoimmune haemolysis, rupture of red blood cells is “possibly the most important mechanism of anaemia during the state of parasitaemia” [113]. Decreased production of red blood cells is mostly due to erythropoid hypoplasia by suppression of the normal response of erythropoietin (EPO), and the imbalance of cytokines which cause bone marrow depression, dyserythropoiesis and erythrophagocytosis [113].

Through passive transfer of maternal immunoglobulins and expression of fetal haemoglobin, infants living in endemic areas are more sheltered from malaria in the first 6 months of life than older ones [171]. The infants of the INFANT COHORT are therefore unlikely to have suffered from malaria at the time of blood sampling and it is been previously shown that malaria in the first three months of life is extremely rare in the study area [96]. For the CHILDREN COHORT, no thick blood smear or other malaria diagnostic procedures were performed at the time of blood sampling and concomittant infections would have been possible. However, at time of blood sampling these children were healthy by definition and are therefore representative of our definition of the local normal population, regardless if they carry Plasmodium parasites at the time of sampling or not.

5.1.1.2 Helminths

Helminths comprise a variety of parasitic worms, including nematodes, cestodes and trematodes [95]. Approximately 20 major helminth infections have public health significance with the most common – the geohelminthiases [133]. More than one-quarter of the world's population are infected with one or more of the follwing parasites: the roundworm, the hookworms, the whipworm and in addition, about 200 million people are estimated to be infected with *schistosome spp*. [133].

Helminths can influence hematological parameters as they are a common cause for both anemia and eosinophilia.

Anaemia is often described as accompanying infections of hookworm and whipworm [47]. Haemoglobin-degrading proteases, produced by the worms in the host's gut, are hypothesized as being responsible for the iron losses during worm infection [28]. "As time progresses, iron intake and absorption […] are unable to compensate for iron loss into the gut" [47] and mostly children at an age when they are both learning and growing suffer from iron deficiency and sustain a reduced physical and intellectual development [133].

First postulated in 1939, it is known for a long time that eosinophils play a role in the immune response to helminth infection [97]. But the role of eosinophils in parasitic infections in vivo remains poorly understood. The role of eosinophils was first shown in histopathologic investigations where eosinophils were surrounding parasites in dying tissue biopsy specimens. In vitro, it was later shown that parasites were killed by eosinophils and eosinophils granule products [97]. Conflicting animal studies and the complex picture of human response to parasite antigens make it difficult to describe exactly the role of eosinophils to helminth infection [97]. However, all helminth infections evoke immune responses that share common features, for example elevations in Th2 cytokines (e.g. IL-4, IL-5, IL-9, IL-13) and following polarization of T-helper cells to Th2 cells, basophilia, mastocytosis, elevations in serum IgE and IgG1 and eosinophilia. The elevation of the eosionphil count depends on parasite species and is detectable within weeks to months post infection [97, 99]. Particulary

during migration, helminths, or their products, come into contact with immune effector cells in tissues and elicit eosinophilia [97, 126]. The mechanism of eosinophil-mediated protection against helminth parasites is not completely understood but “antibody-induced release, complement-induced release, or both of toxic granule proteins and reactive oxygen intermediates” [97] seem to play an important role.

For clinical use, elevated eosinophil counts are an important and significant diagnostic marker for helminth infections in children living in tropical or low-income countries [133].

In Lambaréné, helminth infections are highly prevalent. Intestinal helminths such as *Ascaris lumbricoides* and *Trichiuris trichiura* are present in most children. *Schistosoma haematobium* pesence is highly focalized and in some areas may be found in 100 % of schoolchildren. *Loa-lao* is common in adults and it is not rare to find infections even in children.

5.1.2 Red blood cell parameters

The Lambaréné red blood cell values are similar to those deriving from Uganda [104] and Mozambique [136]. Especially the Ugandan parameters of HGB, HCT and MCV of children aged 1-5 years are nearly of same value. In the younger age group, the lower limits are, however, consistently lower in the Ugandan population compared to Lambaréné. This present analysis confirms the lower values of the major erythrocyte parameters in populations of Central Africa compared to a Caucasian population. In the INFANT COHORT, MCV, MCH and MCHC were similar to reference ranges in western-based medical textbooks [87, 157], whereas in the CHILDREN COHORT this was true only for MCHC.

Previous studies have also shown decreased levels of red blood cell parameters in African populations in contrast to Caucasians (HGB [9, 13, 16, 21, 36, 37, 45, 48, 68, 104, 125, 128, 136, 138, 140, 186], HCT [9, 16, 21, 36, 37, 45, 104, 136], MCV [9, 16, 21, 36, 37, 45, 104, 128], MCH [16, 37], RBC count [16, 37, 104, 128]). These differences in haematological values have been known for a long time and are discussed by several authors. Socioeconomic status [72], nutritional factors [21] and other factors, such as genetic differences and iron deficiency,

may induce variation in haemoglobin [9]. Further studies claim unknown genetic influences on haemoglobin values rather than iron status, alpha or beta thalassaemia trait or environmental influences [21, 140]. Excluding children with Haemoglobin S, Haemoglobin C and those with a mean cell volume of more than 5% below the normal mean for age (to exclude iron deficiency or thalassaemia minor), Dallman et al. [48] were able to show that haemoglobin concentrations averaged 0.5 g/dl less in black children aged 5 to 14 years. Dallman estimates that about 10% of the “normal blacks would be mistakenly designated anaemic” due to genetic or environmental circumstances.

In this present analysis, infants had nearly the same values of MCV and MCH as the reference values from western populations. This observation is corroborated by another study [51] which showed that Kenyan infants, aged 0 to 2 months, had haemoglobin values similar to healthy reference children from developed countries. The author confirms that very young infants who are sheltered have the same levels as their western counterparts, but that these levels decrease starting at 2 months of age. Hence, approximately two-thirds of the infants had become anaemic over a period of 3-5 months compared to their Caucasian counterparts. Due to various influences, the haematologic differences between black and Caucasian populations widen over time. However, in Lambaréné only MCV and MCH, but not HGB, weas similar to western reference values and therefore it is not clear if the same mechanisms are at work in this setting.

5.1.3 Platelets

The PLT counts were found to be somewhat higher in the present analysis than in western medical textbooks. Whereas the lower limit is comparable to other populations, the upper limit is consistently higher than elsewhere. In the present side-by-side comparison of reference values over populations no obvious pattern can be discerned. Previous studies have reported similar difficulties or discripancies. In Zambians [69], Ugandans [104], Nigerians [60] and Kenyans [120], platelet counts tend to be lower, with the cause for this being unknown. The upper value of the platelet count found in Mozambique was comparable to those of Caucasian textbooks, though the lower level showed a decreased value.

In contrast, in one study black American women were found to have significantly higher platelet counts than white women [145], but after exclusion of women with either microcytosis or iron deficiency, no differences in platelet counts between black and white women could be noted. These findings led the authors to conclude that differences in platelets were secondary to common RBC differences, like iron deficiency and other causes of microcytic anaemia. Another reason for the high platelet counts could be an error in measurement caused by WBC fragments that may interfere with the proper counting of platelets and could cause elevated PLT counts [84]. It is impossible to say if these "pseudoplatelets" bias the true platelet values in the absence of manual counts which are still recognized as the gold standard [26].

5.1.4 White blood cells

The total white blood cell count of Lambaréné, Mozambique and Uganda were comparable and had nearly the same ranges as Caucasian values. In Lambaréné, there were less neutrophils in both age groups compared to a Caucasian population. The cause of lower neutrophil counts was described in other studies [14, 61, 63, 67, 75, 85, 104, 118, 125, 129, 138, 150, 152-154] and remains unknown. By comparing African, mixed or western diets in Zambians, one study [63] suggested an environmental cause. Further studies [74, 92, 93, 110] suggested an increased retention in the marginal pool. Showing a significantly lower neutrophil increase after corticosteroid exposure in “neutropenic” blacks than in whites, it was hypothesized that there exists a “defect” in the release of mature granulocytes from the bone marrow to the circulating blood [74, 110].

The monocyte count was, on average, higher in our study population than in western medical textbooks. These results conflict with previous studies that showed lower monocyte counts in blacks. Some studies [15, 67, 164] found a significantly lower mean monocyte count in blacks contrary to other studies [92, 130] who could not report lower monocyte counts in black populations. One study [15] found “that the ratio of mean neutrophil count to mean monocyte count in

blacks was similar to the ratio [they] had previously found in whites". However, reasons for these differences are unknown.

Lymphocytes had an increased level in the INFANT COHORT compared to Caucasian and Ugandan ranges. The lymphocyte parameters in the CHILDREN COHORT had nearly the same values as corresponding western parameters and Ugandan parameters though the upper level exceed both Caucasian and Gabonese values. Some authors [15, 85] could not find differences in the lymphocyte counts between black and white adults as well. Quoting Bain et al. [15] there are many studies on lymphocyte counts [62, 77, 94, 130, 150, 151, 170] with only some studies [63, 67, 125, 169] showing higher counts in South African Bantu and African blacks. Given that the majority of these studies do not observe any ethnic variation in lymphocyte counts it can be assumed that no differences exist.

Regarding the eosinophil analysis, the INFANT COHORT showed the same range as western EOS counts. However, the CHILDREN COHORT had considerably higher values. As in other African studies [13, 45, 63, 104, 125, 143], the eosinophil count in this present analysis was found to be higher than in Western subjects [157]. A multitude of different diseases such as allergic disorders, infections with protozoa and ectoparasites, fungal diseases, lymphomas, immunodeficiency disorders or Addison's disease can provoke eosinophilia. Furthermore, many medications cause eosinophilia, but helminth infections are the most common cause for eosinophilic disorders, especially in children living in tropical areas [103, 126]. Furthermore, previous studies ascribed higher levels of eosinophils in black populations to filariases, schistosomiasis and hookworm infections [33, 131]. Elevated values cannot be found in African residents in Europe [14], and therefore the author suggests an environmental rather than a genetic or other aetiology for higher eosinophil counts in African blacks.

5.1.5 Biochemistry

Comparing the biochemistry results of Lambaréné with those of other populations is hampered by the lack of published reference values for various populations,

especially Africans. In addition, the normal values used to in standard texts for Caucasians differ considerably. No pattern could be distinguished by comparing Lambaréné values with those other populations for any parameter. One study examined the levels of liver enzymes in 50 healthy Senegalese and was not able to identify differences between their values and those of Europeans for GOT, GPT and GGT [144]. The findings suggest that no differences exist between the races.

With regard to ethnic differences in creatinine levels, one study [7] demonstrated significant differences between black and white races, but the authors ascribed these race-sex differences in serum creatinine levels to body mass. One American study [90] found high creatinine levels in blacks compared to whites. On the contrary, one study [112] investigated similar reference values for creatinine in Kenyan subjects 50 years of age or older compared to those of Caucasians. However, in this present analysis, creatinine reference values have lower upper limits than those quoted for Caucasians and creatinine levels of both African studies from Lambaréné and Mozambique were of nearly same values. In the absence of comparable data and publications, the validity and generalizability of this observation is unclear. Furthermore, without controlling for glomerular filtration rate (GFR) or lean body mass, it is difficult to say to what extent the variability by sex, ethnicity, and age reflects normal physiological differences rather than the presence of kidney disease [90]. Until this information is known, the use of a single threshold by which to define elevated serum creatinine values may be misleading.

In this present analysis, total bilirubin reference ranges were of the same magnitude as those for these children’s white counterparts and those from Mozambique. One author could find lower rates of values for total bilirubin in black than in white adult participants and considers therefore race specific reference ranges [108]. In addition, Carmel et al. [31] found lower bilirubin levels among 1538 healthy black Americans compared to whites and Latin Americans.

5.2 Applicability of reference values for other populations

The determination of reference values for the population in Gabon is the first of its kind published for the Central African region following the NCCLS guidelines. They are derived from a homogeneous population of Central Africa with environmental influences, which are typical for this region. Therefore, these reference values are preferable to rough estimates or to those derived from a different population [39].

Investigators and clinicians must consider several factors when applying these reference ranges outside of the exact population and methods used for the present analysis: the influence of population genetics, environmental and socio-economic factors, age and of the laboratory equipment used for analysis.

Genetic and environmental influences:

The data in the present analysis derive from a small population of a geographical limited area. Most of the children were born or grown up in Lambaréné and only few come from the more rural sites up to 40 km outside from Lambaréné. From the west coast to the Great Rift Valley in the east, Gabon, the Republic of Congo and parts of the Democratic Republic of Congo comprise a great area with nearly the same altitude and same equatorial climate.

The people, sharing a common language family, the Bantu languages, are genetically fairly homogeneous. The Bantu group derive from a proto-Bantu group living near the Cameroun-Nigerian border and spread out around 5,000 years (3000 BC) ago to populate a geographically wide area, ranging from Cameroon to South-Africa and East Coast Kenya. These populations are also exposed to similar diseases, all living, for example, in a high risk malaria transmission setting [174] .

Socio-economic influences:

Due to the export of petroleum, manganese and wood products, Gabon is one of the richest countries in Africa, but its social indicators are comparable to those of several low-income African countries [135]. “The relatively high income per capita masks wide disparities in standards of living. Though poverty in its most acute

forms (under-nutrition) is now rare in Gabon, most of its people remain poor" [135]. In 1994, 23% of Gabon's population lived below the extreme poverty line (=US$ 1 per person per day) and 62% below the poverty line (=2/3 of average consumption) [135]. In Cameroon, for example, nearly same indexes are to be found. In 2000, 48% of the population of Cameroon were estimated to live under the poverty line [39]. For the whole central African region it can be said, that children at birth have the same life expectancy of 54 years in Gabon as well as in Cameroon, the Democratic Republic of Congo and the Republic of Congo.
With genetic and socio-economic factors having a minor influence, it is likely that the present reference values are applicable in the whole of Central Africa.

Influence of age:

For this analysis, children and infants were divided into specific age-based groups for practical reasons and then those groups were used to derive age-based reference intervals. The inclusion criteria for both trials defined the range of age for its participants and consequently for this analysis. The CHILDREN COHORT ranged from 18 to 60 months and the INFANT COHORT from 4 weeks to 9 weeks.

The present analysis for the CHILDREN COHORT is based on the pooled values of children aged 18 to 60 months. Dividing this age group into further small groups (e.g. 1-2 years, 2-3 years and so on) was not possible. Only 185 data sets were available with which to establish the present reference values and subclassing would reduce the raw data to an amount insufficient to meet the NCCLS criteria for "well-founded statistical analysis." Strictly speaking, the reference values deriving from the CHILDREN COHORT only count for the investigated age group. It is, however, well established that the values for red blood cells, platelet indices and white blood cells in children aged 12 months differ barely from those aged 18 months and more [11, 20, 29, 49, 80, 91, 98, 105, 132, 142]. Furthermore, differences between 5 years olds and elder children are rare and it may be possible to apply them to elder children. As the prevalence of helminth infections increase with age, it is conceivable that EOS values might

differ between the age extremes, although this was not tested in the present analysis.

The reference values computed for infants (4 to 9 weeks) are restricted to this age and cannot be readily transferred to younger newborns or far older infants. Neonatal erythrocyte values change significantly within the first 3 to 6 months of life [149, 157] and therefore the data from the age group on which we focused for infants only demonstrates one moment during their physiological progress. Though further raw data from later blood draws during the vaccine trial for the INFANT COHORT existed at the age of 4 to 5.5 months and 9.5 to 11 months, it was not possible to use this data to expand the infant's age group or to create further age groups. This is due not only to the statistical difficulty of using values derived from the same children but more so because of the statistically insufficient number of parameters for subclassification.

Influence of equipment:

All specimens were analysed via the ABX Pentra 60 for haematological testing and with the Cobra Mira Plus for biochemical testing. Caution needs to be exercised when using reference values in a setting with measuring different analysers. However, the NCLLS has published guidelines that describe how to transfer reference intervals to populations other than the reference population or when using an analyzers different than that used in measuring reference values: Firstly, "reference population demographics and geographics must be adequately described and be available for review" and secondly, "the preanalytical and the analytical procedural details, analytical performance, the complete set of reference values and the method of estimating the reference interval must be stated. If these factors were consistent with the receiving laboratory's operation and test subject population, then the reference interval may be transferred without a requirement for any receiving laboratory validation studies (p.24, [124]). For more accurate validation of transference, the NCCLS recommend the method proposed by Horn et al. [83] by testing 20 specimens according to the inclusion and exclusion criteria of the original study. Outliers should be detected by the Dixon test and "any apparent outlier should be discarded and new patient specimens obtained to replace it so that 20 test results with no outliers are finally

secured” (p.24, [124]). Afterwards, the analytes of the 20 healthy participants are compared to these values computed in this present case. If 3 or more of the 20 values lie outside the interval, then these reference limits are not applicable to the local population.

In conclusion, this thesis shows that in Lambaréné as well as in other African sites the values for apparently healthy children do not match values derive from western subjects. They are observations of values found in a population where malaria, environmental factors, and other infectious diseases are more common than in Europe. This knowledge may help practicing physicians, laboratory professionals and scientists to better interpret clinical findings and thus make more accurate decisions regarding patient management, thereby improving the quality of research data and, more importantly, the quality of care provided to patients in Lambaréné.

6 Summary

Previously studies show ethnic differences in haematological reference values, but the reference intervals used in African countries often derive from Caucasian or Western populations. The main aim of this thesis was to establish haematological and biochemical reference intervals for children in Lambaréné, Gabon, aged 4 weeks to 9 weeks and 1 year to 6 years.

The children found healthy by physical examination were seen at the Medical Research Unit at the Albert Schweitzer Hospital in Lambaréné, Gabon, from April 2006 to January 2008. The following parameters were measured: leukocytes (white blood cell count [WBC]), red blood cell count (RBC), haemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW), platelets (PLT), lymphocytes (LYM), monocytes (MON), neutrophils (NEU), eosinophils (EOS), basophils (BAS), the biochemical parameters glutamate-pyruvate-transaminase (GPT) and creatinine. For children aged 1 year to 6 years, total bilirubin was measured as well.

On the basis on the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) “How to Define and Determine Reference Intervals in the Clinical Laboratory” (NCCLS document C28-A2, 2nd Edition) the reference intervals were developed using the 2.5th and 97.5th quantiles of the data set. To evaluate gender influences a partition test (z-test) was performed. There was no evidence for differences in gender. For outlier detection, Dixon’s D/R ratio test was used.

In general, values for red blood cells were lower than those of corresponding western ones. PLT ranges were found to be higher. White blood cell counts were similar to those in Western textbooks. However, eosinophils were found to be elevated in the elder age group, most likely caused by intestinal helminths. In general, biochemical reference values were similar to published Caucasian values.

Like previous studies this thesis confirms the importance of establishing population based reference intervals in order to avoid harm by misinterpreting haematological or biochemical findings. These values are not intended to redefine “normality”. They are the product of environmental and genetic influences acting on both the population and the individual level.

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8 Appendix A

Normal ranges for infants and children at the Medical Research Unit Lambaréné

Parameter	Range	
	4 to 9 weeks	12 months to 60 months
WBC [10^3/µl]	5.8 – 13.5	5.4 - 14.8
RBC [10^6/µl]	2.89 - 4.77	3.66 - 5.81
HGB [g/dl]	8.4 – 12.8	8.5 - 12
HCT [%]	25 - 38.3	26.5 - 37.2
MCV [µm³]	70 - 96	54 - 80
MCH [pg]	23 – 33	17 - 27.1
MCHC [g/dl]	32 - 35.3	30.7 - 34.8
RDW [%]	11 - 15.2	11.7 - 20.9
PLT [10³/µl]	143 - 638	192 - 646
MPV [µm³]	6.8 – 10.1	6.9 - 10
PCT [%]	0.145 - 0.498	0.16 - 0.488
PDW [%]	9.9 – 22.1	9 - 20.6
LYM [%]	42.9 - 74.8	27.4 - 64.2
LYM [10³/µl]	3.42 - 8.69	2.34 - 7.11
MON [%]	7.7 – 23.4	4.7 - 13
MON [10³/µl]	0.65 - 2.51	0.36 - 1.62
NEU [%]	8.1 – 34.9	18 - 54.2
NEU [10³/µl]	0.54 - 3.54	1.27 - 6.85
EOS [%]	0.8 - 6.1	1.3 - 24.4
EOS [10³/µl]	0.07 - 0.58	0.12 – 3.05
BAS [%]	1 - 3.1	0.6 - 1.9
BAS [10³/µl]	0.07 - 0.37	0.04 - 0.22
ALY [%]	3.1 - 11	0.8 - 2.4
ALY [10³/µl]	0.22 - 1.09	0.06 - 0.28
LIC [%]	0.7 - 3.9	0.5 - 2.2
LIC [10³/µl]	0.04 - 0.42	0.03 - 0.27
GPT [U/L]	8 – 41	6 - 32
CREA [µmol/l]	11.7 - 39.6	7.3 - 40.5
BILI [µmol/l]		0.44 - 22.12

9 Abbreviations

µl	microlitre
ALT	alanine aminotransferase (= GPT)
ALY	atypical lymphocyte
ANSI	American National Standards Institution
AST	aspartate aminotransaminase (= GOT)
BAS	basophile count
BCG	Bacillus Calmette-Guérin vaccine
BILI	total bilirubin
CBC	complete blood count
CHILDREN COHORT	Phase II randomized, double-blind bridging study of the safety and immunogenicity of GlaxoSmithKline Biologicals. candidate Plasmodium falciparum malaria vaccine RTS,S/AS01E (0.5 mL dose) to RTS,S/AS02D (0.5 mL dose) administered IM according to a 0, 1, 2- month vaccination schedule in children aged 18 months to 4 years living in Gabon Children are aged from 18 months to 5 years
CLSI	Clinical and Laboratory Standards Institute
CREA	creatinine
CV	coefficient of variation
DTP3	diphtheria-tetanus-pertussis vaccination third vaccination
EDTA	ethylenediaminetetraacetic acid
EOS	eosinophil count
EPO	erythropoietin
EPTRV	Expert Panel on Theory of Reference Values

fL	femtoliter
GCP	Good Clinical Practice
GGT	gamma-glutamyl transpeptidase
GOT	glutamic oxaloacetic transaminase (= AST)
GPT	glutamic-pyruvic transaminase (= ALT)
HCT	haematocrit
HDI	human development index
HGB	haemoglobin level
ICSH	International Council for Standardization in Haematology
IFBLS	International Federation of Biomedical Laboratory Sciences
IFCC	International Federation of clinical chemistry
INFANT COHORT	Phase II randomized, open, controlled study of the safety and immunogenicity of GlaxoSmithKline Biologicals. Candidate Plasmodium falciparum malaria vaccine RTS,S/AS01E, when incorporated into an Expanded Program on Immunization (EPI) regimen that includes DTPwHepB/Hib, OPV, measles and yellow fever vaccination in infants living in malaria-endemic regions Infants are aged from 4 to 9 weeks
IRB/IEC	Institutional Review Board/Independent Ethics Committee
LIC	large immature cell
LYM	lymphocyte count
MCH	mean corpuscular haemoglobin
MCHC	mean corpuscular haemoglobin concentration
MCV	mean corpuscular volume
ml	millilitre
MON	monocyte count

MPV	mean platelet volume
MRU	medical research unit
NCCLS	National Committee for Clinical Laboratory Standards
NEU	neutrophil count
NHANES	National Health and Nutrition Examination Survey
NIST	National Institute of Standards and Technology
OPV	oral polio vaccine
PCT	plateletcrit
PDW	platelet distribution width
PLA	platelet count
PRF	patient record form
RBC	red blood cell count
RDW	red distribution width
SD	standard deviation (=Std Dev)
SOP	standard operating procedure
Std Dev	standard deviation (=SD)
TDS	*Trichuris* dysentery syndrome
WBC	white blood cell count

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11 Curriculum Vitae

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