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TOPIC HIGHLIGHT

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Clinical application of DNA ploidy to cervical cancer screening: A review

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Abstract

Screening for cervical cancer with DNA ploidy assessment by automated quantitative image cytometry has spread throughout China over the past decade and now an estimated 1 million tests per year are done there. Compared to conventional liquid based cytology, DNA ploidy has competitive accuracy with much higher throughput per technician. DNA ploidy has the enormous advantage that it is an objective technology that can be taught in typically 2 or 3 wk, unlike qualitative cytology, and so it can enable screening in places that lack sufficient qualified cytotechnologists and cytopathologists for conventional cytology. Most papers on experience with application of the technology to cervical cancer screening over the past decade were published in the Chinese language. This review aims to provide a consistent framework for analysis of screening data and to summarize some of the work published from 2005 to the end of 2013. Of particular interest are a few studies comparing DNA ploidy with testing for high risk human papilloma virus (hrHPV) which suggest that DNA ploidy is at least equivalent, easier and less expensive than hrHPV testing. There may also be patient management benefits to combining hrHPV testing with DNA ploidy. Some knowledge gaps are identified and some suggestions are made for future research directions.

Key words: Cervical cancer screening; DNA ploidy; Automated quantitative image cytometry; High risk HPV testing

Core tip: Although application of automated quantitative image cytometry to screen for cervical cancer was first developed in Canada, the United States and Europe, it is most widely used clinically in China where it is applied to about one million tests annually. Over sixty papers reporting the clinical results have been published in Chinese since 2005. As the first review of this topic in any language, in addition to the usual goals of a review, it has the opportunity to increase the awareness of the Chinese clinical experience for those outside of China and to increase awareness of the technology background for English readers in China.

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INTRODUCTION

In a 2005 paper based on a study conducted in China[1], this reviewer and coworkers stated the study objective as: "To establish if measurements of DNA ploidy could be used to assist cytopathologists and cytotechnologists in population based cervical cancer screening programs in countries where manually reading the slides is impossible due to the lack of sufficient skilled cytotechnologists." Based on the study results, we concluded that it could work. Since that time, DNA ploidy technology has been approved for cervical cancer screening, endorsed by several medical societies and fairly widely disseminated throughout China. The goal of this paper is to review the current status of automated quantitative image cytometry

(AQIC) to measure DNA ploidy as applied to cervical cancer screening.

The structure of this review is: (1) A very general introduction to the technology, with some historical perspective and with discussion of some practical issues; (2) A detailed development of a framework for evaluation of screening tests, mostly as a suggestion for how published data analysis might be made more clinically relevant. This section also attempts to alert readers to various well known and less well known pitfalls these evaluations are prone to and to estimate them to discriminate those that are important from those that are not; (3) A summary of existing published results, mostly from China, including reworking some of the published data in terms of the proposed framework; and (4) A discussion of some research still to be done especially in light of the huge advances in cervical cancer control made in the past decade due to the developments of human papilloma virus (HPV) vaccines and HPV testing. Does the objective from the 2005 paper still apply? Does DNA ploidy still have a potential cervical cancer screening role to substitute for nonexistent cytologists in this world of HPV vaccines and HPV testing?

TECHNOLOGY BACKGROUND

DNA ploidy

"Ploidy" is the genetics term for the number of basic sets of chromosomes in the nucleus of a cell. Cells that have an integer multiple of the basic set of chromosomes are "euploid". Most human cells are euploid and have 46 chromosomes or two times the basic set of 23 chromosomes (one set from the mother and one from the father), referred to as "diploid". Some human heart, liver and other cells are euploid with 92 chromosomes or 4 times the basic set and are known as "tetraploid"[2]. Human gametes have one set of the 23 chromosomes, unpaired, and so are also "euploid". Curiously, mature red blood cells in mammals have no chromosomes.

Cells which do not have an integer multiple of the basic set of chromosomes are "aneuploid", simply meaning "not euploid". Most human embryos that have an extra or missing single chromosome do not survive gestation, but some do[3]; for example, Down's syndrome occurs when there are three instances of chromosome 21 in all cells.

Some degree of aneuploidy is observed in virtually all solid tissue cancers[4] in a "mosaic"; that is, the normal cells remain "euploid" but the cancer tumor cells are "aneuploid". (This contrasts with "non-mosaic" aneuploidy like Down's syndrome in which almost all cells are aneuploid.) Elucidation of the role of aneuploidy in cancer has a fascinating history, briefly sketched next, and remains an active area of study today. The key point is that aneuploidy is the hallmark of cancer cells in general and, in the case of cervical cancer, is present both in the early "pre-cancer" or "pre-invasive cancer" phases as well as in the later "invasive" phases. Generally, to detect aneuploid cells is to detect cancer cells.

Aneuploidy and cancer: A brief history

By the year 1890, chromosomes had been discovered and, although their function was not yet proven, it was known that the material responsible for heredity was contained in the cell nucleus where chromosomes are found. The German pathologist, Hansemann[5], published a paper in 1890 entitled "About asymmetric cell division in epithelial cancers and its biological significance" and another in 1891[6] entitled "About pathological mitoses"; both papers are available online and contain wonderful hand drawn illustrations of asymmetric mitoses, as well as other phenomena, such as what is now known as apoptosis. Cells in the process of division are more commonly seen in tumors than in normal tissue and the division in normal tissue is almost always symmetric, producing identical daughter cells, whereas in cancer, one daughter cell often has more chromosomes than the other. These observations had been noted earlier by others, but Hansemann suggested that the defining characteristic of cancer cells is that they lose their ability to divide symmetrically and, in the process, cease to have tissue specialization and increase their ability to live more autonomously, as cancer cells do in metastasis[7].

Although both cameras and light bulbs were invented before 1890, they were not commonly available for microscopy and the illustrations in the journals were usually hand drawn by the author, sometimes assisted by "Abbe's drawing apparatus", rather than printed photomicrographs. Electric light commercialization slowly started in the 1880s, but incandescent light bulbs were not very practical until the invention of the tungsten filament in 1904. Also, it was not until 1893 that Köhler[8] published the method that bears his name and is still used on most modern transmission microscopes to evenly illuminate a microscope slide without also seeing an image of the light source superimposed on the specimen image. Yet, with minimal technology and very limited knowledge of chromosomes, genetics and cancer, Hansemann was able to make accurate observations and to formulate valuable hypotheses on the cellular mechanism of cancer.

A German zoologist named Boveri[9] studied multicentric cell division in double fertilized sea urchin eggs and, when he learned of the work of Hansemann on asymmetric cell division in cancer, he proposed a chromosomal theory of cancer in 1902 (an English translation is available online[10]). In essence, the theory is that cancer cells come from normal cells that divide asymmetrically for various reasons and, although this is usually fatal for the daughter cells, sometimes a daughter cell will survive and become the progenitor for all the subsequent cancer cells. The Boveri theory of cancer is that aneuploidy, resulting from an error in the division of a normal cell, is the cause of cancer.

Boveri was jointly credited with the American, Walter Sutton (working independently), in 1903 with the discovery that chromosomes are the vectors for heredity.

Boveri[11] published a more complete theory of cancer in 1914 (an English translation is available online[12]) and

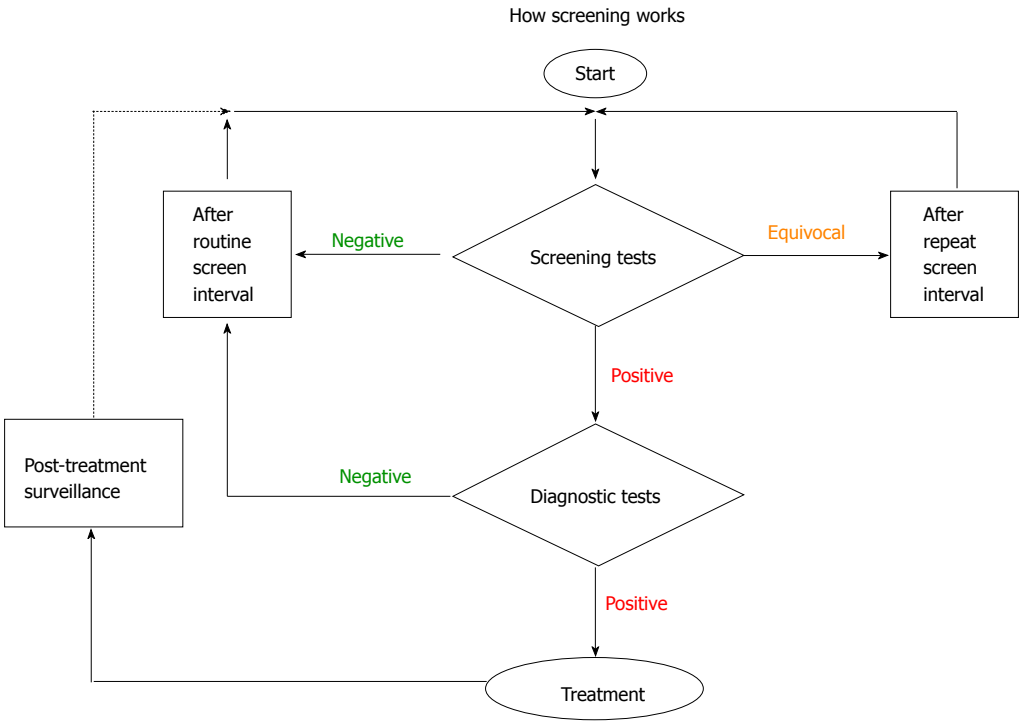

Figure 1 A simplified flow diagram of the relationship between screening and diagnosis showing that screening generally has not 2 but 3 outcomes: positive, negative and equivocal, which is managed by time and re-testing.

he died a few months later. Partly due to World War 1, this work was largely ignored. A translation into English was published by his wife in 1929[13] but received only limited attention outside of Germany until the 1970s. This paper included 20 important insightful hypotheses about cancer, anticipating such concepts as the existence of oncogenes, tumor suppressor genes and cancer induced by infectious agents. Arguably, all 20 hypotheses were subsequently shown to be substantially correct, starting some 50 years after his death[14]! The zoologist Boveri never studied or experimented with tumors or tumor cells and it has been speculated that it was because he was an "outsider" that he was able to form intuitive, imaginative and correct insights, uninhibited by the medical orthodoxy of the day[14].

Boveri's hypothesis that aneuploidy is the cause of cancer remains unresolved today, but is a topic of active study[3,15-17]. Recent work shows that aneuploidy can be both a promoter and an inhibitor of cancer, depending upon the degree of chromosomal instability[18].

Again, the key point for this review is that generally to detect aneuploid cells is to detect cancer cells.

It should be noted that "definitive diagnosis", including tumor type, is usually determined by a diagnostic test, not by the screening test (Figure 1). In the case of cervical cancers, colposcopy directed biopsy is the usual diagnostic test. Screening by detecting DNA aneuploidy alone cannot determine what type of tumor is present, although advanced cytometry techniques, beyond the scope of those discussed here, could make such determination.

DNA Cytometry

"Cytometry" means "cell measurement" which comes in two basic technology flavors: "flow" and "image" (sometimes called "static") cytometry. There are a broad range of techniques to measure chromosomes, both in flow and image cytometry, ranging from the simple measurement of the total DNA content of a cell nucleus to the complex and sophisticated enumeration of small segments of individual chromosomes.

This paper is limited to consideration of AQIC that measures the total DNA content of cell nuclei, along with features that describe the distribution of the DNA within the nucleus and the morphology of each nucleus. These cytometer systems compare the DNA content of each cell nucleus measured to the average DNA content of the measured normal cell population[19]. Cervical "Pap" samples are predominantly comprised of normal cells, even when taken from a woman with invasive cervical cancer. This comparison identifies aneuploid cells even though the technique does not actually identify or count individual chromosomes. A cell with a DNA content 2.5 times that of a normal cell is reliably determined to be aneuploid, even though it is not possible to say that cell has, *e.g.*, 115 chromosomes. This technique is also known as "quantitative DNA Cytometry" or just "DNA Cytometry".

When AQIC was first developed, it was common to apply the genetic language of "ploidy". Unfortunately, this can be misleading, especially in today's world of genomics, because one will be inclined to infer that individual chromosomes are identified and counted. The

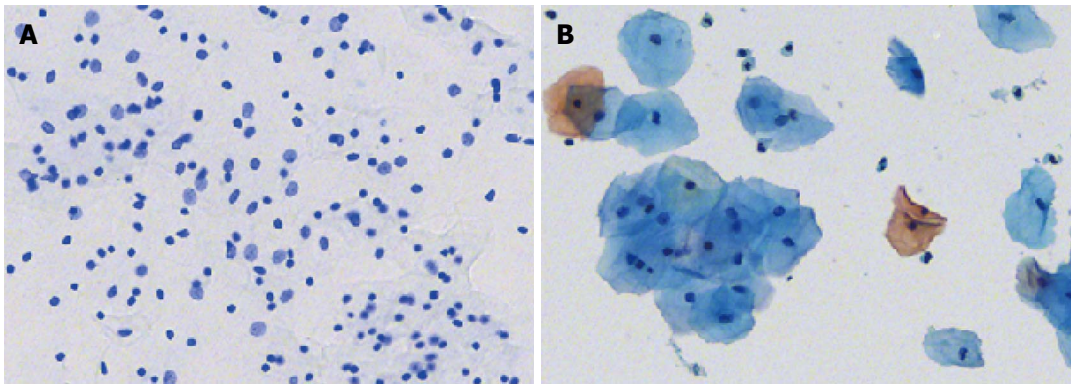

Figure 2 Liquid based cervical samples stained with A: Feulgen thionin and B: Pap stain.

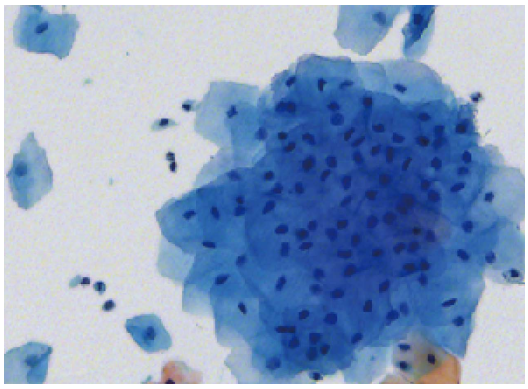

Figure 3 Liquid based cytology cervical sample example of a cell cluster obscured by cytoplasm.

term "DNA ploidy" was coined to be distinct from simply "ploidy" or "chromosomal ploidy" in an attempt to correct this sloppy language, with only middling success because today's young life science students will certainly have encountered the terms of genetics and genomics, but are much less likely to have encountered DNA Cytometry. So it is important to understand that the AQIC technology discussed here does detect aneuploidy by cell measurement, but without explicitly enumerating chromosomes as a geneticist might expect[20].

How DNA is measured

Automated quantitative image cytometers are comprised of a microscope fitted with a digital camera, motorized X-Y stage, robotic slide loader and automatic focus mechanism, all under computer control. The cytometers of this review operate on absorbance microscopy.

Most cytometry relies on staining particular molecules in the specimen and then quantifying the stain. For the automated quantitative image cytometry considered here, the Feulgen[21] reaction stains the nuclear DNA specifically and proportionately to the amount of DNA present, everything else remains clear and unstained[22]. For cervical samples, this greatly simplifies the scene on the slide because the cell cytoplasm is left unstained, as is any red blood (no DNA). Only the DNA contained in the nuclei of epithelial cells, white blood cells and, if present, sperm cell nuclei are stained; the possibility of "false aneuploidy" arising from epithelial cells being overlaid by other epithelial cells, white blood cells or sperm cells is discussed later. Several microorganisms are commonly found in cervix samples, especially Döderlein's bacillus which can completely cover the cervix epithelial cells; the staining protocol does not stain any of these microorganisms either.

Figures 2 are liquid based cytology (LBC) cervical samples with the blue DNA stain thionin (Figure 2A) and with the Pap stain used by conventional cytology (Figure 2B). The images were taken with the same optics (magnification, *etc.*) on the same cytometer. Notice that there are many more cells in the 2A image than in the 2B image and, even so, the scene is much simpler. Notice also that not all cells are in focus at the same time in either image due to the 3-dimensional stacking of the cells. Figure 3 shows a cluster of cells that is very difficult to examine due to the overlapping stained cytoplasm; this would not be a problem with only the DNA stain. Figure 4 shows "low grade abnormality" in the 3 Pap stained photos (arrows), which is especially difficult to see in Figure 4D. The large blue nucleus (Figure 4A) is aneuploid with a DNA content about 2.7 times normal. The key message is that the DNA stained slides are much simpler to measure and interpret than the conventional Pap stained slides.

Measurement of the amount of DNA with a cytometer is identical to the measurement of a chemical with a spectrometer. The basic idea of a spectrometer is shown in Figure 5-light is selected by a slit into a beam which directs it onto a monochromator. A particular color is selected by another slit, the beam is passed through the chemical sample which absorbs some of the light and any remaining light is detected with a photosensor. The concentration of the chemical in the sample can be precisely measured by applying a rule of physics known as Beer's Law.

A quantitative cytometer is a very simplified spectrometer (Figure 6). The optimum light color is selected with color filter and passes through the glass microscope slide. Some of any light that passes through stained DNA is absorbed and the remaining light is detected by the pix-

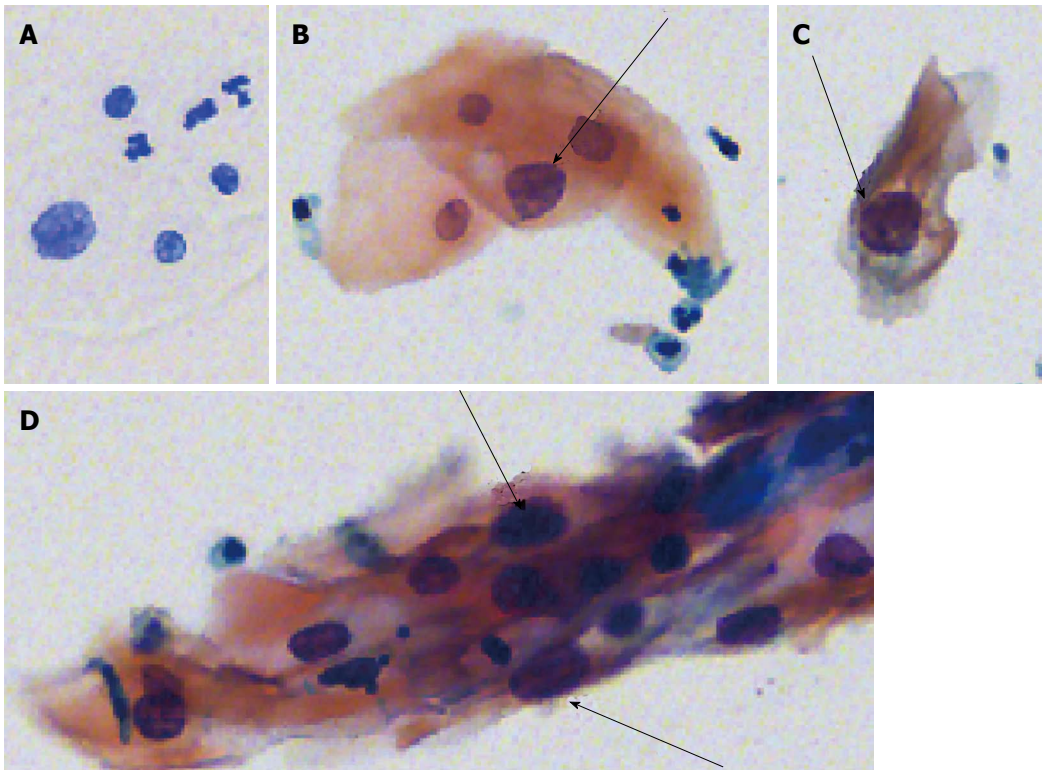

Figure 4 Abnormal cell nuclei. A: Feulgen thionin stained; B, C, D: Pap stained (arrows).

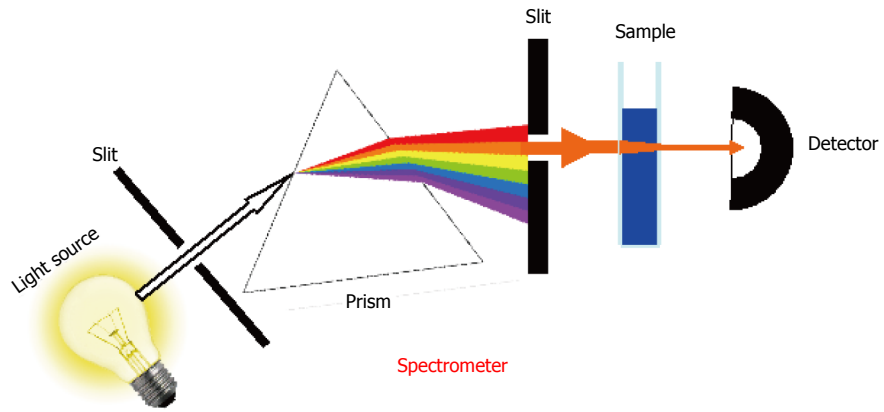

Figure 5 Basic principle of a spectrometer.

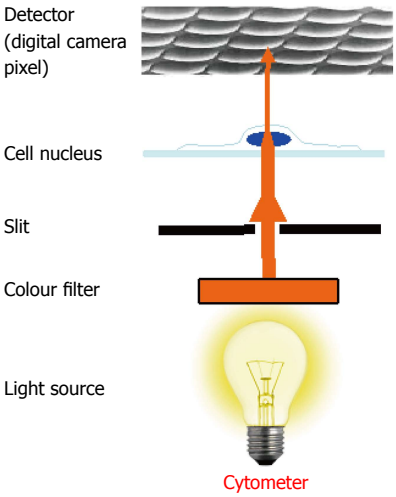

Figure 6 Basic principle of an image cytometer.

els of a digital camera. Beer's law applies separately to every pixel of the camera-in effect, a cytometer is a "Mega-Micro-Spectrometer"-that is, millions of tiny, pixel-sized spectrometers. The total DNA present in a nucleus is obtained by adding up the DNA measured by each pixel of the image of that nucleus. A recent extensive review discusses all of the technical considerations for quantitative cytometry[23].

AUTOMATED QUANTITATIVE IMAGE CYTOMETRY: OVERVIEW

Scanner operation

This review is focused on AQICs that feature "walk away automation" where barcoded stained slides are placed in a slide loader and the operator initiates scanning on a supervisor computer and the cytometer operates without

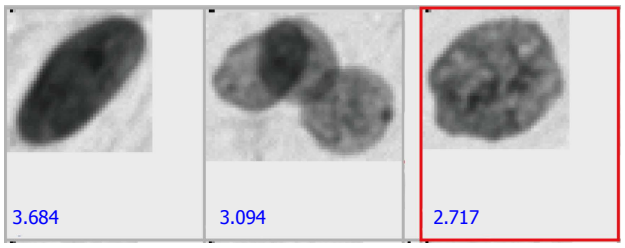

Figure 7 Gallery of potentially aneuploid cell nuclei.

further intervention. Although such machines can scan smears, they are generally employed with LBC samples; indeed, LBC was invented by Cytyc Corp. (now Hologic) with the intended goal to simplify the task of automated imaging[24]. All of the operations of focusing, image capture, segmentation of the scene into foreground and background, feature measurement *etc.* are performed without operator intervention.

Modern AQICs scan a LBC cervical slide in typically 5-10 min and are constantly getting faster, especially due to improvements in computer and digital camera speeds. While the scan rate is typically between 5 and 10 samples per hour, the throughput is generally defined by the slide loader capacity for overnight scanning and so, depending on the particular vendor's design, can range from 75 to 250 per cytometer per day or 15000 to more than 50000 slides per cytometer per year, based on 225 working days per year. If a large volume of samples is available, as is the case for tests in an organized screening program, automated quantitative image cytometry can be very efficient.

Scan review and report generation

Reporting with an automated quantitative image cytometer is done at the conclusion of an interactive review of the scan data for each slide which is comprised of stored images of the cell nuclei, counts of various cell types, and histograms and scatter plots of cell DNA content and other features of the cell nuclei. The reviewer follows a very simple checklist procedure to systematically examine the data, looking: (1) first to check that the DNA scale (normalization) is valid, then; (2) checking any images that could be of aneuploid cell nuclei, then; (3) looking for aneuploid "stemlines", then; (4) assessing cell proliferation; and (5) if none of these are present, then the case is negative and an assessment of scanning adequacy is made.

The idea that a scan has examined enough cells to be deemed to be a "satisfactory" or "adequate" assessment only applies to slides that are free of abnormality because slides deemed to be positive for abnormality are considered to be satisfactory regardless of how many cells were measured.

More than 90% of slides are clearly negative or clearly positive and so working through this checklist for those slides typically takes 1 min or less, including generating the report, once the technician has several days of training and experience. The remaining < 10% of cases are more problematic because their result hinges on the detailed assessment of typically 5 or fewer images of what could be aneuploid cells. The central issue is not "exactly what kind of cell is this an image of?", but the much simpler question of "is this the image of a single cell nucleus or an artifact caused by the overlap of two or more cell nuclei or white blood cells or sperm?"

Figure 7 shows a mini image gallery. The leftmost image is clearly a single nucleus and the next image is clearly of overlapping nuclei as evidenced by the distinct overlapping boundaries and dark overlap area. In fact, the scan computer has no difficulty identifying the second image as being of overlapping cells and automatically places it into a separate group, so in "real life" the reviewer would be unlikely to ever look at this second image.

The last image in the red box (previously shown in Figure 4A) is possibly more difficult because the shape suggests two overlapping ovals but the distinct boundaries and dark overlapping areas are absent. This is actually a single nucleus and the reviewer has the option of clicking on the image and the slide is automagically moved to place that cell into the center of the field of view of the review microscope and it is easily determined to be a single nucleus by panning slightly through focus.

Even when some images must be revisited with the review microscope, the overall task is simple and fast. Most revisit cases take less than 5 min each, so a typical reviewer can work through 40 cases per hour on a sustained basis (4 slides requiring revisiting plus 36 slides without revisiting). Some vendors provide review/revisit microscopes that are separate from the cytometer, while for other vendors the cytometer serves both scan and review/revisit functions.

The report that is generated summarizes the data showing a gallery of images of the most significant cell nuclei, a histogram of the DNA content, a scatter plot of area *vs* DNA content and a summary of cell counts in different DNA ranges.

In conventional cytology of the Pap test, most of the world reports according to TBS 2001 (The Bethesda System, revision of 2001[25]) which is very rich, very nuanced, very complicated and with rather poor inter- and intra-observer agreement[26]. From the viewpoint of patient management, there are really only 4 basic recommendations: (1) Return for another test at the routine screening interval (typically 3-5 years), the test is negative (no abnormality found); (2) Go to colposcopy within a few weeks for a detailed gynecological examination and possible biopsy, the test is positive (abnormality clearly found); (3) Return for another test at an interval shorter than the routine screening interval (typically 6 mo), the test was equivocal (too atypical to call "negative" but not enough to call "positive")-such cases are usually resolved by time; and (4) Return for another test at an interval shorter than the equivocal repeat interval (typically 1 mo), there was no valid test result due to some process failure.

The basic scheme for screening is shown in Figure 1

(recommendation #4 above is not shown). The key focus and goal of the DNA ploidy test is to provide the correct management recommendation from this set of 4 for each woman. A set of objective rules is applied to the data in each report to make the correct follow up patient management recommendation.

These rules can be adjusted to adapt to the reality of each local situation. For example, in a setting where a woman is unlikely to be tested more than once or twice in her lifetime, it may be decided to adjust the rules to make the "negative predictive value" (the chance that a woman who has a negative test result is actually free of cervical cancer or significant pre-cancer) higher than it would be where more testing is done per lifetime. Or, if there are very limited gynecology and colposcopy resources, it may be required that the "positive predictive value" (the chance that a woman who has a positive test result actually has cervical cancer or significant pre-cancer) be adjusted so as to not overwhelm the diagnostic and therapeutic resources available.

The key advantage of DNA Cytometry is that it takes typically only 2-4 wk to train a technician (good high school graduate with some work experience) to competently perform all tasks: cell deposition onto glass slides, staining, operation of the DNA scanner, scan data review and reporting. This contrasts with the 1-2 years of special training for cytotechnologist[27,28] and 3-6 years of specialization training following receipt of an MD degree for a cytopathologist[29-32]. This is not to suggest equivalence between a minimally trained technician and skill of well trained cytotechnologists and cytopathologists, but it is to suggest comparable cervical cancer screening test performance, as summarized later in this review. It is this key advantage that makes automated quantitative image cytometry a candidate for screening in low resource settings where the conventional cytology Pap test cannot be performed due to the lack of trained cytotechnologists or cytopathologists. In such settings, AQIC could mean the difference between screening and not screening.

AQIC DNA PLOIDY: CLINICAL CONSIDERATIONS

Scanner operation: What constitutes a scan?

Two different endpoints are used to define what constitutes an adequate scan for AQIC of liquid based cervix slides: (1) scan all cells deposited on the slide; and (2) scan a preset number of epithelial cells on the slide. Option (1) is the approach primarily motivated by litigation-if an abnormal cell is present on the slide there could be liability. Option (2) is based on science and is completely consistent with current international cervical cancer screening guidelines[33].

What minimum number of epithelial cells must be measured for a scan to be satisfactory? A starting point to answer this question is to consider the guidelines for conventional cytology. TBS 2001[25] stated "Minimal squamous cellularity requirements for a specimen to qualify as "satisfactory" …(is) 5000 squamous cells for liquid-based preparations." It has been reported[34] that this guideline was based on "...personal communications from the authors of two (subsequently published) papers[35,36]." In fact, reference[36] showed a threshold behavior of a jump in sensitivity for ASCUS+, LSIL+ and HSIL+ on Surepath (Becton Dickinson) LBC samples with < 5000 epithelial cells compared to those with > 5000 epithelial cells, supporting this guideline. However, reference[35] concluded that "Cellularity does not provide assurance of adequacy. Any cellularity criterion should be based on measurement of the prevalence of abnormal cells on abnormal slides." McQueen[34] seems to be the only publication so far to take this approach with ThinPrep slides (Hologic) and yet they also conclude: "We have demonstrated that the range of ratios of dyskaryotic to total squamous cells in ThinPrep® preparations is such that it is not feasible to set a minimum acceptable total squamous cellularity so that there is an acceptable probability that all specimen vials containing dyskaryotic cells will be identified. In the light of this, a pragmatic approach should be adopted by deciding on an arbitrary minimum acceptable total squamous cellularity which ensures a rate of detection of abnormality that is at least as good as that for (smears) and which does not impose undue burdens on the users and providers of the screening program." The 2008 American Society for Colposcopy and Cervical Pathology (ASCCP) committee review[33] fell just short of endorsing this recommendation but noted that the TBS 2001 guidelines have been in wide use for many years and stated: "The Bethesda 2001 squamous cellularity criteria provide an acceptable threshold of unsatisfactory results for most patient populations and laboratory settings, although additional studies and data would be useful."

McQueen[34] estimated that for ThinPrep slides there must be at least 16 "targets" (scenes of abnormality) and comprised of at least 87 dyskaryotic cells in total in order to reduce the probability of a false negative result to 2%. This is for Pap stained samples screened visually by experienced cytotechnologists. What is the situation for DNA Cytometry?

Automated quantitative image cytometry is measurement-based and so the detection of even a single aneuploid cell is done with a high degree of confidence, as discussed more in the next section. If we assume that LBC mixes the sample so that the subsample of both physiological cell clusters and isolated cells deposited on the slide is statistically randomized and representative of what is in the vial, then it is possible to calculate from the Poisson distribution, the probability of a real false negative case, defined as having no aneuploid epithelial cell present among the epithelial cells measured. Figure 8 shows the probability of a real false negative occurring given that 5000 epithelial cells are measured. Note that the figure is a log-log plot. Figure 8 shows that when the ratio of normal to abnormal cells is less than about 1000:1, the probability of a real false negative is less than

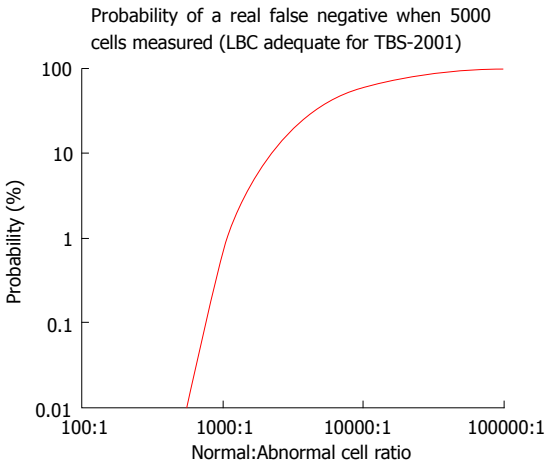

Figure 8 The probability of a real false negative occurring given that 5000 epithelial cells are measured.

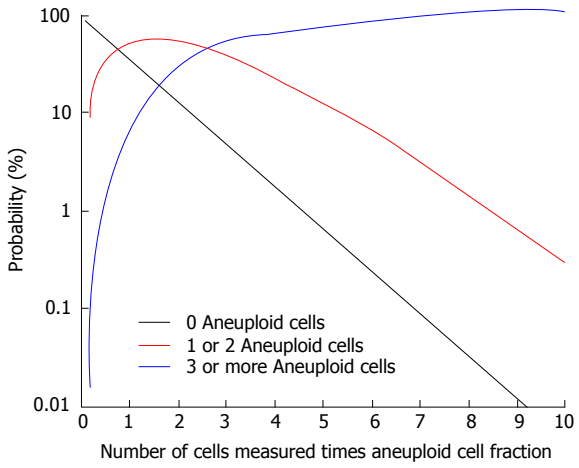

Figure 9 General relationship between number of cells measured and the probability of a real false negative. The red line is considered "equivocal".

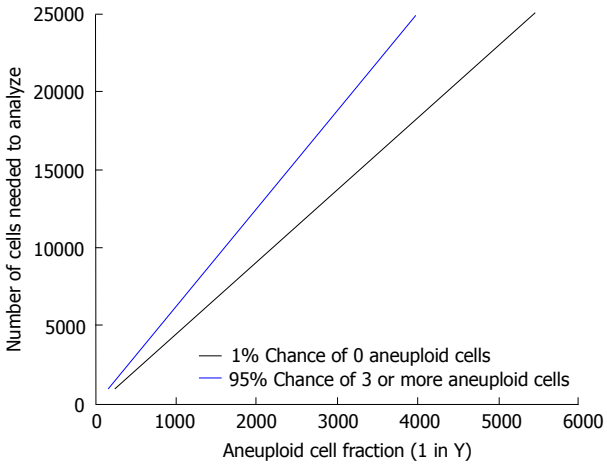

Figure 10 Given an aneuploid cell fraction, how many cells must be measured to get 1% chance of a real false negative or a 95% chance of a positive test result.

about 1% when 5000 epithelial cells are measured. McQueen *et al*[34] counted the number of dyskaryotic cells and total squamous cells on 23 HSIL slides and found only 1 (4.3%) to have a normal:abnormal ratio of > 1000:1-that case was 4600:1.

A more general way of looking at this is shown by the black line in Figure 9 (note the log-linear scale). The X-axis is the aneuploid:normal cell ratio times the number of epithelial cells measured, so it does not just apply to the case of 5000 cells as in Figure 8. The simple "rules of thumb" are: (1) if the aneuploid:normal cell ratio times the number of cells measured is about 5, then the probability of a real false negative is just under 1%; and (2) if this product is about 7, then the probability of a real false negative is approximately 0.1%, which is well into the realm of diminishing returns. That is, to get a ≤ 1% chance of a real false negative case when the ratio of normal to abnormal cells is 1000:1 requires measuring 5000 epithelial cells; if the normal to abnormal ratio is 5000:1, then 25000 epithelial cells must be measured and so on. Setting a goal of a real false negative rate of ≤ 1% is probably very aggressive for screening.

A final way to look at this is Figure 10 which shows how many cells must be measured for a 1% chance of a real false negative case (black line) or for a 95% chance of the being 3 or more aneuploid cells measured (blue line). Most publications on AQIC applied to cervical cancer screening report measuring 6000 or 8000 epithelial cells and so theoretically have a < 1% probability of having a real false negative when the normal:aneuploid cell ratio is less than 1300:1 and 1700:1, respectively.

SCAN REVIEW AND REPORT GENERATION

Normalization and DNA scale

Previously, it was mentioned that Beer's law is applied to measure DNA for each pixel of the image and that the DNA content of each cell is determined by simply adding up the DNA content of each pixel of the image of that cell nucleus. This procedure quantifies the DNA on an arbitrary scale, but it is more useful to convert this to a relative DNA scale by normalizing to the normal diploid cell population. Again, cervical "Pap" samples are predominantly comprised of normal diploid cells, even when taken from a woman with invasive cervical cancer, and so for Pap samples such an internal reference cell population always exists. The normalized scale described here is called "DNA Index" or "DI" in which diploid cells have a DI = 1, tetraploid have DI = 2, *etc.* Figure 11 is an example of such a histogram measured for a pig liver "touch" preparation. The green peak represents purely single diploid hepatocytes, but the other peaks include mixtures of single tetraploid cells and clusters of diploid and/or tetraploid cells used to check the linearity of the DNA measurement. Arguably, the DNA Index is the natural scale to use for cancer screening which is primarily concerned with deviations from diploid for somatic cells.

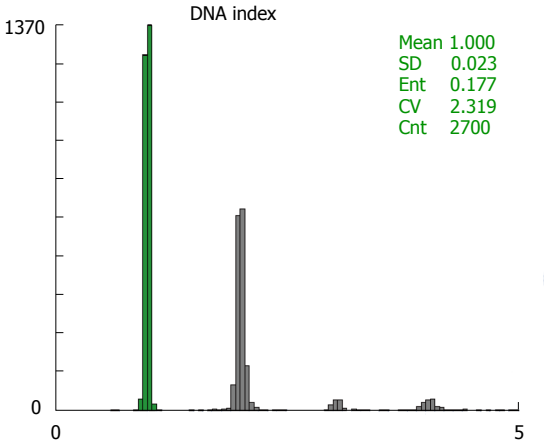

Figure 11 DNA index for pig hepatocytes for DNA linearity calibration.

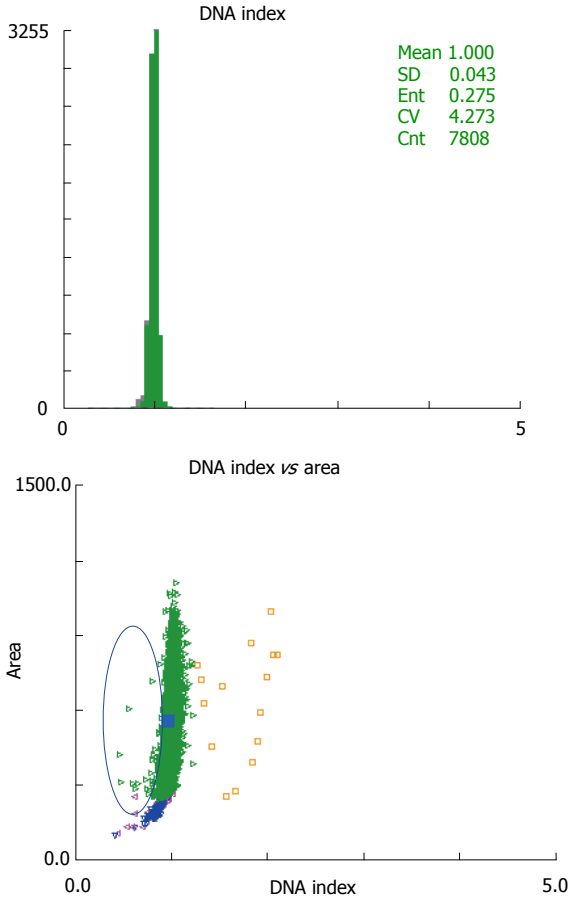

Figure 12 Typical DNA ploidy data for sample taken from a healthy cervix.

However, the original DNA scale is the "C-index" scale in which DI = 1 is designated as "2C", DI = 2 is "4C" *etc*. This scale was first used in 1950 by the American, Swift[37], in a study employing DNA measurement by microphotometry of Feulgen stained cells of various plant species (basically the same technique as AQIC). Swift found that within the non-dividing cells of a species, the DNA content of cells tended to be in quanta that he labeled "C" for "classes": "Non-Dividing Tissues: Photometric measurements made on tissues where mitoses were uncommon tended to fall in certain well marked classes. Means of these classes fit in the series 1:2:4:8:16:32." On this scale, 1C is the quantum of DNA in a (plant or animal) sperm cell. The DNA amount corresponding to 1C varies from species to species. An earlier similar study of various animal species[38] noted the ratio 1:2:4 for DNA in cells from rat liver, but did not describe this in terms of the C-index, which would have been 2C:4C:8C. Arguably, the C-index is the natural scale for biology, in general, as opposed to the narrow field of cancer.

The first step in the data review checklist is to determine that the DNA normalization is valid, meaning that the normal diploid cell population was correctly identified and that the normalization was correctly applied. Figure 12 is a typical, correctly normalized DI histogram of the cervix sample taken from a healthy woman. The scatter plot 12 shows almost all diploid epithelial cells to be in a tight distribution except for a very few hypo-diploid epithelial cells highlighted by the blue oval. This hypo-diploid skewing is a general feature of cervix samples and is comprised of dead epithelial cells in which the DNA has degenerated. The amount of skewing increases with the presence of infection, particularly, but not limited to, trichomonas vaginalis. The skewing is much more obvious in Figure 13 where it does not affect the normalization. The last example (Figure 14) is of a case of trichomonas vaginalis and shows a peak of cells with degenerated DNA that is bigger than the normal diploid peak that should be used to normalize and define the DNA scale. It is beyond the scope of this review to discuss automatic normalization algorithms and technician training strategies to deal with this. However, this problem is minimized if the sample taker cleans the cervix of excess discharge and secretions prior to taking the sample, as is called for by most sampling protocols[33,39]. Some gynecologists believe this to compromise the sample, but an Randomized controlled trial (RCT) that involved taking two consecutive samples has shown the results to be independent of sampling sequence[40].

High DI aneuploid cells and DI > 2.5 (or > 5C)

The second step in the data review checklist is to examine any images that could be of aneuploid cell nuclei, most usually defined as having DI > 2.5 or "5C exceeding". Why is this the practical definition of aneuploidy?

Since AQIC does not identify and count individual chromosomes, it is usually not possible to discriminate normal cells in cell cycle (mitosis), for which the DNA content ranges from $1 \leqslant DI \leqslant 2$, from aneuploid cells with similar DI. Given that no measurements are exact, it is usual to apply a 10% uncertainty[20] meaning that normal cell cycle ranges from $0.9 \leqslant DI \leqslant 2.2$ and hence the likelihood is that cells with DI > 2.2 are aneuploid. The convention of DI > 2.5 (> 5C) was probably a "padding" to the 10% DNA measurement uncertainty, for broad, conservative generalizability. However, Guillaud *et al*[41] looked directly at the DNA ploidy sensitivity and speci-

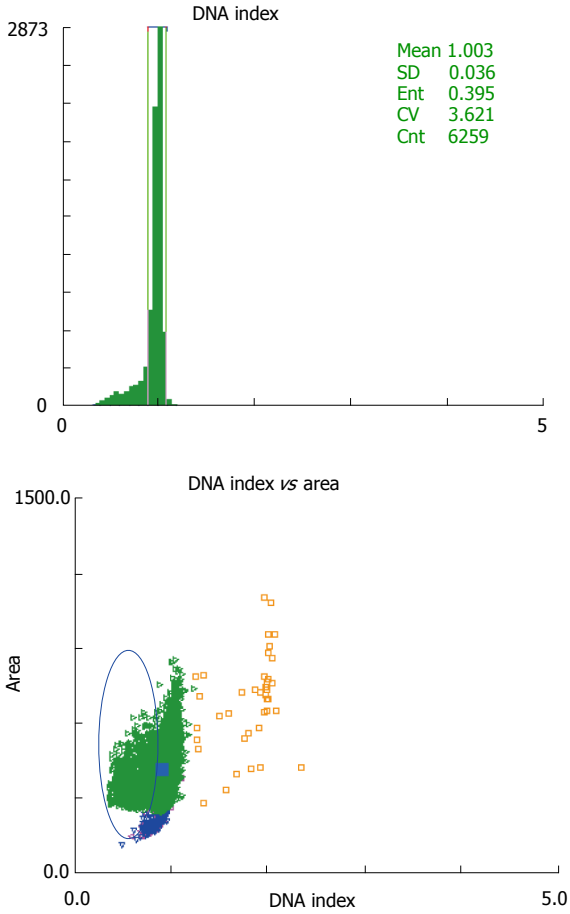

Figure 13 Typical DNA ploidy data for sample taken from a cervix without removing excess secretions or discharge.

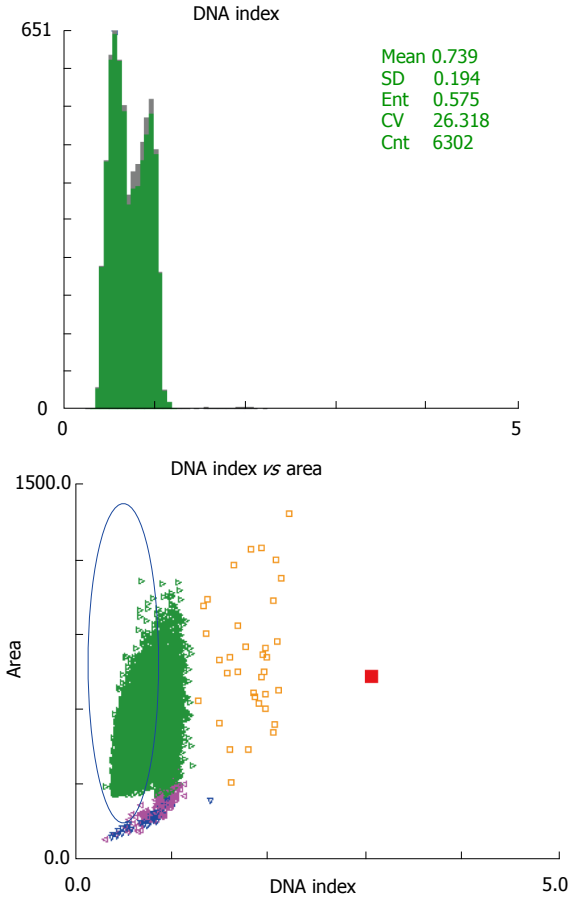

Figure 14 DNA ploidy data for sample taken from a cervix infected with trichomonas vaginalis without removing excess secretions or discharge.

ficity for cervical cancer screening by AQIC as a function of DNA content of putative aneuploid cells. When 3 or more aneuploid cells were present, the threshold converged to DI ⩾ 2.2 (4.4C), but when only 2 or 1 aneuploid cells were present, the threshold rose to DI ⩾ 2.3 (4.6C) and DI ⩾ 2.4 (4.8C), respectively. These results may be specific to their cytometer and their particular laboratory operations standards; other labs may want to calibrate their performance if they choose to adjust the definition of high DI aneuploidy.

Another potential ambiguity is due to polyploidy which refers to euploid cells with DI = 2, 4, 8, 16, *etc.* (4C, 8C, 16C, 32C…). As mentioned previously, this condition does occur normally in some tissues[2] and, even when abnormal, does not necessarily indicate cancer. Concern for polyploidy induced by koilocytosis led Chatelain *et al*[42] to propose setting the threshold for aneuploidy in cervical cancer to DI ⩾ 4.5 (⩾ 9C). However, for cervical cancer screening, such restrictively defined aneuploid cells are too rare to give the test reasonable sensitivity, even in high risk HPV (hrHPV) positive cases[41,43-45] and no evidence of threshold behavior at DI = 4.5 was observed by Guillaud *et al*[41]. For the purposes of cervical cancer screening, the effective definition of an aneuploid cell is one with DI ⩾ 2.5 (5C). Although the 9C aneuploidy threshold is not useful for cervical cancer screening, it may have utility in diagnosis.

Figure 15 shows the histogram, scatter plot and gallery of the 8 aneuploid cells found in this typical positive case.

Aneuploid stemlines

The third step in the data review checklist is to check aneuploid stemlines which appear as a peak in the DNA histogram, but not at DI = 1 or 2 (2C or 4C) where normal cycling cell peaks appear[20]. The fact that stemline cells are in a DI peak means that they have more or less the same DNA content and so they must divide coherently and with a relatively minor amount of chromosomal instability. By definition, such stemlines are aneuploid. Stemline cells are usually in mitotic cell cycle and so a smaller G_2/M peak is usually present at twice the DI of the stemline G_1/G_0, which is usually but not always found between 1 ⩽ DI ⩽ 2. Figure 16 is an example of a stemline histogram with G_1/G_0 at DI = 1.6 and with cells consistent with G_2/M at DI = 3.2, highlighted in blue in the right histogram with the broken vertical scale. Figure 17 is the quite rare case where the stemline G_1/G_0 is at DI = 2.3 (⩾ 2 is rare) and with cells consistent with G_2/M at DI = 4.6 also highlighted in blue in the right histogram with the broken vertical scale.

Although looking for aneuploid stemlines is a step in the review checklist, it is rare for a sample to have a stem-

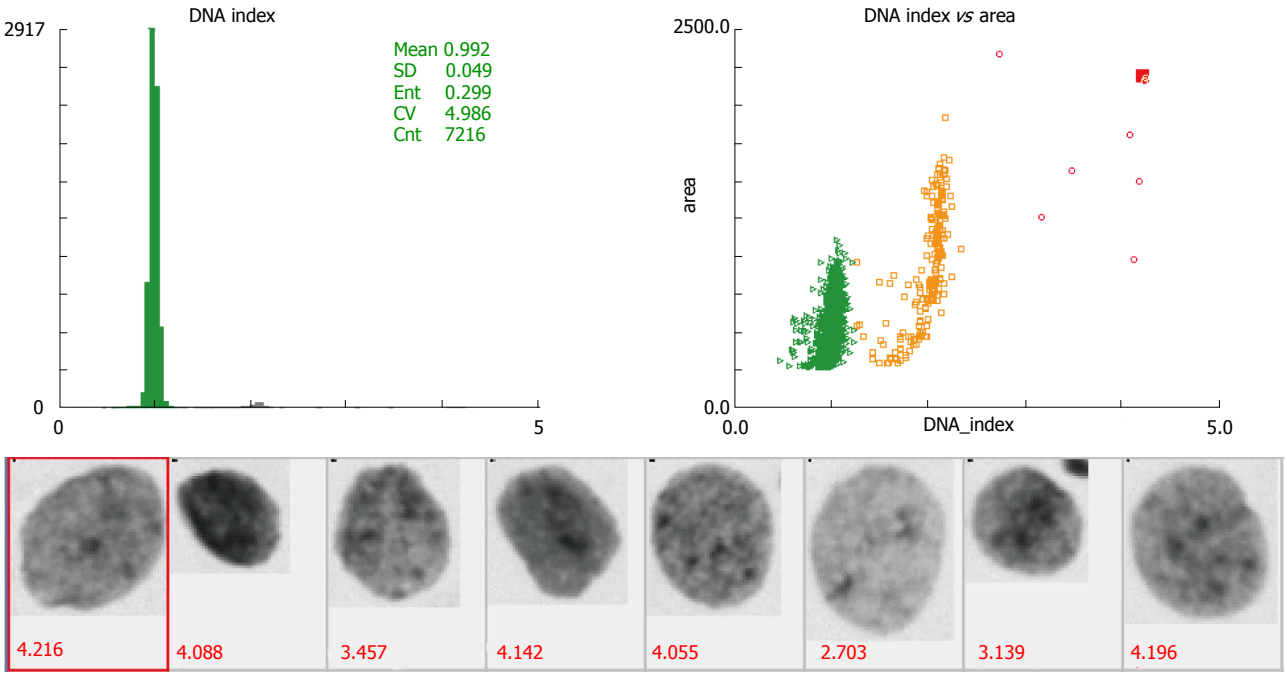

Figure 15 DNA ploidy data from a typical positive case with several aneuploid nuclei with DI > 2.5, the aneuploid nucleus gallery is shown with measured DI indicated in red.

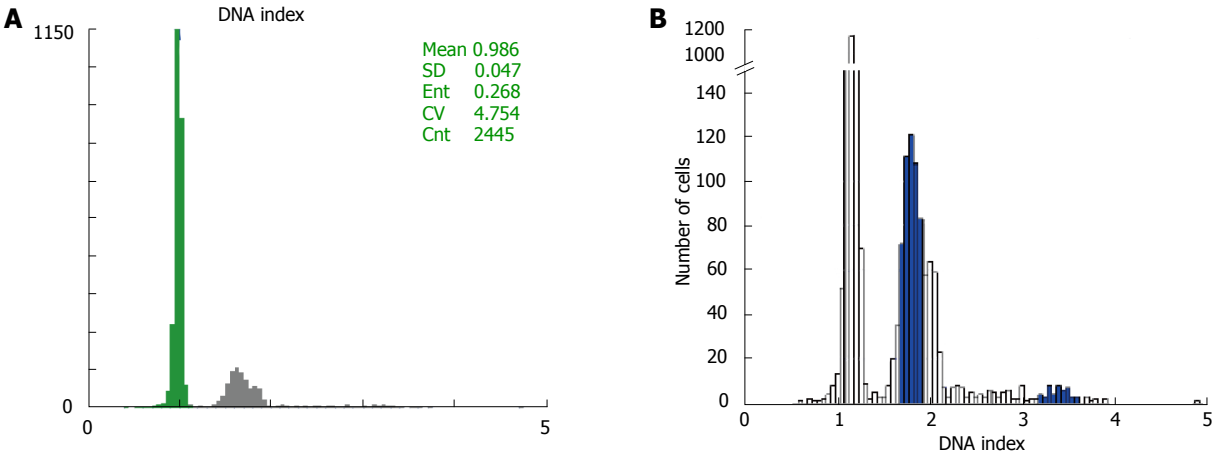

Figure 16 DNA ploidy histograms of aneuploid stemline at DI = 1.6. A: Full histogram; B: Histogram with broken vertical scale.

line without there also being aneuploid cells with DI ⩾ 2.5, so identifying stemlines is usually redundant.

The relative lack of chromosomal instability on an aneuploid stemline may mean that the cell morphology looks rather normal and a biopsy may be interpreted as displaying hyperplasia rather than neoplasia, causing the stemline to be seen as an apparent false positive. However, hyperplasia is genetically normal but stemlines identified by DNA Cytometry are genetically abnormal and so are neoplastic. Presumably, the presence of identifiable stemlines occurs early in the process of neoplastic transformation. This author is not aware of any publications on the malignant potential or aggressiveness of lesions of the uterine cervix that are primarily characterized by aneuploid stemlines.

Cell proliferation

The fourth step in the data review checklist is to determine if there is unusual cell proliferation. Epithelial cells in a properly taken sample from a healthy cervix should almost all be completely differentiated and so do not divide. As mentioned previously, in the range 0.9 ⩽ DI ⩽ 2.2 it is not possible by simple DNA Cytometry to discriminate aneuploid cells from normal cells in mitotic cell cycle. Li *et al*[46] added anti-Ki67 immunostain (marker of cells in mitosis) to Feulgen stained cervical samples, but found no improvement in sensitivity and specificity compared to DNA Cytometry alone.

Excessive cell proliferation could indicate, among other things: (1) repair of wounds or repair processes due to fungal, bacterial and other infections; (2) response

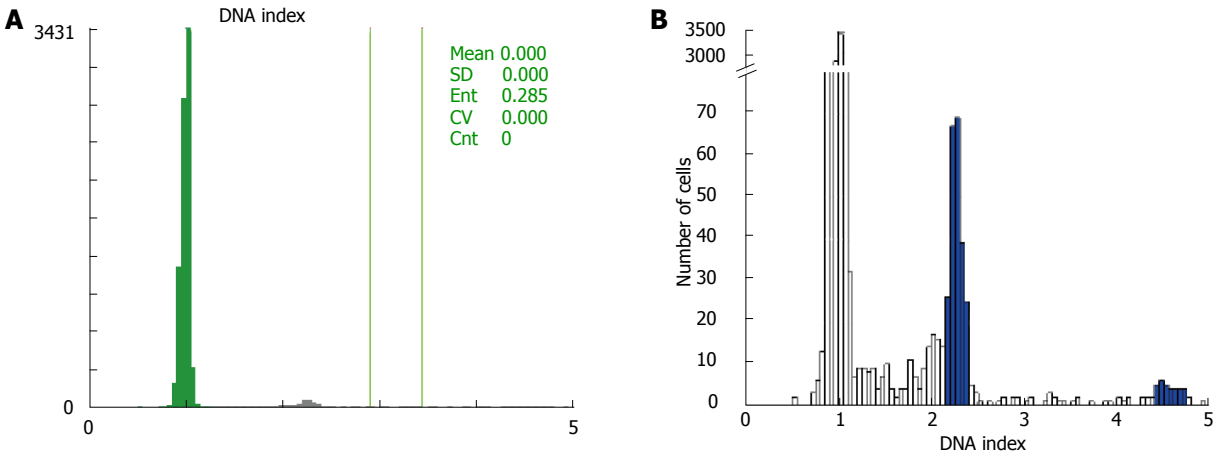

Figure 17 DNA ploidy histograms of a rare aneuploid stemline at DI = 2.3. A: Full histogram; B: Histogram with broken vertical scale.

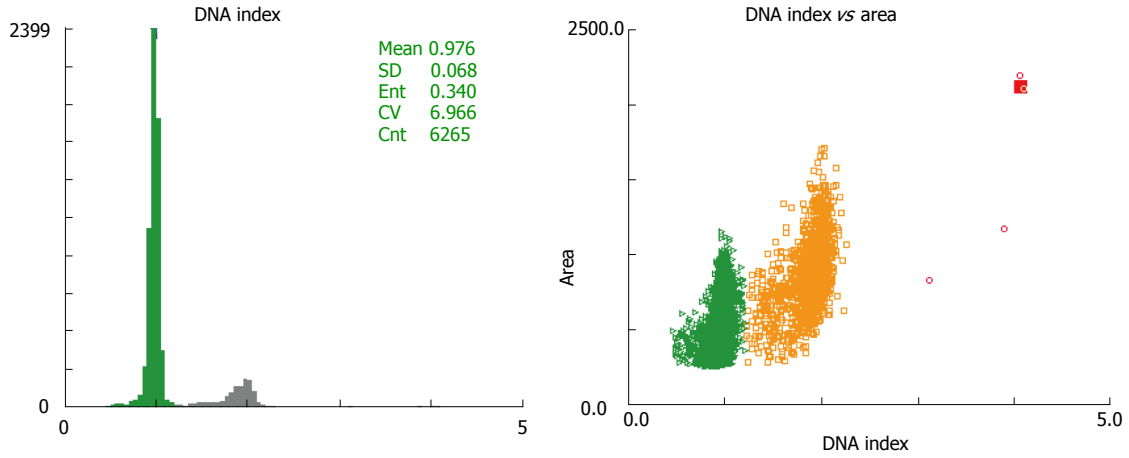

Figure 18 DNA ploidy data of a high proliferation case which also shows high DI aneuploid cells.

to hormones, drugs or radiation, especially in cancer treatment; (3) response to viral infections like HPV; (4) hyperplasia as an epigenetic response to some chemical, mechanical or other stress; and (5) neoplasia.

One expects that applying this criterion alone for cancer screening will have a relatively high false positive rate with so many non-neoplastic causes for cell proliferation. This criterion is usually applied by comparing the number of epithelial cells with $1.25 \leqslant DI \leqslant 2.5$ to the total epithelial cell count. Figure 18 is a typical example showing over 15% proliferation and, as is usually the case, there are 4 aneuploid cells also present, making the proliferation rate determination redundant. Figure 19 is a rare case without any measured aneuploid cells and has a proliferation fraction of 8.7%.

The original proliferation rule was derived from unpublished data of the author at the British Columbia Cancer Agency (BCCA) about 20 years ago (Figure 20), which set a threshold of 10% proliferation for calling a case positive, which is approximately the boundary between LSIL and HSIL. Most of the papers published in China in the past 10 years report using this value. A second "equivocal" threshold is set at 5% proliferation which is approximately the value for ASCUS and is used as an "equivocal" diagnosis, discussed later.

No evidence of abnormality-sample adequacy

The fifth and last step in the data review checklist only applies when none of the preceding criteria for positivity are present. When no abnormality is found, the assessment of sample adequacy must be applied-were enough cells examined for a high probability that the case is actually negative? There is always some reluctance to declare a sample to be "unsatisfactory" with a recommendation of a return visit of the woman for another sample, which must balance the increased but unknown risk to the woman with an unsatisfactory test[33] against "… undue burdens on the users and providers of the screening program"[34], including costs. Although the background of this has already been discussed at length, none of the publications reviewed here have included a definition of what their laboratory uses for an adequacy standard. Guillaud *et al*[41] determined 2000 or more epithelial cells on ThinPrep slides to optimize the sensitivity and speci-

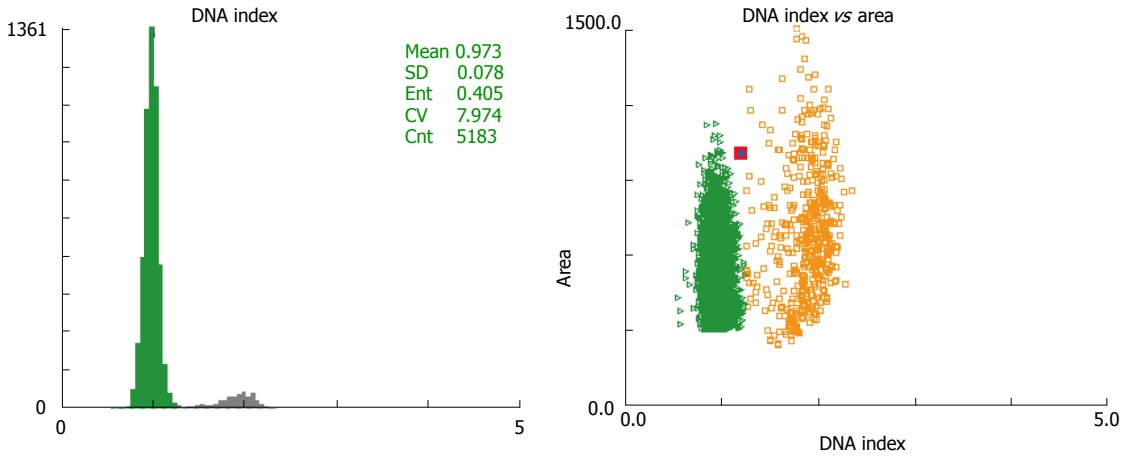

Figure 19 DNA ploidy data of a case with "equivocal" proliferation and no high DI aneuploid cells.

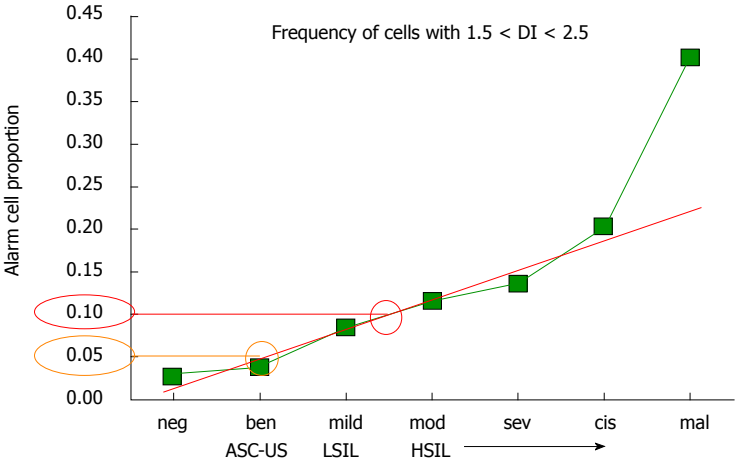

Figure 20 Original data from British Columbia Cancer Agency used to formulate the "proliferation" rules, based on measurement of > 500 samples.

ficity in a research setting.

FRAMEWORK FOR EVALUATING SCREENING TESTS

Arbyn *et al*[47] and Adriaensen *et al*[48] reviewed how to evaluate cervical cancer screening technologies through their various phases, in 2009 and updated in 2013, with extensive references.

However, most of the papers available for examination for this review are cross-sectional, observational studies that compare automated quantitative image cytometry to liquid based conventional cytology and the analysis centers on 2 × 2 contingency tables and the extraction of comparative sensitivity, specificity, positive and negative predictive values, usually also reported in the paper abstract. The literature has similar analyses for screening for other cancers with other technologies[49]. In the opinion of this reviewer, these analyses are confusing at best and highly misleading at worst and most are singular in their absence of insight into genuine clinical utility of the tests. This section attempts to provide some suggestions to improve the data analysis with particular focus on the clinical meaning.

A typical study reviewed here is observational, applied to 10000 subjects screened with both Tests 1 and 2; the 600 who are positive by either test receive a recommendation to attend colposcopy; and the 250 available biopsy results become the exclusive focus of the analysis- the other 9750 cases of the study are ignored! In this analysis example there are "excluded" data (the 10000 - 600 = 9400 cases that test negative by both Tests 1 and 2) and "missing" data (the 600 - 250 = 350 cases who tested positive by at least one of Test 1 and 2 but with no follow-up result). Typically, there are 5 biopsy outcomes: negative (or cervicitis or inflammatory), CIN1, CIN2, CIN3 and invasive cancer. Tests 1 and 2 also typically have several diagnostic grades, such as NILM, ASCUS, LSIL, *etc*. The study applies some thresholds to collapse Test 1 *vs* histology and Test 2 *vs* histology to 2 × 2 contingency tables and the well-known formulae are applied to calculate sensitivity, specificity and predictive values. Typically, the reported specificities are very low as are the negative predictive values (NPVs), which is entirely an artifact of the failure to include the other 9400 negative cases in the analysis. Some statistics software even extracts the area under the receiver operator characteristics (ROC) curve from this single measured point, which is also often reported in the paper abstract. Finally, many papers also report the result of combining Tests 1 and

2 based on these same 2 × 2 tables, almost always as the logical "or" of positive results (the combined test is positive if either 1 or 2 is positive).

What is the interpretation of the results based solely on the 2 × 2 tables for the small subset of cases that have biopsy results available? For the moment, let us ignore the problems of verification bias, missing data and imperfect "gold standard" reference diagnosis-they are discussed later. Because the table is comprised only of cases testing positive by one or both screen tests, both the sensitivity and positive predictive value (PPV) are legitimate estimates of the screen test values and the PPV can be interpreted as the probability that a woman sent for biopsy will have a positive result. The specificity and negative predictive values are meaningless, however, because the "excluded" data is the vast majority of negative cases. In fact, the negative cases that are included in this 2 × 2 table are very strongly biased because the cases negative by Test 1 were positive by Test 2 and *vice versa*-otherwise colposcopy would not have been recommended (a few cases may be negative by both Test 1 and 2, but a biopsy resulted from other clinical evidence, which is not usually consistent with the notion of "screening"[50]). In this fictional example, the vast majority of the 9400 subjects who tested negative by both tests are indeed negative and with less doubt than the highly biased negative cases included in the 2 × 2 table of biopsy results. The only value this "specificity" and "NPV" have is to show them to be higher in one test than the other, but the real magnitude of this difference cannot be estimated from these results. This reviewer believes that authors who perform such analysis should not report these as "specificity" and "NPV" but should find new terms, such as "reduced" or "biopsy biased" specificity and NPV, or better yet, not report them at all as they have no clinical meaning. It is ironic that for this kind of observational study the specificity and NPV are so grossly miscalculated and reported when, in fact, they can usually be quite reliably determined, as discussed later.

Given a 2 × 2 contingency table, the mathematical definitions of sensitivity, specificity, positive and negative predictive values are crystal clear, but the same is not true for their meaning. It is very common to have different definitions of "sensitivity", "specificity" or "predictive" values", even though the formulae by which they are extracted for the 2 × 2 tables are invariant; that is, the meaning depends on the table content. For example, in the world of HPV testing, there is the "analytic sensitivity" which refers to the ability of the test to detect the presence of HPV as distinct from "clinical sensitivity" which refers to the ability of the test to predict the presence of CIN[51-53]; in general, too high analytic HPV sensitivity causes very low clinical specificity. As another example, in the screening mammography program of British Columbia[54], the positive predictive value of a single screening mammogram ranges from about 2% to 20%, depending on the age of the woman, averaging to about 6.5% across all ages. This is the "test PPV". However, the screening program does not send women with a positive screen mammogram to biopsy but instead performs other tests, primarily diagnostic mammograms and/or ultrasound. Of the women actually sent for biopsy, about 33% are found to have cancer or DCIS-this is the "program PPV" and its clinical interpretation is "the probability that a woman sent for biopsy actually has breast cancer."

Excluded data

While a screening test ideally should have both high sensitivity and specificity, high specificity is more critical. A screen test with a sensitivity of 50% and specificity of 95% would be inefficient but could be useful; the conventional Pap test has approximately this performance and it has reduced cervical cancer mortality by 70% in countries where it has been effectively applied. A screen test with a sensitivity of 95% and specificity of 50% would not be useful by itself because half of the screened population would have false positive test results. Depending on the follow-up consequences of a positive screen test result, most screen tests must have a specificity of at least 90% and probably many require specificity > 98%. In many of the papers reviewed here, the specificities are reported as "test" specificities in the abstract and are very low, often 40%-80% and even as low as 2%-3%[55], entirely an artifact of failing to include most of the negative test data. On seeing these low values, those who understand screening might dismiss both Tests 1 and 2 as being completely useless.

These considerations show that the excluded cases that tested negative by both Test 1 and 2 should be included in the contingency tables (the effect of verification bias is discussed later) in order to obtain reasonable estimates of the specificity and NPV.

Screening does not have binary results

A key problem with 2 × 2 tables is the clinical reality that there are not two but three screening test results[56]: positive, negative and equivocal, as shown in Figure 1, and defined as "too atypical to call 'negative' but not enough to call 'positive'." It is common to use time and re-testing to resolve equivocal cases, which is especially important for cervical cancer screening given that most "pre-cancers" resolve without medical intervention, especially in younger women[57-64]. A recent study estimates that < 2% of CIN2/3 lesions progress to cancer within 10 years[65]. At BCCA, ASCUS and LSIL are considered to be equivocal and the management recommendation is for repeat Pap testing at 6 mo intervals up to 4 times; if there is no resolution of the case by then, colposcopy is recommended[50]. The most recent ASCCP management guidelines[66] treat cytology ASCUS and histology CIN1 in all women and LSIL and CIN2 in young women as equivocal, with follow-up intermediate between how negative and positive cases are managed. While there is concern for loss of the patient to follow-up and undue burden on the patient, this must be balanced against the burdens

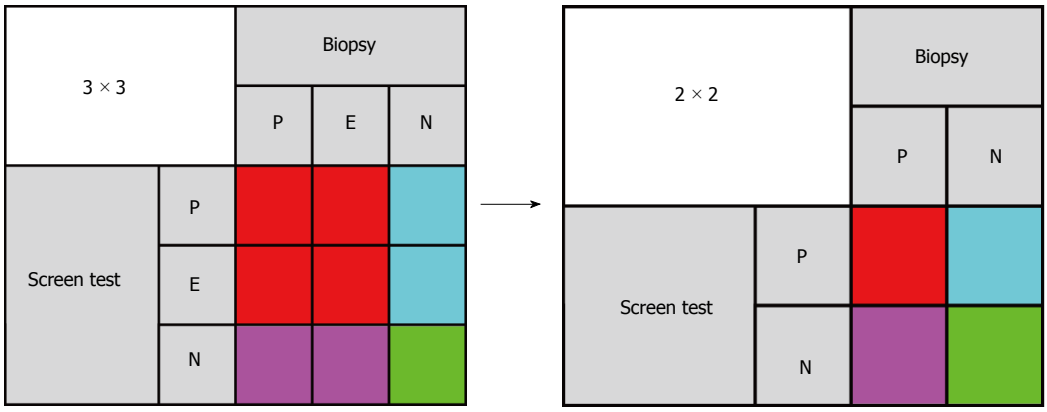

Figure 21 Mapping of 3 × 3 to 2 × 2 tables for sensitivity, specificity and negative predictive value.

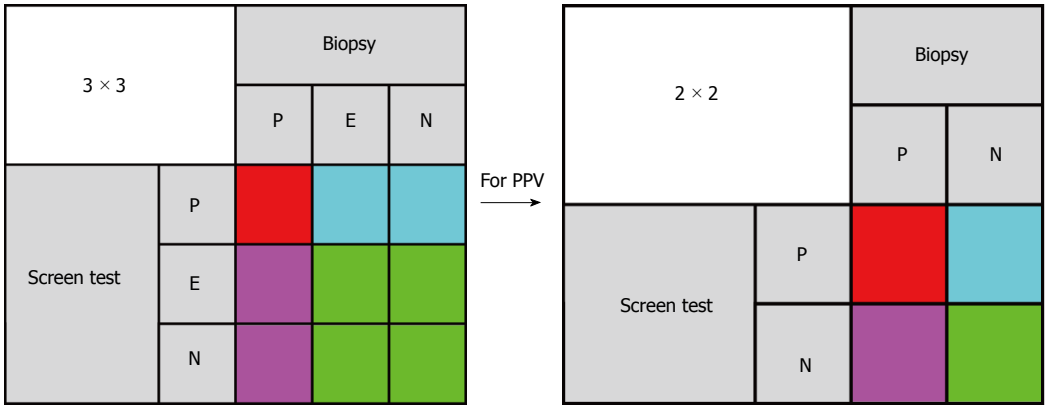

Figure 22 Mapping of 3 × 3 to 2 × 2 tables for positive predictive value.

and possible negative consequences of overtreatment, especially in younger women[67-75]. Various strategies are possible to mitigate the burden of repeat testing.

For DNA Cytometry, most authors follow the recommendation that cases with the detection of 1 or 2 high DI aneuploid cells should be considered "equivocal" while ⩾ 3 are considered positive (Figures 9, 10). Some laboratories also call cases equivocal that have between 5 and 10% proliferation fraction, as shown in Figure 20.

Unfortunately, there are no standard formulae for managing 3 × 3 tables, so some consideration should be given to the clinical meaning of what is calculated and reported. In collapsing the screen Test 1 or 2 and histology "gold standard" 3 × 3 tables to 2 × 2 contingency tables, one possible clinically relevant approach is to set the thresholds between "negative" and "equivocal" for sensitivity, specificity and NPV as shown in Figure 21.

Here the clinical interpretation of the screen test performance is: Sensitivity-the probability that positive gold standard cases had screen test results of positive or at least of equivocal. The notion is that equivocal screen cases cannot be called negative since they do carry a recommendation for follow-up; equivocal screen tests may represent delayed diagnosis cases but not missed diagnosis cases; Specificity-the probability that screen Test 1 negative cases are also screen Test 2 negative and biopsy negative (when biopsy is available) cases; and Negative Predictive Value-its complement, (1-NPV), is the probability that a woman who is told she is disease free is actually not.

For PPV, the collapse of the 3 × 3 to 2 × 2 table follows a different scheme (Figure 22) because its usual clinical interpretation is: Positive Predictive Value-the probability that a patient sent for biopsy will have a positive biopsy result. Women with equivocal screen test results will not generally be recommended for colposcopy and possible biopsy.

A worked example

To illustrate the magnitude of the differences between these different approaches to analysis we work through an example based on data by Bao[76] from 2009, selected because it is one of the larger studies published and because it has reasonably complete reporting of the data. Table 1 shows the overall results for cytology and DNA Cytometry for almost 20000 cases.

The screen test *vs* biopsy results are Table 2 for Cytology and Table 3 for DNA Cytometry.

The 2 × 2 tables used in the publication are shown in Table 4 using CIN2+, LSIL+ and DNA Positive as the thresholds for histology, cytology and DNA Cytometry, respectively.

Table 1 Cytology and DNA Cytometry results from Ref[76]

Cytology[1]		DNA Cytometry	
NILM	18503	Negative	17855
ASCUS	720 (0.40)	Equivocal	1395
LSIL	296 (0.89)	Positive	371
HSIL	59 (1.00)		
Total	19621	Total	19621

[1]Number in parenthesis is the fraction with at least 1 aneuploid cell by DNA Cytometry. NILM: Negative for intraepithelial lesion or malignancy; ASCUS: Atypical squamous cells of undetermined significance; LSIL: Low grade squamous intraepithelial lesion; HSIL: High grade squamous intraepithelial lesion.

Table 2 Biopsy results *vs* Cytology from Ref[76]

		Cytology			
Histology	Total	NILM/inflam	ASCUS	LSIL	HSIL
Ca	15		6	5	4
CIN3	43	5	10	14	14
CIN2	66	12	15	23	16
CIN1	98	42	22	21	13
CC/Neg	401	285	86	18	12
Total	623	344	139	81	59

NILM: Negative for intraepithelial lesion or malignancy; ASCUS: Atypical squamous cells of undetermined significance; LSIL: Low grade squamous intraepithelial lesion; HSIL: High grade squamous intraepithelial lesion; Ca: Invasive cancer; CIN: Cervical intraepithelial neoplasia; CC: Cervicitis.

Table 3 Biopsy results *vs* DNA Cytometry from Ref[76]

		DNA Cytometry		
Histology	Total	Neg	Equiv	Pos
Ca	15		2	13
CIN3	43	1	12	30
CIN2	66	3	19	44
CIN1	98	15	55	28
CC/Neg	401	87	280	34
Total	623	106	368	149

Ca: Invasive cancer; CIN: Cervical intraepithelial neoplasia; CC: Cervicitis.

The test performance results are given in Table 5. The authors did not report the NPV and PPV in their publication. Based on these numbers, the performance of the two tests is quite comparable, although cytology missed 6 of 15 invasive cancers using the LSIL+ threshold while DNA Cytometry missed 2.

Next are 3 × 3 tables that include the previously excluded negative cases, but also with the missing data still deleted (as with the author's analysis above); biopsy results are compared with Cytology in Table 6 and with DNA Cytometry in Table 7. The definitions of Positive, Negative and Equivocal are arbitrary and arguable but used for illustration.

Following the approach outlined above to collapse the 3 × 3 tables to 2 × 2 tables according to the clinical interpretation described above yields new test performance results shown in Table 8.

Table 4 2 × 2 Contingency Tables for Biopsy results *vs* Cytology and *vs* DNA Cytometry from Ref[76]

		Cytology		DNA Cytometry	
Histology	Total	LSIL+	NILM/ASCUS	Pos	Neg/Equiv
CIN2+	124	76	48	87	37
Neg/CIN1	499	64	435	62	437
Total	623	140	483	149	474

NILM: Negative for intraepithelial lesion or malignancy; ASCUS: Atypical squamous cells of undetermined significance; LSIL+: Low grade squamous intraepithelial lesion or higher; CIN: Cervical intraepithelial neoplasia.

Table 5 Cytology and Cytometry test performance based on data and analysis from Ref[76]

	Cytology	DNA Cytometry
Sensitivity	61.3%	70.2%
Specificity	87.2%	87.6%
PPV	54.3%	58.4%
NPV	90.1%	92.2%

PPV: Positive predictive value; NPV: Negative predictive value.

Table 6 3 × 3 Contingency Table for Biopsy results *vs* Cytology from Ref[76]

		Cytology		
Histology	Total	Positive (LSIL+)	Equivocal (ASCUS)	Negative
Positive (CIN2+)	124	76	31	17
Equivocal (CIN1)	98	34	22	42
Negative	18560	30	86	18444
Total	18782	140	139	18503

ASCUS: Atypical squamous cells of undetermined significance; LSIL+: Low grade squamous intraepithelial lesion or higher; CIN2+: Cervical intraepithelial neoplasia, grade 2 or higher; CIN1: Cervical intraepithelial neoplasia, grade 1.

Table 7 3 × 3 Contingency Table for Biopsy results *vs* DNA Cytometry from Ref[76]

		DNA Cytometry		
Histology	Total	Positive	Equivocal	Negative
Positive (CIN2+)	124	87	33	4
Equivocal (CIN1)	98	28	55	15
Negative	18150	34	280	17836
Total	18372	149	368	17855

CIN2+: Cervical intraepithelial neoplasia, grade 2 or higher; CIN1: Cervical intraepithelial neoplasia, grade 1.

The increase in sensitivities is due to considering equivocal cases as positive since they include a recommendation for follow-up. The PPVs are unchanged. The specificities and NPVs are much higher when the negative cases are included and are consistent with the

Table 8 Cytology and Cytometry test performance based on data from Ref[76] and revised analysis

	Cytology	DNA Cytometry
Sensitivity	73.4%	91.4%
Specificity	99.4%	98.3%
PPV	54.3%	58.4%
NPV	99.68%	99.89%

PPV: Positive predictive value; NPV: Negative predictive value.

requirements for screening test performance.

Many authors interpret the high NPVs as being almost equal and so to be uninteresting. In fact, the clinical significance of the NPV is seen from its complement (1-NPV), which relates to "false negative" cases. In this example of 10000 women who are advised that they are disease free, in fact, 32 or 11 are not, based on cytology or DNA Cytometry, respectively. In this regard, the performance is quite different between the two tests. This is especially important when routine screening is infrequent, say once or twice during a woman's lifetime.

An alternative approach to compare the tests is to simply tabulate the results descriptively as in Table 9.

This study was primarily of rural women who had not previously been screened.

The impact of uncorrected verification bias

Most observational screening studies suffer from verification bias because the "gold standard" of colposcopy guided biopsy is only applied to the cases that test positive by the screen test, so the true disease state of those who test negative is not verified. There are statistical methods to correct for this kind of bias (*e.g.*[77]). RCTs sometimes manage this by performing colposcopy and biopsy on a random sample of the negative cases, under proper informed consent and ethics board approval (*e.g.*[78]), although this also has its pitfalls[47].

It may be instructive to look at the impact when this kind of verification bias is left uncorrected. Figure 23 plots the "DNA Cytometry" data of the previous example (Table 8) as a function of the fraction of positive and equivocal cases detected in the study; the left axis is where all positive and equivocal cases were detected and it declines to where only 50% were detected at the right axis. PPV is completely unaffected because it relates only to ratio of positives that the screen correctly identified to the total that it tested positive; hence, any false negatives do not affect this ratio. NPV and specificity decline only slightly over the range plotted. It is the sensitivity, of course, that is potentially most impacted by this kind of verification bias, with an inverse proportional linear dependence; that is, if only half of the positive cases were detected in the study, then the measured sensitivity is double the correct value.

In summary, when this kind of verification bias is left uncorrected it has no impact or almost no impact on predictive values or specificity but it can significantly inflate the measured sensitivities. However, in studies such as those described above, it is likely that well over 90% of the true positive cases were detected by one or both of the screening tests[47,79], so the impact of uncorrected verification bias in this type of study is unlikely to be very large.

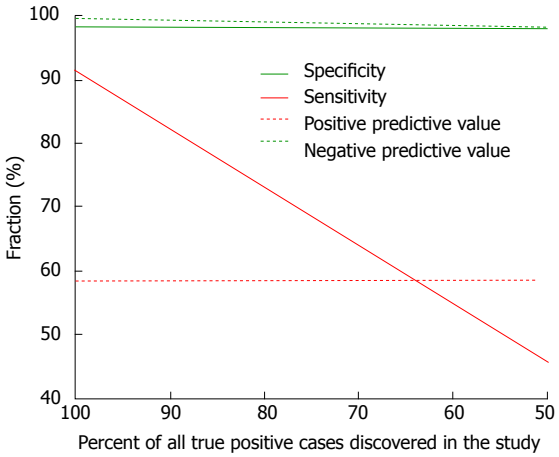

Figure 23 Effect of uncorrected verification bias on test performance indicators.

Unbiased comparisons of test performance

It has been shown that the ratio of (Test 2) : (Test 1) for true positive rate and for false positive rate determined just from the complete biopsy results (with no missing data) does not suffer verification bias of the type discussed above (not subjecting negative cases to the "gold standard" test)[80,81]. This may be enough to rank Test 1 *vs* Test 2.

Table 10[80] summarizes the biopsy data required and the relative true positive rate, *relTPR*, and relative false positive rate, *relFPR*, are simply[81]:

relFPR (Test 2/Test 1) = {(a + b)/[n]}/{(a + c)/[n]} = (a + b)/(a + c)
relFPR (Test 2/Test 1) {(A + B)/[N]}/{(A + C)/[N]} = (A + B)/(A + C)

The key point is that the unknown [n] and [N] are eliminated, leaving only known values from the biopsy result table. If *relTPR* increases and *relFPR* decreases, then Test 2 is easily judged to be the better test, although as Arbyn *et al*[47] points out, such cross-sectional results do not necessarily translate into longitudinal improvement in the screening program. It is more generally the situation that both *relTPR* and *relFPR* increase at the same time, in which case it is necessary to consider the disease prevalence in the target screen population to determine if the cost of increased false positives justifies the benefit of the increased true positive detection. In the special case where the target population and the study population have the same prevalence (this is generally true for observational studies of routine clinical data), then the *FP/TP* ratio is given by: FP/TP = (B-C)/(b-c). This ratio is one "figure of merit" in deciding if Test 2 is better than Test

Table 9 Descriptive summary of the data of Ref [76] as re-analyzed *n* (%)

	Clinical result	Cytology number of cases	DNA Cytometry number of cases	Cytometry-Cytology number (%)
1	Number of women immediately returned to routine screening	18503	17855	-648 (-3.5)
2	Number of CIN1+ cases (false negative cases) per 10000 women returned to routine screening	32	11	-21 (-66)
3	Number of women referred to immediate colposcopy	140	149	9 (6)
4	Number of cases of invasive cancer immediately diagnosed	9	13	4 (44)
5	Number of CIN3+ cases immediately diagnosed	37	43	6 (16)
6	Number of clinically positive cases (CIN2+) immediately diagnosed by colposcopy	76	87	11 (15)
7	Number of women requiring 6 mo follow-up due to equivocal result	139	368	229 (165)
8	Number of women with potentially delayed clinically positive (CIN2+) diagnosis	31	33	2 (6)
9	Number of CIN3+ cases missed	5	1	4 (80)
10	Number of CIN2+ cases missed	17	4	13 (76)

Table 10 Definitions of the biopsy data required for comparison of two screening tests independent of simple verification bias, Ref[80]

	Gold standard positive			Gold standard negative		
	Test 1 +	Test 1-	Total	Test 1 +	Test 1-	Total
Test 2+	a	b	a + b	A	B	A + B
Test 2-	c	[d]	[c + d]	C	[D]	[C + D]
Total	a + c	[b + d]	[n]	A + C	[B + D]	[N]

Variables in square brackets have unknown values.

Table 11 Biopsy data from Ref[82] for simple verification bias free comparison of Cytology *vs* DNA cytometry

	Biopsy +			Biopsy -		
	Cytology +	Cytology -	Total	Cytology +	Cytology -	Total
AQIC+	130	37	167	30	23	53
AQIC-	1	0	1	0	0	0
Total	131	37	168	30	23	53

AQIC+: Positive by automated quantitative image cytometry; AQIC-: Negative by automated quantitative image cytometry.

1 and comes down to the judgment of "how many extra false positive cases are acceptable for each additional true positive case detected?" The value of this ratio will depend upon the undiagnosed disease prevalence.

Exactly the same results are obtained by inspection of the appropriate 2 × 2 tables for each test *vs* the biopsy results; both calculation methods are illustrated next.

Tian *et al*[82], provided the case by case data for that paper to allow illustration of the method here. The first look at this is based on "positive" defined as ASCUS+, Equivocal+ and CIN1+ for cytology, DNA Cytometry (AQIC) and pathology, respectively, summarized in Table 11. The *relTPR* = 1.3 and *relFPR* = 1.8, both increasing as expected. However, the *FP/TP* ratio is just 0.64, meaning that for every extra true positive detected by AQIC, 2/3 additional false positive cases occur, which is probably an acceptable trade-off.

The same result is obtained from the published[82] 2 × 2 tables, using the same thresholds for positive, as shown in Table 12. By inspection *relTPR* = 167/131 = 1.3 and *relFPR* = 53/30 = 1.8 and *FP/TP* = (53-30)/(167-131) = 23/36 = 0.64.

These ratios may provide a useful, unbiased "figure of merit" with which to compare one test to another. The key message is that these ratios-the relative true positive rate, *relTPR*, relative false positive rate, *relFPR* and *FP/TP*-are not subject to verification bias of the type due to failing to perform the "gold standard" reference test on the large number for cases that test negative by both Test 1 and Test 2. However, they may be subject to bias due to missing data, discussed next.

Missing data

But what about the missing data-the cases recommended for colposcopy for whom there is no follow-up information? This is a particular kind of verification bias and there are formal methods to correct for it (for example[77,83-87]) that involve using statistical methods to impute the missing data with minimal bias, but they are beyond the scope of this paper. The simplest method is to delete these cases from the analysis, as was done in the example above and in all of the DNA Cytometry papers reviewed here. This is approximately equivalent to assuming that the missing data is the same as the known data. Because the missing data is all from cases that tested positive by either or both Tests 1 and 2, this has no effect on the NPV, for reasons analogous to why the PPV was unaffected in Figure 23. There is no simple, general statement one can make about the impact of the missing data on the sensitivity, specificity or PPV except that the range of possible deviation between the uncorrected measurements and the true values is proportional to the amount of missing data. Each of the three performance measures could rise, fall or stay the same if the missing data were not missing. The key message is that it is extremely important to organize studies so as to minimize the amount of missing data. In the Bao paper, they did not report the total number of colposcopy follow up recommendations but, depending on the correlations between cytology and cytometry, at least 65% of the biopsy follow up data is missing, which is very typical for this series of papers from China.

Table 12 Alternative presentation of Biopsy data from Ref[82] for simple verification bias free comparison of Cytology *vs* DNA Cytometry

	Cytology				DNA Cytometry		
Histology	Total	ASCUS+	NILM	Histology	Total	Pos/equiv	Neg
CIN1+	168	131	37	CIN1+	127	167	1
Neg	53	30	23	Neg	121	53	0
Total	221	161	60	Total	221	220	1

NILM: Negative for intraepithelial lesion or malignancy; ASCUS: Atypical squamous cells of undetermined significance; CIN1+: Cervical intraepithelial neoplasia, grade 1 or higher.

Table 13 The test performance limits for the combination of two binary valued tests

	Sensitivity		Specificity	
	Minimum	Maximum	Minimum	Maximum
Logical "AND"	$(Se_1 + Se_2) - 1^1$	Min (Se_1, Se_2)	Max (Sp_1, Sp_2)	$(Sp_1 + Sp_2)^3$
Logical "OR"	Max (Se_1, Se_2)	$(Se_1 + Se_2)^4$	$(Sp_1 + Sp_2) - 1^2$	Min (Sp_1, Sp_2)

[1]0% if $(Se_1 + Se_2) \leqslant 100\%$; [2]0% if $(Sp_1 + Sp_2) \leqslant 100\%$; [3]100% if $(Sp_1 + Sp_2) \geqslant 100\%$; [4]100% if $(Se_1 + Se_2) \geqslant 100\%$. Se: Sensitivity; Sp: Specificity.

Effects of an imperfect "gold standard" reference

The "gold standard" reference diagnosis for cervical cancer studies is usually colposcopy directed biopsy, which a number of studies over the past decade have shown to be imperfect[88-92]. More worrying were studies that showed that the measured sensitivity of Visual Inspection with Acetic Acid (VIA) were substantially inflated (by 1/6 to 1/3) due to use of colposcopy directed biopsy as the "gold standard' reference[93-95]. Theoretical studies[96] of such use of imperfect references have shown that if the screen and reference test errors are statistically correlated, then the test accuracy (sensitivity and specificity) measurements are over-estimated; conversely, if the screen and reference test errors are statistically independent, then the test accuracy measurements are underestimated. VIA is essentially a variation of colposcopy and so is highly correlated.

It has also become clear over the past decade that colposcopy sensitivity improves the more biopsies that are taken[88,93,95,97,98], independent of the skill[99] or medical training[90] of the person performing the colposcopy, ranging from nurse-practitioner to gynecological oncologists. Many propose supplementing any lesion biopsies with a random biopsy from any quadrant without visible lesions[97,99]. However, while this increases the rate of detection of CIN2+, it does not appear to affect the measured sensitivity of cytology, although it may improve the specificity slightly[94]. It is unknown if taking more biopsies has any effect on the DNA Cytometry accuracy measurements but one might expect any effect to be small based on the cytology situation.

Combining binary test results

A great many papers, including several reviewed here, look at combining the results of two binary (or dichotomous) tests with the hope that the combined test result is an improvement over either of the component Tests 1 and 2. This is also applied to tests like Hybrid Capture 2 (HC2) HPV test that measures continuously valued viral load but which is made binary by applying a threshold, $\geqslant$ 1 RLU/Cutoff is positive, otherwise it is negative[100].

It can be proven that there are only two non-trivial ways to combine 2 binary tests: as the logical "and" of positive cases, in which the result is positive only if both Tests 1 and 2 are positive, and as the logical "or" of positive cases, in which the result is positive if either or both Tests 1 and 2 are positive. It is also possible to prove that the combined test results will conform to the limits in Table 13, where "Min" and "Max" are the minimum or maximum of the two entries in parenthesis and where Sp = specificity and Se = sensitivity.

For the logical "and" of positive results, the specificity of the combined test will be at least as high as the specificity of the most specific component test and could be 100%; however, the sensitivity will be no better than the sensitivity of the least sensitive component test and could be zero.

Conversely, for the logical "or" of positive results, the sensitivity of the combined test will be at least as high as the sensitivity of the most sensitive component test and could be 100%; however, the specificity will be no better than the specificity of the least specific component test and could be zero.

Examples of "and" and "or" combinations of binary tests and their compliance to these limits can be found in refs[101-103].

Furthermore, it can also be proven that the result of combining these tests does not depend on the test order (they are commutative).

In either combination, it is not necessary to perform both tests on all subjects[104]. For the logical "and" of positive test results, if the first test result is negative then the combined test result will also be negative, regardless of the second test result; similarly, for the logical "or" of positive test results, if the first test result is positive, it does not matter what the second test result is, the combined test result will be positive. This, plus the fact that the test order does not affect the results, means that a test strategy can be adapted to minimize costs in clinical practice (both must be done for comparative performance studies, of course).

There is one caveat with these rules: they apply for analytical tests that are independent in the sense that one test does not impact the other. For example, if one test compromised the sample for the other test, these rules might not apply. It is not so clear that cytology is a test that is independent of DNA Cytometry or HPV status, if the cytologist is aware of the DNA Cytometry or HPV results. Since most of the papers reviewed here are observational studies looking at both tests in routine use, it would be expected that the cytologist would, in general,

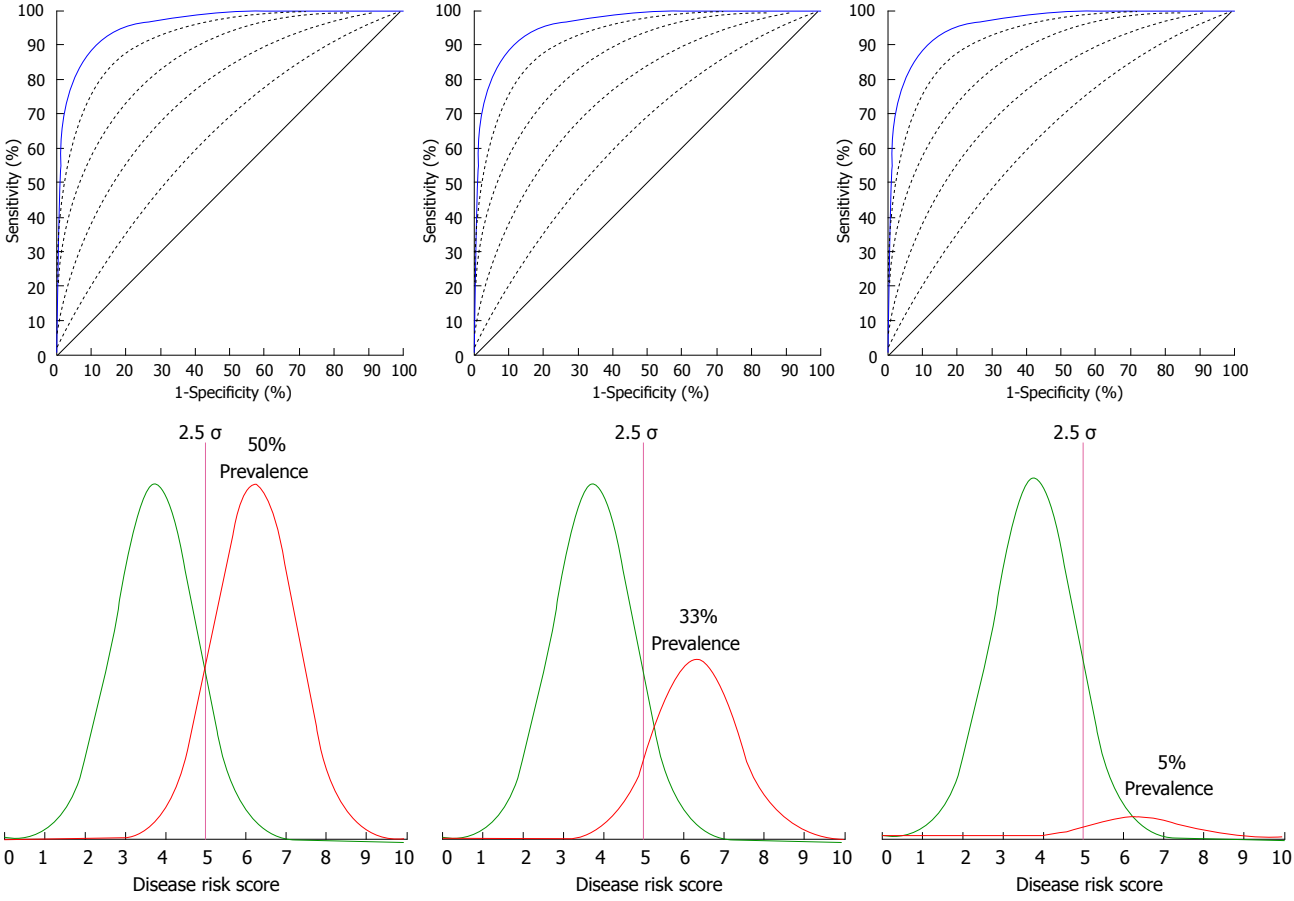

Figure 24 Sensitivity and specificity are independent of disease prevalence when the change in prevalence is only a scalar multiplier that does not change position, width or shape of the population distribution.

be aware of the results of the other tests since the goal is to get the correct answer for the patient, not to do a strict clinical study.

It does not seem to be appreciated by many authors that the limits in Table 13 seriously constrain any potential benefits from combining binary tests. Since one of sensitivity and specificity will rise as the other falls when the tests are combined, it usually only makes sense to combine tests that have very similar and high values for the one that will fall, so that it does not fall much and stays in range of what is useful. For example, cytology has poor sensitivity, but excellent specificity; hrHPV testing has excellent sensitivity, but only good specificity. Combining these tests will result in either excellent sensitivity and less than good specificity, or excellent specificity and poor sensitivity. There is no simple combination of HPV testing and cytology that works well. The work around for this until a better test or test combination is found is in the patient management algorithms, which are becoming increasingly complex[66]. These algorithms manage "equivocal" cases with a combination of time and re-testing, in recognition that screen tests, even in combination, have 3 outcomes, not 2, as already discussed.

Impact of disease prevalence on estimates of test performance

A key issue in appreciating published study results is to determine their degree of generalizability. A basic concept of epidemiology is that while predictive values explicitly depend strongly on disease prevalence, sensitivity and specificity are completely independent of it[105]. Although this prevalence independence of sensitivity and specificity is certainly true, it is a fragile truth that may not survive test generalization. That is, if a published study demonstrates that Test A has sensitivity X and specificity Y with a particular disease prevalence Z, it does not mean that when applied to a different population with a disease prevalence of *1.5 x* Z, Test A will have the same sensitivity and specificity found in the study, except if the mean, width and shape of the underlying distributions of positive and negative populations is also the same as in the study. Figure 24 illustrates ROC curves, which should be independent of disease prevalence since they essentially plot sensitivity *vs* specificity, for the case where the mean, width and shape of both positive and negative populations remains the same and only the positive distribution is multiplied by a scalar to reduce the distribution area without distortion or shifting of the peak position. As expected, the change in prevalence has no effect on the ROC curve or sensitivity and specificity, indicated by the blue line.

However, as a practical matter, "real life" changes in disease prevalence rarely occur this way. If the prevalence increases, and especially if it is a large increase, it also

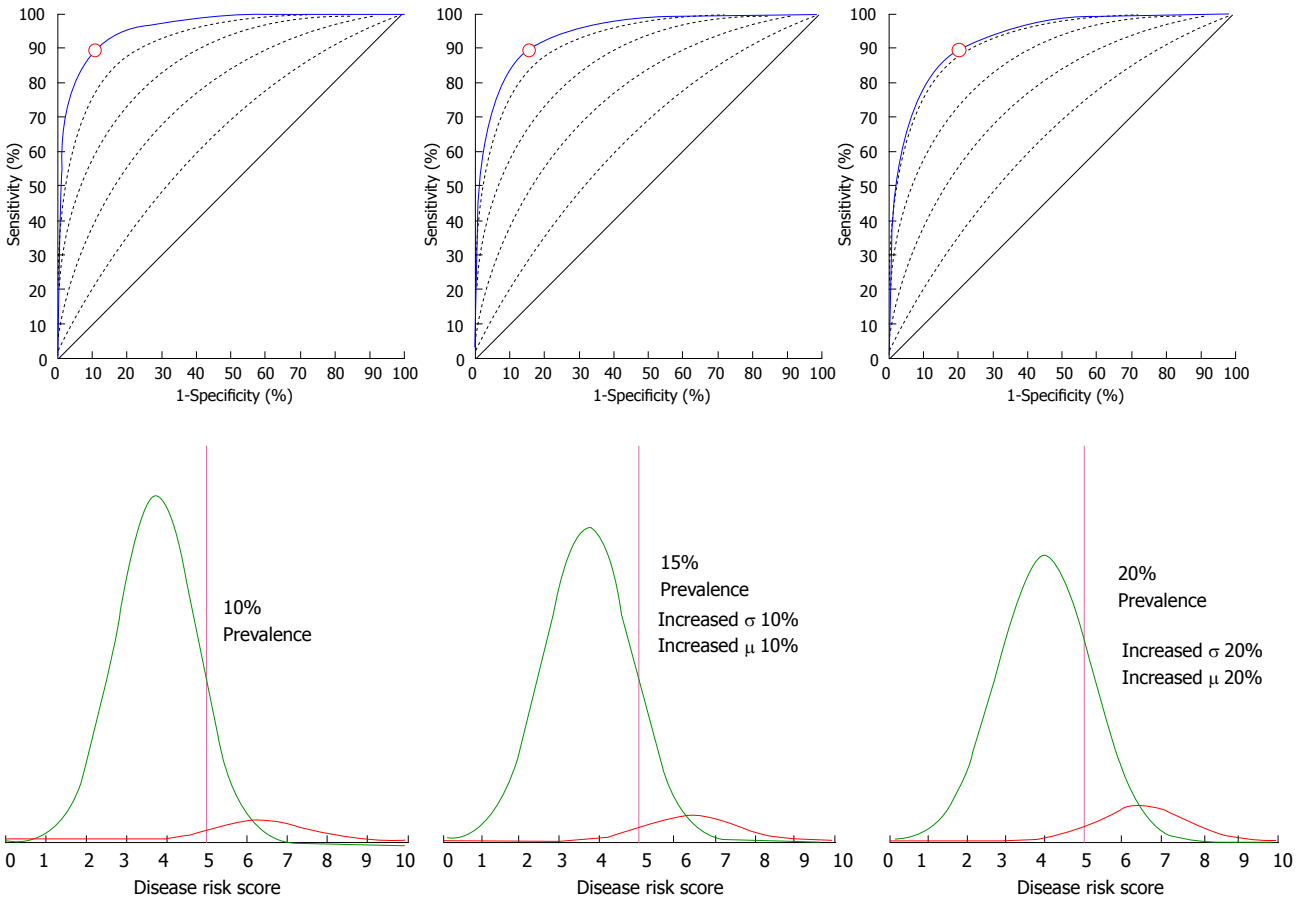

Figure 25 "Real life" changes in disease prevalence are usually accompanied by changes in both positive and negative population distribution positions, widths and shapes which do change the sensitivity and specificity.

probably causes changes in the mean, width and overall shape of both positive and negative distributions as the whole population is somewhat sicker, as illustrated in Figure 25. In scanning the figure from left to right, the prevalence increases, but the positions (μ) and widths (σ) of both distributions also increases. In this example, both distribution means (μ) shifted to the right the same amount-this would not cause any change in the ROC curve itself-the change in the ROC curve is caused by the change in widths (σ) of the distributions. The magenta line represents the Test threshold and the red point on the ROC curve corresponds to test performance at that threshold. Unless the test threshold is adjusted by re-calibrating the test to the new populations, then the test performance moves from optimal, as indicated by the shift of this operating point. This figure is based on symmetric analytic distributions for ease of calculation and drawing-there is no requirement that the underlying distributions for ROC curves are symmetric, analytic or even single peaked; hence, "real life" shifts in disease prevalence may cause different shifts in test performance than the simple ones illustrated here.

This result is technology independent and will apply not just to AQIC but also to hrHPV testing, for example. One situation where there is a large difference in undiagnosed disease prevalence is when a test is applied for screening (testing people without symptoms) *vs* diagnosis (testing people with positive screen test results or with symptoms). Several recent publications have experimentally verified this for colposcopy[106], hrHPV testing[107], the conventional Pap test[108], as well as a recent review of several meta-analyses of various diseases[109]. The previous discussion of BCCA screening mammography *vs* diagnostic mammography is another example. Many physicians erroneously think that test sensitivity and specificity are invariant properties of the test and therefore independent of whom the test is applied to; this is not necessarily the case.

Although it is well understood that predictive values explicitly depend upon undiagnosed disease prevalence, it is less widely appreciated that this imposes significant limits on the positive predictive value. Why is the PPV not 100%? For a low prevalence disease, such as cancer in a screening setting, PPV is determined mostly by the false positives (or 1- specificity); PPV is only weakly dependent on sensitivity. Figure 26 shows the maximum possible PPV (under the condition that sensitivity is 100%) as a function of undiagnosed disease prevalence for various very high values of specificity. Even at a disease prevalence of 1% (1000/100000 persons) and with a test specificity of 99%, the PPV will only be 50%; if the test specificity falls to 98%, the PPV drops to about 33%.

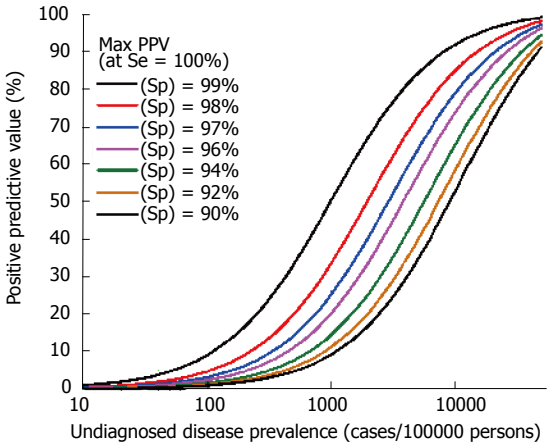

Figure 26 Positive predictive value at 100% test sensitivity as a function of undiagnosed disease prevalence for various high values of test specificity.

This is another demonstration of the crucial importance of high specificity for screening tests. It also illustrates that cancer screening is more than a test-it is a process by which negative cases are progressively removed from the population under surveillance until the disease prevalence of the remainder is increased to levels that allow acceptably high PPVs.

In summary, undiagnosed disease prevalence has a big impact on screening test performance, firstly by affecting the real world generalizability of sensitivity and specificity and secondly by limiting the maximum possible test PPV.

The large Table 14 summarizes the observed crude disease prevalence in several automated quantitative image cytometry studies from China from 2005-2013. The studies were selected as having the word "screening" in the title, abstract or introduction; studies with only the word "diagnosis" were excluded. As previously discussed, there is often substantial "missing" biopsy result data in studies from China. The column "follow-up ratio" is an estimate made by this reviewer of the extent to which high grade cytology had corresponding biopsy data-the blank entries indicate that it was not possible to estimate this. With few exceptions, the listed disease prevalences are underestimated, in many cases by a factor of 2 or more. The heterogeneity of the results is quite alarming and certainly justifies the warnings just made concerning generalizing the study test results for sensitivity and specificity. The very high disease prevalence in some studies that are claimed to be "screening" is quite vexing.

PUBLICATIONS ON THE APPLICATION OF DNA PLOIDY TO CERVICAL CANCER SCREENING

Since 2005, the use of AQIC testing for cervical and other cancers has continuously expanded to about 1 million tests per year in 2013. At least 60 publications on various comparisons for AQIC performance to other means of screening or diagnosing cervical cancer have appeared since 2005, mostly in Chinese language journals. This section reviews some of these papers.

Disease prevalence

Perhaps a good starting point is Table 14 on measured crude prevalence rates of invasive cervical cancer and CIN. As shown previously, disease prevalence is a very important epidemiological quantity that impacts the performance and deployment of tests, especially screening tests. However, prevalence is very difficult to measure and there are two very different flavors of prevalence that can cause confusion. Some cancer monitoring agencies report cancer prevalence meaning as "How many people diagnosed with cancer are alive today?" These are estimated either as "limited-duration" prevalence (limited to cases diagnosed over, say, the past 10 years)[143] or "complete" prevalence which is independent of when the cancer was diagnosed, as is done by SEER[144]. Neither of these is relevant to cancer screening which is focused on and influenced by "undiagnosed" cancer prevalence. From here on, the term "prevalence" will be understood to mean "undiagnosed prevalence," unless explicitly modified.

If the cancer is undiagnosed, how do we know how much there is? In a well screened population, the disease prevalence is less important than it is in poorly screened populations. An effective screening program will detect the "prevalent" cases of cancer within several years of its introduction (depending on the screening program effectiveness and deployment details) and will remove them from the pool of undiagnosed cancers, leaving mostly what are known as new "incident" cases, approximately equal to the incident rate for that cancer among that population times the screening interval: *P* (cases/100000 people) is approximately equal to I (cases/100000 people/year) × Screen Interval (years). In an unscreened population, the introduction of screening is usually the time when the disease prevalence can best be estimated by measurement; otherwise, it can only be estimated from cancer natural history and population models. In the absence of screening, cancer is diagnosed either by investigation of symptoms or incidentally during some other medical investigation. There is usually a delay between the onset of symptoms and diagnosis because the patient, physician or both ignore the symptoms for some time, as they are rarely specific. A crude "back of the envelope" estimate of undiagnosed invasive cancer in an unscreened population would be the incidence rate times the delay between invasion and onset of threshold symptoms-that is, symptoms strong enough to lead to diagnostic investigation: P (cases/100000 people) is approximately equal to I (cases/100000 people/year) × Symptom Threshold Interval (years). What is the incidence rate of cervical cancer in China? The official rate from the 2004-2005 "Third National Survey" is 3.2/100000/year and the mortality is 2.4/100000/year, age standardized to the world population[145]. If it takes on average 10 years between the onset on invasive cervical cancer and the onset of symptoms significant enough to seek

Table 14 Crude cancer prevalence and other data from 36 published DNA Cytometry studies from China, 2005-2013

Ref.	Invasive cancer cases	CIN2+ cases	Total screened	Crude cancer prevalence (per 100000 persons)	Crude CIN2+ prevalence (%)	Follow-up ratio	Ca/CIN2+ (× 10)
[110]	3	14	500	600	2.8		2.14
[82]	7	100	23698	29.5	0.42	0.38	0.7
[111]	48	103	4020	1194	2.56	0.78	4.66
[112]	1	22	1200	83.3	1.83		0.45
[113]	2	17	673	297.2	2.53		1.18
[114]	4	12	3551	112.6	0.34		3.33
[115]	2	15	3162	63.3	0.47	0.39	1.33
[116]	14	78	4109	340.7	1.9	0.93	1.79
[117]	4	84	9261	43.2	0.91	0.36	0.48
[118]	2	30	2599	77	1.15		0.67
[119]	3	65	1200	250	5.42		0.46
[120]	9	111	6793	132.5	1.63	1	0.81
[121]	0	16	2003	0	0.8		0
[122]	4	87	2153	185.8	4.04		0.46
[123]	3	56	12079	24.8	0.46		0.54
[124]	5	26	3000	166.7	0.87		1.92
[125]	8	142	5886	135.9	2.41	1	0.56
[126]	11	147	7735	142.2	1.9	1	0.75
[127]	1	41	4598	21.7	0.89		0.24
[128]	4	34	12278	32.6	0.28		1.18
[129]	2	11	3589	55.7	0.31		1.82
[130]	3	17	1806	166.1	0.94		1.76
[131]	0	10	1206	0	0.83		0
[132]	8	51	3603	222	1.42	1	1.57
[133]	106	168	23993	441.8	0.7		6.31
[134]	6	36	1220	491.8	2.95		1.67
[76]	15	124	19621	76.4	0.63		1.21
[55]	2	21	3070	65.1	0.68		0.95
[135]	1	15	2000	50	0.75		0.67
[136]	0	5	1256	0	0.4		0
[137]	30	172	18097	165.8	0.95		1.74
[138]	1	7	451	221.7	1.55		1.43
[139]	10	53	430	2325.6	12.33		1.89
[140]	6	12	2832	211.9	0.42		5
[141]	15	95	8670	173	1.1		1.58
[142]	30	187	22169	135.3	0.84		1.6

CIN2+: Cervical intraepithelial neoplasia, grade 2 or higher.

medical attention, then this would predict invasive cervical cancer prevalence of 30-60/100000, allowing for differences in age standardized rates. This is a factor 10-40 less than what is reported in several entries in Table 14.

Although the Chinese registries consistently report such cervical cancer incidence rates[146-150] and the international reports (based on the same registries) only slightly inflate them presumably due to different age standardization[151,152], there are good reasons for skepticism, including the fact that the registries span only 2%-6% of the population of China[145]. Pooled and population based studies have shown prevalence rates of CIN2+ and hrHPV infection to be similar to or higher than in other Asian and south Asian countries with much higher cervical cancer incidence rates[145,153-156]. Although the official mortality rate is slightly higher than in countries like the United States, United Kingdom or Canada with long established, effective cervical cancer screening programs, the official incidence rate in China is only 1/3-1/2 of that in these countries, even though a great many women in China have never been screened[145,157]. In Hong Kong, opportunistic cervical screening was introduced in 1970 at which time the age standardized incidence rate was measured to be 25-35/100000/year[158] and even today, with a comprehensive cervical cancer screening program, has a crude rate of 9.7 and age standardized rate 6.9/100000/year[159]. One of the requirements of screening is that the disease must be common enough to justify the expenditure of resources and it has been suggested that a crude rate of about 3/100000/year is a reasonable threshold[160]; below this incidence rate, screening is not justified. This is likely not the situation with cervical cancer in China, but only if cervical cancer is being seriously under-reported by the registries, putting the official incidence rate in serious error. There is a clear need to resolve if the surprisingly low official incidence rate for cervical cancer in China is correct or not.

Two pooled population based analyses[154,161] found crude prevalence for invasive cervical cancer of 170/100000 and for CIN2+ of 2.6% for combined rural and urban populations not screened within the past 5 years, including a significant proportion of women who had never been

screened. These data are consistent with many of those in Table 14.

In Table 14, in which most of the prevalences are lower limits due to missing biopsy follow-up data, 8 of 36 studies found invasive cancer prevalence > 250/100000; these tended to be small hospital-based studies that were possibly contaminated by diagnosis cases. In the author's experience in China, the Pap test is widely used for diagnosis rather than for screening, even though there is evidence that samples from symptomatic women are often unsatisfactory due to blood and other reasons and have a higher false negative rate than from asymptomatic women[39]. As previously discussed, test performance can vary considerably due to differences in disease prevalence in the population tested. Laboratories that routinely use the automated quantitative image cytometry method for differential diagnosis may require re-calibration of the operating point for their instrument.

Comparisons of AQIC with LBC

The diffusion of the automated quantitative image cytometry technology in China has primarily been through existing pathology laboratories which are generally hospital based. Although the cytometers of the various vendors have regulatory approvals[162,163] and endorsement by various medical societies and expert groups, it is natural that each lab would compare performance with existing conventional liquid-based cytology. Consequently, most of the publications from China are cross-sectional, observational studies from routine clinical practice in which both liquid based conventional cytology and AQIC are done on the same sample and the test performances compared, as detailed previously. Most of these studies found substantially increased sensitivity (up to a factor of 2)[76,82,111,112,117,119,120,125,126,129-131,133,134,136,137,140-142], although some found essentially equal[55,115,116,118,120,121,124,132,135,138,139] for AQIC at a slight loss of specificity compared to conventional LBC. These reports are not summarized in a table due to the great variability in analyses and reporting as outlined previously. There would be great benefit if future publications followed a more clinically meaningful standard analysis and reporting.

One RCT conducted in China spanning some 23000 women was published and claimed substantial superiority in sensitivity and PPV comparing AQIC to conventional LBC[133]. Unfortunately, this reviewer cannot understand the analysis done in this paper[164] and cannot even say with confidence how many cases of cancer were detected in the study, even following clarification[165]. The study also had some disturbing longitudinal results that were not explained. On face value, the claims made in the publication of this trial are consistent with those of the observational studies listed above.

These papers, to varying degrees, speak to three intertwined issues: (1) that AQIC is simple and effective and could be applied in low resource settings where skilled cytologists are in short supply; (2) that AQIC is a good "second opinion" or "adjunct" to add on to the conventional LBC Pap test; or (3) that the combined test (AQIC positive or LBC cytology positive = case positive) is best because of the increased sensitivity. To this reviewer's knowledge, issue (1) has really not been deployed in practice in China, although there seems to be a consensus belief that it would work well; most AQIC in China is deployed in hospitals with trained cytologists. Issue (3) is connected to issue (2) but generally ignores the performance cost in combined test specificity and, as previously discussed in detail, most of these papers incorrectly calculate the test specificity. Also as discussed previously, combination tests have the potential to work best when both have very high and very similar values for, say, specificity, so that the combination will greatly improve the sensitivity but only negligibly reduce specificity, or *vice versa*. This is generally not the case for conventional cytology and AQIC, so it is more likely that it is optimal to only use the best test.

Is it necessary to do both conventional cytology and automated quantitative image cytometry? First, here is some evidence that it is not necessary to do both. The biopsy data of Tian *et al*[82] was previously used to illustrate the unbiased method to compare two tests[80,81] and demonstrated that AQIC compared to conventional LBC increased the true positive (TP) count at the cost of less than 1 false positive (FP) case each, which is excellent for screening. When both tests are combined as the "or" of positive results, depending on what thresholds are used, the number of TPs increased by at most 1 case (a CIN1) while the number of increased FPs range from 0-19, indicating that even the combination test provides no added value. Similarly, an earlier paper by Yu *et al*[126] spanning almost 8000 cases, found that conventional LBC found one case (CIN2) missed by AQIC, but AQIC found 29 cases of CIN2+ missed by cytology using the ASCUS+ threshold. As expected, combining the two tests also added nothing.

The results of other papers show that doing both tests is beneficial. For example, the paper by Bao[76], already discussed, spanned almost 20000 women and conventional LBC found 4 cases (1 CIN3 and 3 CIN2) missed by AQIC, a marginal improvement. This comes down to a question of resource availability. As mentioned in the introduction, the conclusion of our 2005 paper[1] was that AQIC made it possible to do large scale screening inexpensively and accurately, even when skilled cytologists are not available. In that situation, doing conventional LBC is not an option.

However, when conventional LBC is an option and both tests can be performed, then the choice comes down to a mixture of the medical consequences of marginally improved sensitivity at the cost of worse specificity, combined with the business consequences, such as customer perception of value, marketing, cost, price, profit, acceptability to the payer, and so on. One Chinese vendor[166] has developed a cytometer designed to scan counterstained slides, so that the blue thionin/counterstain amounts to a fake Papanicolaou stain. This cytometer conceptually scans the slides twice-once with

Table 15 Biopsy data for DNA Cytometry and hrHPV test results for 294 cases of Cytology ASCUS, from Ref[169]

	hrHPV	Histology		
		Neg	CIN1	CIN2+
hrHPV Pos	216			
Ploidy Neg		90	10	1
Ploidy Equ		22	25	8
Ploidy Pos		5	12	43
hrHPV Neg	78			
Ploidy Neg		35	0	1
Ploidy Equ		12	0	0
Ploidy Pos		14	16	0

hrHPV: High risk HPV; CIN2+: Cervical intraepithelial neoplasia, grade 2 or higher; CIN1: Cervical intraepithelial neoplasia, grade 1.

the optics set to minimize the visibility and interference of the counterstain to allow reasonably good DNA measurement, and once in full color for qualitative visual assessment by the reviewer. This system, in principle, has significant advantages over, for example, Hologic's ThinPrep Imager which uses a similar approach[167] to staining and imaging slides but fails to present any DNA quantitative information to the cytologist, operating instead only as qualitative assessment aid.

In summary, in most settings in China, automated quantitative image cytometry provides substantial increase in sensitivity and relatively slightly decreased specificity compared to conventional LBC. However, most labs that offer AQIC also perform conventional cytology on the same liquid based sample with the consequence of an incremental increase in sensitivity accompanied by an incremental decrease in specificity.

AQIC, cytology and hrHPV testing

Management of women with ASCUS, an equivocal cytology result and the most common non-negative result, has always been problematic. The ASCCP guidelines of 2006[168] for the management of women with ASCUS cervical cancer screening tests called for: (1) immediate colposcopy; or (2) repeat cytology testing at 6 mo intervals until two consecutive negative follow-up Pap tests; or (3) "reflex" hrHPV testing, in which case women with hrHPV positive result are managed as if they have LSIL, or with hrHPV negative result have repeat cytology testing in 1 year.

However, these guidelines were revised in 2012[66]. The new guidelines for management of women aged ⩾ 25 with ASCUS cytology are: (1) immediate colposcopy is not recommended for women of any age; (2) hrHPV testing is preferred, in which case women: (a) with hrHPV positive result are managed as if they have LSIL, or (b) with hrHPV negative result are repeat co-tested (cytology and hrHPV) in 3 years; or (3) repeat cytology testing (not preferred) in one year, and (a) if the cytology is NILM, return to routine screening interval (3 years), or (b) if the repeat cytology is ASCUS+, attend colposcopy.

For women aged 21-24 with ASCUS, the management is much more conservative: (1) immediate colposcopy is not recommended for women of any age; (2) repeat cytology testing in one year is preferred, and (a) if the first repeat cytology is ASC-H+, attend colposcopy, but (b) if the first repeat cytology is LSIL, ASCUS or NILM, repeat cytology in 1 year, and (i) if the 2nd repeat cytology is ASCUS+, attend colposcopy, or (ii) return to routine screening interval (3 years) when 2 consecutive annual repeat cytology test results are NILM; (3) hrHPV testing is not preferred, but women; (a) with hrHPV positive, repeat cytology in 1 year-immediate colposcopy or repeat hrHPV testing are not recommended, and (b) with hrHPV negative result are returned to the routine screening (cytology only) interval of 3 years.

A recent cross-sectional study by Zhang *et al*[169] looked at DNA ploidy in 875 cases of ASCUS who had biopsies (of which 157 were CIN2+) and a subset of 294 of these cases (53 of which were CIN2+) were also tested for hrHPV. The raw results of the 294 cases are given in Table 15.

The performance indicators (prefaced with "b" to indicate "biopsy") for DNA ploidy for the whole data set and for hrHPV, DNA ploidy and hrHPV combined with DNA ploidy as an "and" of positive test results are given in Table 16 (the combined test result was not included in the Zhang paper). In general, DNA ploidy had better specificity and hence better PPV than hrHPV testing and both had similar NPV complement. As the authors concluded, AQIC is as effective as hrHPV testing for managing ASCUS patients, while being cheaper and easier to use.

As discussed previously, for the logical "and" of positive test results the specificity of the combined test will be at least as high as the specificity of the most specific component test and could be 100%; however, the sensitivity will be no better than the sensitivity of the least sensitive component test and could be zero. In fact, the combined test sensitivity did not fall below the least sensitive test which indicates that for detecting positive cases, DNA ploidy and HPV testing are very highly correlated. This is also an example of why it is generally the case that combining binary tests only works well if both have similarly high values for one performance indicator (either sensitivity or specificity); the idea is that this high valued performance indicator will drop only minimally when the tests are combined, while the lower valued other performance indicator will rise substantially. That is exactly what happened in this example. For reference, the PPV of cytology for all 875 ASCUS cases is only 18%.

If it is accepted that management of ASCUS will inevitably involve some equivocal cases who must be monitored over time, similar to the ASCCP guidelines above, then the 3 × 3 table analysis previously discussed could be applied. In this situation, only the "biopsy Positive Predictive Values" (bPPV) are revised upwards for each test, as shown in Table 17.

In the case of the combined test: (1) 60 cases would be identified as positive of which 43 will have CIN2+,

Table 16 Test performance indicators for DNA Cytometry, hrHPV tests and combined DNA Cytometry and hrHPV tests, for Cytology ASCUS cases, Ref[169]

	875 Cases (157 CIN2+)	294 Cases (53 CIN2+)		
Performance indicator for CIN2+ (%)	DNA Ploidy	hrHPV	DNA Ploidy	DNA Ploidy and hrHPV
bSensitivity	98.8	98.1	96.2	96.2
bSpecificity	47.5	32.0	56.0	73.4
bPPV	29.1	24.1	32.5	44.3
bNPV	99.4	98.7	98.5	98.9

hrHPV: High risk HPV; CIN2+: Cervical intraepithelial neoplasia, grade 2 or higher; PPV: Positive predictive value; NPV: Negative predictive value. Prefix "b": From among biopsy cases only.

Table 17 Test performance indicators following re-analysis of the data of Table 16 from Ref[169]

	875 Cases (157 CIN2+)	294 Cases (53 CIN2+)		
Performance indicator for CIN2+ (%)	DNA Ploidy	hrHPV	DNA Ploidy	DNA Ploidy and hrHPV
bSensitivity	98.8	98.1	96.2	96.2
bSpecificity	47.5	32.0	56.0	73.4
bPPV	40.8	24.1	47.8	71.7
bNPV	99.4	98.7	98.5	98.9

hrHPV: High risk HPV; CIN2+: Cervical intraepithelial neoplasia, grade 2 or higher; PPV: Positive predictive value; NPV: Negative predictive value. Prefix "b": From among biopsy cases only.

including 100% of the cancer cases, 88% of the CIN3 cases and 70% of the CIN2 cases, (2) 179 cases would be identified as negative, of which 2 would have CIN2+, and (3) 55 cases would be identified as being equivocal of which 8 would have CIN2+.

For comparison, if the hrHPV test was not done, the 3 state analysis outcomes would be: (1) 90 cases would be identified as positive of which 43 will have CIN2+, exactly as in the combined case above, (2) 137 cases would be identified as negative, of which 2 would have CIN2+, and (3) 67 cases would be identified as being equivocal of which 8 would have CIN2+.

The addition of the hrHPV test correctly removed 30 negative cases from the positive group and 12 from the equivocal group into the negative group.

The same research group more recently published a smaller prospective study[170] of 327 cases of ASCUS, 193 of whom had the AQIC test and the remaining 134 had the hrHPV test. The performance indicators were essentially the same as the previous study and no statistically significant difference was detected between AQIC and hrHPV. Again, if the patient management followed the 3 × 3 table guidelines discussed previously, the PPV of DNA ploidy would have been boosted from 32% to 41%.

A tiny study from France using interactive (as opposed to automated) image analysis found similar concordance between DNA ploidy and hrHPV for ASCUS cases[171].

A few additional studies comparing hrHPV with AQIC, but not focused on ASCUS, have been published in China[172-174] and generally they show reasonable concordance between the two techniques but are not large enough to draw more conclusions.

The previously mentioned study of Guillaud and coworkers[41] compared conventional LBC (ThinPrep) with DNA ploidy and with hrHPV testing by QiaGen HC2 on more than 1500 high grade samples and concluded: "DNA ploidy shows comparable sensitivity, specificity, PPV and NPV values to conventional cytology and HC 2."

In summary, there is evidence that AQIC is at least as useful as hrHPV testing for managing ASCUS cases and probably for other abnormal cytology grades and is simpler and cheaper to perform.

CONCLUSION

Like all countries, China has low-, mid- and high-resource settings. Referring to the 2005 study objective recounted in introduction, the key resource under discussion here is the availability of experienced and highly trained cytotechnologists and cytopathologists-if they do not exist, then the conventional Pap test cannot be performed. The other key resource, of course, is money which constrains public health initiatives in all countries. This is not a discussion about the availability of electricity, water or similar resources. The objective cited in the introduction was related to this definition of low resource settings and not just in China. This review will look separately at the low resource and mid-/high-resource settings.

Training

A key element of AQIC technology is that it can be taught much more quickly than cytology and in the hands of the trainees, can perform comparably to conventional

LBC, performed by experienced and highly trained cytologists. The record in China has demonstrated that it is routinely possible to teach the technology from slide preparation and staining, to operation of the cytometer, review of the DNA ploidy data and report generation in 10 working days, especially when dedicated training facilities are available, as is the case with vendors in China. It is somewhat more difficult and inefficient to teach the technology in the end-user facility because the trainees usually have other routine work to do and so their attention is divided. Even so, this reviewer did have the opportunity to teach this technology to technicians in Morocco about 1 year ago at their facility; competition with their routine work was a big challenge, as were language and other issues, but the training was successful in a relatively short period of time (3 wk total). In this case, the trainees were experienced pathology lab technicians, so they were quick studies for sample preparation and staining. However, they had absolutely no experience in microscopy which is only done by the pathologists there and so all aspects of data and image review had to be mastered by them.

Various AQIC products from different vendors are in wide use in China in the hands of many hundreds of different operators and yet the review of publications in the previous section indicates that reasonably consistent results are being obtained under widely disparate conditions. This speaks to the inherent robustness of the DNA ploidy technology.

Low resource setting deployment

In every country, public health programs are first and foremost political initiatives, a fact that strongly shapes what is done and how. China started a "Two Cancer" screening program (cervical and breast) targeted at rural women in central and western China in 2009 that will continue until at least 2015[175]. This is apparently a follow-on to a 2002-2010 cervical cancer screening program[150], possibly to enjoy the anticipated high popularity and expected efficiencies of combined *vs* single disease screening[176]. One curiosity with this initiative is that the target population is aged 30-59 for cervical cancer screening, which is appropriate, but 35-69 for breast screening by clinical breast exam[177]; I have direct knowledge of the target age being 25-59 for breast cancer screening in Ordos under this program. Few outside of China advocate screening for breast cancer before age 50[178] (although some, such as BCCA, initiate breast cancer screening starting at age 40[54]) which, combined with the fact that Chinese women both inside and outside China have 1/3-1/2 the breast cancer incidence rates as other women[179,180], has led a Hong Kong expert group, who have twice reviewed the evidence recently[181,182], to conclude that there is insufficient evidence to recommend for or against breast cancer screening.

This reviewer has personal experience that AQIC was implemented for this program in the city of Ordos (metropolitan population 600000) in Inner Mongolia but it was required that a cytometer and support staff be provided to each of the 8 or 10 participating hospitals rather than setting up a central lab with two technicians and only two cytometers, which would not only make QA easier to establish and maintain, but would enjoy the economy of scale that comes from a high volume, high throughput operation. The point is that the concepts of efficiency can be mixed and contradictory when public health programs are implemented because of many competing interests.

This is a point of great disappointment for this reviewer because the opportunity has so far been lost to learn in real practice how affordable cervical cancer screening could be in China. The 2012 study by Li *et al*[176] determined the affordability limit for rural women for the "two cancer" screening to be 50 RMB, which is probably achievable with an efficient program implementation. Money remains a major constraint to this Two Cancer public health initiative[177].

So far, China is the center of DNA ploidy application to cervical cancer screening. However, a pilot project is underway in Morocco. The first phase was to demonstrate that the technology could be taught and learned, the second phase was to compare the results with split samples and conventional LBC to determine if the technology had enough merit to move forward into phase three: the actual screening project, which is now underway. A similar project is being conducted in the Philippines[183] but no reports on it are yet available.

Mid-and high-resource setting deployment

Most of the preceding papers reviewed are from laboratories that also perform cytology and so do not lack the key resource of trained and experienced cytologists. However, there does not seem to be the profession of "cytotechnologist" in China, except in Hong Kong[184]. In many countries, screening by cytology is divided into 2 tasks: (1) the locator function, performed by a cytotechnologist who can sign out negative cases; and (2) the interpreter function performed by a cytopathologist who is the only one who can sign out positive cases. The locator skills of cytotechnologists are generally superior to those of cytopathologists[185]. In China, normally these two tasks are performed by a cytopathologist. Although it is very rapidly improving, as recently as 2005 only 1/3 of licensed doctors in China held the equivalent of a bachelor's degree (only 1/8 in rural China)[186], so there is a case to be made for AQIC technology even where cytologists are available. This is confirmed by the wide acceptance of the technology there to date.

One frustration facing the authors of most published studies in China is the previously discussed "missing data"-the inability to "close the loop" and gain access to biopsy and treatment data necessary to gauge relative success of the screening. All medical databases suffer from some "persons lost to follow-up" but there is a big difference between 60% and 6% missing data. There is no effective coordination and cooperation between the various medical providers and the various professional medical

societies and regulators seem to lack the clout to make such cooperation happen, even at the city district level, let alone the city, county, province or national level in China. There are undoubtedly many factors contributing to this situation. It is impossible to know how well any technology or program is doing if it cannot be measured. Missing data makes even cross-sectional studies, such as those reviewed here, very difficult. This situation will make longitudinal studies virtually impossible and longitudinal studies are a crucial element in the pathway to evaluating a screening strategy[48]. Other countries, even the United States, have found ways to measure and share outcome data while still respecting patient autonomy and confidentiality. A great opportunity is being lost in China due to this lack of coordination and cooperation.

The revised ASCCP patient management guidelines[66] were substantially informed by the remarkable database of 1.4 million women managed by Kaiser Permanente Northern California (KPNC) with both LBC and hrHPV testing from 2003 to 2010[187-194]. This substantial longitudinal database allowed the formulation of "risk adapted" patient management guidelines. KPNC is a not-for-profit, private Health Maintenance Organization-essentially an activist insurance company that is also involved in the medical service delivery. Screening for cancer is a very easy way to spend lots of money because it involves testing people, of whom 90+% are healthy. So KPNC is highly motivated to evaluate what it does and to consider what it could do by adapting to maximize the health of its clients and minimize the cost. Perhaps, this is generalizable-that the insurer is the most motivated to effect the kind of change required to measure screening on the required scale.

FUTURE WORK

Much remains to be done, according to the framework for evaluating screening strategies outlined by Arbyn and co-workers[48].

To this reviewer, the most exciting results to emerge from this review are the comparisons with hrHPV testing with DNA ploidy that seem to show that

DNA ploidy = LBC + hrHPV testing

The work on ASCUS needs to be repeated and expanded to all of the other categories of cytological abnormality. It would be especially interesting to conduct a study involving CareHPV[195], the low priced hrHPV test from QiaGen for low resource settings. Combinations of DNA ploidy with HC 2 or other widely used hrHPV tests in "non-low resource" settings would also be valuable.

Much of the world has started vaccinating girls and in some cases, boys, against HPV and this is expected to expand throughout the world, including low resource countries[196]. As the vaccinated cohorts reach screening age, existing well established cytology based screening programs will become stressed because the frequency of abnormality will drop significantly (nominally 70% with current vaccines) which will make it increasingly more difficult for cytologists to maintain both competence and vigilance. DNA Ploidy by automated quantitative image cytometry would greatly relieve this stress.

hrHPV testing requires an accompanying "triage" test to manage the 10%-15% of women who will test positive[197]. The ASCUS hrHPV/DNA ploidy data of Zhang *et al*[169], reviewed above, seemed to be the ideal case for combining tests because both have comparably high sensitivity. However, in this example, both tests were made into binary tests which are constrained to increasing either sensitivity or specificity, while decreasing the other, even though both are inherently continuously valued tests. It would also be interesting to look at what happens when hrHPV and DNA ploidy are combined, not as binary tests but as continuously valued tests, for example, using Bayes' theorem. It may then be possible to find conditions where both sensitivity and specificity increase at the same time. Nothing is free in this world, so it is unrealistic to expect that the joint improvement would exceed that of the binary test, but it might be enough to optimize the test combination for some screening applications.

The value of cervical cancer screening has been essentially limited to squamous cell carcinoma and has done little to reduce the mortality and morbidity from adenocarcinoma, which is increasing in incidence[198-200]. It is unclear how much sampling contributes to the low sensitivity of cytology to adenocarcinoma, but studies using hrHPV testing suggest that many cases of adenocarcinoma are hrHPV positive and cytology negative, indicating that the samples are adequate[191,201,202]. It would be interesting to compare the sensitivity for detection of adenocarcinoma for DNA Cytometry with that of conventional LBC and hrHPV testing.

A number of other issues and opportunities for further research have been brushed against in this review. The important issue of official cervical cancer incidence rates needs to be reconciled with those of the rest of the world and with the very high prevalence rates seen in many studies in China. To this end, it would be beneficial if papers more clearly defined their test populations, especially if they are an admixture of screening and diagnosis cases.

The question of whether conventional LBC done in addition to AQIC provides a net benefit remains unresolved, although it may be both vendor product and laboratory staff skill dependent.

The problem of the imperfect "gold standard" test and the possible impact, if any, that has on DNA ploidy results would be useful to know. Is there a difference in measured DNA ploidy sensitivity when the gold standard is colposcopy directed biopsy *vs* random biopsy?

Much was written here about a definition of sample adequacy but the question is not resolved. What is the best number of cells to measure to get a reliable nega-

tive test result, where "best" is a trade-off between scan time and false negative rate? How much is this influenced by sample taking? If the adequacy number is increased to, say, 50000 epithelial cells, what does this do to the diagnostic rules-that is, will 1 or 2 aneuploid cells still be "equivocal" and 3 positive? In fact, at any epithelial cell count threshold, it would be good to review all of the diagnostic rules including the proliferation rules for positive, negative and equivocal. While they seem to be working reasonably well now over a huge range of undiagnosed disease prevalence, are they optimal?

In a related matter, the concern expressed by Chatelain *et al*[42] on HPV infection induced polyploidy may be worth looking at. That is, should cells with DI of 2, 4 or 8 be grouped separately from other DI > 2.5 cells to determine if they have different value in screening? This could be coupled to any HPV testing project.

Another similar issue is the malignant potential of aneuploid stemlines. This requires good longitudinal data and also poses possible problems with ethical approval. However, if such stemlines routinely result in negative colposcopy and/or biopsy (seen as hyperplasia), it would be interesting to follow such patients over 5 years to see how much it progresses.

Finally, one of the weaknesses in DNA ploidy by the methods described here comes from Feulgen staining which is very slow (3 to 4 h procedure) and can also be somewhat finicky. Guillaud *et al*[203] developed a modified Feulgen process for the dye Azure A (a thiazine like thionin, methylene blue, Azure B *etc.*) that takes 30 min but produces a slightly wider diploid peak. Another rapid Feulgen reaction was reported from China[204] but it seemed to be partly a re-discovery of acid hydrolysis at a temperature of 60C, which was used by Feulgen[22] originally and has been not recommended for routine work due to the need for precise control of all staining conditions. Claims are made from time to time that variations of the hematoxylin staining are quantitative, but this has been rejected experimentally by Biesterfeld[205]. The need for an improved (faster) staining protocol remains.

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