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Thirty-five years of CD4 T-cell counting in HIV infection : from flow cytometry in the lab to point-of-care testing in the field

Reference:
Kestens Luc, Mandy F..- Thirty-five years of CD4 T-cell counting in HIV infection : from flow cytometry in the lab to point-of-care testing in the field
Cytometry: part B: clinical cytometry - ISSN 1552-4949 - Hoboken, Wiley, 92:6(2017), p. 437-444
Full text (Publisher's DOI): https://doi.org/10.1002/CYTO.B.21400

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Second revision

Title: Thirty-five years of CD4 T-cell counting in HIV infection: from flow cytometry in the lab to point-of-care testing in the field.

Short title : Thirty-five years of CD4 T-cell counting.

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Keywords : Flow Cytometry; Immunophenotyping; Immune monitoring; HIV; CD4+; T-cell; Point-of-care .

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1002/cyto.b.21400

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Abstract

CD4 T-cell counting was introduced in clinical laboratories shortly after the discovery of the human immune deficiency virus (HIV) in the early eighties. In western clinical laboratories, improvements in the CD4 T-cell counting methods were mainly driven by progress in the field of flow cytometry and immunology. In contrast, the development of dedicated CD4 T-cell counting technologies were needs driven. When antiretroviral treatment was made available on a large scale by international Acquired Immune Deficiency Syndrome (AIDS) relief programs to HIV+ patients living in low income countries in 2003, there was a distinct need for simplified and affordable CD4 T-cell counting technologies. The first decade of 2000, several compact flow cytometers appeared on the market, mainly to the benefit of low income countries with limited resources. More recently, however, portable point-of-care (POC) CD4 T-cell counting devices have been developed especially to improve access to affordable monitoring of HIV+ patients in low income countries. The accuracy of these POC instruments is not yet very well documented as many are still under development and clinical validation but preliminary evidence is encouraging. The new HIV treatment guidelines to be released by the World Health Organization (WHO) in 2016 give CD4 T-cell counting a less central role in the management of HIV infection. It is therefore to be expected that CD4 T-cell counting will be phased out as a tool to assess eligibility of HIV+ patients for anti-retroviral treatment in the future. However, CD4 T-cell counting will remain a valuable tool for directing treatment against opportunistic infections.

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The 35 years history of CD4 T-cell counting in the management of HIV infection

AIDS was recognized for the first time as a new disease when increasing numbers of young homosexual men with unusual opportunistic infections and rare malignancies died in 1981 [1]. It was clear from the beginning that AIDS was associated with loss of peripheral blood helper-inducer T-cells (CD4 T-lymphocytes) [2]. The cause of AIDS, a new T-lymphotropic retrovirus, was discovered in 1983 and renamed human immunodeficiency virus (HIV) in 1986 [3,4]. Almost simultaneously, the CD4 molecule, present on T-helper lymphocytes and monocytes, was identified as the main receptor of the AIDS retrovirus [5]. Reagents and instruments to count CD4 T-cells were already available[6]. Scientists started analyzing leucocyte subsets in blood specimens to measure the degree of immune deficiency in HIV-infected patients from the beginning of the AIDS epidemic [7]. The benefit of counting CD4 T-cells in HIV+ patients was explained in the 1986 World Health Organization/Centers for Disease Control and Prevention (WHO/CDC) AIDS case definition [8]. CD4 T-cell counting in peripheral blood of HIV+ patients soon became common practice in clinical laboratories as an important tool in the management of HIV disease. First, to measure the degree of immune deficiency, then to assess eligibility of HIV+ patients for anti-retroviral treatment (ART) and anti-microbial prophylaxis, and finally to monitor immune restoration in patients receiving ART.

The AIDS case definition was revised several times, once in 1987 and again in 1993 [9,10]. In 1993, the AIDS case definition included the number of CD4 T-cells (<200 cells/µL) as a formal AIDS defining criterion [10]. After the discovery of the anti-retroviral drugs, CD4 T-cell counting also became a tool to take decisions on ART initiation. Azidothymidine (AZT) was the first anti-retroviral drug approved by the US government for the treatment of AIDS patients in 1987 [11]. Unfortunately, AZT was very expensive and initially only given to patients with AIDS and low CD4 T-cell counts. However, a few years later (1990), leading clinical studies suggested that AZT could also be safely used in pre-AIDS patients with less than 500 CD4 T-cells/µL [12]. Consequently, the CDC advised to consider ART initiation in all patients with less than 500 cells/µL in the HIV treatment recommendations of 1993

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and 1998 [10,13]. In 2002, however, clinical experts recommended to restrict the use of ART to HIV+ patients with less than 350 CD4 T-cells/µL [14]. The CDC recognized that ART had serious side effects, posed difficulty with adherence, and carried serious potential consequences from the development of viral resistance. Therefore, the 2002 CDC treatment guidelines recommended starting ART in HIV+ patients with less than 350 CD4 T-cells/µL instead of less than 500 [15]. In line with the CDC guidelines, the 2006 WHO consolidated treatment guidelines recommended treating HIV+ patients with less than 200 cells/µL, and only to consider anti-retroviral treatment initiation in patients with 200-350 CD4 T-cells counts [16]. With time, however, antiretroviral cocktails were optimized to improve their efficiency and reduce the side effects. The benefit of starting ART earlier was further documented over the years. In 2010, WHO formally recommended to initiate ART in all HIV+ patients with less than 350 CD4 T-cells/µL, and in 2013 the treatment initiation threshold was increased again to 500 CD4 T-cells/µL [17,18]. The most recent consolidated 2016 WHO treatment guidelines recommend to treat all HIV+ patients, irrespective of their CD4 T-cell count, in line with the ambitious UNAIDS 2020 treatment target of the Joint United Nations Programme on HIV/AIDS to help end the AIDS epidemic [19,20].

The use of CD4 T-cell counts to monitor immunologic treatment responses and immune restoration became more common practice after the mid-90s. AZT monotherapy did not appear to be very effective on the long-term. It lasted until the introduction of highly active antiretroviral therapy (HAART) in the 1997, also referred to as combination anti-retroviral therapy (cART), before recovery of CD4 T-cells was clearly documented in treated patients [21-23]. From then on, CD4 T-cells counts were also used as a more common tool to monitor immunologic response to ART. CD4 T-cell counting was also used to take decisions on antimicrobial prophylaxis treatment. All the AIDS case definition reports included the presence of opportunistic infections (OI), and the progressive loss of CD4 T-cell counts was considered an important risk factor for developing tuberculosis (TB) and *Pneumocistis carrinii* infection. In 1989, the CDC recommended, for the first time, to provide

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preventive pneumocystis pneumonia therapy to HIV+ patients with less than 200 CD4 T-cells/µL [24]. Over the years, the CD4 T-cell counts were also used to initiate preventive treatment of other OI associated with HIV infection. The current OI treatment recommendations include antimicrobial prophylactic therapy for tuberculosis at all CD4 T-cell counts in patients with latent TB, for coccidioidomycosis at CD4 T-cell counts ≤250 cells/µL, for pneumocystis at CD4 T-cell counts ≤200 cells/µL, for histoplasmosis at CD4 T-cell counts ≤150 cells/µL, for *Toxoplasma* and *Cryptococcus* at CD4 T-cell counts ≤100 cells/µL, and *Mycobacterium avium complex* (MAC) at CD4 T-cell counts ≤50 cells/µL [25].

Guidelines for performing CD4 T-cell counting in HIV+ patients have evolved.

Technical progress in the field of flow cytometry had a significant impact on CD4 T-cell immunophenotyping over the years. Prior to the start of the AIDS epidemic, CD4 T-cell counting was done either by fluorescence microscopy or by flow cytometry using anti-CD4 specific monoclonal antibodies to identify helper/inducer ($CD4^+$) T-lymphocytes in whole blood [26]. Originally, T-helper cells ($CD4^+$ T-lymphocytes) were counted in density gradient purified peripheral blood mononuclear cells (PBMC), instead of whole blood, using an unlabeled mouse anti-human CD4 antibody in combination with a second fluorescein-conjugated goat anti-mouse antibody to visualize the $CD4^+$ T-lymphocytes (single color flow cytometry) [27]. This method was quite laborious and time consuming. As flow cytometers became more powerful and versatile, and directly fluorochrome-conjugated monoclonal antibodies became available, dual- and triple-color flow cytometry was introduced in lymphocyte subsets analysis in research in the early 80s [28,29]. It was just a matter of time before those technical improvements were implemented in counting CD4 T-cells in clinical laboratories. The CD4 T-cell assay was significantly simplified after the implementation of red blood cell lysis step to avoid the laborious purification procedure of mononuclear cells. In addition, CD4-positive monocytes confounding the CD4 T-cell counting results could more easily be excluded from

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the analysis by multi-color analysis. This enhanced resolution capacity became possible because a T-cell specific antibody (anti-CD3) was added to the reagent cocktail to identify $CD3^{+}CD4^{+}$ cells (CD4 T-lymphocytes) [30,31]. In 1992, the CDC published guidelines for the performance of CD4 T-cell determinations in persons with HIV based on dual-color flow cytometry and red blood cell lysis [32]. An important next step was the addition of a third anti-CD45 (pan leucocyte) antibody to the reagent cocktail in the early 90s [33]. This improved the quality of the analysis and allowed the analysis of aged blood samples and the guidelines were updated accordingly [34,35]. Over the years, flow cytometry instruments became more sophisticated and 4-color flow cytometry was introduced in lymphocyte subset analysis by the end of the 90s [36]. In 2002 and 2003, new guidelines for performing single-platform absolute CD4+ T-cell determinations in persons infected with HIV were released based on CD45 gating (panleucogating) using 3-color or 4-color flow cytometry [37,38]. Not much later, technical evolution made it even possible to use more than 4 colors but many laboratories still adhere to those 3 and 4-color CD4 T-cell counting guidelines today [39,40]. With 6-color flow cytometry, it is possible to obtain a reliable complete lymphocyte differential including %B cells, % natural killer (NK) cells and %T cells including % CD4 T cells and CD8 T cells from a single test tube [39]. A more detailed description of the several technical improvements in CD4 T-cell counting over the first 25 years of the HIV epidemic was reviewed 2008 [41]. The CD4 T-cell counting procedures used today in western clinical laboratories do not differ that much from those of 10 years ago although there is quite some methodological heterogeneity between clinical laboratories [40]. As clinical laboratories today strive to more automation, it is not a surprise that the first fully (100%) automated high-throughput flow cytometers start to make their appearance [42]. Figure 1 gives an overview of important milestones in history of CD4 T-cell counting, in parallel with the emergence of HIV treatment milestones.

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The HIV pandemic was driving the biotechnical evolution in dedicated CD4 T-cell enumeration.

In the beginning of the AIDS epidemic, flow cytometers were large and expensive stand-alone research instruments and required dedicated postgraduate personal in large dust and vibration free air-conditioned facilities. Flow cytometers were used in clinical research laboratories for various applications such as cell cycle and ploidy studies, and leukaemia immunophenotyping [43,44]. With time, the size of lasers was reduced dramatically. In 1995, one of the first compact flow cytometers dedicated to CD4 T-cell counting , the FACSCount, appeared [45]. At first it had limited success, because western laboratories preferred more versatile instruments which could be used for a variety of different research and clinical applications. ART was virtually inaccessible to people living in low income countries. However, in 1997, the combination ART (cART) was introduced [21]. It was an important step but things really started to change in 2003. It was the initiation of a major international AIDS relief programs, which provided ART at low cost to those countries most affected by HIV, in particular in sub-Saharan Africa [46]. From early 2000, the interest in small and simplified CD4 T-cell counting instruments increased significantly as these were needed to roll-out (decentralize) antiretroviral treatment [47-49]. Small and more remote hospital laboratories, which could not afford a CD4 T-cell counting instrument either shipped blood samples to HIV reference centers for CD4 T-cell counting, or used manual CD4 T-cell counting test kits such as Dynabeads (Dynal) and Cytospheres (Beckman Coulter) which were developed as affordable and simple manual CD4 T-cell counting tools [50]. Between 2000 and 2010, several affordable small flow cytometers, dedicated to CD4 T-cell counting, appeared on the market (Cyflow Counter, Pointcare Now, Apogee A40, Guava CD4 etc) . Some of these instruments were originally developed for different applications (e.g Apogee A40 and Guava), but were modified to accommodate the increased CD4 T-cell counting demand. While they were promising (affordable, small and mobile), only a few reached wide acceptance. They were all compact instruments but reliable performance in the field remained a challenge [51,52]. Most of these devices remained delicate optical instruments requiring a

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laboratory environment with skilled technicians and regular maintenance, and their use was limited to small to midsize hospital laboratories. Instruments like Partec's Cyflow Counter (now Sysmex) were more successful, mainly because of the relative low instrument and reagent cost [53,54]. Although this technical evolution was an important step in the right direction, affordable CD4 T-cell counting remained a challenge for most low income countries [55].

Since 2010, a new generation of simple point-of-care (POC) CD4 T-cell counting technologies have been developed or are in the pipeline [56,57]. Several of those technologies are based on microfluidic cassettes and digital image analyzers. However, clinical performance data are still lacking for most of those new technologies. Some semi-quantitative CD4 T-cell counting tests were also developed to assess treatment eligibility at different treatment initiation CD4 T-cell thresholds (eg. 200, 350 and 500 CD4 T-cells). One example of an instrument-free disposable POC test for the determination of CD4 T-cell counts in whole blood is Visitect CD4 (Omega Diagnostics). Several other similar POC CD4 T-cell counting tests have failed to reach the market. A detailed overview of technologies, including semi-quantitative CD4 T-cell counting tests, has been published in the UNITAID report in 2014 [58]. In 2015, a systematic update of the performance of several of those CD4 T-cell counting technologies for determining ART eligibility was published [59]. Until today, only two POC CD4 T-cell counting devices have been prequalified by WHO, which are Alere PIMA CD4 (Alere) and FACSPresto (Becton Dickinson) [60-62]. Both instruments are very compact, portable and use disposable cartridges for CD4 T-cell counting in both venous and capillary blood. Multiple internal quality control checks add to the robustness. Capillary or venous blood is loaded directly in the cartridge. The devices are designed to be used by nurses or phlebotomists with minimum instrument oriented training. The use of capillary blood has several advantages: a blood sample is obtained from a simple finger prick, which is less invasive than venipuncture. No need for a skilled phlebotomist and the only materials required are a lancet and an alcohol swap to disinfect the finger. The required blood volume of 5 to 15µL increases the acceptability and accessibility of the

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CD4 T-cell counting test at most point-of-care locations. Although CD4 T-cell counting in venous and capillary blood has been shown to yield similar results [63], several recent studies reported that capillary blood results are not as accurate as venous blood results on POC instruments [63-65]. This discrepancy might be related to blood sampling errors (e.g aggressive finger squeezing, poor mixing of blood with anticoagulant etc). Nevertheless, the use of capillary blood on POC CD4 T-cell counting instruments is an important step towards further simplification of CD4 T-cell counting. POC CD4 T-cell counting time is less than 30 minutes. This significantly reduces the number of patients lost to follow-up, as CD4 T-cell counting results are made available at the time of testing [66-68]. The performance specifications of CD4 T-cell counting instruments including POC devices is highlighted in the next section.

CD4 T-cell counting today: specifications related to precision and bias of CD4 T-cell counts are the same for point-of-care instruments.

Even after 35 years of CD4 T-cell counting, there is still a lack of concordance amongst CD4 T-cell counting laboratory methods despite the availability of international guidelines [40]. The introduction of new methods and technologies for CD4 T-cell counting is not simplifying the harmonization process. The assessment of new CD4 T-cell counting instruments including POC technologies is usually done by comparing the CD4 T-cell counts obtained on a new instrument with those obtained on a flow cytometer, preferably in a certified (accredited) clinical laboratory. The performance assessment generally includes analytical precision (coefficient of variation or CV) and trueness (bias or deviation from the reference) measurements [69]. International standards in the area of CD4 T cell counting are not yet available but efforts are now being made to provide CD4 T-cell reference preparations for proficiency testing to new instruments [70]. Unfortunately, it is not always clear to the end-user if the quality specifications of a new CD4 T-cell counting technology, provided by the manufacturer, are satisfactory.

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In 1997, Fraser et al introduced three quality categories of analytical precision (CV_A) of a test result derived from the intra-individual biological variation (CV_I) [71]. The “optimum” analytical precision, the most stringent one, is defined as $CV_A < 0.25 \times CV_I$; the “desirable” analytical precision is defined as $CV_A < 0.50 \times CV_I$; and the “minimum” analytical precision, the least stringent one, is defined as $CV_A < 0.75 \times CV_I$. The CV_I value differs per analyte and an up-to-date database of “desirable” analytical precision of the most common analytes is published online on the Westgard website (https://www.westgard.com/biodatabase1.htm). This database is updated regularly (most recently in 2014) and is based on the original publication by Ricos et al [72]. According to the published biodatabase, the %CV of a CD4 T-cell count is “desirably” smaller than 12.5%. Applying the formulas provided by Fraser and coworkers, the %CV of a CD4 T-cell count should “minimally” be smaller than 18.75%, and “optimally” smaller than 6.25%.

The Westgard database does not provide any information on allowable bias of CD4 T-cell measurements but according to the database, the relative bias of a total lymphocyte count is “desirably” smaller than 9.19%. The WHO Prequalification of Diagnostics Programme (PQDx) accepts a maximum relative bias of 10% (e.g 500 ± 50 CD4 T-cells) for a CD4 T-cell count [73], which is close to the “desirable” quality specification of allowable bias for total lymphocyte counts in the Westgard database. The determination of precision and bias is usually measured following international guidelines such as the Clinical and Laboratory Standards Institute (CLSI) approved guideline—Third Edition (EP15-A3) [74]. Most studies compare two (or more) CD4 T-cell counting technologies and report the average absolute bias of all CD4 T-cell counts. However, the confidence intervals of the average bias estimate, also known as limits of agreement, are at least equally important as they provide an indication on the precision (or lack thereof) of the bias estimation. A literature overview of reported absolute bias of several CD4 T-cell counting technologies has been published recently [59].

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An important criterion with more clinical relevance than precision or bias of a new CD4 T-cell counting technology, is the calculation of the proportion of misclassified patients at clinically relevant CD4 T-cell count cut-off points for initiating treatment in HIV+ patients [69]. Less accurate CD4 T-cell counting instruments lead to a higher percentage of misclassified individuals. Those misclassified patients will either receive treatment earlier (false positive) or later (false negative) when antiretroviral or OI treatment is to be initiated at CD4 T-cell counts below a particular CD4 T-cell count cut-off point (e.g current <350 or <500 cells/µL for ART). For example, it has been shown that patients with more than 350 CD4 T cells/µL, monitored using the dual platform (DP) technology were 20% more likely to start therapy prematurely than those monitored with the more accurate single platform (SP) technology [75].

Similarly, several studies report sensitivity and specificity as performance parameters to identify treatment eligibility at a clinically relevant CD4 T-cell threshold. However, sensitivity and specificity do not have the same meaning for CD4 T-cell counting as for other diagnostic tests. Misclassification of a patient for treatment eligibility, based on CD4 T-cell counts, is less dramatic than for instance misclassification of patients by diagnostic tests that distinguish infection from no infection (e.g HIV serology). In the future, misclassification, sensitivity and specificity will probably become obsolete performance criteria for CD4 T-cell counting instruments criteria when CD4 T-cells counts are no longer used to initiate ART.

Most high-end flow cytometers measure CD4 T-cell counts which meet the desirable or optimum quality goals for precision and bias [59]. For many new point-of-care CD4 T-cell counting instruments, data from independent clinical (field) studies are not yet available. The point-of-care CD4 T-cell counting technologies prequalified by WHO report precisions and bias which meet the minimum (e.g PIMA CD4) or the “desirable” quality specifications (e.g FACSPresto) of CD4 technologies [62-64]. This is encouraging and illustrates that point-of-care CD4 T-cell counting

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instruments can be produced without a significant compromise in precision and accuracy. However, and most importantly, CD4 T-cell counting devices should be evaluated for their intended use.

Other important aspects, which are generally neglected during evaluations but which should be part of an assessment of CD4 T-cell counting POC devices, are ease of use, running cost, dependence on a cold chain for reagent shipping and storage, reagent shelf life, instrument maintenance, and throughput as POC CD4 T-cell counting is rarely done under ideal laboratory conditions.

CD4 T-cell counting in HIV infection in view of the new 2016 WHO treatment guidelines.

The new 2016 consolidated treatment guidelines of WHO recommend provision of ART to all HIV+ patients, regardless of CD4 T-cell counts, in line with the ambitious UNAIDS 2020 treatment target [19,20]. In the near future, this will undoubtedly reduce the importance of CD4 T-cell counts as ART eligibility criterion of HIV+ patients, in particular in low and middle-income countries. Studies indicate that CD4 T-cell counting can be reduced once patients have reached a stable suppression of viral replication [76,77]. It is therefore not a surprise that there is an increasing consensus to reduce the frequency of CD4 T-cell counting in stable treated HIV+ patients to a yearly test [78]. In spite of the important technical evolution in the CD4 market towards more POC CD4 T-cell counting in HIV+ patients in low and middle income countries, the use of CD4 T-cell counting at the point-of-care may progressively being phased out as an indispensable laboratory tool in the management of HIV infection on the longer term. However, as long as affordable and reliable POC viral load instruments are not yet widely available in regions with a high HIV prevalence, it is expected that CD4 T-cell counts will continue to play a role in HIV treatment programs. Furthermore, CD4 T-cell counts will remain a valuable tool in the management of preventive antimicrobial therapy in HIV+ patients with a risk of attracting opportunistic co-infections [25]. CD4 T-cell counting will also remain useful for follow-up and diagnostic evaluation of primary cellular immune deficiencies, including severe

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combined immunodeficiency, monitoring of immunosuppressive therapy and immune reconstitution after hematopoietic transplantation to name a few applications of CD4 T-cell counting unrelated to HIV.

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Legend to Figure

The changing needs for CD4 T-cell counting in the management if HIV infection over the years had a significant impact on the development of new CD4 T-cell counting instruments and technologies. On the right: The ever changing needs of CD4 T-cell counting in HIV+ patients such as (1) assessment of immune deficiency, (2) AIDS defining criterion (<200 CD4 T-cells/µL), (3) treatment initiation at different CD4 T-cell thresholds, (4) monitoring of treatment response, (5) prophylactic anti-microbial treatment at different CD4 T-cell thresholds. On the left: The use of flow cytometers from the beginning of the AIDS epidemic followed by the introduction of dedicated compact cytometers and finally the emergence of novel small point-of-care CD4 T-cell counting instruments and technologies using digital image analyzers and disposable cassettes.

AIDS: acquired immune deficiency syndrome, ART: anti-retroviral treatment, AZT: azidothymidine, cART: combination antiviral therapy, CDC: Centers for Disease Control, HAART: highly active antiretroviral treatment, HIV: human immune deficiency virus, LICs: Low-Income Countries, WHO: World Health Organization

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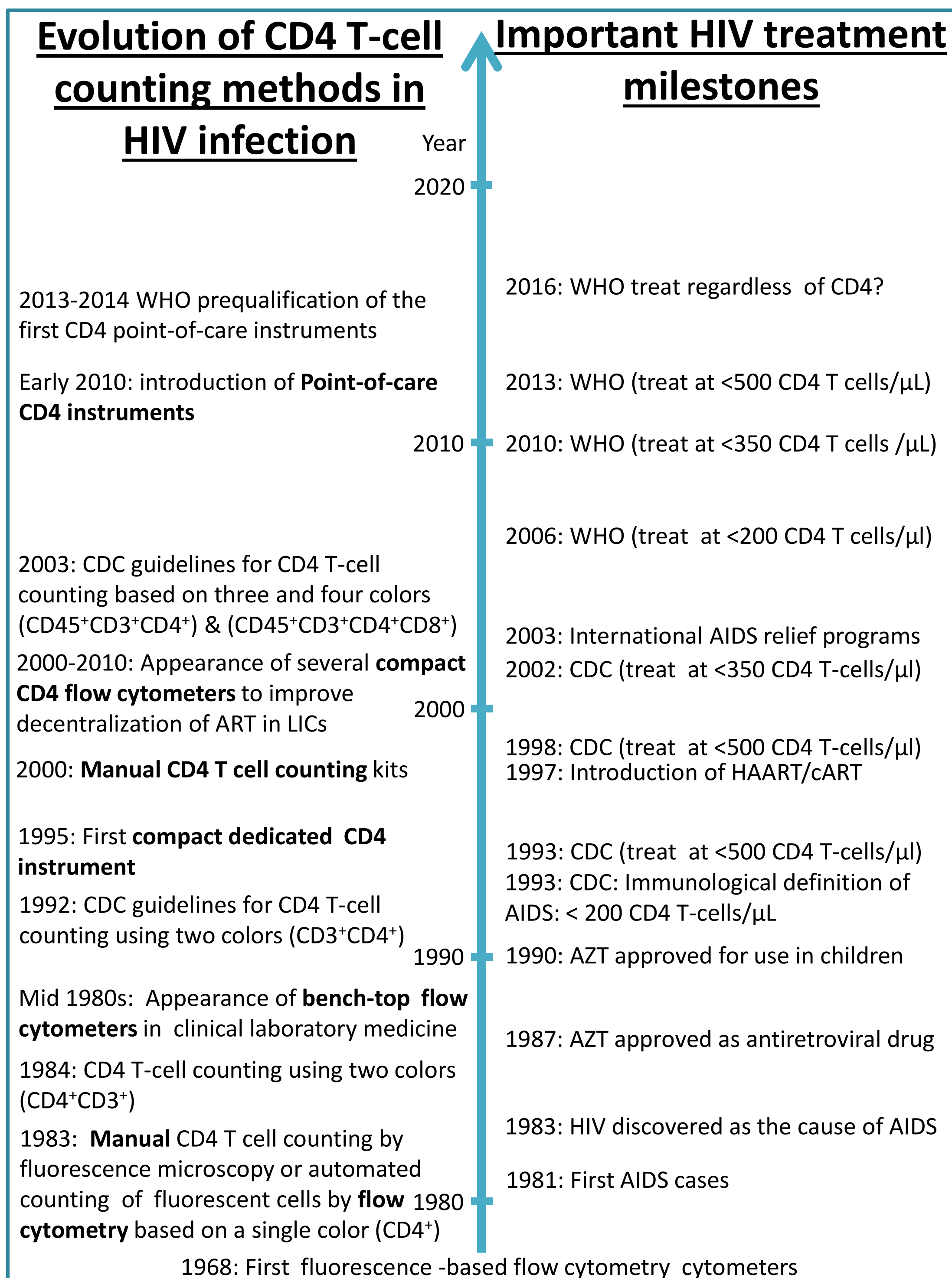
Figure 1



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