MANAGING LYMPHOMAS IN AFRICAN SETTINGS: IMPROVING DIAGNOSTIC ACCURACY AND CARRYING OUT RESEARCH PROJECTS

GIULIA DE FALCO, DEPARTMENT OF MEDICAL BIOTECHNOLOGY, UNIVERSITY OF SIENA, ITALY, **EMILY A ROGENA,** DEPARTMENT OF MEDICAL BIOTECHNOLOGY, UNIVERSITY OF SIENA, ITALY AND DEPARTMENT OF PATHOLOGY, UNIVERSITY OF NAIROBI, KENYA, **KIKKERI NARESH,** DEPARTMENT OF CELLULAR PATHOLOGY HAMMERSMITH HOSPITAL CAMPUS, IMPERIAL COLLEGE LONDON, UK, **MARTINE RAPHAEL,** DEPARTMENT OF HEMATOLOGY, BICÊTRE HOSPITAL, PARIS, FRANCE, **IAN MAGRATH,** INTERNATIONAL NETWORK FOR CANCER TREATMENT AND RESEARCH, BRUSSELS, BELGIUM AND **LORENZO LEONCINI,** DEPARTMENT OF MEDICAL BIOTECHNOLOGY, UNIVERSITY OF SIENA, ITALY

Diagnostic accuracy may still be difficult in developing countries due to the lack of infrastructure. It is imperative to bridge the existing gaps as diagnostic accuracy is a critical factor in determining patient outcomes. In addition, a correct diagnosis is crucial to meaningful research. Twinning between institutions in developing and developed countries may have a positive impact on both the quality and quantity of research. However, technology transfer is necessary if personnel from developing countries are to improve their skills and knowledge and actively participate in local research. This review summarizes the research experience about lymphoma pathogenesis performed with collaborating African institutions.

Gaps and challenges in the management of lymphomas in Africa

Although accurate estimates are difficult, given the paucity of information, it is likely that approximately 30,000 non-Hodgkin's lymphomas (NHL) occur in the equatorial belt of Africa each year and these tumours are among the top ten causes of cancer in this geographical region. The fraction associated with AIDS is not available, and varies with geography, but in some regions may be as high as 50%. Since the beginning of the AIDS epidemic, the incidence of NHL has increased by 2–3 fold in some countries, and as much as 13 fold in others. A realistic estimate of the incidence of Burkitt Lymphoma (BL) is 30–70 per million children in equatorial African countries (Source Globocan), although the evidence for a major impact of the AIDS epidemic in children is sparse, and a recent report from Malawi and our own unpublished data suggest that there is little or no increase in BL in HIV positive children with BL.

At least one component of the worse outcome in African patients with lymphomas is incorrect or incomplete diagnosis. If, therefore, we are to increase the survival rate, it will be necessary to improve diagnostic accuracy in Africa – which is presently impaired by a lack of phenotyping – and develop a clearer picture of how the incidence of NHL varies in different African regions and in relationship to other diseases such as AIDS and malaria. The lower cure rate of NHL in Africa suggests that the difference in mortality will become even more pronounced in the future as populations continue to outgrow their health resources, while the burden of disease continues to climb. Already there are striking gaps between Equatorial African countries and regions such as Europe and the USA with respect to survival; paediatric oncology being the most dramatic example. However, the information available about cancer incidence, treatment and follow-up in many sub-Saharan African countries is generally incomplete at best, since medical records are either rudimentary or do not exist. The importance of establishing and maintaining population-based cancer registries, to support and evaluate cancer control programmes is clear, and currently, the INCTR's Cancer Registries Programme, led by Max Parkin, is

working to improve the quantity and quality of data from several African registries – soon, hopefully, to cover several quite different regions in Africa. Cancer registration also provides an important foundation for developing cancer control programmes, such that simultaneous improvement in pathology as well as population-based cancer registration would do much to improve infrastructure in these countries, so that basic diagnostic and treatment facilities would become more widely available. Many African countries still contain large lacunae in such services, and a few lack them altogether.

Although in the developed world the importance of the correct diagnosis is widely recognized, this is still an evolving concept in some of the developing countries, especially in Africa. In particular, there are striking differences in the turnaround times from biopsy to report, and in the accuracy of lymphoma diagnosis; differences that have a profound impact on the patients' ultimate outcome. The current problems in the practice of lymphoma diagnosis include poor quality histology in the minority of cases where biopsies are performed, and a complete lack of immunohistochemistry and other investigations in a high proportion of patients. Because of these limitations and a lack of continuing education, it is generally not possible to apply the current diagnostic criteria for most lymphoid malignancies. In Africa, a majority of the laboratories still use the Working Formulation for Clinical Usage, a lymphoma classification from the early 1980s, which is based on morphology alone and does not include many entities recognized in the last 20 years.

In addition to inadequacies in diagnosis accuracy, a number of additional factors influence patient outcome. These include health service structure, ultimately derived from the national health policy which in turn influences the ability to detect and diagnose cancer at the earliest possible moment, thus giving the greatest chance of cure. Gender, socioeconomic and demographic issues are also relevant. Methods of improving the early diagnosis of cancer will also have an impact on therapy, since the earlier a cancer is detected and diagnosed, the simpler, as a general rule, is the therapy likely to be. Thus, early detection programmes could well lead to improved morbidity and mortality, as well as psychological outcomes.

What can be done?

Though an issue of this magnitude cannot be addressed in a simplistic manner (because of the realities of resource limitations there are some conditions that will, unfortunately, continue to be misdiagnosed for the foreseeable future), there is much that can be done and some clear targets that constitute the most pressing issues. First of all, the improvement of diagnostic accuracy is essential. This may be done by improving infrastructure, and providing the necessary resources and training in modern diagnostic techniques, as well as reproducible criteria for diagnosis, such as those established by the WHO classification of Haematopoietic and Lymphoid tissues, 2008[1]. Those involved would be able to improve the quality of histology, and to introduce essential tools for modern diagnosis, such as immunohistochemistry, molecular cytogenetics and other molecular methods.

Twinning between institutions in the developed and the developing countries can be a successful long-term approach

Improving diagnostic accuracy

Twinning between institutions in the developed and the developing countries can be a successful long-term approach. However, substantial costs are involved in providing infrastructure, manpower, consumables, follow-up and surveillance programmes and owing to their limited health care resources, developing countries cannot afford the models used in developed countries. Most of the sub-Saharan Africa countries have neither the resources nor the capacity to organize and sustain any kind of improvement programme. Twinning between institutions, assuming the partner in the high-income country is willing to support the cost or a grant is obtained to provide the necessary funding, is one way of improving pathology in Africa. In addition, twinning programmes result in a constant and continuous training of African colleagues, through short courses, audits on diagnoses, diagnostic referrals and feedback, telepathology and establishing diagnostic centres with greater capabilities. This approach can lead to African clinicians receiving constant training and the necessary support to deliver high quality clinical services – as long as the programme is sufficiently well resourced and lasts for a sufficient time.

Several twinning programmes relating to childhood cancer treatment have been in place in Central and South America, Northwest Africa and Southwest Asia for as long as 10 years. These programmes have reduced the rates of abandonment of treatment, relapse, and death due to toxic effects of treatment, and the investments that they have attracted have led to improvements in access to treatment and hospital infrastructure. One such programme that is presently on-

going in Africa and aimed at the improvement of both diagnosis and research was sponsored by the International Network for Cancer Treatment and Research (INCTR) and included several African institutions all of which were visited by several pathologists from European and American institutions (Figure 1) to assess the infrastructure, audit the diagnoses offered by the African investigators, introduce capacity-building measures, provide logistical support for telemedicine and assist with treatment protocols specifically designed for the African setting. This review demonstrated the considerable variation in infrastructure, including the available equipment and personnel[2]. The annual biopsy number varied from 1,500 to 16,000 among the pathology departments visited. Some of the departments had a substantial cytology load and fine needle aspiration cytology (FNAC) was widely utilized. The availability of equipment was variable as was the quality of histology and cytology preparations. In addition, although a proportion of the pathologists in sub-Saharan Africa had worked in centres abroad, the laboratory technical staff had not. Diagnostic accuracy, after the review of lymphoma cases by these lymphoma experts, was shown to be 76% of BL cases in which only cytology was available. The diagnosis of other lymphomas in which histological samples were available was confirmed in 78% of cases, irrespective of the subtype. It was difficult to make diagnoses of specific lymphoma subtypes, as lymphoma subtyping had not been attempted in many cases partly due to the fact that basic immunohistochemistry was not available in most centres[2].

BL is particularly important, as it is endemic in equatorial Africa, Papua and New Guinea, locations where the required infrastructure and technical expertise are not currently available, and are unlikely to be in the near future. Because of this, the construction of a diagnostic algorithm that would ensure a reliable diagnosis of BL with greater ease and fewer resources would be of great value. Such a systematic approach is also relevant in developed countries, as none of the parameters currently used in the diagnostic evaluation can clearly distinguish the entities BL, DLBCL/BL and DLBCL on an individual basis. Therefore, in a collaborative study with African colleagues we have proposed a scoring system-based algorithm in which only three antibodies (CD20, CD10 and BCL2) are used to distinguish between BL cases and non-BL cases[3]. These three antibodies are relatively easy to use and can be employed in laboratories with limited experience in immunohistochemistry. We hope that this small panel of antibodies would make a great impact in the setting of developing countries, especially in Africa. In addition, other parameters such as EBV detection and *MYC* translocation by

Figure 1: The group of five pathologists during the central review

in situ hybridization and FISH, respectively, could be useful to address a diagnosis of BL, although these will require more resources and might only be used when the three-antibody system fails to make a definitive diagnosis[4].

Performing research in developing countries: A challenge that can become a reality

As the susceptibility to and pathogenesis of lymphomas are heavily influenced by genetic background, environmental exposure and lifestyle, there would be a considerable advantage in including the developing countries in this effort, since they are, in general, much more heterogeneous than high-income countries, and may differ markedly from each other as well as high-income countries. In particular, the causative role of infectious agents may be crucial in countries such as Africa, where the burden of infectious diseases is quite high. Infectious agents, especially viruses, account for several of the most common malignancies – up to 20% of all cancers. Some of these cancers are endemic, with high incidence in certain geographic locations, but have sporadic/lower incidence in other parts of the world. Lymphomas are very common in Africa, a large number of which arise as a result of infectious agents. Several pathogens and environmental factors have been detected in association with lymphomas, including the Epstein-Barr virus (EBV), the Human Immunodeficiency Virus (HIV), the Human Herpes Virus 8 (HHV-8), the causative agent of malaria *Plasmodium falciparum*, *Helicobacter pylori* and *Chlamidia psittaci*, just to mention a few, suggesting that they may contribute to lymphomagenesis. They may have a direct role in lymphomagenesis, through dysregulation of regulatory mechanisms of the host cell, or promote an "indirect lymphomagenesis", which involves sustained stimulation of the immune system, eventually linked to malignant transformation. Mechanisms as pathogen-induced B-cell hyper-activation, B-cell stimulatory activity and alteration of the expression of growth factors within the host cell, thus

unbalancing signal transduction, may be used by these pathogens and determine transformation. In addition, viruses may also encode products, which may mimic cellular proteins (i.e. v-cyclins), thus disturbing mechanisms of cell cycle regulation. This leads to the concept that a complex interplay between viruses and the host cell machinery does exist. Epidemiologic differences have been accounted for lymphomas and the substantial differences in incidence patterns among the lymphoid neoplasms may suggest that there is an etiologic heterogeneity by disease subtype[5].

A good example of how infectious agents and environmental factors may participate in lymphomagenesis is offered by the endemic form of BL, as both malaria and the EBV are thought to have an important role for this malignancy. Endemic BL is almost universally associated with infection of Epstein-Barr, though co-infection with malaria also appears to be important in the pathogenesis of BL. Acute malaria infection inhibits EBV-specific immune responses and leads to an increase in the number of EBV-carrying B-cells in the circulation[6]. The pattern of malaria infection appears to be important in lymphomagenesis. Holoendemic, or perennial and intense transmission of malaria, appears to induce much higher circulating EBV viral loads than sporadic transmission of malaria or no transmission of malaria[7]. This immune dysregulation probably contributes to the pathogenesis of BL and explains why EBV-related BL occurs with a much higher frequency in areas where malaria is common and malaria infection is more intense. Despite the recurrent studies on this association, there is still no conclusion as to the exact mechanisms, and no practical approaches have been developed to de-link the two pathogens in order to prevent disease occurrence[8]. Significant spatial clustering of elevated eBL risk in high-malaria transmission regions and its reduced incidence where malaria is infrequent definitely suggests that malaria plays a role in the complex eBL etiology, but additional factors may also be likely involved[9]. Other agents have been proposed as cofactors in BL lymphomagenesis, including arboviruses and plant extracts commonly used in traditional medicine, which can explain the shifting foci and space-time clusters of the lymphoma observed in endemic regions[10].

Due to the high spread of the AIDS epidemics in Africa, the role of HIV in lymphomagenesis is quite important, as HIV infection is often detected in NHL. Three major factors promoting the development of lymphoma are HIV-induced immunosuppression, chronic antigenic stimulation, and cytokine over-production. Though the introduction of highly active antiretroviral therapy has changed the epidemiology of AIDS-related lymphomas, it is likely that AIDS-associated malignancies will continue to be a major clinical manifestation of HIV-infected individuals, as there is still a lack of widespread antiretroviral therapy in Africa where AIDS is epidemic and HIV continues to spread. Better tools for detection and better regimens for management of these malignancies are necessary. Moreover, a better understanding on how infections by these viral agents can lead to tumors will lead to the development of methods to enhance the immune response to control these agents as well as development of therapeutic agents to treat these malignancies. A strong association with the human herpesvirus-8 and lymphomas has also been described in HIV-positive patients[11,12].

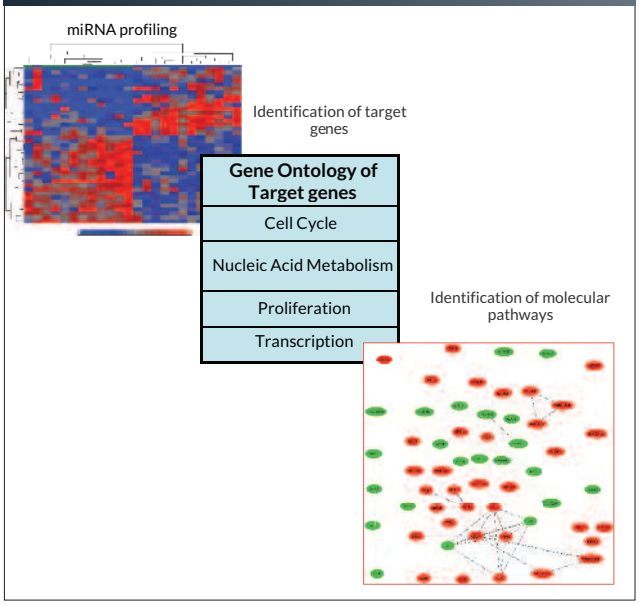
Therefore it is crucial performing research in developing countries trying to highlight environmental factors which may act as risk factors in such settings. Large multinational pharmaceutical and biochemical companies could underwrite the costs of programmes such as this if convinced that the developing world represents a potential future market for them. We have demonstrated the feasibility of this by collaboration with several African institutions in the performance of several research projects in which have African colleagues have been actively involved. A number of young African doctors have, as a result, been enrolled in a PhD programme at our institution in Italy.

Research achievements in collaboration with African institutions: An overview

Understanding the genetics of Burkitt lymphoma

Earlier studies from our group, in collaboration with African colleagues, revealed that differences exist between endemic (eBL) and sporadic BL (sBL), especially in terms of mitotic and apoptotic indexes (MI, AI) and in the association with EBV[13]. These collaborations allowed the detailed characterization of BL at the genetic level, which revealed that eBL and sBL cases carry mutations in a gene belonging to the retinoblastoma family of proteins, the *RBL2* gene[14], whereas no mutations were detected in the AIDS-related BL variant. This observation led to the identification of a novel mechanism of inactivation of this member of the retinoblastoma family by the HIV-encoded Tat protein through the physical binding of Tat to the pRb2/p130 protein and subsequent inactivation[15,16]. In a later study a signature downstream the *RBL2*/p130 gene was identified as relevantly altered in endemic Burkitt lymphoma cell lines and primary tumors[17]. A percentage of BL cases (about 10%) have been shown to lack *MYC* translocations, suggesting that alternative methods to translocation may sometimes be responsible for the up-regulation of the c-MYC protein. We have shown this to be the case in a study involving a recently described class of small non-coding RNAs, the microRNAs (miRNAs)[18,19].

Figure 2: Flow chart illustrating how alterations in molecular pathways can be identified starting from a miRNA profiling



MicroRNAs are able to regulate gene expression by mRNA cleavage or translational inhibition[20]. They are usually expressed in a tissue specific manner and play important roles in apoptosis, differentiation and cell proliferation[21]. MicroRNAs are transcribed by RNA Pol II as capped and polyadenylated transcripts, either mono- or polycystronic, of several kilobases in length. Pre-miRNA are precessed in the nucleus, by RNase III Drosha to produce pre-miRNAs that migrate into the cytoplasm. Here, a protein called Dicer cuts them at specific sites to produce mature miRNAs[22]. Experimental evidence has reported miRNA involvement in cancer and their association with fragile sites in the genome[23-25], suggesting that these molecules could act as tumor suppressors or oncogenes[26]. Even though increasing evidence highlights a possible role in malignant transformation, little is known about their expression and their deregulation in malignant lymphomas. We recently performed a miRNA profiling comparing *MYC* translocation-negative BL cases with classical BL cases, to identify whether a differential expression of specific miRNAs could be detected between these two groups. This study identified several differentially expressed miRNAs which could be related to the absence of *MYC* translocation (Leoncini, unpublished).

Exploring the role of infectious agents and environmental factors

A strong association between EBV and the onset of specific types of lymphomas has been observed, especially in the so-called "lymphoma belt" in Africa. To unravel the molecular mechanisms underlying such a condition, we have investigated the possible role of EBV in inducing lymphomagenesis in collaboration with African colleagues. Results from our studies have shown that the expression of some microRNAs is deregulated in samples of Burkitt lymphoma due to the presence of EBV[18, 27]. In EBV-positive cases, microRNA imbalance seems to specifically rely on the expression of EBV-encoded proteins which deregulate microRNA expression[28,29]. Furthermore, we have performed both Gene Expression Profiles (GEP) and microRNA profiling, since the latter has permitted even more discriminative than GEP in identifying tiny differences among subtypes. In particular, we focused on the possible role of miRNA dysregulation. Figure 2 illustrates the steps that lead to the identification of altered molecular pathways starting from a miRNA profiling. Our GEP studies have demonstrated that the molecular signatures of BL cases associated with EBV differ from those lacking EBV, suggesting a direct role for the virus in dysregulating cellular gene expression[30]. In addition, a microRNA profiling of BL cases, in collaboration with the African institutions and with the Charité University of Berlin, has demonstrated that a few microRNAs are differentially expressed between sBL and eBL[31]. To evaluate the extent to which the expression of EBV-coded miRNAs may affect host gene expression, we have more recently performed a study taking into consideration also the EBV-coded miRNAs, in collaboration with the CNIO of Madrid, Spain. In this study, endemic BL cases collected at Lacor Hospital, were compared with sporadic BL cases with respect to miRNA expression. This study further confirmed that differences in the expression of cellular microRNAs exist between sBL and eBL cases (Leoncini, unpublished results), and identified viRNAs of EBV that may affect the expression of cellular genes involved in key physiological processes, such as signalling and cell control (Leoncini, unpublished results). This finding suggests that EBV may actively interfere with the mechanisms of control of the host cells, through its encoded proteins and/or miRNAs.

The role of Human Immunodeficiency Virus (HIV) in the pathogenesis of AIDS-related lymphomas is still not completely understood. HIV infection is associated with strong polyclonal B-cell/plasma cell activation/expansion. To date, the mechanism whereby HIV leads to malignant transformation remains unknown, although several events have been proposed as co-factors in HIV-related tumorigenesis.

In our collaborative studies, we have previously demonstrated that Tat is able to interfere with the control of cell proliferation, through physical interaction with members of the retinoblastoma family[15,16]. In addition, we have demonstrated that Tat diminishes the promoter activity of VEGF promoter in vitro, suggesting that Tat may modulate the

level of expression of VEGF. In the studied primary HIV-1 positive or HIV-1 negative diffuse large B-cell and Burkitt lymphomas, low expression of VEGF in HIV-1 positive compared to negative cases was observed[32]. However, the microvessel density (MVD), assessed by CD34 expression, was intriguing, being higher in HIV-1 positive as compared to negative cases. This suggests that Tat plays a wider angiogenic role that is related to the observed increase in MVD. From our findings, it is possible to argue that Tat may increase angiogenesis through the direct binding to VEGFR2/KDR, thus inducing its downstream signaling rather than increasing angiogenesis through the enhancement of VEGF expression[32].

Our recent observations show that members of the DNA Methyltransferase family are differentially expressed between HIV-positive and HIV-negative lymphoma patients. In particular, we have identified the upregulation of the DNMT3a and DNMT3b proteins that are involved in de novo methylation, which occurs quite often in cancer in HIV-positive patients (De Falco, et al. unpublished results). Over-expression of DNMTs seems to be related to microRNA dysregulation in HIV-positive tumors as well as in Tat-transfected cell lines. The aberrant expression of DNMT3a and DNMT3b leads to the dysregulation of key cellular regulatory genes such as *INK4/p16*, whose a key regulator of the cell cycle. Silencing of *INK4/p16* may result in the loss of the G1/S checkpoint, resulting in increased and unrestrained cell proliferation (De Falco, et al. unpublished).

Other infectious agents and/or environmental factors may be relevant for lymphomagenesis in Africa. We have recently reviewed African Ocular Adnexal Lymphoma (OAL) cases for their association with *Chlamydia psittaci*, in comparison to Italian OAL cases. Our results demonstrated the lack of association between *C. psittaci* and OAL in all of the Kenyan samples analysed, whereas *C. psittaci* DNA was detected in tumour samples of Italian patients, even though in a lower percentage than that reported in previous studies[33], suggesting that other environmental, genetic and epidemiological factors may be relevant to lymphomagenesis.

Other factors associated with the pathogenesis of lymphomas include chemicals, such as herbicides, hair dyes and various environmental factors. Fisher and Fisher also describe autoimmune diseases, organ transplants, and primary or acquired immunodeficiencies and ultraviolet radiation as co-factors in lymphomagenesis[34]. Among the environmental factors thought to be implicated in lymphomagenesis of eBL the *Euphorbia tirucalli* plant, which is currently used as medicinal plant material, wood fencing or as an ornamental plant, has been suggested to have a role. It has been shown that *Euphorbia tirucalli* is capable of reactivating the EBV lytic cycle in a dose-dependent manner at dilutions as low as 10–6. It has also been reported that the environmental exposure to the latex of *E. tirucalli* can directly activate the EBV lytic cycle, such that the possibility of a role for *E. tirucalli* in the aetiology of eBL cannot be excluded[35]. Very recently, we have conducted an *in vitro* study to assess the possible role of *E. tirucalli* in inducing genetic alterations in non-transformed cell lines, and its transforming capability in B-cells. Our results demonstrated that exposure to *E. tirucalli* induces EBV reactivation[35]. In addition, though no chromosomal alterations have been detected, simultaneous increased expression of c-MYC and BCL2 have been observed. BCL2 may inhibit the apoptotic effect of cells which bear a *MYC* translocation, thus increasing the likelihood of unrestricted proliferation and the potential for tumorigenesis. Interestingly, a five-day treatment with *E. tirucalli* induced chromosome 8 polysomies, leading to genetic instability[35].

Conclusions

Challenges and gaps in lymphoma management in Africa still exist. In particular, a striking gap in terms of the time between biopsy and report exists, and diagnostic accuracy is often poor, in part due to the failure to use monoclonal antibodies. Improving diagnosis is also crucial if meaningful research is to be conducted. We have demonstrated that valuable research can be conducted when investigators work closely with their African colleagues. This is important, since there is much to learn in Africa. These objectives can be achieved through twinning between institutions, which allows technology transfer and constant training of local health personnel as well as exchange visits between doctors from developing countries and those from the high-income countries. This permits African doctors to be actively involved in research projects.

In this article we have recounted the work we have performed in trying to address the relative importance of genetic versus environmental factors in the genesis of BL, with particular attention to viruses, such as EBV and HIV that are wide-spread in African countries. Improving our knowledge of the molecular mechanisms underlying lymphomas would not have been possible without the continuous and active collaboration of many African institutions. Sharing expertise and education led to mutual benefits, and has helped us to shed more light on the complexities and intricacies of the pathophysiological processes of human diseases. ●

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Professor Lorenzo Leoncini did his internship at the Institute of Pathological Anatomy and Histology, University of Siena and was awarded his medical degree in 1978 after which he chose to specialize in pathology receiving his second degree from the University of Bari in 1982. He has had gained a wide experience through his research and diagnositic activities at the Institute of Pathology of the University of Bern (CH), Institute "Gustave Roussy" Paris, Institute of Pathology of the Free University of Berlin, Department of Immunology of the University of Cape Town, Institute of Human Pathology, University of Nairobi (Kenya), Bugando Medical Center, Mwanza (Tanzania), Lacor Hospital, Gulu (Uganda). In 1998 he was appointed Associate Professor, Institute of Pathological Anatomy and Histology, University of Sassari and from 2000 he has been a full professor at the Department of Human Pathology and Oncology, Section of Pathological Anatomy, University of Siena. Professor Leoncini's professional memberships include the European Society of Pathology, International Academy of Pathology, European Society for Haematopathology (where he has been a Board member since 2002), Italian Society for Molecular Biology, International Society of Diagnostic and Quantitative Pathology and the American Association for Cancer Research. He is the author of over 70 articles in peer reviewed journals.

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