Revisiting tumour aneuploidy — the place of ploidy assessment in the molecular era

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Abstract | Chromosome instability (CIN) is gaining increasing interest as a central process in cancer. CIN, either past or present, is indicated whenever tumour cells harbour an abnormal quantity of DNA, termed 'aneuploidy'. At present, the most widely used approach to detecting aneuploidy is DNA cytometry — a well-known research assay that involves staining of DNA in the nuclei of cells from a tissue sample, followed by analysis using quantitative flow cytometry or microscopic imaging. Aneuploidy in cancer tissue has been implicated as a predictor of a poor prognosis. In this Review, we have explored this hypothesis by surveying the current landscape of peer-reviewed research in which DNA cytometry has been applied in studies with disease-appropriate clinical follow up. This area of research is broad, however, and we restricted our survey to results published since 2000 relating to seven common epithelial cancers (those of the breast; endometrium, ovary, and uterine cervix; oesophagus; colon and rectum; lung; prostate; and bladder). We placed particular emphasis on results from multivariate analyses to pinpoint situations in which the prognostic value of aneuploidy as a biomarker is strong compared with that of existing indicators, such as clinical stage, histological grade, and specific molecular markers. We summarize the implications of our findings for the prognostic use of ploidy analysis in the clinic and for the theoretical understanding of the role of CIN in carcinogenesis.

Almost without exception, the somatic tissues of mammals, including humans, are genetically diploid — each nonreplicating cell nucleus contains two sets of chromosomes (corresponding to two DNA-content units; 2c), one maternal and one paternal. The tetraploid state (four DNA-content units; 4c) necessarily occurs transiently in the later stages of the mitotic cell cycle (during G_2 and M phase), once the complete complement of chromosome pairs — that is, the entire genome — has been replicated but before the cell has divided (FIG. 1); however, persistent tetraploidy occurring in the germ-line during embryogenesis is typically lethal in early development[1]. Despite the lethality of germ-line tetraploidy in mammals, polyploidy (multiples of 2c) does occur normally at the cellular level in a few cell types, such as megakaryocytes, hepatocytes, and placental trophoblasts. Polyploidy is also seen in some cell types during organ hypertrophy (in uterine smooth muscle and breast glandular epithelium during pregnancy, for example) and can also occur in association with cellular stress (for instance, within fibroblasts during wound healing)[2,3]. Cellular DNA content is normally tightly controlled by the cell-cycle-associated apparatus to prevent genomic aberrations, including polyploidy and aneuploidy (either DNA or chromosomal; BOX 1), with numerous checkpoints in place that enable DNA replication and subsequent cell division to be monitored and regulated[2,4,5]. The addition or deletion of individual chromosomes (numerical aneuploidy)[6], in particular, has been seen as uniquely characteristic of the large-scale genomic instability of cancer until the demonstration in 2005 of constitutional mosaic chromosome aneuploidy in ~4% of glia and post-mitotic neurons in the neuraxis of mice and humans[7], and the demonstration by Westra *et al.*[8] of an elevated DNA content in frontal-cerebral-cortex neurons in 2010.

Indeed, abnormalities of cellular DNA content (polyploidy and aneuploidy) have long been associated with tumorigenesis. Such abnormalities were originally implicated in cancer development over 100 years ago by German biologist Theodor Boveri[9], although by the year 2013 it had become clear that cellular aneuploidy is a driving force in the process of carcinogenesis[10]. Currently, a wealth of literature suggests that failed regulation of DNA replication[11] and cell division (centrosome separation[5,12], and chromosomal segregation[13,14]) is associated with both the development of an abnormal

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Key points

- Academic interest in the role of chromosomal instability (CIN) in cancer development and the influence of the resultant large-scale genomic alterations on clinical outcomes is increasing
- Aneuploidy — that is, the presence of an abnormal amount of cellular DNA, is an inevitable result of CIN, and this characteristic can be detected and quantified using DNA cytometry
- DNA ploidy (cellular DNA quantity) is an independent prognostic marker in patients with node-negative invasive breast, early stage endometrioid endometrial, early stage ovarian, prostate, or colorectal cancers
- In patients with Barrett oesophagus, DNA ploidy can be combined with other biomarkers to identify disease that will progress to high-grade dysplasia and/or carcinoma, and to improve the diagnostic sensitivity of pulmonary cytology
- In cervical screening tests, detection of aneuploid cells in Pap smears or using liquid-based cytology is a reliable, cost-effective indicator of the early stages of neoplastic progression toward squamous-cell carcinoma

cellular DNA content (tetraploidy or other forms of aneuploidy) and tumorigenesis. Findings from mouse models have also demonstrated that chromosomal mis-segregation leads to cancer development[15]. Large-scale genomic instability (chromosomal instability (CIN); BOX 1) that inevitably results in aneuploidy is now implicated not only as a driver of carcinogenesis, but also as a principal contributor to the genetic heterogeneity seen within and between cancers[16], and to the development of tumoral drug resistance[17]. Evidence also indicates that tetraploid cancer cells possess increased tumorigenicity, compared with diploid cancer cells, and are often resistant to conventional therapies[18,19].

Current opinion regarding the hallmarks of carcinogenesis is that damage to the cellular apparatus of mitosis within epithelial tumours at an early stage can produce cells with CIN, and that these genetically unstable cells drive tumorigenesis by producing progeny with diverse genetic alterations conferring survival advantages[10,20,21]. Intratumoural phenotype heterogeneity is an inescapable consequence of CIN, with cells arising that have both cytogenetic aneuploidy (abnormal metaphase chromosome count) and DNA aneuploidy (abnormal quantities of interphase DNA). Many of these cellular aneuploidies are probably fatal at a cellular level, but evolutionary selection of viable aneuploid cells that are capable of cell division might give rise to stable aneuploid cell lineages. The detection of such aneuploid clones within an early stage lesion can be taken as a sign of previous or ongoing CIN. In this context, Duesberg *et al.*[22] proposed the 'aneuploidy theory of carcinogenesis' a decade ago, suggesting that aneuploidy is the primary cause of genomic instability and carcinogenesis, challenging the more mainstream gene-mutation theory — which posits that mutations in specific genes are the key 'drivers' of tumorigenesis. At present, mutations and aneuploidy are, however, considered as co-conspirators in carcinogenesis[23].

During the 1990s, evidence steadily accumulated that flow cytometry (FCM), which until then had been the main method of DNA cytometry, was not well-suited for analysis of archival tissue, owing mainly to the low signal to noise ratio of this approach[24–29]. At the same time, the first studies using automated image-based cytometry (ICM) to investigate tumour aneuploidy and its influence on patient survival were being carried out[30]. Thus by 2000, technical barriers that had previously hindered the evaluation of cellular ploidy in both archival formalin-fixed, paraffin-embedded tumour specimens and tumour imprints, and in fresh or fresh-frozen biopsy material were removed. Herein, we review the results from a large number of clinical studies, with data published since 2000, that examined the use of ploidy estimation as a predictive and prognostic marker in a range of epithelial malignancies. This Review provides an up-to-date summary of the evidence that the presence of an abnormal amount of cellular DNA is not only a statistically significant, but also clinically applicable, marker of a poor prognosis.

Detecting and measuring aneuploidy

Background

Ploidy analysis has long shown promise as a cost-effective tool to facilitate the early diagnosis of cancer and as a prognostic biomarker[31]. This approach has not, however, been widely adopted in routine clinical practice. This failure to exploit ploidy analysis probably reflects uncertainties relating to variations in methodology, the lack of consensus criteria, and the existence of conflicting results in the scientific literature. Substantial progress has, nevertheless, been made in our understanding of how to best apply ploidy analysis, in part as a result of a better appreciation of the technical caveats of the various approaches used in the measurement of nuclear DNA content.

This article does not aim to be a technical review, although some of the main principles and considerations underlying the measurement of nuclear DNA content by the use of DNA cytometry are summarized. Caspersson[32] was the first author to propose the use of stoichiometric DNA staining to enable quantitative measurement of the DNA content of individual cell nuclei in a cell sample, and his DNA-content-histogram method continues to be used as the accepted approach to describe the population frequencies of nuclei containing different quantities of DNA in the context of the cell cycle of diploid cells (FIG. 1)[33–35]. The term 'DNA Index' (DI) is often used to refer to the normalized modal DNA content of histogram 'stemlines' (cell populations with nuclei possessing a characteristic DNA content indicated by a peak on the histogram; FIG. 1), and this value can also be used to describe the relative DNA content of a single cell nucleus.

Different approaches are available for the assessment of the nuclear DNA content of cells in a tissue sample. Many studies have confirmed the superiority of ICM over FCM when working with formalin-fixed tissue samples[24–29], but FCM of nuclear suspensions from fresh or fresh-frozen tissue provides a fast and sensitive means of multiparametric cell assessment and cell sorting, and is particularly useful in the research setting (SUPPLEMENTARY FIG. 1)[8,33,36]. Fluorescence *in situ* hybridization (FISH) techniques based on the use of

centromere probes can also be utilized in cytometric analyses and can represent a sensitive method for detecting particular chromosome aneuploidies[37–39]. Compared with FCM, routine ICM enables the analysis of smaller tissue samples (such as fine-needle aspirates), subclassification of nuclei into epithelial and stromal cell groups on the basis of morphology, and identification of small subpopulations of aneuploid cells. The option to perform quantitative nuclear morphometry (QNM), which provides measurements of nuclear morphology that have been shown to vary significantly between patients with and individuals without cancer[40], is an additional advantage of ICM. The alterations in nuclear morphometry associated with cancer have been attributed to aberrant expression of nuclear matrix proteins and transcriptional co-activators, such as p300 (REF. 41).

Reporting the ICM DNA-ploidy histogram

A preponderance of cell nuclei of a 2c stemline (DI = 1.0) is the dominant histogram feature of samples reported as diploid (FIG. 1). In addition, a small population of cells stalled at the G_2/M cell-cycle checkpoint is seen as a peak at the 4c position (DI = 2). If cell turnover (proliferation rates) are high, the presence of S-phase nuclei can be seen in bins between the 2c and 4c peaks. Note that the scatter in apparent DNA content around the modal value of 2c is an artefact caused by instrument noise and imperfectly uniform staining. This scatter is the limiting factor for the sensitivity of DNA-ploidy analysis, and is described by the coefficient of variation (*cv*), which is typically >1%.

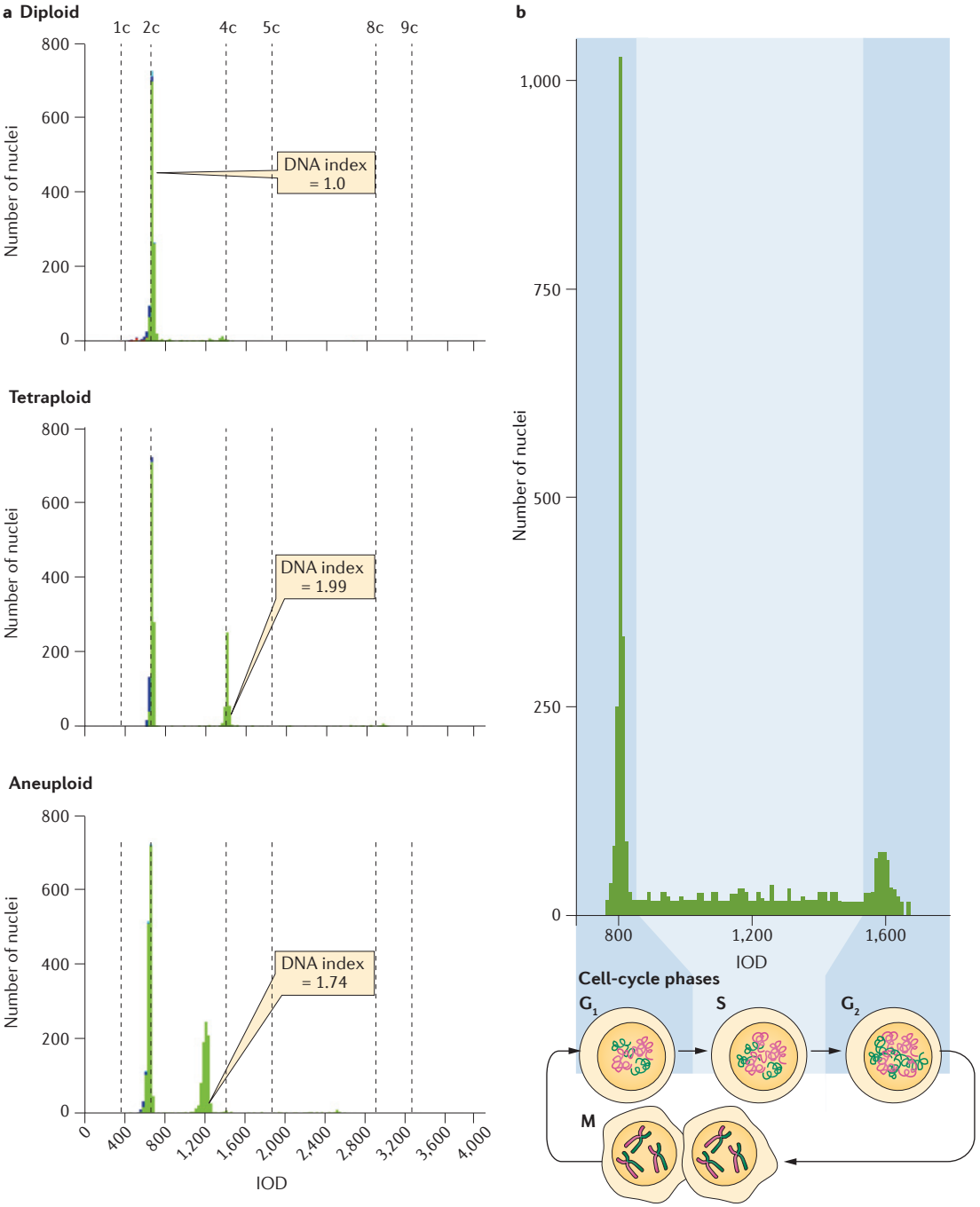
Tumours are considered to be tetraploid if a distinct population is evident at the 4c position, together with a smaller 'tetraploid G_2/M population' at the 8c position, in the histogram, and/or if at least 10% of the total nuclei in the sample have a 4c DNA content; indeed, a 'tetraploid' tumour sample is rarely made up exclusively of tetraploid cells. In the example shown in FIGURE 1a, 24% of the cell nuclei were tetraploid.

Tumour tissue is reported as being DNA aneuploid when the DNA histogram reveals the presence of aberrant cellular phenotypes, whereby the G_0/G_1 population of an aneuploid cell lineage is represented by an abnormal histogram peak with a modal DNA content that is not an integer multiple of the 2c value (DI = 2,4,8,16 and so on). In the example of a histogram for an aneuploid sample shown in FIGURE 1a, the aneuploid cell population has a modal DI of 1.74. The areas under the histogram peaks express the relative abundance of the cell populations in the tissue sample. As with tetraploidy, tumours are rarely entirely DNA aneuploid. In the example in FIGURE 1a, the aneuploid fraction is ~22%. Sometimes two or more such 'aneuploid peaks' are found (known as multiploid aneuploidy).

In normal tissues, no nuclei have a DNA content that exceeds the tetraploid value (DI = 2), with the exception of the examples of 'normal' polyploidy noted previously[2,3]. However, tissue samples from epithelial cancers are sometimes found to contain a small number of aberrant cell nuclei with a higher than tetraploid DNA content. These nuclei are known as 'rare events' or 'single-cell aneuploidies', and they suggest the presence of tumour cells with damaged mitotic machinery. Many reports indicate that a poor prognosis is associated with the presence of these highly aberrant tumour cells in biopsy and resection material, with these cells discriminated by thresholds set at a DI of >2.5 or >4.5 — termed the '5c exceeding rate' (5cER) and '9c exceeding rate' (9cER), respectively[42–45]. Of note, estimation of rare-event aneuploidies is only feasible with the use of ICM, whereby visual examination of suspect nuclei can remove the potential for error owing to clumping of nuclei (which could explain rare-event aneuploidies detected using FCM); to date, many authors who have published studies incorporating ICM have not reported such rare-event aneuploidies.

A tissue sample can sometimes be reported as 'near diploid' if the DNA histogram reveals an aneuploid peak close to the diploid value (for example, DI <1.2)[46]. Histogram reclassification is the process of using thresholds in DI and/or aneuploidy fraction to reclassify 'minor' aneuploidies as DNA diploid, to obtain a stronger prognostic result[46–48]. The 'S-phase fraction' (SPF) is a term applied to the relative number of nuclei that are in S-phase, as indicated by the count of nuclei found between the G_0/G_1 and G_2/M histogram peaks. SPF featured in many early DNA-cytometry studies[49], although cyclin A or Ki67 quantification, for example, is now used more often in studies of cell proliferation.

The resolving power of DNA cytometry is determined largely by the degree of scatter, or *cv* in DNA-content values, which is why the measured *cv* should be included in all reports of DNA-ploidy data[50,51]. Under the best conditions (*cv* = 1%; aneuploidy fraction = 50%) a difference in DNA content of about 1% is detectable, corresponding to addition or deletion of a single average-size chromosome[52]. Thus, a biopsy sample considered to be DNA diploid (a tissue sample without evident DNA aneuploidy) does not necessarily exclude the presence of minor chromosomal aneuploidy, and balanced chromosomal rearrangements will not be detected. Furthermore, an abundance of both normal cells and tumour cells with diploid genomes can potentially 'dilute' out cellular subpopulations with abnormal amounts of DNA. Additionally, detection of the aneuploid fraction limit depends on the DI of a tumour-cell population[52]. This limitation is an important consideration because in some early stage cancers, a poor prognosis has been shown to be associated with 'rare-event' aneuploid subpopulations making up as little as 1% of the tumour-cell population[43–45]. In addition, the analysed tissue area should, ideally, comprise >75% of all tumour cells to avoid small populations of aneuploid cells being missed. This requirement is emphasized by findings from patients with prostate cancer, which demonstrate that the more tumour tissue present in the sample, the greater the probability of finding aneuploidy[53]. The incorporation of areas of the tumour with a high histological grade and regions with marked nuclear pleomorphism in cytometric analyses should also be considered, so as not to miss aneuploid subpopulations and thus potentially falsely predict a more-indolent clinical course.



Ideally, assessments of DNA ploidy should be performed on nuclear suspensions made from thick sections of tissue (>40 μm), as obviously the proportion of cut nuclei increases with decreasing section thickness. The problem of cut nuclei is particularly pertinent when working with standard (5 μm) histology sections and with small samples, for which the combination of a low overall number of tumour cells and a high proportion of partial tumour nuclei makes distinguishing aneuploid tumour nuclei from background S-phase nuclei difficult. In such a scenario, the use of proliferative markers, such as Ki67, can complement DNA-ploidy analysis[54,55]. These considerations apply to ICM only, as FCM is not possible with histological sections or FNA material.

Standards and guidelines

Attempts have been made to develop consensus guidelines with the aim of standardizing diagnostic and prognostic uses of DNA cytometry. In particular, the European Society of Analytical Cellular Pathology (ESACP) task force has produced recommendations on factors including nomenclature for nuclear-DNA measurements, sample-preparation methodology, data measurement and interpretation, quality assurance, and reporting[31,51,56–58]. To date, these guidelines have not been universally applied and important differences in the histogram classification criteria used by different research groups remain; thus, direct comparison of results from different studies is not always possible.

◀ Figure 1 | **Assessment of DNA-ploidy status by DNA cytometry. a** | Illustrative examples of DNA-ploidy histograms obtained by automated image-based cytometry from different tissue samples that would be reported as 'diploid', 'tetraploid', and 'aneuploid'. In each graph, the *x*-axis shows nuclear DNA content expressed in units of IOD; superimposed on this axis is the scale of DNA-ploidy values (2c, 4c, and so on), where 2c represents the nuclear DNA ploidy for nuclei of diploid cells in the G_0/G_1 phases of the cell cycle — reference cells, such as tissue fibroblasts, lymphocytes, or diploid epithelial cells, are normally used to establish the 2c value. The *y*-axis shows the number of nuclei corresponding to each bin (interval) in the histogram. A typical population of normal diploid cells with 23 chromosome pairs will produce a histogram with a major peak at the IOD corresponding to the DNA content of the 46 chromosomes (point 2c; DI of 1); however, a small proportion of the cells in the population are captured at stages of the cell cycle at which DNA is being, or has been, replicated and, therefore, possess anywhere up to double the normal amount of DNA (up to the 4c point on the *x*-axis). An abnormally intense 'tetraploid' peak at the 4c point indicates that the sample contains an increased number of cells with double the normal complement of chromosomes (92, or 4 sets of 23; DI 1.99) which can either be proliferating cells at G_2 phase, or viable cells with tetraploid genome, or a mixture of both. Some cell populations, particularly those comprising neoplastic cells, can possess an 'aneuploid' peak (in this case, with a DI of 1.74), indicating that some cells in the population possess an abnormal DNA content that is greater than that of diploid cells, but is less than that expected if the whole chromosome set was duplicated. The degree of aneuploidy (that is, the DI) can be variable and depends on the extent of chromosomal losses or gains. **b** | This image relates the features of a DNA-content histogram derived for a proliferating diploid-cell population with corresponding phases of the cell cycle, to highlight the relationship between the cell-cycle stages and ploidy. The illustration emphasizes that the DNA content of cells in S-phase, which are in the process of synthesizing DNA in order to replicate their chromosomes, lies along a continuum between diploid (2c) and tetraploid (4c), as well as the tetraploid DNA content of cells in G_2 phase that have completed DNA replication, but have not completed mitosis and divided. DI, DNA index; IOD, integrated optical density.

Most studies have implemented qualitative classification of DNA aneuploidy, based on the DI of stemline or single-cell aneuploidy[59,60]. Histograms demonstrating the presence of one or multiple aneuploid stemlines or single-cell aneuploidy have typically been classified as aneuploid[25,61]. Moreover, in many studies, DNA-ploidy results have been dichotomized into diploid versus nondiploid (comprising both aneuploid and tetraploid histograms), or euploid (diploid and tetraploid) versus aneuploid, in order to simplify statistical analysis[62,63].

Aneuploidy and patient prognosis

That most advanced-stage cancers are aneuploid is now widely recognized. For this reason alone, interpreting the relationship between aneuploidy and patient prognosis based on studies that consider consecutive series of tumours without stratification according to clinical stage or grade is extremely difficult. As a result, a consistently significant correlation between clinical outcome measures and DNA-ploidy status has typically been obtained only in large studies comprising sufficient numbers of consecutive cases to permit stratification according to tumour stage; for example, in 17 published studies that involved patients with R0-resected stage II colorectal carcinoma (TABLE 1), better clinical outcome was reported for patients who presented with diploid tumours compared with any other type of ploidy status, although only in the larger cohort studies was the difference significant on multivariate analysis. Conversely, studies that mixed cohorts of patient with tumours of all clinical stages have provided conflicting results, as illustrated in an insightful review of pre-2000 studies of ploidy status and survival of patients with gastrointestinal cancers by Grabsch *et al.*[64]

As a very general rule, the risk of relapse and/or cancer-related death increases with increasing DI of any aneuploid clones in resection material, but the relationship between DI and risk of relapse in specific patient groups with particular cancers is not always simple. Survival studies have found that 'near-diploid' aneuploid lesions (with a DI <1.3, for example) have limited prognostic significance in a number of patient groups, including those with node-negative carcinomas of the breast[47], endometrium[46], and urothelium[30]. Reclassifying aneuploid lesions with a low DI as 'near-diploid' can reveal a strong prognostic value for 'high-aneuploidy'; a study by Jonas and colleagues[48] provides a striking example. Applying ploidy analysis to the optimal selection of patients for liver transplants, these investigators stratified 246 patients with hepatocellular carcinoma arising on a background of cirrhosis into two groups according to the tumour DI: a near-diploid group ($n = 159$), comprising patients who presented with diploid and DI <1.5 aneuploid tumours; and a 'high-aneuploid' group ($n = 87$) of patients, who presented with DI ≥1.5 aneuploidy[48]. Following complete liver transplantation, 10-year overall survival for the 'near-diploid' group was 80%, compared with 8% for the 'high-aneuploid' cohort ($P < 0.0001$); the corresponding 5-year survival figures were 86% and 27%, respectively[48]. In this study, DI (odds ratio (OR) 11.9; $P < 0.0001$) and venous infiltration (OR 1.7; $P < 0.01$) were significant outcome predictors in the multivariate analysis[48]. As in the aforementioned study, receiver operating characteristic (ROC) analysis of cytometry histograms is often used to find the optimum DI cutoff for use in prognostication[42,47].

Aneuploidy in breast cancers

Intratumoural heterogeneity, genetic instability, and chromosomal imbalances all characterize early stage breast carcinomas[65]. Whole-genome sequencing of early stage breast carcinomas, as well as surrounding neoplastic tissue and healthy 'control' tissue, has revealed that CIN and aneuploidy are both dominant evolutionary features, occurring in the common ancestor of both neoplastic and cancer tissues[66]. Much effort has been devoted to the search for empirical gene-expression profiles of breast cancers that could be used as prognostic markers to enhance the currently used algorithms, which are based on standard histological criteria, such as tumour grade, stage, and oestrogen receptor (ER), progesterone receptor (PR), HER2 and Ki67 status[67]. Prognostic gene-expression signatures that reflect CIN in breast cancer have been developed[68,69].

Breast cancer was the first cancer type to be extensively investigated using DNA cytometry, with many reports of follow-up studies appearing in the 1990s. Most studies used FCM with suspensions of cell nuclei made from formalin-fixed resection material, with no stratification of patients according to clinical tumour stage or grade. Given that grading and staging for cancers of the breast includes mitotic count and tumour size,

Box 1 | **Definitions of key cytometry terms**

- DNA aneuploidy: aberration in the DNA content of a cell population measured by DNA cytometry; DNA aneuploidy can be stemline aneuploidy or single-cell aneuploidy
- DNA-ploidy status: summary of the results of cytometric analysis of the DNA content of a cell population; DNA content is classified as diploid (normal amount of cellular DNA), tetraploid (twice as much cellular DNA as that present in most normal cells) or aneuploid (an abnormal amount of cellular DNA)
- Large-scale genomic instability: a term that has been used interchangeably with 'chromosomal instability' by many authors to described a high frequency of mutations, which can include changes in the DNA sequence, chromosomal rearrangements, and loss and/or gain of whole chromosomes or parts of chromosomes, within the genome of a cellular lineage; aneuploidy acts as a marker of large-scale genomic instability
- Chromosomal instability (CIN): a dynamic process in which cells within a population gain or lose whole, or parts of, chromosomes during each cell division, resulting in cell-to-cell variability; tumours with CIN are usually DNA aneuploid
- Chromosomal aneuploidy: the state of a cell with aberrations in the number or structure of its chromosomes; chromosomal aneuploidy can be stable, as is observed in congenital genetic disorders (such as Down syndrome) or unstable, as is the cases in many malignancies

findings of many studies unsurprisingly emphasized the prognostic relevance of the SPF, as well as ploidy status[47,49,70]. Since 2000, one study involving consecutive cohorts of women with stage I–III breast tumours ($n = 393$) has confirmed aneuploidy as a multivariate indicator of inferior long-term disease-specific survival (DSS), with a hazard ratio (HR) of 2.0 ($P = 0.002$)[71]; two other such studies involving 2,752 and 603 patients have also shown that aneuploidy is a multivariate indicator of metastasis-free survival with a HR of 1.2 ($P = 0.0370$) and 2.5 ($P = 0.001$)[72], respectively. An FCM study of 770 consecutive FNA samples by Gazic and colleagues[73] found that DNA aneuploidy (found in 68% of all samples) is a univariate predictor of poor DFS and overall survival (HR 1.8, $P < 0.001$, and HR 1.5, $P < 0.001$, respectively), but ploidy did not emerge as an independent factor in a multivariate analysis that included ploidy status, tumour size, lymph-node status, and SPF. SPF itself was not prognostic in patients with N0 disease (46% of all samples)[73].

Despite some discordant results[74], use of ploidy analysis could help to address the present need for prognostic indicators in patients with small primary breast tumours without axillary-node involvement[72,75]. A well-controlled, retrospective study in women with N0 tumour resections ($n = 961$), in which the investigator used FCM with a standardized methodology that included robust histogram classification, demonstrated statistically significant prognostic results for relapse-free survival (RFS; HR 1.8, $P < 0.0001$)[47]. Importantly, the results of this US-based study included independent validation at hospitals in France and Sweden[47]. In a study of women with stage I–II (T1–2) invasive breast cancers ($n = 271$) the negative effects of tumour aneuploidy were restricted to node-negative tumours (HR 2.1, $P = 0.008$)[76]. Li and colleagues[77] found that among women with T1N0M0 tumours, aneuploidy was an independent and stronger predictive marker of early relapse than proliferation rate; 24% of the patients with aneuploid tumours relapsed during the 9-year follow-up period, compared with only 3% of patients presenting with diploid tumours ($P < 0.05$). Similarly, Pinto *et al.*[75] reported that aneuploidy and a high cell-proliferation rate were powerful predictors of poor DFS (univariate HR 16.7, $P = 0.007$, and HR 23.1, $P = 0.004$, respectively) in a selected cohort of 135 women with pT1–2N0, grade II breast cancers — a setting in which decisions on the use of adjuvant therapy are critical in determining disease outcome. Interestingly, Auer and colleagues[78] have extended the traditional ploidy-status classification by recognizing a 'short-survivor' group of patients with DNA-diploid breast cancers with a high cell-proliferation rate and a genetic profile similar to that of DNA-aneuploid tumours using representational oligonucleotide microarray analysis.

One exception to the rule of a better prognosis for patients with diploid breast tumours is provided in a report that DNA-diploid status in breast tumours from germ-line *BRCA2*-mutation carriers was associated with significantly worse 10-year DSS (HR 4.86; $P = 0.001$)[70]; however, aneuploid tumours in both carriers and noncarriers of germ-line *BRCA2* mutations were associated with the same somewhat worse prognosis compared with diploid tumours in *BRCA2*-wild-type women (HR 1.2, $P = 0.04$).

Among the many reports of the prognostic effects of aneuploidy in different types of cancer, hypodiploid aneuploidies are rarely reported — presumably because hypodiploidy is usually lethal at the cellular level. Nevertheless, hypodiploidy, defined variably by DI thresholds of <0.85–0.95, has been reported in up to 7% of breast cancers in the pre-2000 literature[47,79]. In a study of ploidy status in fresh resection material and the 10-year overall survival of 584 women with operable breast cancer, univariate predictors of poor overall survival included hyperdiploid aneuploidy, hypodiploidy, tumour size, lymph-node status, and receptor status[79]. Upon multivariate analysis, only hypodiploidy emerged as an independent predictive factor; the mean 5-year and 10-year overall survival rates for patients presenting with hypodiploidy were 23% and 0%, compared with 98% and 98% for patients with diploid tumours ($P < 0.0001$)[79]. In summary, studies indicate that DNA cytometry clearly has the potential to be clinically useful, as an adjunct to traditional grading and gene-expression assays, for prognostication in patients with node-negative breast tumours (SUPPLEMENTARY TABLE 1)[68].

Aneuploidy in gynaecological tumours

Endometrial carcinoma

Patients with endometrioid endometrial carcinomas (EEC) often present with early stage disease, and surgical resection results in a high probability of cure in these patients. These tumours are very common, however, and thus the ability to identify the small proportion of patients with stage I disease who are at an increased risk of relapse is desired. Histological features alone do not enable the identification of women with high-risk, low-grade EEC, which has led to an active search for prognostic biomarkers (reviewed elsewhere[80–82]).

Table 1 | **Findings from studies of the association of ploidy status with survival of patients with stage II CRC**

Study	Number of stage II CRCs (proportion that were diploid)	Survival end point	Follow-up duration (years)	DNA-cytometry method	Survival in patients with diploid vs those with aneuploid tumours	Aneuploidy associated with survival after multivariate analysis	*P* value for association of aneuploidy with survival in univariate analysis
Hveem *et al.* (2014)[62]	348 (34%)	OS	10	ICM	52% vs 34%	Yes; HR 1.46	0.003
Hveem *et al.* (2014)[62]	278 (53%)	TTR	10	ICM	74% vs 49%	Yes; HR 2.19	0.006
Takanishi *et al.* (1996)[164]	210 (45%)*	OS	5	FCM-A	80% vs 64%	Yes; HR NR	0.02
Sinicrope *et al.* (2006)[121]	158 (49%)*	OS	5	FCM-A	78% vs 64%	Yes; HR 2.27	0.026
Sinicrope *et al.* (1999)[165]	154 (48%)*	OS	5	FCM-A	93% vs 84%	NS	0.06
Armitage *et al.* (1991)[166]	139 (42%)	DFS	4.5	FCM-A+F	84% vs 64%	Yes; HR NR	0.01
Chapman *et al.* (1995)[167]	136 (42%)	OS	5	FCM-A+F	84% vs 65%	Yes; HR NR	0.01
Lanza *et al.* (1998)[168]	107 (28%)*	DFS	4	FCM-F	97% vs 80%	NS	NS
Buglioni *et al.* (2001)[169]	94 (48%)	OS	5	FCM-F	NA	NS	NS
Buglioni *et al.* (2001)[169]	94 (83%)‡	OS	5	FCM-F	~85% vs 50%‡	Yes; HR 4.48	0.002
Kay *et al.* (1996)[170]	92 (43%)	OS	7	ICM	76% vs 61%	Yes	0.008
Garrity *et al.* (2004)[171]	91 (NR)	DFS	5	FCM	NA	NS	NS§
Zarbo *et al.* (1997)[172]	91 (NR)	OS	5	FCM-A	80% vs 75%	NS	NS
Kokal *et al.* (1989)[173]	89 (46%)	DFS	4	FCM-A	97% vs 67%	NR	<0.001
Tomoda *et al.* (1998)[174]	81 (32%)	DFS	3	FCM-F	86% vs 85%	NS	NS
Cosimelli *et al.* (1998)[175]	68 (34%)	DFS	4	FCM-F	63% vs 55%	NS	NS
Bondi *et al.* (2009)[176]	66 (39%)*	CSS	5	ICM	NA	NS	0.065
Ahnen *et al.* (1992)[177]	66 (53%)*	OS	7	FCM-A	80% vs 68%	NS	NS
Nori *et al.* (1996)[178]	20/20 (60% /20%)‖	OS	9	ICM	NA	NR	<0.002

*Only colon-cancer tissue was analysed. ‡Comparison was between patients with nonmultiploid tumours versus those with multiploid tumours; the number in parentheses is the percentage of nonmultiploid tumours. §$P = 0.002$ for comparison between patients with aneuploid and Ki67-high tumours and those with diploid and Ki67-low tumours. ‖Colon-cancer tissue from 40 matched patients, 20 with relapsed disease and 20 nonrelapsed disease, was studied. CRC, colorectal cancer; CSS, cancer-specific survival; DFS, disease-free survival; FCM-A, flow cytometry based on fixed archival material; FCM-A+F, flow cytometry based on fixed archival material and fresh or fresh-frozen tissue; FCM-F, flow cytometry based on fresh or fresh-frozen tissue; HR, hazard ratio; ICM, image-based cytometry; NA, not applicable/available; NR, not reported; NS, not significant; OS, overall survival; TTR, time to relapse.

Studies of the relationship between aneuploidy in resection and curettage materials and the clinical outcome of patients presenting with stage I–II EEC were reviewed in 2014 by Mauland *et al.*[83] With one exception[84], all of the studies that we reviewed found that aneuploidy was a significant predictor of a poor prognosis in univariate analyses, but they did not universally report that ploidy status provides additional prognostic value over that of traditional parameters (SUPPLEMENTARY TABLE 2). For example, Pradhan *et al.*[46] found 728 diploid tumours among a consecutive series of 937 women with stage I–II EEC; 170 women had tumours with either tetraploidy or near-diploid aneuploidy with a DI of ≤1.2, and the remaining 39 women presented with tumours with DI >1.2 aneuploidy[46]. The 5-year recurrence rates were 8% in the diploid group, 12% in the tetraploid group, 14% in the patients with DI ≤1.2 aneuploidies, and 20% among the women presenting with DI >1.2 aneuploid tumours ($P = 0.02$ for diploid versus aneuploid tumours with DI ≤1.2; $P = 0.01$ for diploid versus aneuploid tumours with DI >1.2)[46]; nevertheless, ploidy status did not emerge as an independent prognostic factor in the multivariate analysis. In addition, results of a routine assessment of DNA ploidy have shown that this characteristic is an independent prognostic parameter that enabled information over that available from routine histological assessment to be obtained[85].

Several reports have detailed clinical experiences in which decisions regarding adjuvant therapy for patients with EEC stage I have been made on the basis of ploidy status[85–89]. Findings from all of these studies support the use of ploidy analysis. For example, Lim *et al.*[86] reported on the results of a clinical trial, in which 406 consecutive patients with low-risk stage I endometrial carcinoma were assigned to one of two treatment groups on the basis of ploidy status: post-surgery vaginal vault radiotherapy for the 91 patients with aneuploid tumours, and no adjuvant treatment for the 315 patients with diploid tumours. In both groups, 5-year DFS was 95%, suggesting that assessment of ploidy status enables the correct prediction of those patients who would benefit from adjuvant therapy, considering that those with aneuploidy would be expected to have a worse outcome[86]. In summary, a number of clinical studies have confirmed that ploidy status, as determined by ICM, can be used to identify the small group of patients who are at risk of relapse following resection of the primary EEC lesion. For other, more-aggressive uterine cancers, insufficient evidence is available to support the prognostic use of ploidy analysis.

Epithelial ovarian tumours

High-grade serous carcinoma is the most-common cancer of the ovaries. This disease is characterized by advanced stage and grade at presentation, aneuploidy, extensive CIN, and intratumour heterogeneity[90]. Low-grade serous ovarian adenocarcinomas are considered as a separate disease with a more-indolent course. Indeed, 10–15% of low-grade ovarian epithelial cancers are diagnosed when they are confined to one or both ovaries (Fédération Internationale de Gynécologie et d'Obstétrique (FIGO) stage I), and patients with these cancers can potentially be cured by surgery alone[91]; however, in 20–30% of these patients the disease will eventually relapse. Early attempts to evaluate aneuploidy as a biomarker of relapse risk in patients with stage I disease produced mixed results (SUPPLEMENTARY TABLE 3)[92]. Using high-resolution ICM of nuclear monolayers from resection specimens, Kristensen *et al.*[25] found that ploidy status, combined with histological grade, predicted the 10-year RFS of patients with stage I ovarian carcinomas ($n = 284$); in patients with diploid grade 1–2 tumours RFS was 95%, compared with 29% RFS for patients with aneuploid tumours[25]. In multivariate analysis, aneuploidy was the strongest independent prognostic factor (HR 9.3) followed by tumour grade (HR 2.7), and FIGO substage (HR 2.0)[25].

Stage I borderline ovarian epithelial tumours (BOTs) are considered by some to be precursors of low-grade carcinoma, and are generally associated with an excellent prognosis[93]. Nevertheless, some BOTs do recur or can progress to invasive tumours; the overall 10-year DFS of patients with stage I BOT is 70–95%[93]. BOTs are predominantly diploid, although about 10% are characterized by near-diploid aneuploidy (DI <1.4)[94,95]. Reported findings, indicating that aneuploidy in BOTs is associated with a 19-fold increased risk of death, therefore making aneuploidy the single most important prognostic factor[96], have not been confirmed in subsequent studies[93].

Lesions of the uterine cervix

Squamous intraepithelial lesions of the cervix are widely known to be associated with infection by high-risk human papillomavirus (HR-HPV) types. Most such infections and associated dysplasias regress harmlessly, but a small proportion progress to squamous-cell carcinoma (SCC) unless treated. Findings of several studies based on liquid-based cytology specimens or traditional cervical smears have indicated a clinically significant link between an ICM-detected DNA aneuploidy in dysplasias and subsequent progression toward cancer of the cervix — that is, cervical intraepithelial neoplasia grade 2+ histology in subsequent cone biopsy (reviewed elsewhere[92,97]); however, the possibility of an effective immune response against the dysplastic lesions in some women means that DNA aneuploidy does not provide a completely definitive prognosis for progression toward cancer of the cervix[98]. DNA aneuploidy in cells from smears of cervical dysplasias has, nevertheless, been reported to be a prospective marker of malignancy[60].

With regard to cervical-cancer screening, evidence exists that ICM-based measures of ploidy (particularly the detection of rare-event aneuploidy) can improve the sensitivity and positive predictive value (PPV) of cytology alone, or of cytology combined with testing for HR-HPV[99–101]. This conclusion is supported by the results of studies in China that involved paired ICM (in one study, for 5cER alone) and cytology assessment of cervix scrape or brush cytology samples from a total of more than 30,000 women[43,102]. The results of these two studies are impressive, although the study design of the larger trial[102] has been criticized[103]. In light of these findings, Garner[42] has published an in-depth analysis of the epidemiological and cost–benefit aspects of applying automated assessment of ploidy measures to cancer screening. Therein, he also reviews contemporary literature on this subject from China[42].

Attempts to demonstrate the prognostic value of aneuploidy in early stage cervical cancer have been hindered by discordant results (SUPPLEMENTARY TABLE 4)[80]. Among studies that have indicated a significant benefit for evaluation of aneuploidy in this setting[104], Susini *et al.*[36] reported on 10-year DSS in a cohort of 136 patients with mainly FIGO stage I–II cervical cancer; in multivariate analysis, independent predictors of DSS were ploidy status (HR 2.26, $P = 0.043$), FIGO stage (HR 2.71, $P = 0.01$), and lymphatic vascular invasion (HR 2.5, $P = 0.039$)[36]. By contrast, lymph-node invasion was without prognostic significance if the primary tumour was diploid[36]. Of note, the rate of 10-year DSS among 64 patients with FIGO stage I disease was 86% and 39% for those with diploid and aneuploid tumours, respectively ($P = 0.003$)[36].

Aneuploidy in gastrointestinal lesions

Barrett oesophagus

Most oesophageal adenocarcinomas are hypothesized to arise from a background of Barrett oesophagus, via progression from simple intestinal metaplasia, through low-grade dysplasia to high-grade dysplasia and invasive adenocarcinoma; however, less than 5% of patients with Barrett oesophagus eventually develop oesophageal adenocarcinoma[105]. Accordingly, considerable effort has been expended in the search for clinically useful predictive markers of cancer development in patients with Barrett oesophagus[106]. ICM-derived DNA ploidy and FISH-based panels (for centrosomes and locus-specific regions of chromosomes 9 and 17, for example) are the most-studied markers[38,107,108]. Both of these approaches are suitable for automation[28,38]. In a phase III trial that analysed routinely collected clinical paraffin-embedded endoscopic-biopsy specimens[109], a biomarker panel consisting of *Aspergillus oryzae* lectin, ICM-determined ploidy status, and clinically determined low-grade dysplasia was predictive of future progression to oesophageal adenocarcinoma and/or high-grade dysplasia. Of note, use of the biomarker panel was effective in predicting the development of oesophageal adenocarcinoma even in patients with no evidence of dysplasia at diagnosis of Barrett oesophagus.

Ulcerative colitis

Long-standing ulcerative colitis is a known risk factor for the development of CRC. In patients with ulcerative colitis who develop cancer, aneuploidy in endoscopy specimens can be detected by ICM up to 10 years before the diagnosis of cancer, and aneuploidy has been proposed

as an independent biomarker to supplement risk assessment using the traditional risk factors of degree of inflammation and dysplasia[110,111], as reviewed previously elsewhere[112]. According to studies by Gerling *et al.*[113,114] CRCs that arise on a background of ulcerative colitis are invariably aneuploid, differ genomically from sporadic CRCs, and carry the same prognosis as aneuploid CRC (SUPPLEMENTARY TABLE 5).

Colorectal cancer

CRC is the second leading cause of cancer-related death in Europe and North America[115]. Most patients with R0 resections of node-negative CRC are cured of the cancer by surgery alone, but an unacceptably large minority experience relapse owing to locoregional recurrence or distant metastasis, or both (5–15% of patients with stage I CRC and 20–40% of patients with stage II CRC)[116]. The problem of disease recurrence is particularly pronounced for patients with Union for International Cancer Control (UICC) stage II (pT3–pT4N0M0) CRC, as these patients are not normally offered adjuvant therapy, despite the fact that up to a third will experience relapse after surgery alone[116]. Molecular classification of CRC is mainly based on microsatellite-instability (MSI) status and mutations in *KRAS* or *BRAF*[117]. With regards to aneuploidy, despite methodological failings and insufficient sample sizes for TNM stratification in many early studies of this characteristic[64], the findings of two meta-analyses support the use of cytometric aneuploidy as a prognostic factor in patients with stage II–III CRC[118,119]. In addition, three large-scale DNA-cytometry studies, in which the authors performed multivariate analyses, have demonstrated an independent prognostic benefit of DNA aneuploidy in defined cohorts of patients with M0 CRC (particular those with stage II CRC; TABLE 1)[62,120,121]. In one of these studies, Sinicrope *et al.*[121] measured the relative prognostic value of FCM-determined aneuploidy and MSI in fixed archival tumour specimens from a cohort of 528 patients with stage II–III colon cancer. The groups of patients presenting with MSI or with diploid microsatellite-stable (MSS) tumours had an almost identical good prognosis (based on 5-year overall survival), compared with the patients with aneuploid tumours (HR 0.65, $P=0.022$, and HR 0.62, $P=0.002$, respectively). In another of these studies, Mouradov *et al.*[120] evaluated the 5-year DFS of patients with stage II–III CRC in the UK VICTOR trial ($n=822$) and an independent Australian population cohort ($n=375$) in relation to measures of MSI, aneuploidy (CIN), and mutations in *KRAS*, *NRAS*, *BRAF*, *PIK3CA*, *FBXW7*, and *TP53*, and loss of heterozygosity (LOH) at chromosome 18q. None of the specific gene mutations showed prognostic value, whereas MSI (HR 0.58, $P=0.021$) and aneuploidy (HR 1.54, $P=0.005$) emerged as independent indicators of relatively good and poor prognosis (5-year DFS), respectively[120]. In this study[120], the negative prognostic value of aneuploidy was greater than that of the Watanabe LOH prognostic indicator[122]. In the most-recently published of the three large-scale studies, Hveem *et al.*[62] evaluated the effect of tumour ploidy status at presentation on 5-year and 10-year overall survival and 10-year RFS in a stage-stratified consecutive cohort of 952 patients with stage I–III CRC at a single Norwegian institution. The overall negative prognostic value of aneuploidy in these patients was comparable to that observed in other studies in patients with stage II–III CRC (HR 1.56, $P=0.007$). As reported elsewhere[123], the probability of relapse was similar for patients with aneuploid stage II and those with aneuploid stage III tumours, although for patients with diploid stage II tumours, multivariate analysis revealed a significantly better prognosis (HR 2.19, $P=0.002$; SUPPLEMENTARY TABLE 6)[62].

Calistri and colleagues[124] have demonstrated the existence of two genetically distinct types of aneuploid CRC that can be distinguished by their characteristic DI, with these 'low-aneuploid' (DI <1.4) and 'high-aneuploid' (DI >1.6) tumour subtypes also demonstrating a higher frequency of *KRAS* and *TP53* mutations than diploid tumours. In 2013, Domingo *et al.*[117] screened a cohort of patients with stage II–III CRC from the VICTOR trial ($n=906$) for somatic mutations; using logistic regression, clustering, and Bayesian network analysis, in which DNA aneuploidy was considered to be a marker of CIN, the investigators identified seven molecular categories of CRC, and correlated these mutations with ICM-derived DNA-ploidy status[117]. As in the study by Calistri *et al.*[124], this analysis revealed the existence of two types of aneuploid CRC, one associated with the presence of node-positive tumours, and the other with a predominantly distal localization of the tumour and preferential occurrence in men[117].

Thus, a strong evidence base supports the prognostic use of ploidy status in patients with stage II carcinomas of both the colon and rectum, with the findings of large studies incorporating multivariate analyses indicating that tumour diploidy is an even stronger marker of a good prognosis than MSI[62,120,121]. In patients with ulcerative colitis, evidence supports the addition of ploidy status to traditional risk indicators for prediction of disease progression towards CRC.

Aneuploidy in lung cancer

Owing to the relatively high incidence, typically late presentation, and poor overall prognosis of non-small-cell lung cancer (NSCLC), the use of many molecular biomarkers has been proposed to improve diagnosis, prediction, and prognostication[125,126], although none of these markers has been adopted into routine clinical practice. Bronchoscopy with cytology of bronchial washings, bronchial brushings, and bronchial FNA samples continues to form the basis of early detection of NSCLC.

Aneuploidy detected using either FISH or ICM has been shown to have potential for reducing the incidence of overdiagnosis of NSCLC based on cytology findings alone[39]. In this study, FISH and ICM-based DNA cytometry were used to evaluate 210 consecutive tissue samples after cytological analysis: 70 with positive cytology results, 70 with negative cytology findings, and 70 with inconclusive or equivocal cytology results[39]. For the entire cohort of 210 patients, cytology, FISH, and DNA cytometry analyses gave sensitivities for the detection of cancer cells of 84%, 78%, 79%, respectively, and had specificities of 70%, 98%, 98%, respectively[39]. For the 70 patients with 'cytology equivocal' samples, FISH and DNA cytometry were

associated with correct classification rates (CCRs) of 79% and 83%, respectively[39]. In their paper, the investigators also considered the cost-effectiveness and most-appropriate application of the two methods concluding that analysis using DNA cytometry carries ~20% of the cost of FISH and is less time-consuming; however, FISH is preferable for the analysis of samples with only a few atypical cells[39].

In addition, findings of several studies have shown that aneuploidy detection using DNA cytometry or chromosome FISH in sputum samples can improve the sensitivity and specificity of cytology for the detection of early signs of NSCLC in populations at a high risk of this disease, including heavy smokers; bronchoscopy and resection histology represented the gold-standard diagnostic approach for the comparisons in these studies[127–131]. Moreover, a meta-analysis of results from cytometry studies published before 2000 revealed the risk of NSCLC-related death to be substantially lower in patients with diploid versus aneuploid tumours (the ORs for death at 2, 3, and 5 years after surgery were 0.51, 0.67, and 0.87, respectively; $P<0.0001$)[132].

In summary, when applied to cancer screening in heavy smokers and in diagnostic pulmonary cytology, ploidy analysis has the potential to improve the sensitivity of cancer detection and reduce overdiagnosis. The use of existing automated 'batch process' ICM can enable low-cost screening-scale processing of samples. For prognostication in patients with lung cancer, the use of ploidy analysis is restricted to NSCLC, where 2-year survival for patients with diploid cancers is reported to be double that for patients with aneuploid lesions[132,133]. For patients with small-cell lung cancers, which are predominantly aneuploid, DNA cytometry is likely to have limited or no prognostic value[134].

Aneuploidy in urogenital cancer

Prostate carcinoma

Significance of aneuploidy in needle-biopsy samples. More than 20% of the prostate carcinomas that are classified as organ-confined (T1 or T2) based on digital rectal examination and ultrasonography are later found to be locally advanced at prostatectomy[135,136]. The strength of the biopsy-based Gleason scoring system is that it takes into account intratumour heterogeneity, which is commonly seen in this cancer; however, this score is a subjective measure and, despite continuous advances in the effectiveness of this approach, considerable room for improvement in both accuracy and reproducibility remains[137]. Aneuploidy assessment offers a more-objective measure, which correlates closely with Gleason score in resection material[136,138,139]. Despite encouraging reports published before 2000 of an important multivariate-independent prognostic role of ploidy assessment[140], findings of more-recent studies suggest that, although aneuploidy is a significant predictor of clinical outcome, analysis of this characteristic does not add much information over that provided by established prognostic factors (SUPPLEMENTARY TABLE 7)[55]. In a single study in which authors reported on the prognostic value of ICM-derived ploidy status based on nuclear suspensions from needle-biopsy specimens from 94 patients undergoing brachytherapy, aneuploidy in the needle-biopsy specimen with the highest Gleason grade was a strong multivariate predictor of relapse (HR 5.13)[141]. The usefulness of DNA-ploidy status, compared with Gleason score, in predicting disease progression in patients with prostate cancer who are under active surveillance remains unknown[140].

In a trial involving 149 patients with advanced-stage prostate cancer (stage T2–T3) who received either radiotherapy or radiotherapy plus short-term total androgen ablation (TAB)[142], DNA aneuploidy in needle-biopsy specimens was an independent predictor of poor overall survival (HR 1.55, $P=0.03$), but not metastatic relapse. This prognostic effect of non-diploidy was entirely attributable to the poor survival of the patients who received radiotherapy plus TAB; the overall survival for patients in the radiotherapy-alone cohort was not significantly influenced by the ploidy status of pretreatment needle-biopsy samples[142,143].

Prognostic value of aneuploidy in prostatectomy specimens. The results of early cytometry studies generally indicated that aneuploidy in radical prostatectomy specimens is associated with a higher probability of relapse (reviewed elsewhere[140,144]). Findings of more-recent studies confirm both the univariate[145] and multivariate prognostic value of aneuploidy in prostatectomy samples (SUPPLEMENTARY TABLE 8)[146–149]. In particular, a potentially clinically relevant prognostic role of aneuploidy assessment has been indicated for the large number of resections performed in patients with a Gleason score of 7 (4 + 3 or 3 + 4): two papers[146,149], in which authors examined the significance of ploidy status in this setting, reported a prognostic benefit of ploidy status for predicting 10-year DSS in multivariate analyses. Within the subgroup of 68 patients with a Gleason score of 7 in a study by Pretorius *et al.*[149], DNA ploidy was found to be the only significant predictor of early relapse, with 10-year DSS rates for patients with diploid ($n=36$) and aneuploid ($n=6$) tumours of 84% and 0%, respectively (HR 11.6, $P<0.001$); the remaining 26 patients had tetraploid tumours and an intermediate survival rate with a 10-year DSS rate of 64%. Surprisingly, Gleason score was not an independent predictor of relapse in this study[149] — as also shown in a pilot study of DNA cytometry in pretreatment biospy material by Keyes *et al.*[141] In a study at the Mayo clinic, Ward *et al.*[143] reported that ploidy status in resection material had an independent predictive value in a mixed cohort of patients with T2–T3 cancers who underwent prostatectomy for persistent cancer after primary radiotherapy (HR 2.03; $P<0.008$).

Most studies of ploidy status in patients with prostate cancer have demonstrated the presence of large numbers of tetraploid tumours. For example, in patients with low serum PSA levels, 36% of needle-biopsy samples contained tetraploid DNA — half of these samples were from lesions with volumes <0.5 ml[150]. Among the Gleason score 7 cancers reported by Pretorius *et al.*[149], 38% were tetraploid. The significance of this finding is uncertain, but might relate to the fact that mitosis in tetraploid cells has been associated with a high probability of generating genomically unstable daughter cells[151].

Thus, all studies published to date indicate that prostate tumours that harbour aneuploid cells are more likely to recur following resection (SUPPLEMENTARY TABLES 7 and 8). What remains less clear is whether ploidy status adds much value to risk assessments beyond that of clinical stage and Gleason score. Following improvements in the procedure of Gleason grading, ploidy status has not always emerged as a significant predictor of outcome in multivariate analyses that include the traditional clinicopathological variables[55]. Of note, however, good interobserver agreement between histopathologists in expert studies might not be reflected when a scoring test is used in the clinic.

Urinary bladder carcinoma

More than half of all early stage (Ta and T1) urothelial cancers recur, and of these, ~20% recur at higher stage[152]. In 2003, Baak *et al.*[30] introduced a method of fast automated ICM for measuring ploidy status in the nuclei of single cells in suspension. The investigators retrospectively studied the relationship between DNA ploidy and disease recurrence and progression based on pretreatment bladder-biopsy samples from 228 consecutive patients with Ta and T1 tumours[30]. With a median of 4-years of follow up, 88 patients experienced relapse and 13 had stage progression; in multivariate analysis, DNA-ICM features predicted recurrence and stage progression more accurately than classic prognostic factors, independent of treatment modality[30]. Similar findings in patients with early stage bladder carcinoma had previously been reported by other groups, based on tumour cells in bladder-biopsy samples, bladder washings, and voided urine[153–155], although in immunocompromised patients, urinary tract polyomavirus infection can produce 'decoy' aneuploid cells that are not associated with neoplasia[37].

Yamamoto *et al.*[156] confirmed ICM aneuploidy as a univariate predictor of recurrence (HR 1.64), but in a multivariate analysis, in which aneuploidy was compared with various other nontraditional candidate markers of recurrence, only tumour stage and tumour shape emerged as independent predictive factors. In patients with muscle-invasive (T2) disease, ploidy status according to FCM was found to be a strong multivariate predictor of DFS following radical cystectomy (for a summary of these and other reports see SUPPLEMENTARY TABLE 9)[57].

DNA-ploidy analysis and DNA sequencing

The availability of high-throughput next-generation sequencing (NGS) has enabled the accurate detection of DNA mutations, copy-number alteration and chromosomal rearrangements; however, widespread adoption of this technology has been hindered by its costs and the expertise required to correctly interpret test results. By contrast, DNA-ploidy analysis is a relatively simple, economical, and robust methodology for detecting gross genomic alterations. DNA-ploidy and DNA-sequencing data are potentially complementary, with ploidy analysis providing an overview of the state of the cellular genome and DNA-sequence analysis providing details of the specific genetic changes. Undoubtedly, a need exists for in-depth mutation analysis, such as that provided by NGS, to unravel the molecular mechanisms underlying carcinogenesis. In many circumstances, however, an overview of gross DNA changes, as derived by ploidy analysis, might be as — if not perhaps more — useful for routine patient management that includes prognostication of relapse risk and survival, than the exhaustive and detailed results provided by NGS analysis. Currently, intratumoural genetic heterogeneity is of great scientific interest, and might be another factor that, in the long term, raises questions regarding the clinical significance of many NGS results[158,159].

For a biomarker to be implemented in clinical practice, the cost–benefit ratio, test objectivity, positive and negative predictive values, turnaround times, and quality assurance all need to be considered. DNA-ploidy analysis certainly holds promise in all of these regards. Studies examining these factors in relation to NGS analyses and, critically, comparing the clinical utility of this approach with that of ploidy analysis are urgently required.

Conclusions

Owing to the extraordinary pace of advances in molecular biology, and in particular DNA-sequencing technologies, we are able to interrogate the human genome in ever-increasing detail. Such investigations produce vast amounts of data, often demonstrate large numbers of mutations and polymorphisms in genes and their promoters, many of which might ultimately be of clinical significance, and produce results that will increasingly be used to direct clinical therapy. At the same time, mitotic dysregulation, which is a characteristic of most cancers, is accepted as a cause of large-scale genomic instability (CIN), which is associated with intratumour heterogeneity and clonal numerical chromosome aberrations and/or aneuploidy. The fact that aneuploidy has been found at the earliest stages of carcinogenesis has been taken as evidence that CIN is a fundamental process in the development of many or most cancers[20,66,160,161].

For many cancers, specific biomarkers are now available that can be used clinically to assess patient prognosis and predict response to treatment, but these biomarkers often have no functionality in other tumour types. By contrast, DNA-ploidy status, which can be assessed in all tumours, acts as a marker of CIN, and has clear potential as a useful prognostic indicator of risk of relapse and/or disease progression in many tumour types — and can thus complement more-detailed gene-expression studies. Findings of early studies examining DNA ploidy in epithelial tumours were blighted by small sample sizes, inclusion of mixed populations of tumours of varying stage, and the use of a number of different analytical methodologies. Over the past decade, however, it has become apparent that, for histological material, DNA ICM is the analytical method of choice for enabling the assessment of samples containing low numbers of cells and also for the identification of tumour cells. The evidence discussed herein demonstrates the utility of DNA cytometry in a large number of tumour types, particularly when examining the prognosis of patients with carcinomas stratified by stage. Indeed, assessment of DNA ploidy has emerged as one of the strongest independent prognostic biomarkers in multivariate analysis for a number of defined patient groups with common

carcinomas[24,25,30,109,120,149,162]. In multivariate analyses, use of ploidy status has occasionally been surpassed as a prognostic marker by other histological parameters (such as Gleason grade in prostate carcinoma), but the fact that expert studies of histological parameters often show levels of intraobserver agreement between histopathologists that are not necessarily applicable when a scoring test is applied more widely by non-experts should be recognized[163]. DNA ICM is a relatively simple and inexpensive test, which can be readily automated, and produces much-less-subjective results than routine histological assessment.

To optimize tumour treatment and management, the personalized-medicine paradigm is expected to develop further and to eventually become the norm. Even in the presence of underlying CIN, detailed interrogation of the genotypes of both tumour and normal tissues is expected to guide selection of the most-appropriate therapy. In the foreseeable future, conventional guidance from clinicopathological information, such as tumour stage and histopathological grade, will remain central to the assessment of patient prognosis and to disease management, although with increasing supplementation using other measures. We conclude that in the tumour types discussed herein, and also in others we have not reviewed, the case for including genome size as one of these measures is becoming very strong.

This Review is timely in light of the current academic interest in the role of large-scale genome instability in cancer development. It is accepted that aneuploidy is an inevitable result of CIN, and that this feature can be detected and quantified using DNA cytometry. From this fact, one might draw the conclusion that ploidy analysis using DNA cytometry should become a key part of the clinical evaluation of the tumour types evaluated in this Review. Despite technical imperfections in some situations, small patient numbers in others, and the clear need for replication studies in some settings, the consistency of results that are emerging from these disparate survival studies in different epithelial cancers is impossible to overlook. The evidence reviewed herein supports the use of DNA cytometry as a potential prognostic tool in patients with most types of cancer.

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Author contributions

All authors contributed substantially to researching data, discussion of content, writing, and review/editing of the manuscript.

Competing interests statement

M.N. is a Medical Director at Room4 Ltd, a UK company that develops and sells ploidy systems. The other authors declare no competing interests.

Review criteria

We performed searches of the PubMed database for original peer-reviewed, full-text, English-language articles published between 2000–2014 using search items "breast cancer", "endometrial cancer", "oesophageal cancer", "ovarian borderline tumour", "ovarian cancer", "Barrett's oesophagus", "ulcerative colitis", "colorectal cancer", "lung cancer", "prostate cancer", "urinary bladder cancer", in combination with "DNA ploidy" and "prognosis". In addition, we searched for relevant articles particularly, review articles. We excluded articles reporting studies that did not perform multivariate analyses, those that reported on mixed cohorts of patients with tumours of all clinical stages or reported result of ploidy analyses performed by noncytometric methods, or wherever results are offered without sufficient description of how they were obtained.

Supplementary information is linked to the online version of the paper at www.nature.com/nrclinonc.

CORRIGENDUM

Revisiting tumour aneuploidy — the place of ploidy assessment in the molecular era

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In the version of this Review originally posted online, the VICTOR trial was inaccurately referred to as the VITOR trial in one instance. This error has now been corrected in the print and online versions of the article.