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PANLEUCOGATED (PLG) CD4 HIV IMMUNE MONITORING

a difference by disruption

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FREQUENTLY USED TERMS AND ABBREVIATIONS

AIDS Acquired Immune Deficiency Syndrome

AFREQAS African Regional External Quality Assessment Scheme

BCR Bead count rate

CD4 Cluster Designation 4 (the scientific terminology for monoclonal antibodies that primarily identifies helper T-cells).

CCMT Comprehensive Care and Treatment programme

CMJAH Charlotte Maxeke Johannesburg Academic Hospital

DP Dual Platform: a method that employs two separate platforms to generate a cell count using a predetermined common denominator (which may be total lymphocytes or white blood cells for example). A specific denominator count is obtained by multiplying the absolute cell counts generated on a haematology analyser by the proportion of the denominator determined immunophenotypically by flow cytometry.

HIV Human Immunodeficiency Virus

LIMS Laboratory Information Management System

'Lymphosum' The prevailing international guideline terminology used in the 1990's for a six-tube, two-colour panel to identify, and account for, all subsets of the lymphocyte compartment, including CD4+ T-cells (as CD3+/CD4+), CD8+ T-cells (CD3+/CD8+), B-cells (CD3-/CD19+) and natural killer cells (CD16+ and CD56+). Contaminating monocytes were excluded with CD14 expression whilst all (total) lymphocytes were established with bright CD45 and low side (light) scatter. Isotypic controls eliminated non-specific binding and negative population cutoff

MSF Médecins Sans Frontières

NHLS National Health Laboratory Service

PLG PanLeucogating (a gating terminology especially coined to describe the newly developed CD4 assay foundationally based on total (all) leucocytes described here in this work).

SAIMR South African Institute for Medical Research

SP Single Platform: a method whereby both the cell counts, and the proportion of chosen denominator determined immunophenotypically, are both determined on the same platform. Counts can be obtained by addition and measurement of commercially prepared beads added in a fixed volume with known concentration. Volumetric counters are also regarded as SP systems

TAT Turnaround time

TLC Total Lymphocyte Count

WCC White (blood) cell count

WHO World Health Organisation

SOME TECHNICAL REFERENCING AND FORMATTING NOTES

There are 21 sections in this thesis. The first section, entitled "Preamble – go with the flow', outlines my early career flow cytometry experience; relevant publications by myself, or colleagues' work where I assisted on the flow cytometry of the respective studies, serve to demonstrate the extent of flow cytometry experience gained before I started working in the CD4 field. I have included this history as I believe it is relevant and contextual to events that followed, and which put me on the path to start the first South African CD4 testing laboratory. The sections that follow tell the story of how CD4 services were developed and grew in South Africa over the next 30 years.

All author-cited references submitted for examination (in bold) are listed at the end of each respective section where mentioned. The Vancouver referencing style is used and each cited reference includes a direct hyperlink (DOI); DOI links may not be available in some older publications before 1999. The works are cited (grouped) according to the topic of the respective section and may be cited more than once; the papers however retain their original numbering according to the order in which they first appear in the text. Occasionally, a congress abstract by the author is cited in the footnotes; these are not for examination but included to describe context for the author-cited papers that follow. A consolidated list of author-references additionally appears at the end of this thesis and follows a declaration of contribution to each work.

All relevant contextual literature is cited in the footnotes on the page where mentioned, also using Vancouver referencing but with a distinctive smaller font. Where possible DOI hyperlinks to these contextual papers are included. An occasional student higher degree submission is also cited in the footnotes where I acted as a sole or joint supervisor, but only if relevant to the discussion of the section in question; these are not included for examination.

ABSTRACT

In 1990, the high cost of implementing a national HIV/AIDS programme was set to overwhelm South Africa's health care budget. Not only were the prices of antiretroviral drugs prohibitive, laboratory tests needed for monitoring response to antiretroviral drugs was also unaffordable, further limiting access to care. By 1998, pleas from local clinicians to lower prices for CD4, as well as other laboratory tests like HIV viral load, were intensifying. Clinicians and local advocacy groups like Médecins Sans Frontières (MSF) and the Treatment Action Campaign (TAC) were no longer asking for, but demanding, cheaper laboratory services for HIV treatment monitoring. Respite to the emergent HIV epidemic could only come from cost-effective therapies, clinical trials, and vaccine and treatment research, as well as a reliable and affordable laboratory monitoring service that was uniquely developed and appropriate for local needs. Urgent attention was needed to deliver affordable and sustainable state HIV laboratory services considering that, certainly for CD4 testing, the prevailing, conventional guide-line based CD4 testing approaches were cumbersome and costly; highly trained and skilled scientists were also expected to do the flow cytometry-based testing.

It is from this background that novel PanLeucogated CD4 testing, invented and further developed by the author and her team, disrupted existing CD4 testing dogma of the time, and addressed the prevailing and emerging HIV challenges of high cost, local lack of technical skill and variable quality of the reported CD4 results. Patented and assigned to the South Africa National Health Laboratory Services, PLG CD4 was implemented into service at the start of the South African Comprehensive Care and Treatment programme in 2004, growing from a single reporting laboratory in 2002 to as many as 75 laboratories by 2010. The method was successfully implemented because it was simple to use; at the time, most personnel who were required to take on the testing, had not heard of CD4 testing, never mind flow cytometry needed to undertake testing. It was also considerably cheaper than existing methods. At the time, the potential costs-savings of implementation were predicted to significantly cut HIV laboratory monitoring costs; CD4 count prices were reduced to 10% of original costs and there were millions of HIV+ patients who were predicted to present themselves for care.

Work subsequently undertaken by the Glencross team, and outlined in this thesis, describes the step-by-step development and expansion of the national CD4 testing programme. The implementation of uniform systems (PLG CD4) was key to the success that followed; standardised operating procedures and quality control protocols were especially developed to meet local requirements. This tailored and specific approach has ensured reliable and harmonised CD4 reporting throughout the state laboratory service. The success of the programme has emphasised the importance of thorough due diligence and planning, as well as coordinated implementation of standardised systems appropriate for the level of care, including instruments and methodologies, that have streamlined and ensured efficient delivery of the Panleucogated CD4 testing across the South African national laboratory services.

Initially local, then extended to a regional scheme, Glencross and her team also introduced a locally directed external quality assessment in collaboration with the World Health Organisation, introduced as the African Regional External Quality Assessment Scheme (aka AFREQAS). The scheme, established as an independent monitoring and evaluation body for PLG CD4 users in the NHLS, subsequently grew and was expanded to support and provide external quality assessment for all CD4 laboratories across the African continent, irrespective of methodology used.

Later, as requirements for additional services grew, and as an increasing number of patients accessed HIV care, new service delivery models that considered local deficiencies and strengths of service were developed. Applying these models to re-examine and appraise national services, ultimately secured nationwide service coverage, extending accessibility of CD4 counts beyond high-volume centralised testing precincts to hard-to-reach community laboratory level, but without incurring additional cost. Economically, all available resources in the national network of integrated state pathology laboratories were utilised to maximise cost savings, including use of a common laboratory management system and transport networks to facilitate transport of specimens to reference laboratories. This hierarchical approach to service delivery has been the foundation for programmatic self-determination that has ensured the success of, and underpinned, the national South African state CD4 service over the past 20 years. As gaps in service delivery later emerged, new systems to review efficiency of PLG CD4 state services were introduced that led the way for national monitoring and evaluation and timely review of, not only PLG CD4 results, but a basket of the top 25 tests offered across the national programme.

Additional work described builds on the capacity and infrastructure created through rollout of CD4 testing and includes the development and integration of tests that can be 'piggy-backed' onto CD4 services (reflecting the versatility of protocol), as well as related ongoing training and technical capacity initiatives. A notable initiative, which has been successfully integrated into CD4 services nationally, has provided for screening of early cryptococcal infection in HIV+ patients with advanced disease. Before this milestone achievement, immune activation identified by CD38 expression on CD8 T cells, also incorporated into the PLG CD4 assay, was also shown to reliably predict response to antiretroviral therapy, offering a cheaper, more accessible alternative to more expensive HIV viral load monitoring.

Last, but not least, work is presented that reveals how longitudinally assembled and curated CD4 laboratory data, collected through delivery of services over the last 17 years, has provided invaluable health programme insights into the effectiveness of programmatic delivery but additionally, enabling important epidemiological review of infectious and non-communicable disease for South Africa.

1. PREAMBLE: GOING WITH THE 'FLOW'

I was first introduced to the field of flow cytometry, as a new haematology registrar, following the donation of two flow cytometers to the Wits Medical school in 1987. The first of these was the Beckman Coulter (BC) 'Epics V', a sizeable, water-cooled instrument which practically filled the small, dedicated laboratory space located on the 7th floor of the medical school and required the expertise of a dedicated scientist[1]. The second machine installed was a smaller, user-friendly, and more practical 'bench-top' analyser, the BC Profile™, that I taught myself to use by analysing patient samples that had been prepared for single immunofluorescence microscopy in our local routine haematology laboratory. Two years later, expanding on this single-fluorescence microscopy work, I introduced the first flow cytometry-based, 2-colour fluorescence leukaemia/ lymphoma immunophenotyping service based in the Wits medical school 7th floor facility, heralding a new era in routine leukaemia diagnostics in Johannesburg and for South Africa.

In the years that followed, I gained experience in using flow cytometry and its applications[2, 3, 4, 5] as I managed the growing repertoire of diagnostic-pathology flow cytometric tests in the new routine service, including immunophenotyping of peripheral blood and bone marrow samples or lymph node biopsies, as well as fine needle aspirates (FNA) and progenitor/ CD34 stem cell enumeration[6, 7]. I also learned a lot by offering an informal medical school core-facility support for pathology and clinical colleagues in our faculty who were interested in pursuing flow cytometry to do their research. The projects I became involved in were diverse and included amongst many others, setting up an assay for the quantitation and assessment of Pneumocystis *Carinii* by flow cytometry[8], developing alternative methods for flow cytometric platelet studies including Bernard-Soulier Disease[9] or describing haematology-related technical artefacts, such as red cell agglutination affecting flow cytometric analysis outcomes[10]. Studies on pre-thrombosis[11] and flow

[1]The scientist who was employed to run the unit was Dr. Clive Grey, who subsequently went on to become Professor and Head of Immunology at the University of Cape Town, later at Stellenbosch University.

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cytometric cell cycle and proliferation assays of human breast cancer, osteosarcoma or lymphoblastoid cell lines[12] were other examples of projects explored. During this period, I also offered, or contributed to, several training flow cytometry workshops[13, 14].

Due to the lack of commercially available diagnostic kits, several in-house flow cytometric assays were developed, many of which are still used in the routine service laboratory at the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH). One of the in-house techniques developed (described in my MMed[15]), enabled the classification and characterization of the variation of CD10[16] surface marker antigen density in acute lymphoblastic leukaemia (ALL), later cited as useful to predict prognosis in common/ pre-B ALL[17, 18]. Cell cycle studies including DNA ploidy evaluation and cell S-phase proliferation, modified from the method of Fried and Perez[19], are still used in the unit in 2021. This latter assay has supported several departmental research projects, including amongst these, delineation of apoptosis in megaloblastic anaemia[20], enumeration of malaria parasites[21] or reticulocytes[22] and another documenting DNA ploidy changes in multiple myeloma[23]. One particularly interesting and challenging project, undertaken in collaboration with a local agricultural research unit[24], involved the processing of orange tree leaves to establish sapling aneuploidy.

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[24]Unpublished study; collaborator now known as the Agricultural Research Institute, Addo, Eastern Cape.

2. THE HISTORY OF HIV, FLOW CYTOMETRY AND CD4 TESTING: A CONVERGENCE OF WORLDS

In the latter half of my intern year, in 1986, I was assigned an extended four-month unit rotation in the Hillbrow Hospital oncology ward under Professor W. Bezwoda. Whilst I was treating patients diagnosed with Pneumocystis *Carinii*[25, 26] infection, or others with Kaposi Sarcoma[27, 28], reports of immune deficiency, associated with these diseases, were appearing in the medical literature that were to have a profound impact on South Africa (and have a direct influence on my career too). Both Kaposi sarcoma and the opportunistic infection with the organism, Pneumocystis *Carinii* had, earlier in 1981, been documented by the Centers for Disease Control (CDC) in the United States (US) to be associated with men who have sex with men[29]. In 1982 the associated syndrome linking these conditions was described by the CDC and termed the Acquired Immune Deficiency Syndrome (AIDS)[30, 31]. The causative agent for AIDS was recognised in the following year, 1983, simultaneously described by two groups: the Montagnier group naming the T-lymphotropic retrovirus[32] and Robert Gallo's group describing the Human T-lymphotropic virus III (HTLV-III)[33]. Later, the essential component receptor for the AIDS retrovirus was revealed to be the helper T-cell receptor, T4[34] (later termed Cluster Designation 4 (aka CD4). A reduction of peripheral blood T-cell counts expressing the T4 antigen was subsequently linked to patients presenting with AIDS[35]. The causative agent of AIDS was later renamed the Human Immunodeficiency Virus (HIV) in 1986[36]. A few years later, CD4 (aka T4) was described as a glycoprotein, located the surface of both T cells as well as monocytes.

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In the context of T-cells, the molecule was described as a co-receptor to assist the T cell receptor in communicating with antigen-presenting cells[37].

Preceding the arrival of HIV and AIDS, significant concurrent but coincident diagnostic technological advances in cellular diagnostics were paving the way for the understanding of human lymphocyte subsets in the newly described HIV/AIDS syndrome. A new technology, later termed 'flow cytometry', was slowly emerging, enabling the study of the pathophysiology and antigen expression in white blood cell populations. Amongst these technological developments, described as early as 1956, was the invention of a high-speed automated blood cell counter by Wallace Coulter[38]. Other early iterations of cell counter technologies invented included Kamentsky and Melamed's 'spectrophotometric cell sorter', in 1965[39] and 1967[40]. Later, in 1976, 'Fluorescence-Activated Cell Sorting' (FACS) was described[41]. Kohler and Milstein's creation of hybridoma technology in 1975[42] was another crucial, and simultaneous and concurrent invention, providing for the formation of continuously-fused cells that were able to secrete specifically-predetermined antibody with unique specificity for leucocyte and leukaemia-related antigens. The development of techniques to conjugate fluorescent compounds to antibodies, produced by the Kohler and Milstein hybridoma technology,[43, 44] boosted the diagnostic capability of monoclonal antibodies that could be used in routine flow cytometry. Monoclonal antibodies could now be fluorescently-labelled and used as reagents[45, 46] to identify specific populations of lymphoid cells in the newly described FACS system, a critical invention that would enable immunophenotypic identification of antigens expressed on lymphocytes in patients infected with HIV.

[37]Wang JH, Yan YW, Garrett TP, Liu JH, Rodgers DW, Garlick RL, Tarr GE, Husain Y, Reinherz EL, Harrison SC. Atomic structure of a fragment of human CD4 containing two immunoglobulin-like domains. Nature 1990;348:411-8.

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[46]Thornthwaite JT, Seckinger D, Sugarbaker EV, Rosenthal PK, Vazquez DA. Dual immunofluorescent analysis of human peripheral blood lymphocytes. Am J Clin Pathol 1984;82:48-56.

In another significant diagnostic development on the FACS, Loken et al[47]outlined how individual white blood cell populations could be differentiated and isolated (termed 'gated') using the light scattering properties of cells (which portrayed the physical characteristics of cell populations). This advancement proved to be especially crucial for cellular enumeration and a key step in subsequent guidelines that followed. Some years later, applying all these techniques, the full characterization of T-lymphocyte subset alterations in HIV-infected patients were described by Giorgi et al[48].

By 1992, the US CDC had revised the classification system for HIV infection, emphasising the clinical importance of the CD4+ T-lymphocyte count for disease staging and categorisation of HIV-related clinical conditions or risk of opportunistic disease[49]. Later that year, the CDC also released its first guideline[50] for performing lymphocyte subsets and T-cell enumeration. The system described six requisite separate flow cytometric analyses to delineate CD4 T-cells and other lymphoid subsets[51]. Highly trained scientific staff with knowledge of the newly described techniques, and skills in the newly developing flow cytometry field, largely took responsibility for this testing.

Additional detail about the history of CD4 testing is published elsewhere[52].

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3. BACKGROUND: THE EMERGING HIV EPIDEMIC AND THE NEED FOR AFFORDABLE CD4 TESTING IN SOUTH AFRICA

Be open to possibility. Serendipitous detours may turn out to be the most important part of one's journey.

In 1990, during the year that the first state flow cytometric CD4 service was started at the South African Institute for Medical Research (SAIMR) in Johannesburg, HIV prevalence in South Africa was ~0.76%; this rate had sharply increased to 4.25% by 1993[53]. Described by Professor Barry Schoub as the silent epidemic[54], HIV was (sic) 'the most formidable public health peril facing mankind' and 'an inexorably expanding epidemic with an ever-increasing reservoir of infection'. These predictions were confirmed in the emerging documented data[55]. The epidemic was rapidly shifting away from predominantly affecting white homosexuals and unmistakably emerging, with a significant expansion of infection, amongst urban black heterosexuals by 1990[54].

By 1994, although the prevalence of HIV[53] was lower in areas like the Western Cape (WC) at 1.6%(CI 0.76-1.56), other regions around South Africa reported higher prevalence, with up to 7.6%(CI 4.62 – 8.8%) reported in both Gauteng and the Free State. In other areas of the country, such as KwaZulu Natal[53], HIV prevalence had risen as high as 14.4%(CI 12.22 – 16.48%). A national estimated-doubling time of 15.5 months was predicted at this time, with ~1.4 million people estimated to be infected by the end of that year[53]. The 'relentless progression' of the HIV epidemic was again predicted in 1991[56]; up to 27% of the population were predicted to be infected before 2010, mainly through heterosexual spread of the disease. McIntyre[57] noted at the time that little information was available to understand the impact of HIV on health services and resources in South Africa. He declared that much work lay ahead and projected that the epidemic, in the future, would likely devastate the health economy of South Africa. Broomberg et al., from the University of the Witwatersrand Centre for Health Policy[58], reiterated this view, indicating that HIV/AIDS expenditure was likely to reach 34-75% of total health care expenditure in South Africa by 2005. By 1994, national HIV/AIDS prevalence had reached 22.4% (and was climbing)[53].

[53]Kustner, H.G., J.P. Swanevelder, and A. Van Middelkoop, National HIV surveillance--South Africa, 1990-1992. S Afr Med J, 1994. 84(4): p. 195-200.

[54]Schoub BD. The AIDS epidemic in South Africa--perceptions and realities. S Afr Med J 1990; 77:607-8. https://www.ncbi.nlm.nih.gov/pubmed/2360112

[55]Schoub BD, Smith AN, Johnson S, Martin DJ, Lyons SF, Padayachee GN, Hurwitz HS. Considerations on the further expansion of the AIDS epidemic in South Africa - 1990. S Afr Med J 1990;77:613-8. https://www.ncbi.nlm.nih.gov/pubmed/2360116

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[58]Broomberg J, Steinberg M, Masobe P, Behr G. The economic impact of AIDS in South Africa. In: Doyle PR. AIDS in South Africa: The Demographic and Economic Implications. University of the Witwatersrand: Centre for Health Policy; 1991.

McIntyre and Broomberg made important and prophetic statements. In South Africa, between 1982 and 1988, during the timeframe when I was working as an intern in 1986, and later whilst beginning my specialist training in haematology, Professor Rubin Sher had diagnosed just ~166 cases of AIDS[59]. He further documented 1857 HIV antibody-positive sera for the same period (collating results from different laboratories around the country, excluding the mining industry). Amongst 710,000 blood donors tested at the time, Sher reported the presence of HIV-1 antibodies in just a small proportion of donor sera (n=244) during the same year. In line with these early predictions, by 2020, Statistics South Africa (STATSSA) reported[60] adult HIV prevalence at 18,7% of South Africans adults(15–49 years), with the total number of people living with HIV estimated at ~7.8 million.

By 1990, Sher was offering regular HIV-clinics at the Hillbrow hospital. In 1991, with the support of the SAIMR, I assisted Sher and setup a more efficient, flow cytometry-based, CD4 testing service[61]. The Centers for Disease Control (CDC) CD4 testing guidelines [62, 63] were implemented; one technologist covering the service was able to set up ~10-20 samples in a single testing session and provide results within 24-48 hours. The method required a 'dual platform' (DP) approach and was labour-intensive, prone to error and overly complex by today's standards. In this method, both a haematology analyser and flow cytometer were needed; lymphoid subset counts were obtained by multiplying the absolute total lymphocyte count (from the haematology analyser) by the respective lymphoid subset percentage of lymphocytes generated by flow cytometry. The quality control recommended was also extensive in order to assure reliable CD4 counting; termed 'lymphosum', testing involved use of a 6-tube, 2-colour panel arrangement to identify, and account for, all lymphocyte subsets, including helper(CD3+/CD4+) and suppressor(CD3+/CD8+) T-cells (CD3+), B-cell(CD3-/CD19+) and natural killer cell (CD16+ and CD56+) immunophenotypes. A fifth tube allowed for exclusion of contaminating monocytes whilst a sixth tube was used for assigning background non-specific isotypic binding. Preparation was cumbersome and impractical to meet the demands of increasingly large volumes of tests that were being received (or anticipated as an increasing number of HIV+ patients accessed care).

[59]Sher R. HIV infection in South Africa, 1982-1988--a review. S Afr Med J. 1989;76(7):314-8. https://www.ncbi.nlm.nih.gov/pubmed/2799575

[60]STATISTICS South Africa (STATSSA), 2020 Mid-year Estimates. Located at http://www.statssa.gov.za/?p=13453 (accessed 11/03/2021).

[61]Before 1991, CD4 testing was performed with a complement-dependent, cytotoxicity assay, and undertaken in the Department of Immunology at the erstwhile South African Institute for Medical Research (SAIMR). The assay was manually intensive and considered exceptionally time consuming and inaccurate.

[62]U.S. Centers for Disease Control (CDC). 1997 revised guidelines for performing CD4+ T-cell determinations in persons infected with human immunodeficiency virus (HIV). Centers for Disease Control and Prevention. MMWR Recomm Rep 1997; 46:1-29, as well as other international, mainly U.K. and U.S.-based guidelines that are cited elsewhere in this thesis.

[63]U.S. Centers for Disease Control (CDC). Guidelines for the performance of CD4+ T-cell determinations in persons with human immunodeficiency virus infection. MMWR Recomm Rep 1992;41:1-17. https://www.ncbi.nlm.nih.gov/pubmed/1350319

Flow cytometry was the only practical technology that could enable scaling up CD4 services, but the convoluted lymphosum-based guideline (recommended) testing method seemed to contradict this notion. Firstly, in a resource-poor context like South Africa, lymphosum-based testing was expensive; aside from the high capital outlay for flow cytometers and equipment needed to prepare samples and store reagents, there was the associated expense of the 12 separate monoclonal antibody reagents needed for the lymphosum quality control of a CD4 count. Secondly, the labour-intensiveness of the approach, with long sample-preparation times, would also not be a workable option considering the anticipated increasing demand for tests as an increasing number of patients accessed care. Longer sample preparation times would also likely negatively impact the turnaround time of the test which, in turn, could impact upon the standard of care proposed[64]. Thirdly, there was also the fact that there were limited numbers of trained staff available to undertake testing; the guideline method was expected to be undertaken by skilled, and experienced, professional scientifically-trained staff (scientists) to set up, analyse and report the CD4 tests, a further barrier in our local, relatively scarce-skills setting. It was therefore not surprising that that there were growing (mis)perceptions amongst governing and regulatory bodies that flow cytometry was too sophisticated and high-priced for a skills-limited context like South Africa. During this timeframe various manufacturers took advantage of the situation and vigorously promoted their manual 'low-tech' products for use in South Africa[65, 66, 67, 68, 69, 70] despite that these products would not realistically meet the requirements for scaling up CD4 services to a national level or potentially offer safe testing.

By the late 1990's, the South African government was yet to implement a national HIV treatment programme despite a burgeoning local HIV+ population needing care. Although the denial by the Mbeki government, that HIV caused AIDS, had further hampered progress[71], there were additional limiting factors limiting implementation too; antiretroviral drugs were expensive, and clinical and laboratory costs needed to support patients in care were high. There was also the fact that the newly formed National Health

[64]World Health Organisation(WHO). World Health Organization Emergency scale-up of antiretroviral therapy in resource-poor settings: technical and operational recommendations to achieve 3 by 5. 2003. Date accessed: July 23, 2021.

[65]Lyamuya EF, Kagoma C, Mbena EC, Urassa WK, Pallangyo K, Mhalu FS, Biberfeld G. Evaluation of the FACScount, TRAx CD4 and Dynabeads methods for CD4 lymphocyte determination. J Immunol Methods 1996;195:103-12.

[66]Johnson D, Hirschkorn D, Busch MP. Evaluation of four alternative methodologies for determination of absolute CD4+ lymphocyte counts. The National Heart, Lung, and Blood Institute Retrovirus Epidemiology Donor Study. J Acquir Immune Defic Syndr Hum Retrovirol 1995;10:522-30.

[67]Denny TN, Jensen BD, Gavin EI, Louzao AG, Vella FA, Oleske JM, Wong W. Determination of CD4 and CD8 lymphocyte subsets by a new alternative fluorescence immunoassay. Clin Diagn Lab Immunol 1995; 2:330-6.

[68]Nicholson JK, Velleca WM, Jubert S, Green TA, Bryan L. Evaluation of alternative CD4 technologies for the enumeration of CD4 lymphocytes. J Immunol Methods 1994;177:43-54.

[69]Carella AV, Moss MW, Provost V, Quinn TC. A manual bead assay for the determination of absolute CD4+ and CD8+ lymphocyte counts in human immunodeficiency virus-infected individuals. Clin Diagn Lab Immunol 1995;2:623-5.

[70]Balakrishnan P, Dunne M, Kumarasamy N, Crowe S, Subbulakshmi G, Ganesh AK, Cecelia AJ, Roth P, Mayer KH, Thyagarajan SP and others. An Inexpensive, Simple, and Manual Method of CD4 T-Cell Quantitation in HIV-Infected Individuals for Use in Developing Countries. J Acquir Immune Defic Syndr 2004;36:1006-1010. https://doi.org/00126334-200408150-00002 [pii]

[71]Heyward M. Civil Society and Uncivil Government: The Treatment Action Campaign (TAC) versus Thabo Mbeki, 1998-2008 Glaser ED, editor: Wits University Press; 2010.

Laboratory Service (NHLS)[72] did not have the necessary infrastructure, nor was immediately ready to handle the large number of HIV related tests that were expected to arrive.

Respite to the emergent HIV epidemic could only come from cost-effective therapies, clinical trials, and vaccine and treatment research, as well as a reliable and affordable laboratory monitoring service. Understanding this, and anticipating the costs predicted by McInytre and Broomberg, local alliance groups such as the Treatment Action Campaign (TAC)[73] and Section 21[74], as well as other international bodies such as Médecins Sans Frontières (MSF)[75], began actively campaigning for affordable treatment and laboratory monitoring approaches to support our local HIV antiretroviral treatment (ART) programmes. By 1998, their influence was felt as pressure mounted for more efficient and cost-effective CD4 testing. Pleas to lower prices for other laboratory tests like HIV viral load (VL) were also intensifying. Clinicians were no longer asking for but demanding cheaper laboratory services for HIV treatment monitoring.

[72]Republic of South Africa. No. 37 of 2000: National Health Laboratory Service Act. In: Government Gazette, editor. Volume 426. Cape Town; 2000.

[73]Treatment Action Campaign. Working for Access to Quality Healthcare in South Africa. 1998. Accessed: 22 November, 2019. Website: https://tac.org.za/

[74]Section 21. Catalysts for Social Justice. Accessed: 22 November, 2019. Website: https://section27.org.za/. SECTION27 is a public interest law centre that seeks to achieve substantive equality and social justice in South Africa. Guided by the principles and values in the Constitution, SECTION27 uses law, advocacy, legal literacy, research, and community mobilisation to achieve access to healthcare services and basic education. SECTION27 aims to achieve structural change and accountability to ensure the dignity and equality of everyone.

[75]Médecins Sans Frontières (MSF). International, independent medical humanitarian organisation. Accessed: 22 November, 2021. Website: https://www.msf.org/

4. THE CHALLENGE OF LABORATORY MONITORING OF HIV AND THE NEED FOR SELF-RELIANCE

"Sweet are the uses of adversity" [76]

By 2002, our group began to fully understand the many challenges associated with scaling-up HIV laboratory services nationally(1). Indeed, it became increasingly apparent that the associated expense of laboratory tests for diagnosing HIV and monitoring treatment would not be sustainable if the prevailing treatment recommendations from the World Health Organisation[77] were implemented into care. Further, the diagnostic technologies needed to deliver such laboratory services were perceived as being too sophisticated for our relatively poorly resourced and skills-limited setting(1). There was only one way forward that would provide the solutions needed for our South African HIV/AIDS treatment programme; embrace a culture of creative problem-solving and lateral-thinking. Notwithstanding that funding was crucial to enable our research, and gratefully received[78], our group called on other South African researchers(2) to join our endeavour to develop novel solutions that would ensure the success of our much-anticipated HIV/AIDS national treatment programme. In this communication, we suggested that adversity, in other words having considerably fewer financial and skills resources than first-world countries, but with a substantially higher burden of patients than anywhere else in the world, could act in our favour - forcing us to think outside the box and be creative with what we *did* have. In other words, bear in mind that the unique challenges faced were an opportunity to develop, not only new, but appropriate, solutions to manage the HIV burden in the South African health care system(2).

With the largely unknown task that lay ahead, inspiration about how to start came from South African artist, William Kentridge. In conversation[79] about problem-solving in painting and drawing with fellow artist Robert Hodgins, Kentridge commented on (sic) 'uncertainty as a virtue', suggesting that one could manifest resourcefulness and originality only if faced with uncertainty; that invention and novelty would emerge as one started *doing* the work. Indeed, Kentridge was right; by digging in and getting our hands dirty, along with some new ideas for CD4 counting (like the novel CD4 assay described in this work), several ground-breaking new ideas also emerged from our group, some with considerable positive social[80] and important clinical impact(3,4).

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[76] Shakespeare W. 'As you like it', Act 1: Scene 1. 1666.

[77] World Health Organisation(WHO). World Health Organization Emergency scale-up of antiretroviral therapy in resource-poor settings: technical and operational recommendations to achieve 3 by 5. 2003. Date accessed: Nov 23, 2021.

[78] **Glencross DK**. Affordable and accessible, quality CD4 testing by Panleucogating (PLG). Bristol Myers Squibb: Secure the Future Grant; 2002.

[79] Atkinson B. Conversations, Collaborations: Robert Hodgins, William Kentridge, Deborah Bell. In Robert Hodgins. First ed. Cape Town: Tafelberg Publishers; 2002. p 55.

[80] Sherman GG, Cooper PA, Coovadia AH, Puren AJ, Jones SA, Mokhachane M, Bolton KD. Polymerase chain reaction for diagnosis of human immunodeficiency virus infection in infancy in low resource settings. Pediatr Infect Dis J 2005;24:993-7.) https://doi.org/00006454-200511000-00014 [pii]

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5. ADVOCACY AND THE POWER OF COOPERATION AND COLLABORATION: HOW COMMITMENT AND SYNCHRONICITY ALIGNED TO ENABLE AFFORDABLE CD4 TESTING

Be bold! Commit! Synchronicity aligns to make things happen!

Johann von Goethe, commonly known as "Goethe", was considered the most remarkable German literary figure of the modern era. A poet, dramatist, novelist, philosopher, lawyer, theorist, painter and scientist, Goethe wrote about the power of commitment and synchronicity… *'Until one is committed, there is hesitancy, a chance to drawback. Always, ineffectiveness concerning all acts of initiative (creation), there is one elementary truth, the ignorance of which kills countless and splendid plans. At the moment one definitely commits oneself then Providence moves too. All sorts of things occur to help one that would never otherwise have occurred. A whole stream of events issue from the decision, raising in one's favour all manner of unforeseen incidents and meetings and material assistance, which no-one could have dreamt would have come their way. Whatever you can do, or dream you can, begin. Boldness has genius, power, and magic in it. Begin it now!'*

5.1 CHALLENGING THE CENTRAL DOGMA

As calls for the implementation of our South African HIV/AIDS treatment programme were intensifying (during the time of the Mbeki government's dissident views about the relationship of HIV and AIDS[81, 82]) increasingly frequent and vehement complaints were being received about the high cost of CD4 testing. The problem was addressed in a first study(5) that investigated an obvious way to reduce costs; abbreviate the guideline-based CD4 assay. Two shortened derivatives of the guideline method were proposed based primarily on lymphocyte identification by light scatter(5). The first option introduced a single triple-fluorochrome combination of monoclonals, CD3/CD4/CD8, where CD3 was retained as a T-cell marker to define CD4+ T-cells within a CD3+ T-cell gate. A second alternative looked at measuring CD4 alone using primary lymphocyte gating i.e., a single-reagent test, also known as 'CD4 only'. Both technical effort and reagent costs were cut in the two proposed alternatives. Later published work revealed that testing on a single (platform) volumetric counter (as opposed to testing by dual platform) could improve the reproducibility of the 'CD4-only' approach (thereafter coined 'primary CD4 gating' by Janossy et al[83] and independently validated by others[84] several years later). Although our controlled experiment showed acceptable precision and accuracy of the abbreviated CD4 cell panels, the obvious limitation of these alternative panels was that there was no quality control to ensure reliable identification of lymphocytes that would (quality) assure accurate cell counts if the assay was still primarily based on lymphocyte identification. Other groups also published similar rationalised, simplified

[81] Treatment Action Campaign. Working for Access to Quality Healthcare in South Africa. Johannesburg. 1998. Date accessed: 22 November 2021. https://tac.org.za/

[82] Heyward M. Civil Society and Uncivil Government: The Treatment Action Campaign (TAC) versus Thabo Mbeki, 1998-2008 Glaser ED, editor: Wits University Press; 2010.

[83] Janossy G, Jani I, Gohde W. Affordable CD4(+) T-cell counts on 'single-platform' flow cytometers I. Primary CD4 gating. Br J Haematol. 2000;111(4):1198-208.

[84] Lynen L, Teav S, Vereecken C, De Munter P, An S, Jacques G, Kestens L. Validation of primary CD4 gating as an affordable strategy for absolute CD4 counting in Cambodia. J Acquir Immune Defic Syndr 2006;43:179-85. https://doi.org/10.1097/01.qai.0000242447.82403.c2

options[85] around this time; however, all of these assays remained reliant on both lymphocyte gating and/ or CD3 immunophenotyping to define T-cells. The positive implications of the potential costs saved with use of the proposed abbreviated panels were notable however, offering a significant cost reduction and representing a saving of ~76% and ~90% respectively over the recommended 6-tube guideline method.

5.2 ADVOCACY

After this initial study(5) that challenged the prevailing guideline dogma, the high cost of CD4 reagents was considered. Taking inspiration from the successes of prominent South African HIV/AIDS treatment advocates who were working to reduce costs of antiretroviral medicines (including amongst others Justice Edwin Cameron[86], Zackie Achmat[81] and their colleagues Mark Heywood[81] and Nathan Geffen[81]), Professor Janossy[87], of London's University College, and I, began to canvas support from prominent and respected scientists in the field of flow cytometry to join us in advocating for reduced prices of monoclonal reagents. Our CD4 advocacy effort began with an international awareness drive for affordable CD4 testing(6). Initiatives included the launch of a website[88] and an invitation to several prominent international flow cytometry experts to join our initiative , a.k.a. the 'AFFORDCD4' group which was formed with a specific aim of creating awareness for affordable and more accessible CD4 testing. Members of the global flow cytometry community, including the late Dr Howard Shapiro[89] from the United States, Dr Frank Mandy[90] (Health Canada), Dr Bruno Brando[91] from Italy and Dr David

[85]Schnizlein-Bick CT, Mandy FF, O'Gorman MR, Paxton H, Nicholson JK, Hultin LE, Gelman RS, Wilkening CL, Livnat D. Use of CD45 gating in three and four-color flow cytometric immunophenotyping: guideline from the National Institute of Allergy and Infectious Diseases, Division of AIDS. Cytometry 2002;50:46-52. https://doi.org/10.1002/cyto.10073

[86]Justice Edwin Cameron (JEC) founded the pioneering AIDS Law Project (ALP) to protect those affected by HIV. The ALP fought the government's inaction on treatment for HIV and later JEC co-founded the Treatment Action Campaign (TAC) with Zackie Achmat. Successful in securing affordable treatment and providing generic medicines, in 2010 the ALP eventually became part of SECTION 27, focusing on wider socio-economic rights when Nathan Geffen and Mark Heywood join the group. See https://timeline.avert.org/?102/AIDS-Law-Project, accessed 19 November 2019.

[87]Emeritus Professor George Janossy was, at the time, Head of Immunology at the University College London and an ISI highly cited research. Prof. Janossy began his career by developing the monoclonals now used across the globe, including the RFT4, RFT8 and 2D1 (CD45) clones that were subsequently donated by the UK-based the National Institute for Biological Standards and Control (NIBSC) and used for generic production in South Africa.

[88]The AFFORDCD4 collaborative was established with its own domain at www.AffordCD4.com (no longer active).

[89]Shapiro HM. Author of the 'bible' of flow cytometry, 'Practical Flow Cytometry'; published by John Wiley and Sons (Wiley-Liss) New York: 1995.

[90]Dr Mandy was head of Health Canada, Quality Assessment Scheme International. With M Bergeron and T Minkus, Dr Frank Mandy, as the lead author, publishes an international review of 'Evolution of leukocyte immunophenotyping as influenced by the HIV/AIDS pandemic: a short history of the development of gating strategies for CD4+ T-cell enumeration', published in Cytometry 1997;30:157-65. Later Dr Mandy is the lead author for the U.S. Centers for Disease Control, and the later versions: 'Guidelines for the performance of CD4+ T-cell determinations in persons with human immunodeficiency virus infection. MMWR Recomm Rep 1992; 41:1-17.

[91]Brando B with authors Barnett D, Janossy G, Mandy F, Autran B, Rothe G, et al., on behalf of the European Working Group on Clinical Cell Analysis, had collated the guideline document entitled 'Cytofluorometric methods for assessing absolute numbers of cell subsets in blood', Cytometry 2000;42:327-46. This work was especially relevant for CD4 cell enumeration. https://doi.org/10.1002/1097-0320(20001215)42:6<327::aid-cyto1000>3.0.co;2-f

Barnett[92, 93] from the United Kingdom National External Quality Assessment Scheme (UK NEQAS), eagerly joined us to advocate for support for cheaper reagents and use of appropriate and simplified laboratory protocols for CD4 testing for resource-limited settings. Our group drew attention to the fact that inappropriate and disproportionately high costs of HIV laboratory monitoring in resource-limited settings were effectively blocking implementation of sustainable HIV treatment programmes recommended in the World Health Organization '3 by 5' initiative[94] and that immediate action was needed.

5.3 A PLEA TO INDUSTRY TO DEVELOP INEXPENSIVE, ACCESSIBLE CD4 INSTRUMENTS

The AFFORDCD4 group additionally proposed the development of small, user-friendly CD4 instruments that could play an important role in making CD4 counting more accessible to under-resourced countries and laboratories (without the financial means, or scientist skills-capacity, to undertake traditional flow cytometry-based CD4 testing). An impassioned plea was made to industry to develop small foot-print, low-cost, volumetric benchtop flow cytometers specifically making use of less expensive, small red diode lasers[95] typically used in compact disc players, to bring down instrument manufacturer prices (as opposed to the more costly typical argon gas lasers used in large conventional flow cytometers). The instrument was proposed with a capability of performing lower volumes of test (up to 25 per day) aimed at smaller sites; it was also proposed that the instrument would be used by operators without flow cytometry skills. In other words, the instrument put forward should preferably be user-independent, with user-friendly software; it was also envisaged to be developed as 'load-and-go', enabling safely testing samples directly from the tube (without exposing the operator to opening samples).

An early study validated a small CD4 testing instrument that utilised microvolume fluorimetry(7); the machine offered promise based on the AFFORDCD4 requirements call. The cost of cartridges and equipment were however expensive, and cartridges had to be manually filled. After a larger manufacturer instrument bought the company (and the instrument), manufacturing was stopped. Many similar instruments followed; almost 10 years later, several small point of care instruments(8), similar to that first conceptualised by the AFFORDCD4 group, were developed, and some commercially released later (a section on the evaluation of the more successful of these instruments was published by my group; details follow in Section 16).

[92]Barnett D, Granger V, Whitby L, Storie I, Reilly JT. Absolute CD4+ T-lymphocyte and CD34+ stem cell counts by single-platform flow cytometry: the way forward. Br J Haematol. 1999;106(4):1059-62,

[93]Whitby L, Granger V, Storie I, Goodfellow K, Sawle A, Reilly JT, et al. Quality control of CD4+ T-lymphocyte enumeration: results from the last 9 years of the United Kingdom National External Quality Assessment Scheme for Immune Monitoring (1993-2001). Cytometry. 2002;50(2):102-10. https://doi.org/10.1002/cyto.10094

[94]World Health Organisation(WHO). World Health Organization Emergency scale-up of antiretroviral therapy in resource-poor settings: technical and operational recommendations to achieve 3 by 5. 2002. Date accessed: Nov 23, 2021. WHO | 3 by 5 documents

[95]Janossy G, Jani IV, Kahan M, Barnett D, Mandy F, Shapiro H. Precise CD4 T-cell counting using red diode laser excitation: for richer, for poorer. Cytometry. 2002;50(2):78-85. https://doi.org/10.1002/cyto.10082

5.4 TAKING ON MONOCLONAL REAGENT MANUFACTURERS AT THEIR OWN GAME BY DEVELOPING LOCAL CAPACITY FOR MANUFACTURING

"Teach a man to fish…" (and learning self-reliance).

Although advocating for lower prices of CD4 monoclonal antibody reagents was one way to reduce reagent prices[96], another way was to become less reliant on supply from multinational manufacturers. Encouraged and supported by Professor Janossy, and locally by Professor Mendelow, I began planning a small cell culture facility to locally produce CD4, CD8 and CD45 monoclonal antibody, the reagents that would be needed for local CD4 testing. The idea behind this initiative was not only to reduce reagent costs, but also avoid being reliant on multinational commercial product and the exploitation that potentially came with it. Negotiations with Professor Janossy and the National Institute for Biological Standards and Control, (NIBSC) in the United Kingdom (UK), secured a crucial donation of the CD4 (RFT4), CD8(RFT8) and CD45 (2D1/anti HeL1) clones that would allow us to produce our own monoclonals. A noteworthy innovation from this newly-established cell culture facility, published by Scott and Glencross in 2001, was the novel use of a commercially available heterogeneous cell cultivation system, the CELLLine1000®, which substantially improved the unit's monoclonal antibody product yield(9)[97]. This significant process modification not only lowered our monoclonal production costs but also simplified the monoclonal production itself through elimination of manual labour-intensive downstream purification steps. Details of the feasibility and comparative data using these home-grown generic monoclonal reagents, in comparison to commercially-available antibody products, were reported later(10,11). Similar positive CD4 testing outcomes, using the same generic monoclonal reagents, was also reported by Pattanapanyasat et al later in 2005[98], (these monoclonal products were provided to the Pattanapanyasat team by my collaborator, Professor Janossy and the NIBSC).

[96]Through our AFFORDCD4 advocacy, word spread that the Glencross' South African unit in Johannesburg was producing enough monoclonal reagent to supply not only the local laboratory, but many other laboratories around Southern Africa and elsewhere on the continent. This action subsequently led to many monoclonal antibody manufacturers reconsidering their position in respect of their pricing. After 2004, despite originally having had the intention to continue long-term production of our own monoclonal antibody reagents, manufacturers and their 'middle-man' agents had begun to significantly lower their costs pricing of reagents to the point that costs were not dissimilar to our production costs. Professor Mendelow and I took the decision to close the cell culture facility to allow my unit to focus on National Health Laboratory Service core function and provide for other aspects of CD4 service delivery which now needed my attention.

[97]This paper was later included as one of the chapters of the work submitted by Lesley Scott for her PhD thesis entitled: 'HIV, AIDS and CD4: Africa's Problem'. Johannesburg: University of the Witwatersrand; 2008. **Supervisors: Glencross DK**, Coetzer T.

[98]Pattanapanyasat K, Shain H, Noulsri E, Lerdwana S, Thepthai C, Prasertsilpa V, Likanonsakul S, Yothipitak P, Nookhai S, Eksaengsri A. A multicenter evaluation of the PanLeucogating method and the use of generic monoclonal antibody reagents for CD4 enumeration in HIV-infected patients in Thailand. Cytometry B Clin Cytom 2005;65:29-36. https://doi.org/10.1002/cyto.b.20052

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7. **Glencross DK**, Scott L, Aggett H, Sonday S, Scott CS. Microvolume fluorimetry for the determination of absolute CD4 and CD8 lymphocyte counts in patients with HIV: a comparative evaluation. Clin Lab Haematol 1999;21:391-5. https://doi.org/10.1046/j.1365-2257.1999.00276.x https://www.ncbi.nlm.nih.gov/pubmed/10671991
8. **Glencross DK**, Scott LE. CD4 cell monitoring for HIV/AIDS: old options; new insights. SADJ 2001;56:602-5. https://pubmed.ncbi.nlm.nih.gov/11887447/
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6. DISRUPTION: INTRODUCING PANLEUCOGATED CD4 TESTING

A challenge is both an opportunity and a point for departure.

6.1 CHALLENGING THE PREVAILING CD4 TESTING PARADIGM: LOOKING BEYOND THE DOGMA OF THE LYMPHOCYTE COUNT DENOMINATOR

The prevailing CD4 guideline testing dogma[99, 100] mainly based on variations of the 'lymphosum' method described previously in section 3, had several superfluous procedural steps that compounded error (leading to poorer quality control); the same procedural quality control steps also made CD4 testing considerably more expensive.

The first major drawback in the conventional CD4 test was its reliance on total lymphocyte count, a component of the fairly widely used, but error-fraught, white blood cell differential generated on a haematology-analyser[101, 102, 103]. The second shortcoming was the so-called quality control to assure that all lymphocytes were accounted for on the flow cytometry side; here, all lymphocyte subsets comprising the total lymphocyte population had to be accounted for in the flow cytometrically derived 'lymphosum'. This added complexity, with multiple preparation and analysis steps, compounding error in the conventional assay. Thirdly, the lack of standardisation of either dual or single platform approaches used in the conventional assay also introduced variability of reporting[104]; for example, several different types of haematology analyser, that were used for dual platform CD4 counting, each had their own unique parameter and platform differences which were known to contribute to white blood cell differential count variability. There were also many variations of manufacturer flow cytometers and monoclonal reagents used to generate a CD4 (reviewed by the author(8)). Additional variation between testing centres was introduced as predicate procedures used by laboratories often deviated from the stipulated

[99] U.S. Centers for Disease Control (CDC). 1997 revised guidelines for performing CD4+ T-cell determinations in persons infected with human immunodeficiency virus (HIV). Centers for Disease Control and Prevention. MMWR Recomm Rep 1997;46:1-29 as well as other international, mainly U.K. and U.S.-based guidelines that are cited elsewhere in this thesis.

[100] U.S. Centers for Disease Control (CDC). Guidelines for the performance of CD4+ T-cell determinations in persons with human immunodeficiency virus infection. MMWR Recomm Rep 1992;41:1-17. https://www.ncbi.nlm.nih.gov/pubmed/1350319

[101] Robinson G, Morgan L, Evans M, McDermott S, Pereira S, Wansbrough-Jones M, Griffin G. Effect of type of haematology analyser on CD4 count. Lancet 1992; 340:485

[102] Bentley SA, Johnson A, Bishop CA. A parallel evaluation of four automated hematology analysers. Am J Clin Pathol 1993;100:626-32.

[103] Simson E, Groner W. Variability in absolute lymphocyte counts obtained by automated cell counters. Cytometry 1995; 22:26-34.

[104] Reilly JT, Barnett D. UK NEQAS for leucocyte immunophenotyping: the first 10 years. J Clin Pathol 2001;54:508-11.

guideline; most laboratories applied variations of lymphocyte and or CD4 gating approach (including light scatter, bright CD45 or CD3 gating,[105, 106, 107] to generate their CD4 counts.

6.2 INTRODUCING THE NEW PANLEUCOGATED CD4 TESTING METHOD

"*Less is more*"[108]

As pressure mounted to reduce the costs of CD4 testing, it became increasingly obvious that the so-called 'lymphosum' quality control of the conventionally applied CD4 method was not only adding huge cost, but it was also neither effective, nor reliable to ensure an accurate CD4 count. As a haematologist (which proved to be a distinct advantage over my immunology colleagues who predominantly managed CD4 testing at that time), I understood that the total lymphocyte count, used as the common denominator certainly in the dual platform method, was the weakest link. Improving the test quality would however require a significant paradigm shift in the prevailing belief about what constituted 'proper' quality control and what represented an acceptable common denominator.

As I thought about it, it became increasingly evident to me that substituting total lymphocyte count with total white cell count (WCC), a much better reported reliable and stable parameter[102, 103] could immediately improve the quality of the existing CD4 test method. In other words, instead of relying on total lymphocyte count with its inherent reporting variability that consequently led to variability of CD4 reporting, the reproducibility, hence quality of the CD4 test would immediately improve by simply shifting the denominator to total WCC. Likewise, higher costs of the conventional method would also address concerns about unaffordability; significantly less monoclonal reagents would be needed to undertake quality control in a simplified version.

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[105]Gelman R, Wilkening C. Analyses of quality assessment studies using CD45 for gating lymphocytes for CD3(+)4(+)%. Cytometry. 2000;42(1):1-4. https://doi.org/10.1002/(sici)1097-0320(20000215)42:1<1::aid-cyto1>3.0.co;2-a

[106]Gratama JW, Kraan J, Keeney M, Granger V, Barnett D. Reduction of variation in T-cell subset enumeration among 55 laboratories using single-platform, three or four-color flow cytometry based on CD45 and SSC-based gating of lymphocytes. Cytometry. 2002;50(2):92-101. https://doi.org/10.1002/cyto.10084

[107]Bergeron M, Nicholson JK, Phaneuf S, Ding T, Soucy N, Badley AD, Hawley Foss NC, Mandy F. Selection of lymphocyte gating protocol has an impact on the level of reliability of T-cell subsets in aging specimens. Cytometry 2002;50:53-61. https://doi.org/10.1002/cyto.10092

[108]Robert Browning, 'Andrea del Sarto', 1855. According to Robert Browning, del Sarto was a renaissance painter who admitted to his painter-model wife, Lucrezia, that he had given up his artistic integrity in favour of a prolific output of faultless paintings. He described his work as 'without soul', and that 'less was more' in the painterly work of his contemporaries, Raphael, and Michelangelo. Later, in the 20th century, the adage 'less is more' was picked up and expanded upon by founder of modern architecture and proponent of simplicity in design, Ludwig Mies van der Röhe (1886-1969). R. Buckminster Fuller (1895 – 1983), an inventor and visionary who worked as a 'comprehensive anticipatory design scientist' to solve global problems, also adopted the slogan as a lifestyle standard to inspire simplified invention and design. The 20th century iconic architect and furniture designer Frank Lloyd Wright later clarified the concept best though by writing that, (sic), "Less is more, only when more is too much." Many other famous designers, including Coco Channel have also used this less-is-more aesthetic to promote their pared-down 'improved' designs.

The new CD4 method, that incorporated this shift of common denominator, was termed the Panleucogated CD4 (PLG CD4) method(12). Several critical features differentiated it from the prevailing guideline conventionally applied method. The first and most important shift was to use WCC as the common denominator(12); this meant that the laborious lymphocyte subset identification, i.e. 'lymphosum' was no longer needed to quality control the lymphocyte differential in the conventional dual platform method. The PLG CD4 method also incorporated our previously introduced idea that CD4+ lymphocytes could be directly identified by primary CD4 gating(5), by relying on their brighter antigen expression of CD4 antigen and distinct low side-scatter of CD4+ lymphocytes(10), obviating the need for total lymphocyte identification in the first instance. This simple modification further removed the need for additional CD3 reagent to identify T cells; it also meant that CD19 and CD56, to respectively identify B-cells and natural killer cells in a lymphocyte gate, could also be dropped. Likewise, as monocytes could be identified with dim CD4 (antigen expression), and by their distinct and separate intermediate side scatter, the PLG CD4 method also made CD14 inclusion redundant (previously used to identify and exclude contaminating monocytes in the lymphosum procedure). The PLG CD4 method further did away with the need for isotypic non-specific background assessment recommended in the prevailing guideline; CD4-negative lymphocytes could be used to establish any isotypic background staining if present. Another important distinguishing feature was a much quicker processing time which markedly streamlined technical effort (time per test) in a single, one-step preparation. A critically important aspect was that reagent costs per tests were immediately and substantively reduced due to significantly fewer monoclonal antibody reagents required to generate a PLG CD4 count, using just CD45 with CD4 (as opposed to ~12 needed for the lymphosum method).

A high correlation of outcomes, with comparable accuracy and precision of PLG CD4 against both conventional dual platform and single platform methods, was reported on both fresh and fixed samples, as well as for stabilised blood product used for external quality assessment(12). The work also introduced a further simplifying step by reporting equivalency of the DP PLG CD4 assay to single platform systems, using either volumetric counting or bead-based technologies(12).

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7. PATENTS REGISTERED: RETAINING INTELLECTUAL PROPERTY IN SOUTH AFRICA

A South African patent describing the PanLeucogating (PLG) CD4 assay was registered in July 2001(13) describing a CD4 counting assay based solely on CD4 and CD45; the patent outline also included a separate description of a method for a white blood cell differential using only CD45 and CD4. A year later, a World Intellectual Patent Organization (WIPO) Patent Cooperation Treaty (PCT) application(14) for an international patent was recorded.

Full patent rights for the PLG CD4 method were issued in Japan in 2008(15). In 2010, USA patent rights were published(16); full patents were registered in the UK and European Union(17) in the same year. In 2011, further patents were published for an antibody reagent kit for enumeration of CD4+ lymphocytes that included monoclonal antibodies CD4 and CD45(18). All patents were assigned to the newly formed National Health Laboratory service (NHLS) and the invention ascribed to the author (DK Glencross).

The PLG CD4 assay was U.S. Food and Drug Administration (FDA) approved in 2008 and 'CE' marked for the European Economic Area, including distribution in Switzerland and Turkey, during 2010.

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8. ADDRESSING THE 'WONDER CD4' CRITICS[109]

Criticism is useful. Listen and act accordingly. But let the data speak for itself.

Several news articles reporting PLG CD4 appeared in both printed and web-based social media sites during 2002. Initially, there were many criticisms and open skepticism[109] leveraged at the newly described PLG CD4 method, with doubts expressed about the claims about improved quality or potential cost savings. The ability of the newly formed NHLS to undertake an extensive national rollout was also questioned. Could the NHLS afford the extensive capitalization of equipment needed? How would local skills levels be considered? On a practical level, who (scientists or technologists) would perform testing? There was also the question raised about which pathology discipline should undertake testing.

Not all media coverage was negative though. Included amongst some of the more positive viewpoints were those expressed in a Nature Medicine news-editorial openly commending the PLG invention[110]. A second significant mention was made in the South African Parliament, in a presentation by Treasury of the potential positive financial implications of a cheaper (PLG) CD4 test on the local treatment programme[111]. Later, by 2007, despite the initial reservations, even amongst members of some established flow cytometry forums, PLG CD4 was endorsed for use by both the international Clinical Laboratory Standards Institute (CLSI)[112] and by the US CDC[113], where it was included as an alternative method for performing both dual and single-platform absolute CD4+ T-cell determinations. The potential and impact of PLG CD4 was also acknowledged with several awards to the author[114].

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[109]Bateman C. Glencross confounds 'wonder CD4' critics. S Afr Med J. 2002;92(8):572-3.

[110]Willyard C. Simpler tests for immune cells could transform AIDS care in Africa. Nat Med. 2007;13(10):113.

[111]Research at Wits leads to cheaper HIV testing (Member's statement) 2005. Prof I J MOHAMED (ANC): "Thank you, Madam Chair. The ANC wishes to recognise and commend Prof Deborah Glencross and her team at Wits University for devising an effective and reliable method of testing and monitoring immune levels in HIV/AIDS patients. The Food and Drug Administration of the USA has accredited the South African Aids technology that radically reduces the cost of monitoring immune levels in Aids patients. The exorbitant cost of monitoring HIV/AIDS is a major obstacle in treating the continent's most devastating disease. With this groundbreaking research, Prof Deborah Glencross will save South Africa, Africa and countries further afield billions of Rands. The World Health Organisation is supporting Prof Glencross in establishing a quality support programme for CD4 testing across Africa. The test will provide a cost saving of 70% to 80% over traditional HIV testing. The test will cost R60, compared to the current test, which costs R1500. We in the ANC join the Department of Health and health workers in general in saluting Prof Glencross for her meritorious contribution in the great battle against this scourge, which afflicts humanity. I thank you."

[112]Clinical Laboratory Standards Institute (CLSI). H42-A2: Enumeration of Immunologically Defined Cell Populations. In: Institute CaLS, editor. Wayne, PA: Clinical Laboratory Standards Institute 2007.

[113]Mandy FF, Nicholson JK, McDougal JS. Guidelines for performing single-platform absolute CD4+ T-cell determinations with CD45 gating for persons infected with human immunodeficiency virus. Centers for Disease Control and Prevention. MMWR Recomm Rep 2003;52:1-13. https://www.ncbi.nlm.nih.gov/pubmed/12583540.

[114](1) International JP Morgan Chase Health award laureate, for Innovation in Health Technology: The Tech Museum of Innovation, San Jose, California, USA, 2002; (2) South African National Productivity Institute - Gold Award, 2003; (3) National Science Technology Forum (NSTF) award for Innovation over the past 5 years, 2004; (4) Checkers Woman of the Year Finalist: Health Laureate, 2002.

The 'wonder-CD4 critics' criticisms and concerns[109] were addressed by letting the data speak for itself. Over the next 8 years, several studies and reports published demonstrated that PLG CD4 was an extremely robust and accurate method; subsequent work also showed that the method was easily translatable into routine national laboratory services, even where technical staff undertaking testing had very little or no flow cytometry skills (Sections 10 and 15). Later studies also confirmed affordability relative to other proposed implementation ideas (Section 15).

Important and notable positive outcomes about the accuracy and precision of PLG CD4 are outlined below:

8.1 CLINICAL EQUIVALENCY (ACCURACY) OF PLG CD4 COUNTING TO TRADITIONAL CD4 METHODS

Firstly, and most importantly, the comparable accuracy of the PLG CD4 method versus other established guideline or state-of-the-art CD4 methods was confirmed in several published studies.

8.1.1 THE PANLEUCOGATING APPROACH ENABLES ACCURACY (EQUIVALENCY) OF HAEMATOLOGY ANALYSER-DERIVED WHITE CELL COUNT (WCC) TO FLOW CYTOMETRY DERIVED TOTAL CD45 WCC

Initial work on fresh whole blood using the volumetric cell counter(12), the Ortho Cytoron™, confirmed the accuracy of the WCC common denominator in the dual platform PLG CD4 method. Excellent comparative results revealed a minimal bias between total CD45+ white cell count by flow cytometry and WCC generated on a haematology analyser; similar outcomes were again confirmed in a later study that looked at accuracy in aged samples(11). Improved overall equivalency of CD45+ WCC, using flow cytometry and PLG, against WCC by haematology analyser, was also confirmed in day-old samples across five United States (US) sites that employed variations of haematology analyser but used a standardised and universal PLG approach(19) .

8.1.2 PLG CD4 COUNTS ARE ACCURATE AND EQUIVALENT TO CD4 COUNTS ON OTHER SYSTEMS

Equivalency of dual platform (DP) PLG CD4 counts(12) was confirmed against traditional DP and single platform (SP) bead-based single platform technologies (in this instance, against the Becton Dickinson Trucount™ bead, described elsewhere by Schnizlein-Bick et al.,[115]). Data from the first year(20) of our local CIPRA SA[116] study showed acceptable accuracy of DP PLG CD4 reporting to another

[115]Schnizlein-Bick CT, Spritzler J, Wilkening CL, Nicholson JK, O'Gorman MR. Evaluation of TruCount absolute-count tubes for determining CD4 and CD8 cell numbers in human immunodeficiency virus-positive adults. Site Investigators and The NIAID DAIDS New Technologies Evaluation Group. Clin Diagn Lab Immunol 2000;7:336-43. https://doi.org/10.1002/cyto.10073.

[116]CIPRA SA Clinical trial entitled 'Safeguard the Household: A Study of HIV Antiretroviral Therapy Treatment Strategies Appropriate for a Resource Poor Country'. Details available at (accessed December 2021): https://clinicaltrials.gov/ct2/show/NCT00080522?term=CIPRA+SA&cntry=ZA&draw=2&rank=2

commercially-available SP bead-based counting method (Beckman Coulter FlowCount™, described elsewhere by Reimann et al[117]). The CIPRA SA study also enabled comparison of both DP and SP PLG CD4 against the more traditional, guideline-based[118] DP and SP CD4 testing approaches(21) in a longitudinal view, where accuracy and precision of all methods was investigated studying 1181 returning patients results over a four-year period. Similar outcomes were reported for the CD4 percentage of lymphocytes parameter (aka CD4%Ly) in both the PLG CD4 method versus guideline methods(21).

Participation in several United Kingdom's National External Quality Assurance Scheme (UK NEQAS) trials(21) provided additional evidence of the longitudinal (reproducible) accuracy of PLG CD4 counts. Study outcomes revealed that all submitted PLG CD4 outcomes fell within ±1 SD of the respective trial pool mean; absolute PLG CD4 counts, on average, were reported within 6% of the pool mean, whilst, in comparison, the group performance of laboratories using typical predicate methods showed wider variation of reporting and were reported, on average, per trial, within 10% of the trial pool mean(21).

Other long-term external quality assessment participation in the African Regional External Quality Assessment Scheme (AFREQAS), over several years, provided additional longitudinal evidence of PLG CD4 accuracy in comparison to the participating AFREQAS scheme user pool(22). Cumulative absolute CD4 Standard Deviation Index (SDI) score analyses confirmed the reported %CV and excellent between-laboratory accuracy amongst Becton Dickinson (BD) FACSCount™ and PLG CD4 users, with excellent Z-score (SDI) outcomes measured at less than 1.2 for both FACSCount and PLG CD4 participants(21). As in the US National Institutes of Health study(19), the PLG CD4 and FACSCount outcomes again emphasised the positive impact and importance of standardised and simple gating in improving the precision of CD4 counts between centres. Similar outcomes were recorded for CD4% of lymphocytes(21).

8.1.3 PLG CD4 SHOWS EQUIVALENCY TO INDUSTRY-STANDARD FACSCOUNT™

The BD FACSCount™[119, 120] is an established propriety, industry-standard SP system capable of producing highly reproducible CD4 counts. Tight equivalency of reporting of DP PLG CD4 to BD FACSCount™ was first reported in a local educator study cohort(11); later, a local study that

[117]Reimann KA, O'Gorman MR, Spritzler J, Wilkening CL, Sabath DE, Helm K, Campbell DE. Multisite comparison of CD4 and CD8 T-lymphocyte counting by single- versus multiple-platform methodologies: evaluation of Beckman Coulter flow-count fluorospheres and the tetraONE system. The NIAID DAIDS New Technologies Evaluation Group. Clin Diagn Lab Immunol 2000;7:344-51. https://doi.org/10.1128/cdli.7.3.344-351.2000

[118]Clinical Laboratory Standards Institute (CLSI). H42-A2: Enumeration of Immunologically Defined Cell Populations. Editor. Wayne, PA: Clinical Laboratory Standards Institute, 2007.

[119]Strauss K, Hannet I, Engels S, Shiba A, Ward DM, Ullery S, Jinguji MG, Valinsky J, Barnett D, Orfao A, Kestens L. Performance evaluation of the FACSCount System: a dedicated system for clinical cellular analysis. Cytometry 1996;26:52-9.

[120]Lopez A, Caragol I, Candeias J, Villamor N, Echaniz P, Ortuno F, et al. Enumeration of CD4(+) T-cells in the peripheral blood of HIV-infected patients: an interlaboratory study of the FACSCount system. Cytometry. 1999;38(5):231-7.

investigated the value of providing FACSCount™ CD4 counts at the point of care in a clinical interface, demonstrated remarkably tight equivalency of reporting between FACSCount™ to SP PLG generated CD4 counts(23). Details about this work are included later in the section under Point of care testing (see Section 16).

FACSCount™ however, only reported a CD4% of T-cells (utilising CD3+/CD4+). A distinct advantage of PLG CD4 over the FACSCount™ system at the time, was that the PLG method was able to additionally report the conventional percentage of CD4 lymphocytes (CD4%Ly) needed for paediatric HIV+ patient assessment of disease progression. (This was achieved by incorporating CD45*bright* and low side scatter gating that enabled reporting the percentage of CD4+ lymphocytes amongst total lymphocytes). Later versions of the FACSCount™ however included a percentage of CD4 lymphocytes[121].

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8.1.4 INTERNATIONALLY COMPARATIVE PLG CD4 STUDIES PUBLISHED BY OTHER AUTHORS VALIDATE THE ACCURACY OF PLG CD4 METHOD

Several international studies from Europe, Asia, and as far afield as the Caribbean Islands, amongst others, have independently validated the PLG CD4 method against various state-of-the-art methods, confirming equivalency of reporting to various predicate CD4 methods [122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137].

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[126] Pattanapanyasat K, Shain H, Noulsri E, Lerdwana S, Thepthai C, Prasertsilpa V, et al. A multicenter evaluation of the PanLeucogating method and the use of generic monoclonal antibody reagents for CD4 enumeration in HIV-infected patients in Thailand. Cytometry B Clin Cytom. 2005;65(1):29-36. https://doi.org/10.1002/cyto.b.20052

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8.2 THE PRECISION OF CD4 TESTING IS IMPROVED USING THE PLG APPROACH

The precision of any laboratory test is essential to ensure safe and meaningful, immediate as well as longitudinal, clinical decision making. Improved reproducibility (within-laboratory precision) and repeatability (between-laboratory precision) of PLG CD4 testing was recorded in two noteworthy studies (19,21).

8.2.1 PLG CD4 LEADS TO A 33% IMPROVEMENT OF BETWEEN-LABORATORY PRECISION AND A 25% IMPROVEMENT OF WITHIN-LABORATORY PRECISION

The most significant outcome demonstrating the improved precision of PLG CD4 came about as a result of a United States (US) National Institutes of Health (NIH) sponsored research study, with preliminary data presented in an international meeting in the United States[138] and the completed study data published two years later in 2008(19). This study looked at DP PLG CD4 against other predicate CD4 testing methods (predicate denoting the local variation and method of choice) in five U.S. based laboratories that used varying DP or SP systems with four-colour CD45/ CD3/ CD4 /CD8 protocols. Analysis of the data showed that the use of the PLG CD4 method led to a significant ($P<0.0001$) 33% improvement of between-laboratory precision over conventional, predicate methods, with a median reported percentage co-efficient of variation (%CV) of 9.3% reported for PLG versus a 13.1% for predicate methods used. Within-laboratory precision was also significantly improved by 25% ($P<0.0001$) (with respective median %CV of PLG outcomes reported at 4.6% versus predicate outcomes of greater than 6.2%). The conclusion of the study was that any laboratory using predicate methods similar to those used in the study, irrespective of whether located in the third or the first world, could improve their laboratory's CD4 counting reproducibility and repeatability by switching to the PLG method.

8.2.2 EXCELLENT AND CONSISTENT LONGITUDINAL BETWEEN-LABORATORY PRECISION IS DEMONSTRATED DURING INTERNATIONAL EXTERNAL QUALITY ASSESSMENT

The CIPRA SA study mentioned above(21), with follow-up on a cohort of 1181 patients, reported excellent longitudinal precision (reliability) of PLG CD4 counts in patient's consecutive CD4 patient measurements. Improved contributing intra-laboratory precision and reproducibility of PLG CD4 (during the period of the study) was also confirmed during participation in five trials on the UK NEQAS, where an improved intra-laboratory longitudinal precision of the South African CD4 laboratories of

[138]Denny T, Gelman R, Bergeron M, Forman M, Landay A, Louzao R, Mandy FF, Schmitz J, Wilkening C, **Glencross DK**. A Multi-Lab study of CD4 counts using flow cytometric PanLeukogating (PLG): A NIAID-DAIDS Immunology Quality Assessment Program Study. Conference on Retrovirus and Opportunistic Infections (CROI). Boston, USA; 2005.

7.2 CV% was reported for PLG CD4, versus 10.7 CV% for laboratories employing other CD4 predicate methods(21).

8.2.3 Excellent and consistent longitudinal Between-laboratory precision is demonstrated during regional External Quality Assessment over five years of scheme participation

The excellent performance of NHLS laboratories using PLG CD4, enrolled on the African Regional External Quality Assessment scheme (AFREQAS), provided yet further evidence of significantly improved longitudinal between-laboratory precision of PLG CD4(21). Excellent PLG CD4 outcomes were only matched by (industry-standard) FACSCount™ users; over five years of participation, between-user precision (again as a calculated %CV) of less than 8% was reported for both PLG and FACSCount users(21). In comparison with laboratories that used other CD4 technologies, i.e., individual group performance varied markedly, ranging between 12%CV and 25%CV where other conventional DP or SP, or specific volumetric-based SP testing methods were used (21). Overall participant between-user precision across all technologies was also less precise than PLG CD4 registered sites, recorded at ~10.5%CV(21).

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8.3 USE OF PLG CD4 IMPROVES ACCESSIBILITY AND EXTENDS CD4 LABORATORY SERVICES TO REMOTE AREAS

In any African or other third-world context, certainly for South Africa, there are often vast distances between where samples are taken (at the clinic or referring hospital site) and where samples are analysed (at a testing laboratory). Transit can potentially take many days, with the consequence that samples may be rejected if too old or disintegrated upon receipt at a testing laboratory. This had been especially relevant for CD4 samples that relied on the DP cell counting method; if not tested within 6 hours of venesection, the integrity (and quality) of the white blood cell count and more specifically, the differential count, both generated on a haematology analyser[139, 140] could not be assured. The implementation of SP PLG CD4 offered a solution to this challenge.

8.3.1 FIXATIVES PRESERVE SAMPLES AND EXTEND THE PRE-ANALYTICAL WINDOW

Initially, short-term fixatives were investigated as a means to preserve the integrity of samples during long transit times to a laboratory from a distant referral site. An early study by Jani, Janossy, Glencross and others(24) reported the use of a short-term sample fixative, aptly named, 'Transfix'[141] that preserved samples for up to 30 days. Transfix™ was also used for the first reported PLG CD4 study(10), enabling coordinated method comparison studies to be undertaken simultaneously in both Johannesburg and in London during 2001. In the longer term though, this was not seen to be a practical solution ('Transfix' tubes would need to be widely distributed and accessible in remote sites potentially adding substantive additional logistical and costs to testing)(24).

8.3.2 CD45+ PANLEUCOGATING FACILITATES AN EXTENDED WINDOW OF TESTING

At one of the early international AIDS meetings, held in Paris in 2003[142], we first reported the observation that total CD45 expression, used in the PLG CD4 method, was reliably retained on all leucocytes to 5 days post-venesection. In fact, at 8 days, total CD45 expression still remained distinct and was an easily identifiable and sufficiently discrete to undertake panleucogating, with only slight

[139]Simson E, Groner W. Variability in absolute lymphocyte counts obtained by automated cell counters. Cytometry 1995; 22:26-34.

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loss of expression noted that did not affect identifying the overall CD45 cluster. This was an important feature of PLG CD4 that set the method apart and meant that CD45+ panleucogating could be used to reliably obtain a white cell count, and hence CD4 counts, by SP, even if the sample was up to 5 days old. Effectively, PLG gating thus 'extended' the acceptable time frame allowable between sample collection and sample testing not possible in conventional testing and gating. Data reported in our local educator study later confirmed this finding(11), but also revealed that proper packaging and temperature control of the samples during transit of samples was equally important to ensure sample integrity. Later, the loss of CD45, together with the loss of forward scatter amongst neutrophils, was also used as practical tool to predict sample age (unpublished data but included in NHLS teaching materials[143]) and assist decision making about sample rejection.

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8.4 PLG CD4 DRAMATICALLY SAVES COSTS

PLG CD4 testing was officially offered amongst the repertoire of new HIV tests offered by the NHLS at the launch of the South African HIV/AIDS Comprehensive Care and Treatment (CCMT) programme in April 2004. At USD2 to USD3 per PLG CD4 test[144], the cost was drastically reduced by ~80% per test(12) ; in 2009, the World Health Organisation had, in comparison, reported other CD4 tests costing between double and ten times the base-cost of PLG CD4[144].

PLG CD4 has been remarkably cost-efficient for South Africa[145, 146]; by November 2020, more than 40 million PLG CD4 tests have been performed, with cumulative effective savings approaching R8 Billion[146] over the past 17 years of the South African HIV/AIDS CCMT programme.

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9. SCALING UP SERVICES FOR A NATIONAL LABORATORY PROGRAMME

Climbing a mountain, one step a time…

A sound laboratory service, fundamental to implementing and growing a national ART treatment programme for HIV/AIDS, should offer quality and timeous pathology reporting; this aspect is key for appropriate and timely clinical decision making. Historically though, the provision and maintenance of laboratory infrastructure has not been given the same level of priority in HIV/AIDS services[147] as provision of medicines, or clinical sites for example.

9.1 LABORATORY SUPPORT FOR AN ANTIRETROVIRAL TREATMENT PROGRAMME IN A RESOURCE POOR SETTING

At the start of the HIV/AIDS CCMT in South Africa, despite the hinderances caused by local HIV/AIDS denialism, (described elsewhere by Heywood[148]) our group took up the challenge for the massive scale-up of scale up of laboratory services that were needed, armed with enthusiasm(16), awareness of the importance of Good Clinical laboratory practice[149] and guidance provided in the World Health Organisation 3-by-5 initiative[150]. There were inspiring examples to follow. Elsewhere, Brazil had reported on their universal access to care policy and inclusive approach to managing large scale support for HIV treatment programmes. Teixeira et al[151] described that coordinated, and specifically designed activities that had considered local needs and resources, had improved health infrastructure in Brazil and was assisting in overcoming many obstacles typically encountered in limited-resource countries where there was an increasing burden of HIV/AIDS patients presenting for care. This work also emphasised that standardised and simplified laboratory testing was fundamental for programmatic success.

With the Brazilian programme in mind, the South African HIV/AIDS laboratory CD4 programme was built, certainly from a CD4 testing perspective, step by step, tackling one problem at a time, by trial and error.

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[151]Teixeira PR, Vitoria MA, Barcarolo J. Antiretroviral treatment in resource-poor settings: the Brazilian experience. AIDS 2004;18 Suppl 3:S5-7. https://doi.org/10.1097/00002030-200406003-00002

Working alongside colleagues[152], several local HIV/AIDS laboratory-based projects[153, 154, 155, 156, 157, 158] (and other early CD4 projects mentioned elsewhere in this thesis) were initiated and aligned to introduce affordable and accessible pathology services in South Africa. Issues related to high costs and appropriateness (or inappropriateness) of certain assays were considered(1); concerns about a general lack of resources, including funding, facilities, equipment, and skilled staff were also addressed (1).

After 18 months of experience gained through building the HIV diagnostic services for the NHLS, our group, led by colleague Wendy Stevens, were invited to present our experience at the first South African AIDS meeting held in Durban in 2005(25). This work included a review of HIV viral load and related drug resistance testing and the progress and developments in the NHLS CD4 service delivery over the previous two years; the successful implementation of 25 CD4 laboratories and ~400 000 CD4 tests performed was also announced[159]. Details about new emerging point of care CD4 technologies, that could be useful to extend services to areas without access to laboratory services, were also presented; specifically at this time, validation outcomes of the PointCARE and Guava Personal Cell Analyser (PCA) systems were reported (these and other point of care CD4 evaluation studies undertaken by the author are discussed later in Section 16).

9.2 CONSOLIDATING ON EXPERIENCE GAINED AND SHARING THE LESSONS LEARNED

Our consolidated experience and lessons learned in the initial scaling-up of HIV laboratory services in South Africa were outlined in a book commissioned through the Elizabeth Glaser Foundation, '*From the ground up: Building comprehensive HIV/AIDS care programs in resource-limited settings*'(26). The chapter by Stevens and Glencross, entitled 'Developing Laboratories to Support HIV-related Service and Research Activities in Sub-Saharan Africa', described a detailed step-by-step approach needed for scaling up of laboratory services aimed at assisting and directing policy makers and HIV/AIDS programme managers.

[152]Including notably, Stevens WS who headed up the programme, the author **Glencross DK**, as well as Sherman GG, NHLS CCMT laboratory coordinator Marshall T, assisted by Cassim N. From 2009 onwards, Scott LE and Carmona SG.

[153]Sherman GG, Stevens G, Stevens WS. Affordable diagnosis of human immunodeficiency virus infection in infants by p24 antigen detection. Pediatr Infect Dis J. 2004;23(2):173-6. https://doi.org/10.1097/01.inf.0000109332.83246.1a

[154]Sherman GG, Stevens WS, Stevens G, Galpin JS. Diagnosis of human immunodeficiency virus infection in perinatally exposed orphaned infants in a resource-poor setting. Pediatr Infect Dis J. 2000;19(10):1014-5.

[155] Sherman GG, Stevens G, Jones SA, Horsfield P, Stevens WS. Dried blood spots improve access to HIV diagnosis and care for infants in low-resource settings. J Acquir Immune Defic Syndr. 2005;38(5):615-7. https://doi.org/00126334-200504150-00016 [pii]

[156]Gilmour JW, Stevens WS, Gray C, de Souza M. Laboratory expansion to large-scale international HIV preventive vaccine trials. Curr Opin HIV AIDS 2007;2:201-6. https://doi.org/10.1097/COH.0b013e3280eec77a

[157]Patton JC, Akkers E, Coovadia AH, Meyers TM, Stevens WS, Sherman GG. Evaluation of dried whole blood spots obtained by heel or finger stick as an alternative to venous blood for diagnosis of human immunodeficiency virus type 1 infection in vertically exposed infants in the routine diagnostic laboratory. Clin Vaccine Immunol 2007;14:201-3. https://doi.org/10.1128/CVI.00223-06

[158]Stevens WS, Marshall TM. Challenges in implementing HIV load testing in South Africa. J Infect Dis 2010;201 Suppl 1:S78-84. https://doi.org/10.1086/650383

[159]NHLS Annual report, 2004/5. http://www.nhls.ac.za

Topics covered included the importance of background programmatic assessment together with the value of laboratory and clinic site-visits, scoping of test repertoires, budget development and provision for procurement of reagents and equipment, all underscoring the need for a thorough understanding of economies of scale and detailed test-unit costing. Further laboratory implementation planning needs were covered including assessment of laboratory infrastructure (existing versus expansion and renovation planning), the importance of laboratory workflow design, information technology setup and choice of laboratory information management systems to match the service needs of the laboratory in question. The importance of appropriate staff recruitment, according to the type of testing applied, and training assessment needs were emphasised. Final implementation stages were also discussed including aspects of equipment installation and calibration and external quality control scheme enrolment, as well as the value of instrument maintenance contracts and service level agreements.

9.3 METHODS FOR VALIDATION AND EVALUATION

In the absence of available appropriate statistical methods for the evaluation of CD4 technologies that accommodated CD4 counts from zero to greater than 1500 cells/µL, Scott and Glencross, with statistician Jackie Galpin, specifically developed a novel statistical method(27) for comparison of two or more CD4 methods simultaneously, entitled 'the percentage similarity model'[160]. The model introduced a methodology to accommodate the evaluation a wide range of CD4 counts that could be expected to be generated when analysing patient data, by effectively removing the mean cell count bias evident in the lower CD4 ranges when high CD4 counts were included in the evaluation (as opposed to the established and widely-used Bland Altman[161] method that enables excellent comparison of methodologies but is best applied for data in a narrow range). The method was also shown to be valuable for centers collating for external quality assessment programmes, enabling differences in results between several laboratories to be compared in the same analysis(21). The 'the percentage similarity model' has been extensively used and cited elsewhere for CD4 method comparison as well as for evaluation of new other non-CD4 laboratory methods.

Extensive description and details about how to go about evaluating different CD4 technologies and methods was later published by Stevens, Gelman, Glencross and others, in 2008(28). The paper offered a concise and detailed approach to evaluation of a new or existing CD4 method, including pointing out the importance of performing prospective testing on a relevant population group, how to select an appropriate comparative reference technology, how to calculate adequate sample size and the choice of

[160]This publication was based on a chapter of the work submitted by Lesley Scott for her PhD thesis entitled: 'HIV, AIDS and CD4: Africa's Problem'. Johannesburg: University of the Witwatersrand; 2008. **Supervisors: Glencross DK**, Coetzer T.

[161]Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 1986;1:307-10.

appropriate statistical methods to facilitate data analysis; also emphasised was the need for testing of both fresh patient specimens and stabilised blood controls or reference assessment material. Information about how to assess site readiness and the importance of on-going monitoring and surveillance was also included. A related publication by Scott et al[162] later revealed that the choice of statistical method, used to compare different CD4 methods, can potentially influence the decision about the practical choice of CD4 technology. This particular paper confirmed that the specific combination of statistical methods applied in our local CD4 evaluation studies, including both the percentage similarity model(27) as well as the Bland Altman analysis mentioned earlier, reiterated that the approach taken to evaluate PLG CD4 against other more conventional methods had been the optimal statistical approach to compare CD4 technologies.

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10. PRACTICAL APPLICATION: COORDINATED IMPLEMENTATION ACROSS A NATIONAL NETWORK

"*There is a crack, a crack in everything. That's how the light gets in.*" [163]

Problems faced by African health laboratories, largely those of limited resources and high burden of infectious disease, can potentially be solved with 'cooperation and networking amongst African CD4 laboratory professionals who take charge and deliver the continent from crisis management to sustained response'(21). Work presented(20,21,29-33) in the sections that follow reiterates the importance of such networking and cooperation - of all levels of technical and professional staff - as the foundation for programmatic self-determination that has ensured the success of, and underpinned, the national South African state CD4 service over the past 20 years.

The following milestone implementations further emphasise the importance of appropriate planning and coordinated use of appropriate standardised systems including instruments and methodologies, as well as developing and adopting standardised protocols and procedures that, together, will streamline and ensure efficient workflow of tests across an organisation.

10.1 SIMPLIFYING WORKFLOW: HOW SINGLE PLATFORM PLG TESTING STREAMLINES WORKFLOW PROCESSES AND ACCOMMODATES HIGH WORKLOADS ACROSS A NETWORK OF LABORATORIES

In 1999, Barnett et al[164], reiterated by others[165, 166], recommended that single platform testing was 'the way forward' to ensure better quality cell counts**.** There were two options available to count CD4 cells by single platform. The first was volumetric CD4 cell enumeration[167], typically requiring a dedicated instrument, such as the Ortho Cytoron™; this was a flow cytometer incorporating precision volumetric

[163]Leonard Cohen, lyrics from his song, 'Anthem', 1992.

[164]Barnett D, Granger V, Whitby L, Storie I, Reilly JT. Absolute CD4+ T-lymphocyte and CD34+ stem cell counts by single-platform flow cytometry: the way forward. Br J Haematol. 1999;106(4):1059-62.

[165]Brando B, Barnett D, Janossy G, Mandy F, Autran B, Rothe G, Scarpati B, D'Avanzo G, D'Hautcourt JL, Lenkei R and others. Cytofluorometric methods for assessing absolute numbers of cell subsets in blood. European Working Group on Clinical Cell Analysis. Cytometry 2000;42:327-46. https://doi.org/10.1002/1097-0320(20001215)42:6<327::aid-cyto1000>3.0.co;2-f

[166]Mandy FF, Nicholson JK, McDougal JS, on behalf of the U.S.CDC. Guidelines for performing single-platform absolute CD4+ T-cell determinations with CD45 gating for persons infected with human immunodeficiency virus. Centers for Disease Control and Prevention. MMWR Recomm Rep. 2003;52(RR-2):1-13. https://www.ncbi.nlm.nih.gov/pubmed/12583540

[167]Janossy G, Jani I, Gohde W. Affordable CD4(+) T-cell counts on 'single-platform' flow cytometers I. Primary CD4 gating. Br J Haematol. 2000;111(4):1198-208.

counting[168, 169] with direct immunophenotypic measurement of surface antigen CD45 to identify white blood cell sub-populations (as opposed to previous iterations of the volumetric counting methodology which relied on physical characteristics to identify cell populations and were used in the volumetric haematology analysers invented by Wallace Coulter[170]). A more accessible and practical option, considering the immediacy that was required to roll out services, was to use commercially-available cell enumeration microfluorospheres, commonly described as 'beads', that could be used on any make of flow cytometer. Commercially available 'beads' include FlowCount™, made by Beckman Coulter, or Trucount™ beads, manufactured by Becton Dickinson, for example.

In 2002 we first reported how PLG CD4 counting could be done by single platform testing, utilising either a volumetric cell counter or by adding beads to the samples in known concentration(12). A separate study(10) described employing single platform bead-based counting with other industry-standard innovations to manage increasingly higher workloads in the network. Specifically, we reported how on-board autobiosamplers could assist in the handling of considerably higher number of samples in consolidated batches of up to 96 tests; automated software-driven gating was also demonstrated to assist with workflow, especially when combined with single platform counting and simplified protocols like PLG. These industry standards, in combination with local innovation (i.e. single platform PLG CD4), allowed the NHLS to massively extend services across the South African national network(21); impact was highest in large regional laboratories that processed a sizeable number of samples every day. Implementation of single platform testing also assisted in streamlining processes by removing the logistical challenges of transferring samples between haematology and CD4 testing laboratories (that would have been necessary if the dual platform method was adopted and where samples would have had to be either split at receipt, or, after WCC enumeration, transferred to the sister flow cytometry laboratory). Training could also be streamlined using the simplified and standardised systems that included notably, the same instrumentation, reagents and procedures and protocols(21).

Following the successful newly-standardised installation of the first two automated flow cytometers, utilising newly written PLG CD4 protocols to report CD4 counts at the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) in 2002, the NHLS CD4 network (using identical technology as initially implemented at CMJAH) was gradually extended to include a further 25 CD4 laboratories during 2005(20). By April 2007, the network had been extended to 52 CD4 testing sites (21), with high volumes of tests accommodated across the programme in scaled-up services established at large regional

[168]Connelly MC, Knight M, Giorgi JV, Kagan J, Landay AL, Parker JW, Page E, Spino C, Wilkening C, Mercolino TJ. Standardization of absolute CD4+ lymphocyte counts across laboratories: an evaluation of the Ortho CytoronAbsolute flow cytometry system on normal donors. Cytometry 1995;22:200-10.

[169]Mercolino TJ, Connelly MC, Meyer EJ, Knight MD, Parker JW, Stelzer GT, DeChirico G. Immunologic differentiation of absolute lymphocyte count with an integrated flow cytometric system: a new concept for absolute T cell subset determinations. Cytometry 1995;22:48-59.

[170]Shapiro HM. Practical Flow Cytometry. New York: John Wiley and Sons (Wiley-Liss); 1995.

facilities. These high-volume centralised facilities on the network were able to perform up to 1500 samples per day and 15 000 samples per month. In 2012, further advancements in streamlined PLG CD4 workflow in very large CD4 testing facilities, offering 24-hour services, were reported. These centralised, single facility sites are able to receive referred samples from up to 100, or more, primary health care and/ or hospital-based clinics, set up with a capacity to process more than 25 000 CD4 samples per month (see Section 15 for details).

10.2 PRACTICAL IMPLEMENTATION OF NOVEL BEAD COUNT RATE (BCR) PLUS PLG ASSURES ACCURATE PLG CD4 CELL COUNTS

The introduction of single platform PLG testing across the NHLS did however pose an unexpected challenge as only semi-automated sample preparation systems were initially commercially available. Newly appointed and relatively inexperienced CD4 technical staff were entrusted with pipetting the cell enumeration beads to enable reporting of single platform PLG CD4 cell counts in all samples tested. Of concern, it had been reported by Bergeron et al[171] that pipetting errors would lead to incorrect CD4 cell counts, but these could only be quality controlled with duplicate or triplicate testing. A replicated testing approach would have, however, crippled our fledging service, effectively doubling or tripling reagent costs as well as technical effort depending on the number of additional confirmatory tests done. To solve this problem, instead, we applied the novel principle of 'bead count rate' (BCR)(31), relying on the stability of modern flow cytometer fluidics to reliably predict pipetting error in samples. The system was flexible in that it could be implemented across multiple machines in a single laboratory, as well as across multiple laboratories in the NHLS network(21), to detect bead pipetting error encountered during routine service delivery (but without adding any additional cost). Individual sample BCR data could then be transferred by instrument interface to the NHLS laboratory information management system which, by algorithm-driven instruction, would identify those samples with pipetting errors that needed re-preparation. Later, by 2016, BCR was also used to assure accurate volumetrically delivered, single platform cell counts on the newer fully automated, user-independent volumetric counter, the Aquios (details follow in point 10.5). Analysis of laboratory management system BCR data also enabled review of the number of repeated tests, useful to document reagent 'shrinkage' (wasted or lost reagents) and establish a true 'cost-of-sales' per test (actual costs of testing divided by the number of tests reported) (21). Observed unique patterns of consecutive BCR could also provide insights into specific machine error, for example, compressor failure or probe blockages that would lead to service-downtime. These patterns were additionally shown to be useful for proactive instrument monitoring as specific patterns could predict imminent flow cytometer component failure before the breakdown actually occurred (details

[171]Bergeron M, Lustyik G, Phaneuf S, Ding T, Nicholson JK, Janossy G, Shapiro H, Barnett D, Mandy F. Stability of currently used cytometers facilitates the identification of pipetting errors and their volumetric operation: "time" can tell all. Cytometry B Clin Cytom 2003;52:37-9. https://doi.org/10.1002/cyto.b.10014

follow in Section 11). BCR precision studies were also shown to be useful to assess training needs and identifying individual technologists whose pipetting skills needed honing(21).

10.3 STANDARDISED TESTING AND OPERATING PROCEDURES IMPROVES QUALITY OF REPORTING ACROSS A NETWORK OF LABORATORIES.

Systems developed(21,30,32,33) underlined the importance and the critical role of standard operating procedures (SOP) in the success of the new national CD4 service; diligent adherence to standardised testing is also emphasised as critical to safeguard the quality of reporting and enable efficient workflow and related processing of samples.

Standard operating procedures, aimed at all new CD4 laboratories taking on testing at the start of the HIV/AIDS CCMT programme, were first introduced into the NHLS in a handbook[172] written by Glencross and Lawrie. The universal use of a single test, across the organisation, was an important new milestone for the newly formed NHLS; PLG CD4 was one of the very first standardised tests to be implemented where the assay itself, the equipment and the same protocols, were used across the organisation to ensure harmonised and consistent quality of testing.

The approaches introduced, while also providing evidence of the successful coordinated transfer of PLG technology into all the laboratories across the South African network, were subsequently shared in a short educational film that was published in the Journal of Video Experiments(29). The 'virtual' video format applied[173], now considered common place in 2021, together with more detailed content in a published paper, was unique in the field in that it provided practical and visual demonstrations of sample preparation and how standardised operating procedures could be applied to ensure quality control in a routine diagnostic setting. The video also shared several specific systems employed to make workflow more efficient in a busy site. Modules specifically focused on PLG CD4 plus BCR, as well as other quality monitoring systems introduced into the South African NHLS CD4 network that enabled testing up to 1500 CD4 tests, or more, per day in a single flow cytometry laboratory. Standard operating procedures introduced included daily practice for (i) standardised instrument set-up, (ii) standardised sample preparation and testing panels (iii) details about application of PLG CD4 plus BCR for monitoring of individual (within) sample quality and (iv) the methodology to further assure accuracy of SP PLG CD4 counts by using a daily comparison of immunophenotypic (CD45-enabled) WCC to traditional WCC obtained on a different platform i.e. direct comparison to white blood cell differential derived counts obtained from a haematology analyser.

[172] **Glencross DK**, Lawrie D. The PLG CD4 Survival Kit: User Standardised Procedures Handbook (not for examination, not peer-reviewed), 2004. Available on request from Glencross DK, University of the Witwatersrand.

[173] As of 30 December 2019, this work (author reference #29, a video), has been viewed < than 10 000 times, with > than 1000 views during 2019 alone, providing evidence of continued relevance and impact in the field: https://doi.org/10.3791/2312/

10.4 ESTABLISHING THE NHLS NATIONAL PRIORITY PROGRAMME

The managed coordination of the South African PLG CD4 national implementation, became the foundation for, and the forerunner of, the NHLS's sixth service tier, the harmonising and overseeing sixth service level (described in more detail in Section 15). Around 2010, our Wits University group consolidated our collective efforts and approached the NHLS to form the National Priority Programme (NPP)[174]. Described in consecutive NHLS Annual reports[175] from 2008 onwards, the NPP has provided proactive CD4 service support since its inception, with ongoing development and improvements of standardised operating procedures and protocols (for instrument setup, sample preparation and analysis and workflow). This programme currently functions as the coordinating, harmonising, and overseeing body to supervise the implementation, ongoing operations, training and quality management for all NHLS HIV/AIDS and tuberculosis(TB) testing services across South Africa. Harmonised service delivery is continually reinforced with active training, offered both onsite in laboratories, as well as in workshops and participation in the NHLS CD4 external quality assessment scheme (for more details, see Section 14). Additional new programmes have also been recently included under the NPP umbrella, including the national implementation of automatic reflexed cryptococcal antigen screening for all patient samples with documented CD4 counts of less than 100 cells/µL (more details about this programme are outlined in Section 19).

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10.5 FULLY AUTOMATED 'WALK-AWAY' AQUIOS WITH PLG CD4 EXTENDS RELIABLE CD4 TESTING AND DECENTRALISES SERVICES TO DISTRICT LEVEL (REMOTE) AREAS.

During 2018, Beckman Coulter released their first fully automated, small-footprint flow cytometer, the Aquios™, which was purposefully designed as a 'walk-away', 'hands-free' CD4 testing system with on-board sample loading and automated data acquisition and analysis. The user-independent format presented was a significant development, especially important in a low technical skills context; a full repertoire of on-board quality monitoring and workload management functions (that would have usually been performed manually by trained and experienced laboratory technologists or scientists) was incorporated, providing for fully automated quality management for precision flow cytometry. Our group published two full evaluations of the Aquios incorporating the PLG CD4 system(34,35). Importantly, we reported clinically insignificant bias of cell count between the volumetric Aquios with PLG and SP PLG CD4 count performed on the larger bench top instruments (with partial automation of sample preparation and analysis). Ease of workflow and improved continuous service was a notable improvement introduced by the Aquios (offering an opportunity for laboratories to introduce 24-hour services utilising technicians with minimal flow cytometry training)(34).

In the South African programme, implementation of the Aquios has been especially advantageous in sites with limited technically skilled CD4 staff and lower CD4 workload. Placement has permitted decentralised routine CD4 services and enabled establishment in remote NHLS laboratories that had not previously undertaken CD4 testing(34), offering a cost- and labour-efficient solution despite small workloads of up to 122-150 samples per instrument, per day. Serial installation of several contiguously-placed Aquios instruments[176] has also been useful to accommodate extended services in busy sites that typically have utilised high-volume orientated instruments for testing, but alternatively enabling operation and authorisation of results[177] by clinical pathology technicians and technologists in a 24-hour service automated 'track' laboratory context.

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11. MONITORING AND EVALUATION - NOVEL INTERNAL QUALITY CONTROL

11.1 INTRODUCING NEW SYSTEMS FOR ENSURING INDIVIDUAL SAMPLE-BY-SAMPLE QUALITY CONTROL ACROSS A NETWORK OF CD4 LABORATORIES

Centralised (national) coordination of quality is vital to ensure that all laboratories, working together as a national service network, provide the same quality of testing in a standardised format, irrespective of where the test was carried out in the network. At the start of the CCMT programme, there were no available systems for coordinated and simultaneous monitoring of CD4 test quality across the CD4 network. There was, however, an embedded solution to coordinated quality assessment which lay in the single platform bead counting method already in use. The uniform suspension of fluorescent microbeads that were added in a fixed concentration to every sample, used to determine absolute counts on the flow cytometer, could provide a novel and standardised means to measure parameters that predicted the quality of testing (over and above the bead function to enumerate cells). This was possible because of the specific physical microbeads characteristics (like uniform forward and side scatter), as well as their specific consistent fluorescence, could be used to simultaneously monitor the operation of the flow cytometer itself, providing information about the instrument fluidics and optical alignment of the sample stream (29). The specific bead measurements could also be used to confirm that the stipulated standardised protocol setup had been correctly applied at the site. The ideas were investigated in a PhD project I supervised, undertaken by Denise Lawrie[178], that investigated documenting alternative and novel applications of fluorescent microbeads otherwise prescribed for the sole determination of absolute counts on a flow cytometer. Outcomes from Lawrie's PhD work confirmed that these 'counting' microbeads could additionally be reliably used to determine fluorescence sensitivity and volumetric operation of flow cytometers; the work also showed that the consistent bead measurements could be used to reliably monitor and evaluate sample-by-sample CD4 count quality across the network and was important to ensure individual sample quality control. Aspects of Lawrie's PhD study were subsequently published by Lawrie, Coetzee and Glencross(29), with a central message that continuous quality control (CQC), incorporating BCR, could be used to implement remote monitoring of both network or local sample-by-sample quality control. The work also introduced the idea of proactive (as opposed to reactive) instrument maintenance and function by highlighting that specifically-identifiable, collated, longitudinal BCR patterns, that could emerge during error and instrument failure encountered during routine service delivery, would be useful to predict instrument error and breakdown(29).

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[178]Lawrie D. Standardisation, Calibration and Development of Novel Flow Cytometry Techniques. PhD. Johannesburg: University of the Witwatersrand; 2009. **Supervisor: Glencross DK.** https://wiredspace.wits.ac.za/handle/10539/13858

12. MONITORING AND EVALUATION – ESTABLISHING LOCAL REFERENCE INTERVAL RANGES

CD4 diagnostic test outcomes can only be properly interpreted, and disease diagnosed, if a patient's CD4 count is reported with an appropriate reference interval (range); this provides necessary insight into expected values in a normal healthy population and allows for interpretation of individual patient laboratory results to discern disease. The practice is in line with Good Clinical Laboratory Practice and International Standards Organisation standards (ISO 15189:2003) and is applicable to all pathology tests.

International reference intervals have been mostly used in South Africa due to a paucity of published local laboratory reference interval information (extensive details about various reported international CD4 and related reference is contained in reference(35)). However, international reference intervals may not be appropriate for use outside of the context in which they were generated, prompting my interest in undertaking studies that established local population reference intervals for South Africans(36,37).

12.1 ADULT REFERENCE INTERVAL STUDIES

My first opportunity to investigate the reference intervals of CD4 counts of local healthy blood donors presented itself in 1992, organised by Professor Ruben Sher in collaboration with the Johannesburg-based blood transfusion service. This was an important first step locally to establish a regional reference range for white blood cell and lymphocyte counts as well as CD3, CD4 and CD8 subsets. The data was presented at the Federation of the South African Societies of Pathology[179] in the same year, with outcomes revealing that our locally derived reference interval data was not dissimilar to other internationally published ranges. Of note especially, there was no significant ethnic differences or CD4 epitope deficiencies reported. These values were subsequently introduced for CD4 reporting at the SAIMR.

Some years later, we had concerns that the 1992 findings may have not been accurate due to the DP technology and gating methodology used at the time of the first study that relied on total lymphocyte count. A second study by Lawrie, Glencross et al in 2009(36) was undertaken, after the formation of the NHLS, using single platform PLG CD4 technology. Despite the reservations about counts generated on the DP methodology, the 2009 study confirmed the accuracy of the 1992 reported outcomes for both CD4 count, and CD4% of lymphocytes reference interval ranges. Two other African studies described similar

[179] **Glencross DK**, Lawrie D, Loubser M, Mendelow BV. T cell subset analysis in healthy South Africans including an evaluation of CD4 epitope distribution. 32nd Annual Congress of the Federation of the South African Societies of Pathology, Durban, South Africa: FSASP; 1992. (p 104).

outcomes [180, 181]. In the same year as our 2009 study, a very large regional study, undertaken by the Clinical Laboratory Standards Institute, reported comparable CD4 reference range outcomes in Southern Africa[182]; this work additionally cited several reports of similar reference interval outcomes from other African-based reference interval studies as comparison. A later collaboration with Beckman Coulter reported the reference CD4 interval ranges in an international cohort of patients that were derived using PLG CD4 on the Aquios instrument; the outcomes confirmed and reiterated local reference intervals in use(35).

12.2 PAEDIATRIC REFERENCE INTERVAL STUDIES

Local paediatric full blood count and lymphocyte subset reference interval ranges were established during 2012 - 2014 in a cohort of clinically healthy South African children attending a well-baby clinic in an informal settlement in Cape Town(37). This study is the largest data set describing healthy children from an African environment; the work revealed that the reference intervals reported by our group for healthy children living in Cape Town, were similar to international paediatric reference ranges[183]. Later, together with our UK study collaborator, Dr Helen Payne, we undertook to compare this data to other available international paediatric data sets(38). Although absolute reference intervals were similar, the important message reported, across all age groups studied, was the dramatic decline in naïve to memory subset ratio, in both CD4 and CD8 T-cells, reaching a 1:1 ratio around the first decade of life in healthy children, much earlier than their first-world counterparts. This finding suggested that our local children are exposed much earlier in life to infectious disease than children in better resourced environments. Increased cellular activation and expanded natural killer cells were also noted amongst younger children in this cohort.

[180]Klose N, Coulibaly B, Tebit DM, Nauwelaers F, Spengler HP, Kynast-Wolf G, Kouyate B, Krausslich HG, Bohler T. Immunohematological reference values for healthy adults in Burkina Faso. Clin Vaccine Immunol 2007;14:782-4. https://doi.org/10.1128/CVI.00044-07

[181]Lugada ES, Mermin J, Kaharuza F, Ulvestad E, Were W, Langeland N, Asjo B, Malamba S, Downing R. Population-based hematologic and immunologic reference values for a healthy Ugandan population. Clin Diagn Lab Immunol 2004;11:29-34. https://doi.org/10.1128/cdli.11.1.29-34.2004

[182]Karita E, Ketter N, Price MA, Kayitenkore K, Kaleebu P, Nanvubya A, Anzala O, Jaoko W, Mutua G, Ruzagira E, Mulenga J, Sanders EJ, Mwangome M, Allen S, Bwanika A, Bahemuka U, Awuondo K, Omosa G, Farah B, Amornkul P, Birungi J, Yates S, Stoll-Johnson L, Gilmour J, Stevens G, Shutes E, Manigart O, Hughes P, Dally L, Scott J, Stevens W, Fast P, Kamali A. CLSI-derived hematology and biochemistry reference intervals for healthy adults in eastern and southern Africa. PLoS One 2009;4:e4401. https://doi.org/10.1371/journal.pone.0004401

[183]Shearer WT, Rosenblatt HM, Gelman RS, Oyomopito R, Plaeger S, Stiehm ER, Wara DW, Douglas SD, Luzuriaga K, McFarland EJ, Yogev R, Rathore MH, Levy W, Graham BL, Spector SA, Pediatric ACTG. Lymphocyte subsets in healthy children from birth through 18 years of age: the Pediatric AIDS Clinical Trials Group P1009 study. J Allergy Clin Immunol 2003;112:973-80. https://doi.org/10.1016/j.jaci.2003.07.003

12.3 RELATED REFERENCE INTERVAL STUDIES

Underlying iron and other nutritional deficiency outcomes were reported in a 2008 cohort of adult female health care workers in South Africa(39). Similar outcomes were also documented amongst 14% of paediatric cohort participants(40), confirmed elsewhere for Southern African patients[184]. Later, in line with these latter studies, another study reported by our group in 2018, revealed iron deficiency amongst South Africans who regularly donate blood, most notably amongst male donors who frequently donated blood at intervals of less than three months(41).

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13. MONITORING AND EVALUATION – INVESTIGATING PARAMETERS THAT LEAD TO VARIABILITY OF REPORTING

13.1 CONTROLLING LABORATORY VARIABLES TO IMPROVE PRECISION AND ACCURACY OF CD4+ T-CELL ENUMERATION ACROSS FLOW CYTOMETRY METHODS

All laboratory tests have a margin of error which is determined by the (within-laboratory) reproducibility of the specific test when performed in the hands of attending laboratory personnel. Many aspects can affect quality and can contribute to reporting variability; pre- and intra- analytical variables need to be identified and minimised in order to produce a reliable and reproducible test outcome. The measure of how these diverse variables affect a test outcome i.e., the test precision, is represented by a coefficient of variation (%CV). A laboratory with a lower within-laboratory precision %CV will, in turn, have a narrower margin of error.

CD4 testing is no different; several important improvements, to both pre- and intra-analytical components, expanded on below, ensured tighter precision of PLG CD4 counts (%CV) reported, both within- and between- state laboratories, and which considerably strengthened the South African CD4 programme overall.

13.1.1 SIMPLIFIED STANDARDISED PROTOCOLS AND OPERATING PROCEDURES FOR SAMPLE ANALYSIS

Standardised testing is key to reducing error and minimising interfering factors that can lead to variable clinical pathology reporting. The first and most important impact for the national CD4 programme, as detailed earlier, was that the PLG CD4 method used was the sole and standardised method across the organisation(21), with all sites applying identical standard operating procedures (SOP), daily instrument quality control (QC) and protocol setup. Use of the simplified PLG CD4 gating approach itself played an important role. The standardised and streamlined, two-step gating flow cytometry protocol, with a clearly defined and established primary total CD45+ (as total WCC or PanLeucogate), and immediate identification of CD4 positive lymphocytes in a second gate, was key to ensure improved within-method reproducibility. Additional standardised within-sample QC further assured accurate and precise bead pipetting by using specific continuous QC systems mentioned previously, including monitoring of BCR. Supplementary further monitoring of measurements including bead channel location and CD4 fluorescence mean intensity, as well as median channel intensity, were also recorded to document standardised protocol setup and ensure technical aspects like fluorescence colour compensation were correctly applied at all CD4 testing sites(29).

13.1.2 MULTIPLE LABORATORY VARIABLES TOGETHER CAN IMPACT TEST PRECISION

Whilst standardisation of testing during the intra-analytical phase is important, there are other pre-analytical variables that need to be identified and documented to limit the impact which led to disparity and differences of laboratory test reporting. Establishing sample integrity is one such example. Following a study where pre-analytical factors had had a profound negative impact on report outcomes(11), I subsequently supervised a master's project for student Wilja Mandy[185], where we interrogated the extent that pre- and intra- analytical variables can impact on, and cause, discrepant reporting. Mandy's work[185] specifically emphasised how certain logistical and methodological factors (for example, how blood samples were handled during transportation or after receipt into the laboratory) can influence precision and accuracy of enumeration of CD4+ lymphocytes. Pre-analytical factors such as motion or transit temperature during transportation, as well as within-laboratory variables such as operator pipetting and analysis skills, storage temperature, different protocols, different gating strategies - or the use of different flow cytometers and cell enumeration platforms - were interrogated in this student MSc project. The majority were found to negatively influence accurate and precise enumeration of CD4+ counts in a single laboratory pointing to a need for careful control over, and monitoring of, any variable that may impact CD4 reporting.

13.1.2 THE IMPORTANCE OF ADHERENCE TO, AND MONITORING OF, STANDARD OPERATING PROCEDURES

Despite procedures and processes in place to control pre- and intra-analytical variables across all laboratories providing CD4 testing across the NHLS, CD4 cell count variability of consecutive patient CD4 counts was documented in a local cohort of HIV+ ART naïve patients(42) (resulting in different clinical classification at primary health care supporting sites versus follow-up treatment classification at the tertiary (metropolitan) centre). This discrepancy of classification meant that, whereas at primary health care level there was an indication to, for example, screen for cryptococcal disease using an absolute CD4 cutoff of 200 cells/µL, at the tertiary centre, the CD4 count, when retested, suggested the opposite (i.e., CD4 count exceeded 200 cells/µL). This finding was especially important to document as discrepancy of CD4 counts, certainly those less than 100 cells/µL in patient's CD4 counts has been documented to impact care[186].

Our paper(42) highlighted two important aspects about discrepancies in CD4 count reporting. Firstly, discrepancies, if assumed correct, might either be disease related or biological, or technical in origin

[185]Mandy WM. Thesis: Controlling laboratory variables to improve precision and accuracy of CD4+ T-cell enumeration across flow cytometry methods. MSc(Med). Johannesburg: University of the Witwatersrand, 2009. **Supervisor: Glencross DK.** https://wiredspace.wits.ac.za/handle/10539/7975

[186]Daneau G, Buyze J, Wade D, Diaw PA, Dieye TN, Sopheak T, Florence E, Lynen L, Kestens L. CD4 results with a bias larger than hundred cells per microliter can have a significant impact on the clinical decision during treatment initiation of HIV patients. Cytometry B Clin Cytom 2017;92:476-484. https://doi.org/10.1002/cyto.b.21366

(tests were however undertaken in a short timeframe where substantial clinical change is not expected). Secondly, if assumed due to technical error, the work again highlighted a need for tighter control in making sure that standardised testing processes in the laboratory were *actually* applied; application is first and foremost. Good laboratory practice dictates that all laboratories should ensure that their within-laboratory precision is within expected ISO 15985 or national accreditation-body standards. The reported outcomes of this work thus emphasised the importance of consistent and reviewed training and competency assessment of technical staff to ensure that standard procedures were diligently and consistently applied during testing. Also reiterated was the importance of ongoing monitoring and evaluation of within-laboratory precision, necessary to ensure that all CD4 sites, across the national network, report results that are within the parent organisation's stipulated reproducibility and within-laboratory precision (%CV).

Despite the lessor role of CD4 count for determining eligibility anti-retroviral treatment(ART) in the context of universal test and treat approaches, tight CD4 threshold cut-offs are still used to determine eligibility for fast-tracking into care, screening for various opportunistic disease or monitoring of immunological outcomes. The Venter et al outcomes(42) stressed the need for awareness and understanding amongst clinicians that all laboratory test results will always have a margin of error (and that no laboratory result is absolute). Armed with this understanding, a more flexible approach to clinical decision making was proposed when a faced with making a clinical decision around a rigid threshold that would otherwise strictly dictate the patient's treatment (for example, being flexible when interpreting a CD4 result to decide whether a patient should be fast tracked into care, for example). The work also introduced a controversial idea that laboratories could perhaps report a 'plausible range' or confidence interval (to indicate the laboratory precision of the reported test, also effectively forcing a laboratory to publish their precision of testing with a reported laboratory test).

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14. MONITORING AND EVALUATION – INDEPENDENT EXTERNAL QUALITY ASSESSMENT

14.1 INTRODUCING 'AFREQAS': THE REGIONAL EXTERNAL QUALITY ASSESSMENT SCHEME TO ENABLE ACCESSIBLE AND AFFORDABLE EXTERNAL QUALITY ASSESSMENT FOR THE AFRICAN REGION

The cost of participation in an international external quality assessment scheme (EQAS) can limit access to EQAS for laboratories in less resourced regions. This was no different for South Africa at the start of the South African national testing programme. The high cost of participation by 52 individual NHLS CD4 laboratories in an established international scheme, such as the United Kingdom(UK) National External Quality Assessment Scheme(NEQAS)[187, 188], was in reality unaffordable for our fledging laboratory service.

As we had already learned from our experience in facing similar issues, the costly exercise of international participation was not so much of a problem but an opportunity to take matters into our own hands and consider starting a local scheme. The power of any external quality assessment scheme (EQAS) lies in the number of participants who register and partake in any given trial, and who simultaneously test the same sample and provide the outcomes to the organisers of the trial. By 2005, there were 52 NHLS laboratories, and counting, that could be enrolled to establish a new and self-governed local scheme. Improved statistical power (and higher enrolment numbers) could be achieved by encouraging other local laboratories to participate in our South African scheme. A CD4 external quality assessment programme was started from my unit in 2005(22), in collaboration with Beckman Coulter South Africa, who initially subsidised the cost to provide stabilised blood materials. Most NHLS CD4 laboratories within Gauteng enrolled, as did many private pathology CD4 service providers in the region. The programme was dramatically expanded after an agreement was secured with the World Health Organisation[189] to start a regional scheme in 2006, with further assistance given by Quality Assessment Scheme International[190] (a Health Canada initiative) who provided the necessary stabilised blood material for trial shipments. Named the CD4 African Regional External Quality Assessment Scheme (AFREQAS)(22) , participation was offered free to all African laboratories to ensure that any CD4 laboratory offering CD4 services on the continent had the opportunity to enroll and have access to CD4 EQAS. A review of the AFREQAS programme was published in 2008(22); the publication included details about participation and the

[187]Whitby L, Granger V, Storie I, Goodfellow K, Sawle A, Reilly JT, Barnett D. Quality control of CD4+ T-lymphocyte enumeration: results from the last 9 years of the United Kingdom National External Quality Assessment Scheme for Immune Monitoring (1993-2001). Cytometry 2002;50:102-10. https://doi.org/10.1002/cyto.10094

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precision and accuracy of specific CD4 user-groups. Outcomes highlighted specific skills deficiencies in certain CD4 user-groups, as well as individual laboratories, and drew attention to areas where training needs could be focused. Participation in the AFREQAS scheme further harnessed South African expertise for participant feedback; remotely driven remedial action and technical training was offered from South Africa to individual participating laboratories that needed input and assistance. The programme had another distinct advantage in that anonymised participant data analysis enabled post-marketing surveillance of CD4 methodologies(22); in other words, analysis of user data provided information about how various CD4 systems and platforms were used in the hands of African users in the field so that a more objective and practical approach could be adopted to facilitate improvement of services in the region. The extent of instrument manufacturer-support in the field could similarly also be established(22).

By 2010, the CD4 AFREQAS had grown substantially, reaching 550 registered sites across 20 African countries[191]; by this time, the AFREQAS programme was also supporting several country-based schemes. In the same year, the CD4 AFREQAS was formally taken over by the NHLS and was renamed the NHLS CD4 PT scheme; the initiative currently runs as an independent service within the division of the Quality Assurance Division of the NHLS.

Through evaluation of sites and methodologies, a novel method of presenting the longitudinal EQAS performance of users was additionally introduced through this work. Participant or user group performance was presented in a concentric radial plot format permitting a visual, at-a-glance 'snapshot' of longitudinal performance. This novel approach of presenting EQAS outcomes was subsequently used to present data in many other published works by Glencross et al(34,43-45) as well as in NHLS Health Technology Assessment reports (not presented here) to describe or predict laboratory EQAS proficiency outcomes.

14.2 Archived External Quality Assessment Scheme Sample Panels Enables Accelerated Virtual Peer Laboratory Review: Categorising and Establishing CD4 Service Equivalency.

Additional innovations were introduced through AFREQAS using archival EQAS materials for 'virtual longitudinal' site assessment (based on initial concepts published through UK NEQAS[192]). The idea employed, specifically, the power of the individual trial consensus outcome (in other words, the power of statistical analysis of a large pool of users, who had previously participated in a specific EQAS trial, was harnessed to establish a 'true consensus value' for the material tested). Samples left over from the

[191]National Health Laboratory Service. Annual Report: Research and Development: Progress of the African Regional External Quality Assessment Scheme (AFREQAS). Sandringham, Johannesburg; 2009 - 2010. p238.

[192]Barnett D, Whitby L, Wong J, Louzao R, Reilly JT, Denny TN. VERITAS?: A time for VERIQAS and a new approach to training, education, and the quality assessment of CD4+ T lymphocyte counting (I). Cytometry B Clin Cytom 2012;82:93-100. https://doi.org/10.1002/cyto.b.20624

historical trials could then be used to 'test' new sites on their ability to produce similar results close to the 'true consensus value'. Thus, unused trial EQAS material, where 'known' values had been already established by a pool of users, could be assembled in up to 25-sample panels (potentially comprising samples gathered from up to 25 historical trials) to enable a real time 'retrospective' review of the new site's performance, including evaluation of accuracy and precision. The real innovation of the retrospective panel testing however is its immediacy, offering a virtual glimpse about how a new testing laboratory would likely have performed, but without the site having participated in any previous trials of the scheme. The approach was found to be especially useful for assigning competency of newly implemented CD4 sites prior to going live in our national programme; it has also been helpful for site-specific trouble shooting in existing services to ascertain where corrective action was needed to align quality of reporting across the CD4 laboratory network(45). Panels could also be used to highlight skills deficiencies and be used for teaching and training purposes(45).

14.3 CONTINUOUS QUALITY ASSESSMENT AND A SHIFT AWAY FROM TRADITIONAL EQAS

In the discussion section of this above mentioned paper(45), an important controversial paradigm shift about the need for conventional EQAS was introduced. Historically, conventional EQAS evaluation emerged in a time where the distribution of identical testable material, to many participating sites, was the only way to simultaneously evaluate the performance of many laboratories. It was also the time where the only form of communication was by post or telephone. The advent of the internet, big data analysis and machine learning-driven artificial intelligence (AI) have changed this playing field. Typically, the materials used in any EQAS programme are stabilised whole blood products issued by manufacturers in production batches. Not widely known, these materials are not uncommonly, the identical materials used for daily internal process quality control, the results of which are monitored and logged daily on both the flow cytometers themselves as well as being downloaded into the laboratory's information management system (LIMS). Theoretically therefore, as the EQAS materials and internal process quality control materials are to all intents and purpose identical, LIMS-extracted quality data could be continuously tracked through the linking of identical material batch numbers - across all users who are testing the material, irrespective of geographic location of the user. This approach could effectively eliminate the need for external quality assessment altogether and replace and update conventional EQAS monitoring with real-time organisational- or manufacture centrally-collated continuous quality control. No extra effort or cost is incurred, as scheme participation costs are effectively eliminated too, especially relevant in a resource limited setting. Response time for corrective action, if needed, can be immediate (as opposed to the conventional snapshot approach with intermittent, two- to four-monthly EQAS assessment that may lead to delays in corrective action if a site's performance is outside of consensus).

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15. ENSURING EQUITABLE ACCESS TO A CD4 COUNT AND WIDESCALE UNIVERSAL FULL-SCALE COVERAGE: OPTIMISING LABORATORY SERVICES TO MATCH LOCAL SERVICE NEEDS USING A TIERED LABORATORY SYSTEM

15.1 DESCRIBING AN INTEGRATED TIERED SERVICE DELIVERY MODEL TO EXTEND LABORATORY SERVICES AND INTEGRATE POINT OF CARE TECHNOLOGIES IN HARD-TO-REACH AREAS

South Africa is a large country, with its people, and hence support services, widely dispersed; a 'one size fits all' approach to manage the growing HIV/ AIDS laboratory service load, with widely varying local service demands, was therefore unlikely to provide a comprehensive solution. Disparate capacity and/technical skills requirements at different levels of service were considered to facilitate meaningful and immediate clinical intervention and improve access, irrespective of the area where the patients presented. In 2014, our group described several inconsistencies of CD4 service that existed across the country(46): up to 14 of 52 districts in South Africa were revealed to have limited access to, or lacked, a local district laboratory CD4 service. In order to attain full coverage of laboratory services, the footprint of existing laboratory amenities needed to be extended away from the existing focus of mainly centralised high-volume testing sites in use at that time. This entailed decentralising HIV services closer to where patients presented for care, irrespective of location (in other words, CD4 and other HIV pathology-related services could be broadened to include such HIV linked services in the repertoire of tests offered by existing smaller community laboratories). Alternatively, tailor-made testing, using either hand-held equipment or alternatively small operator-independent technologies, could be directly implemented at the clinical interface/point of care (POC) or used as multiple POC technologies in especially created laboratory clinical pathology 'hubs'(46).

An integrated tiered service delivery model (ITSDM)(46) was described that addressed the above challenges whilst fulfilling service and related quality requirements to enable widescale, full-service coverage across South Africa. In essence, the model provided the framework for extending the footprint of nationwide CD4 services to include laboratory services in outlying areas and hard-to-reach sites; this ensured more accessible and scalable CD4 services across South Africa, whilst maintaining best practice and cost effectiveness. The overall aim of the ITSDM was to ensure optimised and efficient CD4 service delivery matched with meaningful clinical turnaround time (TAT) across all levels of clinical service; it was described as a comprehensive, hierarchical but local demand-driven model (as opposed to a fully centralised laboratory testing with optimised logistics or on the opposite spectrum, widespread, universally implemented POC testing at each and every health care facility[193]). Importantly, the proposals outlined were in line with local Department of Health primary health care drives and with earlier World

[193]Médecins Sans Frontières (MSF) South Arica. New Roots for South Africa: Challenging Unequal Access in South Africa. The Musina Model of Care. 2013. Date accessed, November 23, 2021. Available from: https://www.msf.org/sites/msf.org/files/musina_new-roots-for-rural-health_south-africa.pdf

Health Organisation calls for 'the development of context-specific service delivery models' [194, 195]. The model further considered the NHLS Act of 2000 and that related government procurement policies require specific regulated national procurement (tenders) and optimal pricing(46). Implementation also ensured the benefits of economy of scale (including equipment, reagents, operating-protocols and related quality control procedures) and thus, a cost-effective national service(46).

Six ITSDM tiers of CD4 service(46) were described that could be immediately responsive to service needs and quality needs across an organisation, including POC testing sites as the most decentralised extension of laboratory-based service. The model thus provided for an integrated network, accommodating both centralised high-volume testing demands whilst also making provision for decentralised service delivery, with flexible systems to reach under-served and difficult-to-reach remote areas through deployment of small, operator-independent laboratory equipment or point of care technologies. A hierarchical custodian supportive framework(46) was additionally included (as 'parental' support so to speak) aimed at supporting each increasingly de-centralised service tier, envisaged to be managed and directed by a team of pathologists, scientists and various levels of business manager who would additionally provide a broad base of support to lower decentralised tiers of service from their positions in higher service tiers.

Briefly, the tiered model prescribes (1), academic support for training initiatives that would be organised through tertiary and quaternary institutions to take on the role of coordinated teaching and technical training, (2), coordination of quality through ongoing monitoring and evaluation of service delivery facilitated through flow of network (LIMS) information about sample quality through centrally coordinated monitoring systems (that provide for real-time monitoring and therefore, proactive (as opposed to reactive) corrective action interventions), and (3), provision of a 'support-services' infrastructure, including transport logistics and provision for appropriate information technology (IT) systems to ensure an adequate and maintained patient demographic and reports data-base, as well as provide a mechanism for timely and secure delivery of reports.

Estimated implementation and operational costs across all tiers of ITSDM CD4 services, including both laboratory-based and provision of point of care service tiers in remote areas, were published in an accompanying paper at the same time(47). This supplementary work showed that implementation of the ITSDM would save money for South Africa if consolidated service delivery needs dictated implementation and if the level of technology used was appropriate for the specific level of service. Further work(48) showed that costs could also be saved if clinicians played their role in ensuring that the tests ordered for patients were compliant with, and ordered according to, the clinical practice recommended in local

[194]World Health Organisation(WHO). World Health Organization Emergency scale-up of antiretroviral therapy in resource-poor settings: technical and operational recommendations to achieve 3 by 5. 2002. Accessed: November 23, 2021. Available from http://www.who.int/3by5/publications/documents/zambia/en/index.html

[195]U.S. Centers for Disease Control(CDC). Consultation on Technical and Operational Recommendations for Clinical Laboratory Testing Harmonization and Standardization. Maputo, Mozambique; 2008. Accessed: November 23, 2021. Available from http://www.who.int/diagnostics_laboratory/3by5/Maputo_Meeting_Report_7_7_08.pdf

treatment guidelines(48); both service efficiency could be improved, and health costs reduced, by clinician's adhering to relevant guidelines and policy(48).

15.2 THE ITSDM ADDRESSES SERVICE GAPS IN HIV/AIDS LABORATORY SERVICES

The ITSDM approach of 'service precinct' identification was used to assess, and address, HIV/AIDS treatment (ART)-related diagnostic service coverage gaps in a manner that balances use of existing services whilst also taking into account costs and equal patient access to care(49). This analysis showed how the ITSDM could be applied to pinpoint areas with deficient laboratory services (in this instance, HIV viral load testing) and identify new candidate sites to supplement, and bolster, existing services. Whilst the ITSDM was described around CD4 service delivery, this latter study(49) provided the evidence that the ITSDM model was universally applicable to a broader base across all pathology testing; in other words, it was also applicable to other ART-related tests or non-communicable diagnostic tests in the NHLS test repertoire basket. An ITSDM approach, also based on extraction of laboratory-based data, has been subsequently described elsewhere in Southern Africa to consolidate and improve access to diagnosis and treatment of Tuberculosis in Lesotho[196].

15.3 DECENTRALISING CD4 SERVICES USING THE ITSDM EXTENDS LABORATORY SERVICES AND SUBSTANTIALLY IMPROVES ACCESS WITH REDUCED TURNAROUND TIME

A proof of concept study(50) revealed how de-centralising services, provided for in the ITSDM, brings services closer to patient care and dramatically improves efficiency, without the need for widespread implementation of costly POC services. This 2012 paper(50) confirmed that the implementation of a local CD4 service into a small community laboratory, in this instance, in Pixley ka Sema of the Northern Cape area, led to a marked decrease in turnaround time of CD4 reporting. Turnaround time was markedly reduced, from 20.5 hours to 8.2 hours for patients who needed a CD4 count, largely due to substantive reduction in the pre-analytical time component (sample logistics were improved as samples had been previously referred to the Kimberly CD4 laboratory for processing and reporting). Encouragingly, albeit it anecdotal, positive feedback and support was received from district client (Department of Health) liaison officers. The implementation of this decentralised CD4 laboratory site also meant that extensive, and very expensive, POC CD4 testing was no longer needed in this region.

[196]Albert H, Purcell R, Wang YY, Kao K, Mareka M, Katz Z, Maama BL, Mots'oane T. Designing an optimised diagnostic network to improve access to TB diagnosis and treatment in Lesotho. PLoS One 2020;15:e0233620. https://doi.org/10.1371/journal.pone.0233620

The value of the contribution of the ITSDM, together with the positive impact of decentralised services noted in Pixley ka Sema, was recognised amongst the top innovative local HIV/AIDS supporting programmes that would enable South Africa to meet its HIV/AIDS 90-90-90 targets. Our group was invited[197] to the National Department of Health[198] and US and Regional African Centers for Disease Control (CDC) 90-90-90 Forum[199]. The data that was presented emphasised how different service modules(tests) could be implemented into existing community laboratories that already offered a basic repertoire of tests, efficiently making use of existing laboratory infrastructure and skilled technical staff already employed; minimal costs are therefore incurred, with maximum benefit and value for money. It was also highlighted how decentralised services could also take advantage of the same (fixed) national tender-secured price-per-test, that included placement of equipment and instrument maintenance, thus avoiding the higher costs associated with decentralising services including extending POC services(47).

Recent work(51) has again confirmed the importance of a decentralised approach to ensure timely provision of pathology reports in another site, Tshwaragano in the Northern Cape. This work also emphasises that success of decentralised testing is dependent on installation of appropriate instrumentation that allows for fully automated, walk-away, and user-independent CD4 systems; reliable hierarchical academic and technical support provided through the ITSDM, specifically the harmonizing 6th service tier described above, is again reiterated as key to long-term successful implementation.

15.4 INVESTIGATING 'IDEAL' GEOGRAPHICAL PLACEMENT OF LABORATORIES

In collaboration with Smith et al. from the department of Mathematical Sciences at the University of Southampton, we first considered the role of precise geographical location of laboratories in the optimisation of service delivery[200]. Later our group described this approach in a paper that interrogated a theoretical spatial laboratory site planning algorithm(52) to determine the ideal placement/ location for HIV/AIDS laboratory services across South Africa; ideal placement was based on volumes of tests

[197]**Glencross DK**, Coetzee LM, Cassim N. Strategies for 'Full Coverage' of CD4 Services in South Africa: An Integrated Tiered Service Delivery Model (ITSDM). 2015; National Department of Health and U.S. CDC 90-90-90 Highlights Forum. South African AIDS Meeting, Durban, South Africa.

[198]Malaza B, Smith J, Mdaka N, Haynes R, Shezi S. The 90-90-90 COMPENDIUM Volume 1: An Introduction to 90-90-90 in South Africa. Westville, South Africa. 2016. Date accessed: 12 December 2021. Available from : https://www.hst.org.za/publications/HST%20Publications/90-90-90%20Vol%201(web)%202019.pdf.

[199]Joint United Nations Programme on HIV/AIDS (UNAIDS). 90-90-90. An ambitious treatment target to help end the AIDS epidemic. Date accessed: 12 December 2021 Available from: http://www.unaids.org/sites/default/files/media_asset/90-90-90_en_0.pdf
The 90-90-90 initiative is driven by UAIDS and refers to HIV Treatment targets: where 90% of people living with HIV know their status, 90% of people who know their status are on antiretroviral treatment(ART) and 90% of people on ART are virally suppressed.

[200]Smith H, Smith J, Coetzee LM, Cassim N, Carmona S, Stevens WS and **Glencross DK**. Geographic location and capacity optimisation modelling to plan effective and efficient diagnostics service placement. 1st meeting of the African Society of Laboratory Medicine (ALSM) Cape Town, South Africa. 2012.

emanating from a particular site studied and the exact geographical location of the demand for services. The modelling enabled predicting ideal siting for new laboratories or placement of CD4 diagnostic equipment in existing (non-CD4) laboratories across South Africa, based on the premise that services should be placed as close as possible to centres of need (i.e., close to where clinical services were offered). Additional analyses performed offered insights about the appropriateness of current laboratories' sample transportation referral routes or the appropriateness of the existing location of established laboratories. The modeling also enabled extracting information about where potential new laboratories should ideally be located if these were to be built. Several scenarios were applied based on the length of travel time from facilities to the closest laboratories. This in turn determined how the algorithm resolved the ideal number of laboratories to provide 'full coverage'.

A second paper(53) looked at the programmatic implications of applying our published algorithm(52) to the existing south African CD4 network. The idea behind the study(53) was to allocate laboratories, and, in particular, point of care sites in hard-to-reach areas using a set coverage precinct model with defined travel times. Three relevant scenarios were investigated: the first looked specifically at the impact of a full centralised service with just 15 laboratories placed in geographical locations determined by the algorithm and a minimum four-hour pre-analytical sample travelling time (but with substantial negative impact on rural service delivery). The second model reduced the maximum travelling time to three hours, incorporating utilising existing laboratories, but additionally added implementation for additional POC laboratory hubs that would utilise multiple POC instruments to provide a clinical pathology service. Reducing the maximum travelling time to two hours produced better CD4 test turnaround time, more in line with local HIV treatment standards of care whilst making use best use of operational laboratories in the existing network referral system. The 2-hour travelling 'precincts' approach further revealed that just twenty additional supplementary POC testing sites were needed to completely ensure widescale service coverage and confirmed the recommended placement of additional sites outcomes reported in the more practically driven ITSDM(46) outlined above.

Work based on similar geospatial tiered pathology service modelling used in our publications(52,53) has been undertaken elsewhere in Southern Africa; Girdwood et al[201] developed a costing model to improve access to HIV viral load (VL) testing through the optimised placement of HIV VL point of care machines in Zambia. Details about diagnostic network planning that enhance and improve diagnostic laboratory networks in low- and middle-income countries has been reviewed elsewhere[202], including the collection,

[201]Girdwood SJ, Nichols BE, Moyo C, Crompton T, Chimhamhiwa D, Rosen S. Optimizing viral load testing access for the last mile: Geospatial cost model for point of care instrument placement. PLoS One 2019;14:e0221586. https://doi.org/10.1371/journal.pone.0221586

[202] Nichols K, Girdwood SJ, Inglis A, Ondoa P, Sy KTL, Benade M, Tusiime AB, Kao K, Carmona S, Albert H, Nichols BE. Bringing Data Analytics to the Design of Optimised Diagnostic Networks in Low- and Middle-Income Countries: Process, Terms and Definitions. Diagnostics (Basel) 2020;11. https://doi.org/10.3390/diagnostics11010022

mapping and spatial analysis of baseline data, the selection and development of scenarios to plan for, and improve, services, as well as measure impact of implementation.

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47. Cassim N, Coetzee LM, Schnippel K, **Glencross DK**. Estimating implementation and operational costs of an integrated tiered CD4 service including laboratory and point of care testing in a remote health district in South Africa. PLoS One 2014;9:e115420. https://doi.org/10.1371/journal.pone.0115420 https://ajlmonline.org/index.php/ajlm/article/view/545/738
48. Cassim N, Coetzee LM, Schnippel K, **Glencross DK**. Compliance to HIV treatment monitoring guidelines can reduce laboratory costs. South Afr J HIV Med 2016;17:449. https://doi.org/10.4102/sajhivmed.v17i1.449 https://sajhivmed.org.za/index.php/hivmed/article/view/449/824
49. Cassim N, Coetzee LM, Stevens WS, **Glencross DK**. Addressing antiretroviral therapy-related diagnostic coverage gaps across South Africa using a programmatic approach. Afr J Lab Med 2018;7:681. https://doi.org/10.4102/ajlm.v7i1.681 https://ajlmonline.org/index.php/ajlm/article/view/681/1084
50. Coetzee LM, Cassim N, **Glencross DK**. Implementation of a new 'community' laboratory CD4 service in a rural health district in South Africa extends laboratory services and substantially improves local reporting turnaround time. S Afr Med J 2015;106:82-7. https://doi.org/10.7196/SAMJ.2016.v106i1.10081 http://www.scielo.org.za/pdf/samj/v106n1/27.pdf
51. Coetzee LM, Cassim N, **Glencross DK**. The impact of implementing a new community CD4 service in Tshwaragano in the Northern Cape Province on result turn-around-time. AJLM - in press:Available on request.
52. Cassim N, Coetzee LM, **Glencross DK**. Programmatic implications of implementing the relational algebraic capacitated location (RACL) algorithm outcomes on the allocation of laboratory sites, test volumes, platform distribution and space requirements. African Journal of Laboratory Medicine 2017;6(1):a545. https://doi.org/10.4102/ajlm.v6i1.545 http://www.scielo.org.za/pdf/ajlm/v6n1/02.pdf
53. Smith H, Smith J, **Glencross DK**, Cassim N, Coetzee LM, Carmona S, Stevens WS. Siting of HIV/AIDS diagnostic equipment in South Africa: a case study in locational analysis. International Transactions in Operational Research (ITOR) 2016;00:1-18 https://doi.org/10.1111/itor.12366

16. ENSURING EQUITABLE ACCESS TO CD4 SERVICES AND WIDESCALE UNIVERSAL FULL-SCALE COVERAGE: EXTENDING LABORATORY SERVICES TO THE POINT OF CARE

16.1 WHY PROVIDE CD4 COUNTS AT THE POINT OF CARE?

The point of 'point of care' CD4 testing was to provide a patient's immediate access to a CD4 result and therefore, to improve chances of a patient's enrolment into care if their CD4 count determined that they were eligible for treatment. Access to state-of-the-art flow cytometers, needed for delivery of services, is not commonplace, certainly in Africa. Skills may also be lacking to take on flow cytometry-based services. The cost of conventional CD4 reagents may also be considered too high for some low-income countries. These factors, together with treatment initiatives aimed at recruiting higher numbers of patients into care, were likely the main drivers that lead to the development of many POC CD4 tests and dedicated POC CD4 equipment. As described earlier in section 5.3, our AFFORD-CD4 group were the first to make the call to industry to develop low-end, affordable, small flow cytometers that were envisaged to be used closer to the point of care. By 2009, many small commercial CD4 instruments were being released in line with the concepts originally put forward by the AFFORD-CD4 group around 2001.

From 2010 onwards, there was increasing international pressure from groups including the US CDC, MSF and the U.S. President's Emergency Plan for AIDS Relief (PEPFAR), amongst others, as well as from the manufacturers and distributors of hand-held or small CD4 counting equipment, to implement POC CD4 testing widely. The groups strongly encouraged HIV/AIDS treatment programme coordinators to provide CD4 testing where patients were first encountered in the health care system, at the point of care. This did change however, with newer treatment guidelines published that provided for universal test and treat initiatives for HIV+ patients (described in more detail below).

16.2 CD4 COUNTS AT THE POINT OF CARE (POC) ENABLE FASTER ENROLMENT ONTO ART BUT DO NOT ADDRESS PATIENT LOSS TO FOLLOW-UP

Early data on the outcomes of providing CD4 counts at the POC (and assessing the impact on patient enrolment onto ART), was first published by Faal, Glencross et al(23). This study provided evidence that the provision of CD4 counts at the point of HIV testing improved early ART initiation, but unfortunately did not assist in retaining patients in care. This study preceded commercial release of POC CD4 instrumentation, (such as those examples that are described later in this section), and on-site testing was provided in a mini-laboratory hub set up within the primary health care clinic itself. Later, this same clinical site was used to undertake Pima™ POC CD4 testing (details below(44)). After 2014, several

additional studies[203, 204, 205, 206, 207] confirmed our findings(23), revealing that, although the provision of CD4 testing facilities at the POC may have facilitated earlier enrolment of patients onto ART, in the longer term, providing immediate access to CD4 testing did not improve overall patient retention in care. Specifically, Stevens et al[205] reported that even a multidisciplinary approach, providing CD4 and other tests needed for following up HIV+ patients on treatment at the POC, did not reduce patient loss to follow-up and or longer-term retention in care.

16.3 UNIVERSAL TEST AND TREAT

By 2016, opinions were changing; it was increasingly becoming apparent that patients would benefit from receiving immediate ART as soon as possible after diagnosis[208] and that immunity in HIV+ patients was more likely to recover to pre-infectious levels (i.e., immune reconstitution) if treatment were given as soon as possible after HIV seroconversion. Rosen and Fox[207], in their systematic review, had also reported that there was poor longer-term retention in care, with up to 41% of patients lost to follow-up in South Africa, even if CD4 counts were provided at the point of care. Awareness of the importance of acting immediately and treating all newly diagnosed and existing diagnosed HIV+ patients, irrespective of their CD4 count grew, so that by 2017, there was a significant paradigm shift away from the use of a specifically determined CD4 threshold to define a patient's eligibility for entry to care and provision of ART[209]. This long-needed change of outlook, adopting a 'Treat All'[210] strategy was included in several guidelines issued

[203]Larson BA, Schnippel K, Brennan A, Long L, Xulu T, Maotoe T, Rosen S, Sanne I, Fox MP. Same-Day CD4 Testing to Improve Uptake of HIV Care and Treatment in South Africa: Point-of-Care Is Not Enough. AIDS Res Treat 2013;2013:941493. https://doi.org/10.1155/2013/941493

[204]Kiwanuka J, Mukulu Waila J, Muhindo Kahungu M, Kitonsa J, Kiwanuka N. Determinants of loss to follow-up among HIV positive patients receiving antiretroviral therapy in a test and treat setting: A retrospective cohort study in Masaka, Uganda. PLoS One 2020;15:e0217606. https://doi.org/10.1371/journal.pone.0217606

[205]Stevens WS, Gous NM, MacLeod WB, Long LC, Variava E, Martinson NA, Sanne I, Osih R, Scott LE. Multidisciplinary Point-of-Care Testing in South African Primary Health Care Clinics Accelerates HIV ART Initiation but Does Not Alter Retention in Care. J Acquir Immune Defic Syndr 2017;76:65-73. https://doi.org/10.1097/QAI.0000000000001456

[206]Gous NM, Scott LE, Potgieter J, Ntabeni L, Sanne I, Stevens WS. Implementation and Operational Research: Implementation of Multiple Point-of-Care Testing in 2 HIV Antiretroviral Treatment Clinics in South Africa. J Acquir Immune Defic Syndr 2016;71:e34-43. https://doi.org/10.1097/QAI.0000000000000872

[207]Rosen S, Fox MP. Retention in HIV care between testing and treatment in sub-Saharan Africa: a systematic review. PLoS Med 2011;8:e1001056. https://doi.org/10.1371/journal.pmed.1001056

[208]Lifson AR, Grund B, Gardner EM, Kaplan R, Denning E, Engen N, Carey CL, Chen F, Dao S, Florence E, Sanz J, Emery S, Group ISS. Improved quality of life with immediate versus deferred initiation of antiretroviral therapy in early asymptomatic HIV infection. AIDS 2017;31:953-963. https://doi.org/10.1097/QAD.0000000000001417

[209]Ford N, Meintjes G, Vitoria M, Greene G, Chiller T. The evolving role of CD4 cell counts in HIV care. Curr Opin HIV AIDS 2017;12:123-128. https://doi.org/10.1097/COH.0000000000000348

[210]Tymejczyk O, Brazier E, Yiannoutsos C, Wools-Kaloustian K, Althoff K, Crabtree-Ramirez B, Van Nguyen K, Zaniewski E, Dabis F, Sinayobye JD, Anderegg N, Ford N, Wikramanayake R, Nash D, Ie DEAC. HIV treatment eligibility expansion and timely antiretroviral treatment initiation following enrollment in HIV care: A metaregression analysis of programmatic data from 22 countries. PLoS Med 2018;15:e1002534. https://doi.org/10.1371/journal.pmed.1002534

both internationally (World Health Organisation)[211, 212] and locally (in the South African ART guidelines[213, 214] in 2019). This approach allowed for ART initiation at the earliest opportunity after diagnosis; substantial clinical, public health and programmatic benefits were anticipated, with a dramatic improvement anticipated in the number of HIV+ patients enrolling onto ART earlier. A 'Treat All' drive aka 'Universal Test and Treat' (UTT) also meant that patients were no longer required to have a CD4 count to be eligible for ART, largely doing away for the need with baseline treatment threshold CD4 testing, including at the POC.

Subsequently, the very high burden of advanced HIV+ disease, documented especially in African countries without access to appropriate laboratory CD4 services (see Section 18), has led to a revival of the need to continue CD4 monitoring, including at the POC where CD4 laboratory services may be lacking; a CD4 test offered at the POC thus continues to afford a patient the opportunity for access to treatment for co-existing opportunistic infection, enabling identification of immunocompromised HIV+ patients who are at risk, for example, to Cryptococcal disease or other infectious opportunistic disease associated with a CD4 threshold of <100 or <200 cells/μL[214].

16.4 PROVIDING CD4 AT THE POC IMPROVES INITIAL ENROLMENT INTO CARE BUT COSTS MORE

From around 2010/2011, there was widescale pressure to implement POC testing in all clinical sites that were considered outside of a service-precinct of a high-volume laboratory. Certainly, in South Africa, one province had already implemented widespread POC CD4 testing, despite the close proximity of the many clinics to NHLS CD4 laboratories[215]. During this period, our work had revealed that the implementation and operational costs of providing CD4 tests at the clinical interface (or POC) were estimated to be between five to seven times higher than a CD4 test performed in a laboratory(46). This data showed that providing widescale POC services was largely unaffordable for South Africa.

[211]World Health Organization(WHO). Consolidated guidelines on the use of antiretroviral drugs for treating and preventing HIV infection. 2nd edition. Geneva: WHO; 2016. Available from: http://www.who.int/hiv/pub/arv/arv2016/en/

[212]World Health Organisation(WHO). Guidelines for managing advanced HIV disease and rapid initiation of antiretroviral therapy. 2017. Accessed: November 29, 2019. Website: http://www.who.int/hiv/pub/guidelines/advanced-HIV-disease/en/.

[213]Department of Health, Republic of South AFrica. National consolidated guidelines for the prevention of mother-to-child transmission of HIV (PMTCT) and the management of HIV in children, adolescents and adults. Pretoria, South Africa; 2015. These guidelines were to be read in conjunction with the "circular of Department of Public Service and Administration (DPSA). Circular 3 of 2016. Date accessed: 2019. Website: https://sahivsoc.org/Files/22%208%2016%20Circular%20UTT%20%20%20Decongestion%20CCMT%20Directorate.pdf

[214]Ford N, Shubber Z, Jarvis JN, Chiller T, Greene G, Migone C, Vitoria M, Doherty M, Meintjes G. CD4 Cell Count Threshold for Cryptococcal Antigen Screening of HIV-Infected Individuals: A Systematic Review and Meta-analysis. Clin Infect Dis 2018;66:S152-S159. https://doi.org/10.1093/cid/cix1143

[215]Van Turha L, Maharaj K, Rose A, Boeke C, Peter TF, Vojnov L, Quevedo J, Tsibolane Y. Point-of-care CD4+ technology implementation in Free State, South Africa, was associated with improved patient health outcomes. S Afr Med J 2020;110:126-131. https://doi.org/10.7196/SAMJ.2020.v110i2.13823

Additional studies were needed to identify specific sites or areas where laboratory services were lacking and falling into service precinct 'gaps'. Our detailed analysis of the South African CD4 NHLS services in 2012/2013(46) revealed that just 17% of clinic sites were not adequately serviced, i.e., not within the service precinct of the nearest (centralised facility) high-volume laboratory that received samples from sites located within a two-hour travelling distance by road. However, even the cost of providing testing for the modest 17% of the total national service, as a POC service, was set to cost as much as the remaining 83% of services that were provided by 'centralised' laboratories(46), practically doubling national CD4 programme costs. This work was confirmed in other local studies reporting higher costs associated with providing clinical pathology services at the point of care[216]. Mobile pathology services, using multiple, on-board installed POC technologies and utilising dedicated operators, were also neither a practical, nor cheaper solution and noted to be even more expensive than clinical site utilising POC[217]. Despite these higher individual test costs shown by our group, point of care CD4 testing has however been justified in a cost effectiveness analysis[218] because it saved lives and potentially recruited patients into care earlier.

The ITSDM (described in detail previously in Section 15) addressed many of the challenges, including the addressing logistical responsibilities posed, and higher costs associated with, widescale implementation of POC CD4 services. The ITSDM simultaneously addressed the service 'gaps' by taking maximum advantage of the infrastructure and services already functioning and available in the existing NHLS lower tier community laboratories(46), offering instead an integrated national service that addressed all levels of service demands. In other words, additional decentralisation of laboratory based CD4 services brought more cost-effective CD4 services closer to the site of clinical need. Adopting this approach potentially saved South Africa the (costly and unnecessary) expense of implementing widescale, universal POC CD4 testing and allowed the laboratory network to reserve POC testing for where it would have most impact i.e. in far-to-reach sites that did not have access to a local NHLS laboratory service(47).

[216]Larson B, Schnippel K, Ndibongo B, Long L, Fox MP, Rosen S. How to estimate the cost of point-of-care CD4 testing in program settings: an example using the Alere Pima Analyser in South Africa. PLoS One 2012;7:e35444. https://doi.org/10.1371/journal.pone.0035444

[217]Coetzee LM, Cassim N, **Glencross DK**. A Cost Analyses of Mobile Laboratory CD4 Testing in a National Health Insurance (NHI) Pilot Site; 2012 African Society of Laboratory Medicine: 1st International Conference. Cape Town. 1-7 December 2012.

[218]Hyle EP, Jani IV, Lehe J, Su AE, Wood R, Quevedo J, Losina E, Bassett IV, Pei PP, Paltiel AD, Resch S, Freedberg KA, Peter T, Walensky RP. The clinical and economic impact of point-of-care CD4 testing in mozambique and other resource-limited settings: a cost-effectiveness analysis. PLoS Med 2014;11:e1001725. https://doi.org/10.1371/journal.pmed.1001725

16.5 INVESTIGATING APPROPRIATE POC CD4 TECHNOLOGIES

Several studies(23,43,44,54) by Glencross et al evaluated the performance of commercially available and manufacturer-supported POC technologies against current predicate laboratory CD4 methods. Investigation included testing both venous and capillary blood samples in a laboratory setting where laboratory staff undertook testing, as well as testing capillary-sampled blood in field sites where ancillary nursing or counsellor staff performed testing. Aspects such as ease of use at the POC, acceptance by attending staff, appropriateness, amongst other aspects, were also reported. Several early validation studies were undertaken that looked at the performance of the first commercially-released iterations of POC technologies for near-patient CD4 testing (these were all presented at various congresses but not formally published). The most relevant amongst these evaluation studies follow.

Initially, our group evaluated of the Guava PCA™ (Personal Cell Analyser) for CD4[219, 220, 221] a small microcapillary flow cytometer which required manual intensive sample preparation and knowledge of flow cytometry for sample analysis as well as spreadsheet literacy for reporting CD4 counts. Around 2005, several early versions of the PointCARE™ system[222] were investigated (initially known as the 'Aurica' and later 'PointCare Now'). This small CD4 counting instrument was the first commercially-available complete end-to-end user-independent system that used EDTA venesected whole blood; importantly, the unit performed all aspects of sample preparation, analysis and reporting in a single closed unit. Later in 2010, further work was presented from our group's initial experience of the Pima™[223] using EDTA whole blood. Other commercially available systems, evaluated on behalf of our NHLS Health Technologies Assessment (HTA) unit, included different small bench-top instruments that were aimed at providing CD4 services in small remote laboratories or in laboratory 'clinical pathology laboratory hubs' inside clinics. Amongst these investigated were (i) the Partec CyFlow® miniPOC[224] (Sysmex-Partec GmbH, Germany), (ii) the Accuri

[219]Scott LE, Lawrie D, Harvey J, Stevens WS, **Glencross DK**. A Comparison of the Guava Personal Cell Analyser with PLG CD4 T-cell Enumeration: A Pilot Evaluation. Abstract no. 962. 11th CROI: Conference on Retrovirus and Opportunistic Infections. San Francisco, California; 2004.

[220]Scott LE, Lawrie D, Harvey J, Stevens WS, **Glencross DK**. A Comparison of the Guava Personal Cell Analyser with PLG CD4 T-cell Enumeration: A Pilot Evaluation. Abstract no. 962. 11th Conference on Retrovirus and Opportunistic Infections. 2004. San Francisco, California.

[221]Pattanapanyasat K, Phuang-Ngern Y, Lerdwana S, Wasinrapee P, Sakulploy N, Noulsri E, Thepthai C, McNicholl JM. Evaluation of a single-platform microcapillary flow cytometer for enumeration of absolute CD4+ T-lymphocyte counts in HIV-1 infected Thai patients. Cytometry B Clin Cytom 2007;72:387-96. https://doi.org/10.1002/cyto.b.20167

[222]Scott LE, Kirkpatrick D, Hansen P, Stevens WS, **Glencross DK**. PointCare CD4 Testing: The New Kid on the Block. Abstract 742, 12th Conference for Retroviruses and Opportunistic Infectionsn(CROI). 2005. Boston, USA.

[223]**Glencross DK,** Coetzee LM, Lawrie D, Stevens W, Osih R. Abstract 947: Encouraging Point-of-Care (POC) PIMA CD4 Testing Performance in a Laboratory Setting, Johannesburg, South Africa.; Abstract #947, 17th Conference Retrovirology and Opportunistic Infection. 2010. San Francisco, USA.

[224]Coetzee LM, **Glencross DK.** Laboratory Evaluation of CD4 Testing using the Sysmex Partec CyFlow® Counter instrument. Sandringham, Johannesburg: National Health Laboratory Service - Health Technology Assessment Unit and NHLS Q-PULSE: March, 2017.

C6 flow cytometer utilising ReaMetrix Reagents[225], (iii) the Blue Ocean flow cytometer with PLG CD4 reagents[226] as well as (iv) the Abbott CELL-DYN Emerald™ hematology analyser that incorporated CD4 counting[227].

Detailed reporting about specific point-of-care instruments, including the PointCare™, Pima™ and Presto™ instruments, or small footprint laboratory-based instruments like the Becton Dickinson FACSCount™ that were evaluated in a POC or clinic 'laboratory hub' context, follow (43,44,54).

16.5.1 FACSCOUNT™ AT THE POINT OF CARE

The FACSCount flow cytometer[228, 229] is a small benchtop CD4 counting instrument that uses a green laser and on-board automated algorithm driven software to provide automatic, user-independent analysis of CD4 and other T-cell parameter reporting (CD3 and CD8); the instrument has a testing capacity of up to 30 samples per day. Excellent reliability of the instrument had been long established in both local(11,21) and other international studies[229, 230]. A FACSCount service system was setup as a mini-satellite laboratory within a busy urban clinic(23) (in the absence of a reliable commercially available CD4 point of care testing technology at that time), to provide immediate access to CD4 counts at the clinical interface and enable a study to assess whether providing a CD4 count at the time of HIV voluntary counselling and testing had a positive impact on early patient enrolment into care(23). Although the service was effective in providing immediate access to CD4 counts, and provided excellent accuracy and precision of CD4 reporting, a full-time medical technologist was required on-site making the service impractical and costly; furthermore, proper GCLP set-up was cumbersome in a clinical setting and not feasible for long-term operations. With the particular version of FACSCount™ assessed in this clinical setting, CD4% of lymphocyte values were not reported (but not considered a limitation in adult-based HIV care). Subsequent versions of this technology did

[225]Coetzee LM, **Glencross DK.** Laboratory Evaluation of CD4 Testing using Accuri C6 Flow Cytometer and ReaMetrix Reagents. Sandringham, Johannesburg: National Health Laboratory Service - Health Technology Assessment Unit and NHLS Q-PULSE: February 2010.

[226]Coetzee LM, **Glencross DK**. Laboratory Evaluation of CD4 Testing using Blue Ocean Flow Cytometer with PLG CD4 reagents. Sandringham, Johannesburg: National Health Laboratory Service - Health Technology Assessment Unit and NHLS Q-PULSE: June 2011.

[227]Coetzee LM, **Glencross DK**. Laboratory Evaluation of CD4 Testing using Abbott CELL-DYN Emerald™ Hematology Analyser. Sandringham, Johannesburg: National Health Laboratory Service - Health Technology Assessment Unit and NHLS Q-PULSE: June 2011.

[228]Strauss K, Hannet I, Engels S, Shiba A, Ward DM, Ullery S, Jinguji MG, Valinsky J, Barnett D, Orfao A, Kestens L. Performance evaluation of the FACSCount System: a dedicated system for clinical cellular analysis. Cytometry 1996;26:52-9. https://doi.org/10.1002/(SICI)1097-0320(19960315)26:1<52::AID-CYTO8>3.0.CO;2-I

[229]Lopez A, Caragol I, Candeias J, Villamor N, Echaniz P, Ortuno F, Sempere A, Strauss K, Orfao A. Enumeration of CD4(+) T-cells in the peripheral blood of HIV-infected patients: an interlaboratory study of the FACSCount system. Cytometry 1999;38:231-7.

[230]Pattanapanyasat K, Sukapirom K, Kowawisatsut L, Thepthai C. New BD FACSCount CD4 reagent system for simultaneous enumeration of percent and absolute CD4 T-lymphocytes in HIV-1-infected pediatric patients. Cytometry B Clin Cytom 2008;74 Suppl 1:S98-106. https://doi.org/10.1002/cyto.b.20415

however include a CD4 percentage (%) of lymphocyte-population reporting, an important parameter needed in an HIV paediatric context[130].

16.5.2 THE POINTCARE INSTRUMENTS

Several iterations of the PointCARE instrument were evaluated by our group[231] including 'AuRICA', 'PointCARE', PointCARE NOW' and 'HumaCount CD4 NOW' (all iterations of the same machine manufactured by Human Diagnostics Worldwide, Germany). The instrument provided an absolute CD4 count, a CD4% of lymphocytes and some basic haematology parameters (haemoglobin and white cell count), with counting enabled by an impedence oriface technique and light scattering based on colloidal gold binding. Following the presentation of our data comparing predicate and PLG CD4 outcomes with 'PointCARE NOW' CD4 at the international Conference for Retroviruses and Opportunistic Infection (CROI)[231] as well as outcomes of local health technology assessment studies, an invitation was received from a large international group to pool our data for publication of a meta-analysis(54) of 'PointCare NOW' CD4 data. Five independently dervied country datasets were included in this study; the metadata analysis outcomes were unfortunately not favourable for the technology, revealing an over-reporting bias of more than 35%, which was noted to be especially prevalent in samples in the clinically relevant range of <350 cells/µL. Similar bias was noted for CD4% of lymphocyte reporting. Repeatability (precision) testing revealed variable reporting with percentage coefficient of variation (%CV) of greater than 11% between predicate methods used and the PointCARE NOW CD4 results. Misclassification analysis, using the CD4 treatment threshold of 350 cells/µL, again confirmed the over-reading bias, with only 47% of individuals who were eligible for ART with reference testing, being eligible for care with a PointCARE NOW CD4 result. Although the evaluation outcomes were not in the interests of patient enrolment into care, the concept of end-to-end analysis in a single 'closed box' unit, with full on-board reagent processing of a capped EDTA venous blood tube, and algorithm-based, user-independent reporting[232], was novel. Further, it was an extremely important landmark development in the field, especially bearing in mind the general lack of flow cytometry skills and the context in which the instrument was envisaged for use in a resource-poor setting.

[231]Scott LE, Kirkpatrick D, Hansen P, **Glencross DK**, Stevens WS. PointCare CD4 Testing: The New Kid on the Block. 12th Conference for Retroviruses and Opportunistic Infections. Volume Abstract 742. Boston; 2005.

[232]Hansen P, Barry D, Restell A, Sylvia D, Magnin O, Dombkowski D, Preffer F. Physics of a rapid CD4 lymphocyte count with colloidal gold. Cytometry A 2012;81:222-31. https://doi.org/10.1002/cyto.a.21139

16.5.3 THE PIMA MOBILE CD4 COUNTER

Around 2010, Alere International (Waltham, MA) released a commercially available, light-weight and portable near-patient CD4 counting machine that did away with the need for EDTA venesected blood. The system, instead, utilised a small cartridge that was filled with patient capillary blood; microfluidics technology, cleverly incorporated into the tiny cartridge, enabled the counting of CD4 lymphocytes. Known as the 'Pima™', the system was envisaged, in a similar fashion to the PointCARE machine, as a total end-to-end solution for the clinical interface; no reagents were necessary other than the cartidge itself and analysis/ reporting was algorithm-software driven. Evaluation against prevailing laboratory predicate CD4 methods revealed a modest CD4 count bias when venous blood-filled cartridges were used, or when a well-trained and practiced health care worker performed the 'capillary cut' to fill the cartridge(44).

The step toward a 'finger-prick' solution was a good idea for the point of care; however, the notion of sampling being a 'finger-prick' may have inadvertently misinformed health care workers who, certainly in our study(44), misjudged the extent of the lancet cut necessary for adequate capillary sampling. This misperception about the capillary 'cut' step therefore made implementation of Pima™ based testing at the clinical interface unreliable. In our study, wide variation of CD4 count reporting, that approached a %CV of 27%, was noted in field studies(44). Use with venous blood draw filled cartridges however demonstrated that the instrument itself produced sound and reliable counts(44) and in phase 2 of our local study, where a properly trained and supervised health care worker attending patients in a local urban clinic undertook patient capillary 'cut' bleeding, better reproducibility of Pima™ CD4 counting was noted. External quality assessment also proved difficult as operators were required to pipette EQAS stabilised blood into cartridges; in consequence, although the EQAS material was compatible with the Pima™ testing, so-called EQAS assessment only evaluated the operators ability to pipette the material into the cartridge and did not evaluate the capillary bleed itself, reported as the cause of variable reporting in field studies(44). Several other studies, including a large meta-analysis[233] revealed similar relatively wide bias to predicate laboratory methods[234, 235].

Another challenge encountered with the Pima™ was the relatively high number of invalid outcomes where a CD4 count could not be generated by the machine. In our study an 'invalid' rate of between

[233]Scott LE, Campbell J, Westerman L, Kestens L, Vojnov L, Kohastsu L, Nkengasong J, Peter T, Stevens W. A meta-analysis of the performance of the Pima CD4 for point of care testing. BMC Med 2015;13:168. https://doi.org/10.1186/s12916-015-0396-2

[234]Diaw PA, Daneau G, Coly AA, Ndiaye BP, Wade D, Camara M, Mboup S, Kestens L, Dieye TN. Multisite evaluation of a point-of-care instrument for CD4(+) T-cell enumeration using venous and finger-prick blood: the PIMA CD4. J Acquir Immune Defic Syndr 2011;58:e103-11. https://doi.org/10.1097/QAI.0b013e318235b378

[235]Sukapirom K, Onlamoon N, Thepthai C, Polsrila K, Tassaneetrithep B, Pattanapanyasat K. Performance evaluation of the Alere PIMA CD4 test for monitoring HIV-infected individuals in resource-constrained settings. J Acquir Immune Defic Syndr 2011;58:141-7. https://doi.org/10.1097/QAI.0b013e31822866a2

9-10% was noted(44); more recent work[236] confirmed this rate of invalid reporting on the Pima™ instrument, with variable invalid reporting documented between 6.6 – 11.2%. Another study from the MSF group reported even higher mean 'invalid' reporting rates, up to 13%[237] where operator proficiency was revealed as an important indicator of higher 'invalid' reporting. Interestingly, despite the issues published concerning use of the Pima™, close to 1 million Pima™ CD4 tests had been performed across 5 sub-Saharan countries between 2011 and 2016[238] underscoring the continued need for near-patient based CD4 testing, especially in the absence of accessible laboratory services.

16.5.4 THE PRESTO MOBILE CD4 COUNTER

The Becton Dickinson FACSPresto™ near-patient CD4 counter was released after the Pima™ and showed better outcomes than the Pima™, especially with regard to capillary bleed sampling in a laboratory or typical field clinic setting. The instrument uses microfluidics technology for cell enumeration and additionally provides for assessment of haemoglobin measurement. Comparative studies from our group(43) revealed acceptable precision to predicate reporting, irrespective of venous or capillary sampling and a very slight but consistent (~7%) over-estimation of counts against PLG CD4 and FACSCount™. End-users anecdotally reported ease of use(43). Confirming our outcomes, a later field study by Daneau et al[238] reported that the capillary blood sampling resulted in a larger bias than that seen when venous blood was used, and that a higher cell count bias was noted in field work than that achieved in laboratory-based testing. This study also reported a similar invalid 'rejection' rate, around 11% on first reading and ~6% rejections after a second reading (but incurring double costs for testing). Other studies[239, 240] also reported better precision using venous blood over capillary sampling, with worse results in the clinically relevant lower CD4 ranges as well as when field tested. Similar comparison of gold standard testing to Pima™ was also reported by Bwana et al[240].

[236]Lamp K, McGovern S, Fong Y, Abere B, Kebede A, Ayana G, Mulugeta A, Atem CD, Elat Nfetam JB, Nzuobontane D, Bollinger T, Jani I, Sitoe N, Kiyaga C, Senyama G, Mangwendeza PM, Mtapuri-Zinyowera S, Sacks JA, Doi N, Peter TF, Vojnov L. Point-of-care CD4 technology invalid result rates in public health care settings across five countries. PLoS One 2019;14:e0219021. https://doi.org/10.1371/journal.pone.0219021

[237]Fajardo E, Metcalf C, Piriou E, Gueguen M, Maman D, Chaillet P, Cox V, Rumaney MB, Tunggal S, Kosack C, Roberts T. Errors generated by a point-of-care CD4+ T-lymphocyte analyser: a retrospective observational study in nine countries. Bull World Health Organ 2015;93:623-30. https://doi.org/10.2471/BLT.14.146480

[238]Daneau G, Aboud S, Prat I, Urassa W, Kestens L. Performance of FACSPresto Point-of-Care Instrument for CD4-T Cell Enumeration in Human Immunodeficiency Virus (HIV)-Infected Patients Attending Care and Treatment Clinics in Belgium and Tanzania. PLoS One 2017;12:e0170248. https://doi.org/10.1371/journal.pone.0170248

[239]Makadzange AT, Bogezi C, Boyd K, Gumbo A, Mukura D, Matubu A, Ndhlovu CE. Evaluation of the FACSPresto, a New Point of Care Device for the Enumeration of CD4% and Absolute CD4+ T Cell Counts in HIV Infection. PLoS One 2016;11:e0157546. https://doi.org/10.1371/journal.pone.0157546

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A detailed meta-analysis of implementation of point-of-care devices in the field are published elsewhere[241]; another work that reviewed feasibility in the field[242] confirmed our experience(43,44) including operational challenges encountered, preference of venous blood over finger-prick sampling, frequent device failures and operator errors, the importance of continued operator training and burden on clinic workers whose workload is increased with use in the clinics.

Lateral flow strip assays like the Visitect™, which provides a visually-interpreted result of above or below 200 CD4 cells/µL, may provide a solution for field testing where laboratory access is limited and where the local burden of advanced HIV disease dictates the need for screening for opportunistic disease[243]. Ndlovu et al[243] and others[244] reported that interpreting a patient's Visitect CD4 result was however subjective and required strict attendance to the multi-stage sample preparation. These papers also reported that the precise incubation times and the multiple procedural steps required for performing the test made some users less confident to use the test, especially where the required multi-tasking could potentially negatively impact on efficient management of a busy clinic. Lay health cadres were recommended to support use in the field, including undertaking all administration, logistics and quality control[243]. Additional studies are therefore needed to confirm robustness in the field.

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17. ENSURING EQUITABLE ACCESS TO CD4 SERVICES AND WIDESCALE UNIVERSAL FULL-SCALE COVERAGE: TIMELY DELIVERY OF RESULTS

The turnaround time (TAT) of any laboratory-reported test is undoubtedly the most perceptible measurement of any pathology service. Regarded by clinicians and pathologists alike as an important indicator of any laboratory's efficiency, the timely delivery of pathology reports is essential to expedite meaningful and appropriate clinical intervention. Equally so, the lack of attention to timely reporting can severely impact patient care, not to mention elicit frustration in supporting clinicians. Not all laboratory tests have the same TAT; both the nature of test, as well as the clinical significance and absolute time to clinical intervention, should be considered when establishing a suitable TAT of individual laboratory tests.

A CD4 result, although not considered a laboratory test with immediate life-threatening consequence if reported outside of TAT, is nevertheless an important outcome needed to determine immune status and the progression of HIV disease as well as determine if patients are eligible for accelerated initiation into care or for screening and treatment of opportunistic infection[245]. In the context of delivering our national CD4 service, it became increasingly evident that the TAT of CD4 tests, issued across the programme, and within provinces, needed attention. Monitoring and evaluation at a national level had become cumbersome and difficult to manage; local, manually-collated CD4 data, assembled at individual laboratory level, was not immediately accessible for centralised review and monitoring. Historically, certainly within the state service before 2017, TAT reporting of most laboratory tests has been static and episodic, with long time-frames between reporting. Reported outcomes have also been largely based on aggregated (data) mean performance, at national or provincial level(55,56) often masking of poor performance of some districts or regions within the overall performance of the majority of adequately performing regions. Such monitoring also meant that existing measures used to judge service delivery, were not sufficiently meaningful to detect, or effect, appropriate and timely corrective action(56). Furthermore, there were areas or regions where even the mean (effectively masked) annualised documented CD4 TAT was documented to clearly fall outside of the NHLS's preferred-provider client needs (the National Department of Health) and was not fulfilling the demands of the HIV/AIDS standard of care, despite a window of seven days for the patient to return for care(56). A fresh approach was needed that simultaneously allowed for coordinated and centralised remote monitoring of all levels of national CD4 laboratory services, in real-time, whilst facilitating closer scrutiny of efficiency at individual laboratory level by individual laboratory managers.

Work to establish such a coordinated framework for monitoring TAT across all state CD4 laboratories was started by my group in 2014. In 2018, our NPP CD4 group published the first analysis of CD4 network performance(55) showing how simple bivariate plot of two parameters, i.e. the 75th centile of performance

[245]World Health Organisation(WHO). Guidelines for managing advanced HIV disease and rapid initiation of antiretroviral therapy. Second Edition. 2017. Date accessed: November 2021. http://www.who.int/hiv/pub/guidelines/advanced-HIV-disease/en/

versus the percentage of reported tests within stipulated organisational TAT. This novel way of presenting TAT outcomes simultaneously (visually) identified CD4 laboratories with excellent performance, as well as those with outlying performance requiring intervention, at a glance. The data presented in this study also revealed that, as a whole, the NHLS national service was delivering a timely CD4 service and was meeting the local HIV/AIDS standard of care.

The 75th centile of TAT performance versus the percentage of reported tests within stipulated organisational TAT was subsequently translated into a practical and user-friendly dashboard(57) in a hierarchical (top-down) format. This allowed various levels of attending business managers as well as laboratory personnel to regularly monitor efficiency not only of CD4 tests, but additionally, a further 24 of the most commonly requested tests offered by the NHLS. The positive impact of regular review of TAT reported in the dashboard was documented in a large academic laboratory(58) where an unexpected, but promptly identified irregularity of TAT of a particular test enabled timely remedial corrective action. This work also highlighted that the separate components of TAT, including pre-analytical, analytical and post -analytical factors, can offer important insights into specific aspects of service and are useful to establish the root-cause of inefficiency needed to effect immediate corrective action(58). The study also underscored the need for consistent and coordinated collection and delivery of samples to ensure a standardised pathology service for patient care, irrespective of the particular tier of laboratory service providing testing. Other recent work from our group(59) specifically interrogated components that affect pre-analytical TAT, and how these components negatively impact on overall turnaround-time across a national laboratory service; specifically, the work focused on transport logistics as an ongoing issue. The underestimated negative impact of longer inter-laboratory referral distance, a crucial but often forgotten component of TAT in a centralised service model, was also considered.

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18. USING CD4 HEALTH DATA TO UNDERSTAND THE BURDEN OF HIV/AIDS DISEASE

Vast amounts of information are generated every day through the delivery of laboratory services across South Africa; the NHLS, as the primary provider of public health laboratory services in our country, is the custodian of this intangible but powerful and invaluable national health asset. Recent work from our group has demonstrated the enormous inherent value of our state laboratory data. Within the NHLS itself, the data can be used to for due diligence and inform important business decision-making to manage efficiency and risk in the organisation. More importantly though, the collection, integration, analysis and presentation of pertinent aspects of the NHLS laboratory health data can offer insights about relevant programmatic health and epidemiological data for our country's main healthcare stakeholder, the South African National Department of Health (NDoH). The work outlined in the next sections (particularly Sections 18 and 19, but also Sections 15, 17 that have preceded) would not have been possible without access to this important national health database.

South Africa has made considerable progress since the implementation of the national HIV treatment programme in 2004. In 2020, 7.8 million South Africans were living with HIV (PLHIV), with a reported prevalence amongst adults aged between 15- 49 years of 19.1%[246] and 230 000 new infections documented the same year; the United Nations Programme on HIV/AIDS (UNAIDS) also reported during 2020 that 92% of people living with HIV in South Africa are aware of their status, with ~72% are on treatment and ~65% of PLHIV virally suppressed. Realising that the treatment (CD4) thresholds were a barrier to care in terms of the UNAIDS 90-90-90 goals[247], the World Health Organisation published their updated treatment guidelines in 2015 and advocated that ART be offered to all patients, regardless of WHO clinical stage, and at any CD4 count. Despite these earnest efforts, the burden of advanced HIV disease has remained unacceptably high in South Africa. In 2017, our group published the first work that documented this high burden of HIV disease amongst immune-compromised patients, classified as advanced (with CD4 counts <200 cells/μL), or very advanced (<100 cells/μL), disease(60). This work was unique in that it characterised the geospatial distribution of South Africa's HIV disease burden; the analysis provided important insights by highlighting specific areas of the country in need of intensified commitment to HIV programmatic support by revealing how some regions, notably, had shown little or no improvement of the proportion of immunocompromised patients over four years of HIV programmatic intervention between 2010 and 2014. In contrast to the analysis highlighting areas with highest HIV disease burden, a second comparison was also reported(60); this data showed, geospatially, those areas of programmatic successes - where the proportion of patients with CD4 counts was above 500 cells/ μL - had increased during the same timeframe (and indirectly reflected an increased number of HIV+ patients who were on treatment and virally suppressed).

[246]UNAIDS(United Nations Programme on HIV/AIDS). Factsheet HIV/AIDS - South Africa 2020. Geneva, Switzerland. 2021. Date accessed: September 23, 2021. https://www.unaids.org/en/regionscountries/countries/southafrica

[247]UNAIDS(United Nations Programme on HIV/AIDS). 90-90-90 - An ambitious treatment target to help end the AIDS epidemic. Geneva. 2017. Date accessed: December 2020. https://www.unaids.org/en/resources/documents/2017/90-90-90

During the same year, 2017, the World Health Organisation published another important treatment guideline concerning the management of patients with advanced HIV disease[248]. By 2018, our group had published leading work based on an intentionally-constructed, laboratory-based patient cohort to determine 'first-ever' CD4 counts across the first 12 years of the South African CCMT(61) (in other words, to categorise disease burden at entry level of care in the context of patients journey onto ART). More than 8 million CD4 case results were included in the review. Importantly though, and of concern, the data revealed that whilst the proportion of HIV+ patients with a CD4 count <200 cells/µL had decreased from close to 50%, down to 36% since 2011, the proportion of patients entering care with advanced disease had remained relatively unchanged over the entire period(61).

Notably, also reported in this paper, was the very high disproportionate number of men who entered care late(61). This finding was re-emphasised in further study(62) where we documented the very late presentation by (migrant) HIV+ men with advanced disease burden, who were accessing (or rather not accessing) HIV care in a rapidly growing economic hub in the northern region of South Africa, namely Lephalele, in the vicinity of the Medupi power plant and large regional coal mines. In this paper we reported a substantive and disproportionately high burden of advanced HIV disease (defined as CD4 counts less than 200 cells/µL) amongst economically active, mostly younger (<45 years) migrant working males, with a corresponding equally disproportionately low representation of men (versus women) attending health care in the local Lephalele clinics. The overwhelming message of the paper was that urgent attention needs to be given to tailoring clinic services to working-men's health and their employment demands, so that working class men can be encouraged and supported to enter HIV care earlier during the trajectory of their disease.

Of additional concern, more recent work[249] has revealed an emerging burden of advanced or very advanced HIV disease amongst young adolescent females, revealing another crisis of younger girls presenting with advanced HIV disease (aged 10 -19 years) and possibly, clear evidence of under-age sexual activity or abuse (or both). Both the late presentation of adult males and unacceptably high numbers of adolescent girls who are sick, reveal a crisis of conscience that demands urgent attention to curtail the HIV epidemic in South Africa.

[248]World Health Organisation(WHO). Guidelines for managing advanced HIV disease and rapid initiation of antiretroviral therapy. Second Edition. Published by WHO Geneva. 2017. Date accessed: 29 November 2020. http://www.who.int/hiv/pub/guidelines/advanced-HIV-disease/en/

[249]**Glencross DK**, Coetzee LM and Cassim N. Assessing late presentation for female adolescents (10-19) with HIV in 2018, South Africa. 16th World Congress on Public Health Online; September 2020. (Draft manuscript in process).

18.1 THE IMPORTANCE OF UNIQUE PATIENT IDENTIFIERS

Unique patient identifiers are crucial to ensure meaningful extraction and interpretation of laboratory health data. One of the major limitations of our national laboratory health data resource, aka the NHLS Corporate Data Warehouse or CDW, is the lack of unique patient identifiers used in other South African national databases including the payment of social grants[250] or electronic Covid-19 vaccine registration[251]. To overcome the lack of information available but still enable extraction of important information about when patients accessed care, early studies were undertaken to encourage submission of programme status with CD4 request forms. However, due to the cumbersome nature of information required and associated workload of entering this information at the time of venesection and test form request, and additional workload at the point of receipt of the sample at the laboratory itself, the data reported revealed this approach was not openly adopted(63,64). Properly establishing the baseline CD4 to define entry into care count (and other many laboratory parameters), therefore remains a challenge and limitation that prevents thorough and accurate national programme and epidemiological data extraction and review.

To overcome the limitations of a lack of a suitably and universally acceptable unique identifier in the database, but still facilitate broader epidemiological programmatic review, probabilistic matching algorithms have been developed that enable a best-fit and a 'linking' of separate patient records to a single, most likely, unique individual record in the national laboratory health database. This approach is not ideal and can only be regarded at best an approximation, but has importantly, allowed for some deduplication of 'similar' patient records in the database(61). Despite the limitations, these valuable algorithms have enabled the construction of important large longitudinal South African cohorts(61) that have enabled the study of the outcomes of HIV+ patients followed over ten years.

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19. INITIATIVES LINKED TO CD4 SERVICES: SOUTH AFRICA'S LEADING NATIONAL CRYPTOCOCCAL REFLEXED SCREENING SERVICE FOR HIV+ PATIENTS WITH VERY ADVANCED DISEASE

Infection with Cryptococcus *neoformans* affects over 1 million people worldwide. More than 600 000 deaths are recorded annually, largely in patients with concomitant advanced HIV infection[252]; the overwhelming majority of patients however, with the largest burden of disease, are documented in sub-Saharan Africa, including Southern and Central Africa. Despite increased availability of HIV antiretroviral treatment (ART) and anti-fungal therapy, infection with Cryptococcus *neoformans* leading to Cryptococcal meningitis (CM), continues to be associated with high mortality rates (20%-70%) in these resource-poor regions[253]. The disease is the leading cause of meningitis and reportedly responsible for up to 20% of early deaths among HIV-infected patients initiating ART in resource-limited settings[253]. Enabling the screening for cryptococcal antigenemia, by testing for cryptococcal antigen (CrAg) in severely immunocompromised HIV+ patients with CD4 counts less than 100 cells/µL, can facilitate early CM disease detection, and save lives[253] if combined with pre-emptive antifungal treatment.

19.1 REFLEXED CRAG SCREENING: A MILESTONE ACHIEVEMENT IN THE CARE OF HIV+ PERSONS

In 2014, after the South African HIV/AIDS treatment guidelines were modified to include screening all HIV+ patients with CD4 counts less than 100 cells/µL for early cryptococcal disease, the South African NHLS, in collaboration with National Institute for Communicable disease (NICD) Mycology unit, embarked on a bold initiative[253, 254, 255] to screen all indigent South African immunosuppressed HIV+ patients for cryptococcal disease. The NHLS NPP CD4 team, under my leadership, began the necessary due diligence and feasibility assessments needed for implementing a 'reflexed' cryptococcal antigen (CrAg) screening approach(65), with a view to automatically testing all CD4 samples received at the NHLS with a CD4 count of less than 100 cells/µL. This investigative work was published over a series of four papers. In the first instance, we established the burden (absolute numbers) of patients with advanced HIV disease eligible

[252]Park BJ, Wannemuehler KA, Marston BJ, Govender N, Pappas PG, Chiller TM. Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. AIDS 2009;23:525-30. https://doi.org/10.1097/QAD.0b013e328322ffac

[253]Govender NP, Chetty V, Roy M, Chiller T, Oladoyinbo S, Maotoe T, Stevens WS, Pinini Z, Spencer D, Venter WD, Jassat W, Cameron D, Meintjes G, Mbengashe T, Pillay Y. Phased implementation of screening for cryptococcal disease in South Africa. S Afr Med J 2012;102:914-7. https://doi.org/10.7196/samj.6228

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[255]Department of Public Service and Administration (DPSA). Circular 3 of 2016. Pretoria, South Africa. 2016. Date accessed: December 2020. https://sahivsoc.org/Files/22%208%2016%20Circular%20UTT%20%20%20Decongestion%20CCMT%20Directorate.pdf

for screening for cryptococcal disease in South Africa(60). Two cost evaluation studies followed, the first included establishing the cost of a laboratory-based testing using a commercial lateral flow assay (LFA) (66), whilst a subsequent work that established the overall national cost-per-result, important for forecasting the impact of the prescribed HIV guideline changes and treatment goals(67). Thereafter, in collaboration with the Boston University School of Public Health, cost effectiveness outcomes of various screening approaches were reported(68). Although this latter study did not show true cost effectiveness of the automated laboratory-based reflexed approach, it did reveal that implementation would be costs-neutral. More importantly, the reflexed laboratory CrAg testing approach would save lives.

In November 2016, the CD4 service-based CrAg reflex screening programme was successfully launched within the NHLS, proving to be an international milestone in the care of HIV+ patients with advanced disease(65). Since then, other countries with a similarly high burden of advanced HIV disease and related opportunistic infection, have introduced similar screening programmes[256, 257, 258]. Various manufacture CrAg lateral flow assays (LFA) and Elisa based immuno-assays[259,] as well as local flow cytometry-based novel methods[260] have also been evaluated for use in the programme. In the absence of networked laboratory support, our group has also proposed CrAg screening at the point-of-care linked to companion CD4 testing (69), an important alternative in resource-poor countries, with little or no laboratory support. Lately, proposals for broadening the screening umbrella to include HIV+ patients with CD4 counts between 100 and 200 cells/µL has been proposed by others [261]; feasibility of this extended screening approach has also been investigated locally[262].

Data collected from the NHLS reflex screening programme has since enabled the important collation of important information about the burden (prevalence) of patients with cryptococcal antigenaemia(70,71). This data has been useful to establish where the burden of cryptococcal disease lies (geospatially) in South

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[258]Hurt WJ, Tenforde MW, Molefi M, Mitchell HK, Milton T, Azama MS, Goercke I, Mulenga F, Tlhako N, Tsholo K, Srivastava T, Leeme TB, Simoonga G, Muthoga C, Lechiile K, Mine M, Jarvis JN. Prevalence and Sequelae of Cryptococcal Antigenemia in Antiretroviral Therapy-experienced Populations: An Evaluation of Reflex Cryptococcal Antigen Screening in Botswana. Clin Infect Dis 2020. https://doi.org/10.1093/cid/ciaa356

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[262]Coetzee LM, **Glencross DK**. Operational Feasibility of Screening Blood Samples with A CD4 Count Of 100-200 Cells/µl for Cryptococcal Antigen in an established Reflex Programme in South Africa. African Society for Laboratory Medicine (ASLM). Online meeting; November 2021.

Africa and can be used to assess areas of clinical services deficiency, especially in areas with a very high burden of cryptococcal disease such as that seen in KwaZulu-Natal province(70), South Africa.

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20. CD4 RECOVERY AND STUDIES RELATED TO IMMUNE ACTIVATION AND RECONSTITUTION IN HIV/AIDS

20.1 THE USE OF CD38+ EXPRESSION OF CD8+ LYMPHOCYTES YIELDS IMPORTANT CLINICAL INFORMATION ABOUT EFFECTIVE ANTI-RETROVIRAL TREATMENT IN HIV+ PATIENTS

CD38 is a novel, multifunctional protein that acts as both antigen and enzyme[263] where the molecule primarily catalyses the metabolism of cyclic ADP-ribose and nicotinic acid adenine dinucleotide phosphate. In the context of HIV/AIDS, CD38 is generally associated with immune activation in lymphoid populations. The chronic over-activation of T-cells[264, 265, 266, 267] in patients with HIV/AIDS, considered as one of the main pathogenic events that drives disease progression, can be measured by markers like CD38 on the surface of CD8+ T cells. Tilling and others[268] first demonstrated that absolute CD38+ expressing CD8+ T cells declined rapidly in parallel with HIV viral load (VL), revealing a clinical response to highly active antiretroviral therapy(ART) to patients on treatment. Various European groups, including Benito et al[269], and later, the Salamanca group[270], subsequently reported that reduced of expression of CD38 on peripheral blood T cells could be used to clinical advantage to monitor response to ART in adults, whilst others reported similar outcomes for HIV type 1-infected children receiving antiretroviral therapy[271, 272] or

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adolescents[273]. At least one European group[274] reported that, although the percentages of CD38+|CD8+ cells appeared to be the best correlate of VL, this measurement lacked specificity for the determination of early virological drug failure and did not appear to be a reliable surrogate of RNA viral load in their Côte d'Ivoire cohort; instead, this group reported that the measurement of CD38 on CD8 T cells was best used as a sensitive estimate of the overall immune recovery.

Our local CIPRA based study[275] offered a unique opportunity to determine CD38 expression as a measure of cellular activation in a local cohort of anti-retroviral treatment naïve patients, including measurement before commencing treatment (as a baseline) as well as follow-up at fixed and relatively closely defined intervals that enabled a unique longitudinal perspective. This study was an important work to establish baseline CD38 levels in in local patients before they started ART; the longitudinal follow-up then enabled the further documentation of reduction of CD38 expression in response to ART. Whilst variable levels of absolute CD8 T-cell activation (CD38 expression) were noted at baseline, all patients showed a similar proportional of decrease of CD38 expression from baseline over time (when measured as a percentage rather than absolute decrease). This reduction was demonstrated in all patients with a gradual but substantial decrease of CD38 mean fluoresce intensity on CD8+ T cells as the patients responded to ART. Importantly, demonstrating the potential use and value of monitoring the CD8+|CD38 expression independently in patients who started ART, was that all reduction of CD38 expression was equally matched by an equivalent reduction of HIV viral load(72).

Later work from our group described the translation and practical implementation of CD38 expression on CD8 T cells in a routine laboratory context(73), by making use of instrument spare capacity and 'piggy backing' the CD38+|CD8+ analysis onto PLG CD4 assay, as a single test. Other details about quality control and biological controls were also outlined(73). Fully decentralised testing and aspects of assay standardisation were later described(74). Work presented at the US-based Congress on Retroviral and Opportunistic Infection (CROI) revealed the CD38+|CD8+ assay in our hands, could be potentially used as an alternative to HIV viral load testing, and as a cost-

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effective and affordable technology to lower costs of HIV viral load laboratory monitoring[276, 277, 278]. These findings were consolidated into our study of a randomised trial of scheduled treatment interruptions of ART, versus continuous ART, in HIV infected patients(75). Unpublished data from this trial, also presented at CROI in 2010[279], consolidated earlier reported findings(72) and confirmed the concomitant decrease of CD38 mean fluorescence intensity on CD8+ T-cells with a rise or decline of HIV viral load in response to treatment interruption in chronically HIV-1-infected individuals. Published data from local colleagues[280] has confirmed the substantial reduction in T cell activation in a different cohort of South African HIV+ female patients on ART, with baseline CD38 expression in HIV+ patients remaining higher than uninfected controls. This latter work also reported that while ART mostly normalises CD4+ T cell memory subsets, interestingly, the CD8+ T cell memory subsets remained significantly skewed compared to HIV-uninfected individuals. The same local group further reported on activation in B cells of HIV-infected women, not studied by our group. Here Tanko et al[281] reported only partially normalised B-cell subsets post-ART, but still, as for CD8 T-cells, B cell activation remaining notably higher in comparison with HIV-uninfected individuals. This group also reported that the extent of baseline B cell activation prior to ART did not correlate with HIV plasma viral load or response to ART.

Not all published studies confirmed ours and other's enthusiasm for measuring CD38 expression of T cell populations as a marker to predict response to ART in HIV patients; positive outcomes were refuted by one group[282].

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20.2 IMMUNE ACTIVATION STUDIES IN HIV+ ADULTS AND CHILDREN

As mentioned earlier, our CIPRA study cohort offered a unique opportunity to study a group of anti-retroviral treatment naïve patients from baseline testing through enrolment onto ART. Several additional linked projects were undertaken on this cohort, with immune activation and CD4 count recovery as a focus. These studies were done in collaboration with our local CIPRA SA study group as well as colleagues from Wistar University in the United States (with all the laboratory flow cytometry immunological sub-study analyses were under my direction). In one study, viral load was closely correlated with CD38-measured immune activation where we reported the association between HIV replication and serum leptin levels in HIV-1 infected women(76). Another study, undertaken with the same group of collaborators, showed similar reduced immune activation with preservation of naïve T-cells amongst HIV infected infants enrolled onto early ART(77) . Our group later reported marked immune activation and T cell exhaustion amongst ART-suppressed HIV-1-infected women in association with HPV infection(78).

Another study from our CIPRA SA study group reviewed the relationship between metabolic parameters, specifically blood lipids, HIV viral load and immune activation in a cohort of South African women(79). Specifically, higher T cell activation associated with high HIV viral load, already documented in our CIPRA study(72) was also associated with lower waist circumference, body mass index and subcutaneous body fat in these women(79). This work(79) also revealed that baseline levels of CD8 T cell activation, measured using both CD38 as well as HLA Dr, were important in predicting CD4 count recovery. Certainly, in our CD38 cohort, CD4 recovery, although initially reasonably rapid, showed only modest recovery after one year on ART, reaching over 300 cells/μL. Similar modest recovery of CD4 T cells was also described amongst HIV-infected patients in sub-Saharan Africa by Lawn et al[283]. Slow CD4 recovery, in HIV+ patients after treatment, was again confirmed in a nationwide constructed cohort study using NHLS CD4 laboratory-based data[284]; here, over 4 years of follow-up, CD4 recovery was again noted to be slow after ART, but most notably amongst patients who initiated treatment when severely immune-suppressed.

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Our CIPRA SA sponsored study also offered a unique opportunity to study immune activation in treatment-naïve HIV+ children. Described on behalf of the CHER study investigators[285], principal investigators Violari and Cotton published important milestone papers in the care of HIV+ infected infants (3,4); these publications subsequently triggered important changes to international treatment guidelines and provided for early ART in neonates and infants as soon as possible after HIV diagnosis. Specific details about the immune reconstitution in the CHER study cohort children, after early diagnosis and treatment, despite data missingness, was published later together with collaborator, Livio Azzoni, et al., in 2015(77). Papasavvas et al later described the associated significantly higher activation of CD8 T -cells in HIV+ infected infants who had had ART deferred(80), matched with increased microbial translocation and rising HIV viral load; these children were also shown to have rapid deterioration of CD4% of lymphocytes and absolute CD4 counts.

20.2 IMMUNE RECONSTITUTION INFLAMMATORY SYNDROME

Reconstitution Inflammatory Syndrome (IRIS) is regarded as the intersection of drug toxicity and ART, with or without opportunistic infection and/ or related treatment[286]. The first important HIV immune activation study work from our group looked at IRIS in the context of HIV infection(81). The study revealed that, during IRIS, CD8 T-cells showed marked and significant increase in activation (including HLA Dr and CD38) in comparison with control HIV+ patients. This work also reported significant reduction of CD4 cell numbers in HIV+ patients experiencing IRIS, affecting predominantly the effector memory cell subset, but with no effect on HIV viral load.

In South Africa, HIV infection has been specifically complicated by the high prevalence of M. *tuberculosis* (M*tb*) infection[287]. An important reported outcome from the CIPRA cohort collaboration was the reported association of M*tb*-mediated IRIS in HIV+ patients with M*tb* co-infection(82). Specifically, our study showed that natural killer cell activation could distinguish M*tb*-mediated IRIS from chronic HIV and M*tb*

[285]**Cher Study investigators**: A Violari, J McIntyre, W Pelser, J Steyn, S Madhi, A Naeem-Sheik, M Budge, M Saleh, S Cassimjee, E Lazarus, S Mashinini, S Dlamini, V Kemese, J Bolton, M F Cotton, H Rabie, A Janse van Rensburg, E Dobbels, G Fourie, M Bester, W Orange, R Arendze, C Andrea, M Smuts, K Smith, T Louw, A Abrahams, K Kelly, A Bohle, I Mong, J Howard, T Cyster, G Solomon, G Benjamin, J Mkalipi, E Barnes, I Sanne, G Gray, R Panchia, C Davies, M Cornell, W Stevens, **DK Glencross**, S Spector, C van der Horst, A G Babiker, D M Gibb, J-M Steens, W X Snowden, N Thoofer, E Loeliger, E Handelsman, K Reese, P Jean-Phillipe, J Nadler, J McNamara, R Hoff, S Lehrman, C Oster, T Peto, L Levin, D Gibb, S Ellenberg, R DerSimonian, A Dodoo, D Harrington, A Kamali, E Katabira, C Lombard, C Luo, M F Marshall, L Mokgatlhe, A Mwinga, A Nunn, H Saloojee, M Sande, J Schoeman, J Singh, R Boss-Victoria, T Chipato, S Emerson, J Mfutso-Bengo, P Mwaba.

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infection. Marked over activation of CD8 T-cells was later confirmed by others[288, 289] whilst natural killer cell degranulation capacity was confirmed to herald early onset of IRIS in HIV-infected patients with M*tb* co-infection[290], in line with our published findings(82). Marked activation of monocytes, with over expression of CD14, was also later described as an additional predictor of M*tb* -mediated IRIS[291].

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21. CONCLUSION

The works presented here for examination describe the novel PanLeucogated (PLG) CD4 test, developed by the author and subsequently used by the South African state laboratory services, the National Health Laboratory Service, to support the national HIV/AIDS Comprehensive Care and Treatment Programme in South Africa since 2004. PLG CD4 has proven itself to be a remarkably robust assay since it was first implemented; a cumulative number of PLG CD4 tests performed for state patients exceeds 40 million tests over the past 16 years, with a cumulative total savings to South Africa of more than R8 billion.

Read together, the papers presented provide a framework for taking a new laboratory test from concept through related research and development into national service. For the most part, the author publications are presented in chronological order as a natural and logical sequence of actions taken and challenges encountered as the test was implemented into national service. Aspects covered include identifying and understanding a clinical need for a particular test, advocacy to promote the ideas and novel concept and patent writing. Proof of concept studies, as well as evaluation against existing state-of-the-art and predicate methods are described with various clinical and multisite validations outlined. Details about novel quality assurance for the new PLG CD4 test across state laboratories follows. Various monitoring and evaluation systems that assist in identifying deficiencies during service delivery are later highlighted, including the development of test-specific novel internal quality control and the provision of locally established, regional schemes that provided for external quality assessment. Important studies undertaken to establish relevant reference interval ranges are also examined.

Universal laboratory service coverage is addressed with descriptions of appropriate (local and regional) service delivery models that facilitate expansion of services across a national network and ensure harmonised coordination of several hierarchical tiers of service. Centralised medium- to high-volume services as well as low-end provision of point-of-care testing, that are key to ensure wide-scale CD4 service delivery, are detailed, ensuring that all levels of service requirement are met, irrespective of geographical location. Examples of requisite due diligence for scaling up services and capacity building, standardisation of testing requirements, and assessing initial and ongoing training needs etc., are also examined. Specific post-implementation practical service monitoring and evaluation systems that were developed during the course of implementation are shared, including models for assessing whether laboratory services are both sufficient (in terms of geographical coverage) and efficient (including pre- intra- and post-analytical test turnaround components that contribute to overall service delivery efficiency).

The inherent value of CD4 programme health data, collected and carefully curated by the National Health Laboratory Service over the last 17 years of programmatic PLG CD4 support, is also emphasised. Several works that have made use of this curated data are described, offering important insights about the South

African HIV treatment programme; the prevalence of and burden of advanced and very advanced HIV disease and the related burden of associated cryptococcal disease amongst patients with advanced HIV are highlighted.

The versatility of the PLG CD4 service is reflected in how other testing modules can be augmented and 'piggy-backed' onto CD4 services. Specifically, work presented reveals how a novel immune activation assay incorporating CD38 and CD8, and coupled onto PLG CD4, can be used to measure CD8 T cell immune activation simultaneously in a in a single assay. Additional studies show how the coordinated CD4 service infrastructure can be utilised to rollout and integrate related laboratory services like reflexed automatic cryptococcal antigen testing. Lastly, the outcomes of several important collaborative studies follow, offering a broader view of CD4 and HIV immunology including lymphocyte subset immune activation and reconstitution in HIV+ patients receiving antiretroviral treatment.

To my knowledge, the body of CD4 work presented has significantly contributed to knowledge in the field; PLG CD4 has influenced the way that the NHLS and other laboratories provide CD4 testing for state patients, both locally and internationally. Moreover, certainly, I have learned many lessons over the course of the past 20 years(83)[292] as I have worked with colleagues to build our highly successful South African national CD4 laboratory programme. Notably, my experience has highlighted that it is important to let the challenges lead the way, trust in the practice and process, generously share ideas and let the data speak for itself. The work presented here further provides evidence that it is acceptable to challenge the dogma, break the rules and develop appropriate systems that best meet local country needs. Without doubt, PLG CD4 broke the rules and defied the dogma. The disruption to the prevailing CD4 testing status quo, not only allowed for a CD4 test that was significantly cheaper but created a better quality CD4 test for South Africa; an important factor in ensuring that the South African programme will be able to continue working towards achieving 95-95-95[293, 294] HIV/AIDS treatment goals.

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POSTSCRIPT

In 1947, Albert Camus in his famous book, The Plague[295], forewarned "that pestilences have a way of recurring in the world… yet always, plagues and wars take people equally by surprise." I believe that Camus' intent was likely metaphorical, interpreted as a contagion that might overtake any society; from corrosive ideologies, like fascism, to actual infectious disease, like HIV/AIDS, or the most recent scourge on our planet, Covid-19. It is the latter, Covid-19, is one that is foremost on our minds in 2021. Camus also wrote to be weary and aware of the "bewildering portents," and be cognisant of not missing their broader significance. From this I took his words also to mean, that despite experience, we soon forget.

When I started to write this thesis in early 2020, local and international daily news-broadcasts forecast the impending catastrophe that was to become the Covid-19 pandemic, with threats of massive shutdown, foreboding economic disaster and predicted crippling of our health care infrastructure. The scenarios, very eerily, echoed what I had written in my introductory section concerning the early days of HIV/AIDS in our country; it was becoming increasingly obvious to me (and I have no doubt many other local colleagues too) that there were significant, and many, parallels of the Covid-19 pandemic that South African health care professionals, active in the late eighties and nineties, had already witnessed in the South African HIV epidemic. History was repeating itself, again, as the Covid-19 pandemic broke in March 2020. The only difference between the two horrific infectious scourges has been the timeframe, that Covid-19 killed people faster and economic breakdown was more rapid.

Nonetheless, Camus also suggested that authorities are liable to minimise the threat of an epidemic, but only to a point that the evidence becomes undeniable, i.e., that underreaction is more dangerous than overreaction. Most people share that tendency, he writes, 'it's a universal human frailty'. This is certainly the case with HIV and TB disease in South Africa. Covid-19 has brought a silver lining of awareness of the burden of infectious and preventable disease across all sectors of society in South Africa. As we enter the fourth wave in 2021 and fully comprehend the impact of Covid-19, it is important that this awareness is not lost on us; that we understand that there are many more unnecessary and untimely deaths caused by HIV and TB infections that should not be overlooked. In 2020 alone, aside from the negative impact of Covid-19 on HIV and TB treatment access[296], more people died of HIV than of Covid-19; the estimated HIV-linked deaths in 2020 in South Africa exceeded 75000[297].

A renewed awareness, for how HIV and TB devastates our country, is desperately needed to save more lives.

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DECLARATION OF CONTRIBUTION

The list of published papers that follow are submitted for the degree of Doctor of Science in Medicine; these represent work carried out by me directly or work in which I actively participated during over the last ~22 years, initially while I was employed in the erstwhile South African Institute for Medical Research and then later from 2002 onwards, under the auspices of the National Health Laboratory services (NHLS), as well as under my joint appointment as a Research Professor in the School of Pathology in the Faculty of Health Sciences of the University of the Witwatersrand, Johannesburg.

The works that are listed below have not been submitted to any University for any degree.
My contribution has been as follows:

A. Editorials, review papers or letters: Work where I took the leading role in writing the manuscript as either the first author, or where relevant, shared the editorial review of the work equally with coauthors(1,2,6,8,32,65,83).
B. Patents where I am named as the sole inventor, but which registered and assigned to the National Health Laboratory Service. I actively participated in the writing and defending of these patents at both the United States and European Union patent offices, together with the attending patent attorney Sandra Clelland from Spoor and Fisher, Johannesburg(13-18).
C. Principal or co-principal investigator-led studies. Work carried out largely by me, in which I contributed substantially to the evaluation of results and preparation of the respective manuscripts for publication, additionally providing leadership and oversight, study design and technical input. Papers(7,12,19-22,44-46,62,72)
D. Work where I provided leadership and oversight as the senior author, as well as contributed to the design the study, developed the methodology, directed the flow cytometry studies, participated in data analysis, as well as contributed to the writing of the manuscripts. Papers include:(5,10,20,29-31,34-37,39,40,43,47-52,55-60,66,67,70,71,73,74).
E. Editorial, book chapters and reviews published where I played an equal role in the writing and final editing of the work(25,26,28).
F. Work which I carried out or shared the task equally with team members in projects or congress papers(23,24,33,38,53,68,69).
G. Papers which I provided senior intellectual input and contributed to the final manuscript draft(41,42,61).
H. Laboratory-based studies, where all preparatory and flow cytometric analytical work was supervised and guided by me, (including writing of respective protocols, laboratory standard operating procedures, bench techniques, managing all quality control, collation of data Excel spreadsheets and

analysis of flow cytometry data and outcomes) (3,4,11,38,54,75-82). In publications(11,75-82), I additionally contributed to the final manuscript before submission for publication.

I. The work presented here has not been submitted to any University for any degree, with the exception of the following works; these publications include a component of N. Cassim's MPH dissertation (UNISA) (63,64)) and specific sections from PhD dissertations (University Witwatersrand – L.E. Scott(9,27) and D. Lawrie(30)). My contribution, as the sole or co-supervisor, and senior author in all these works, was to conceive and plan the project, share in the responsibility for evaluation of results and prepare of the manuscript for publication. Papers by Cassim et al.(64), Lawrie et al.(30), were submitted and published after the candidates had received their degrees and were not submitted for examination.

CONSOLIDATED LIST OF PUBLICATIONS INCLUDED FOR EXAMINATION

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