

DNA Histogram Analysis and Breast Cancer Prognosis

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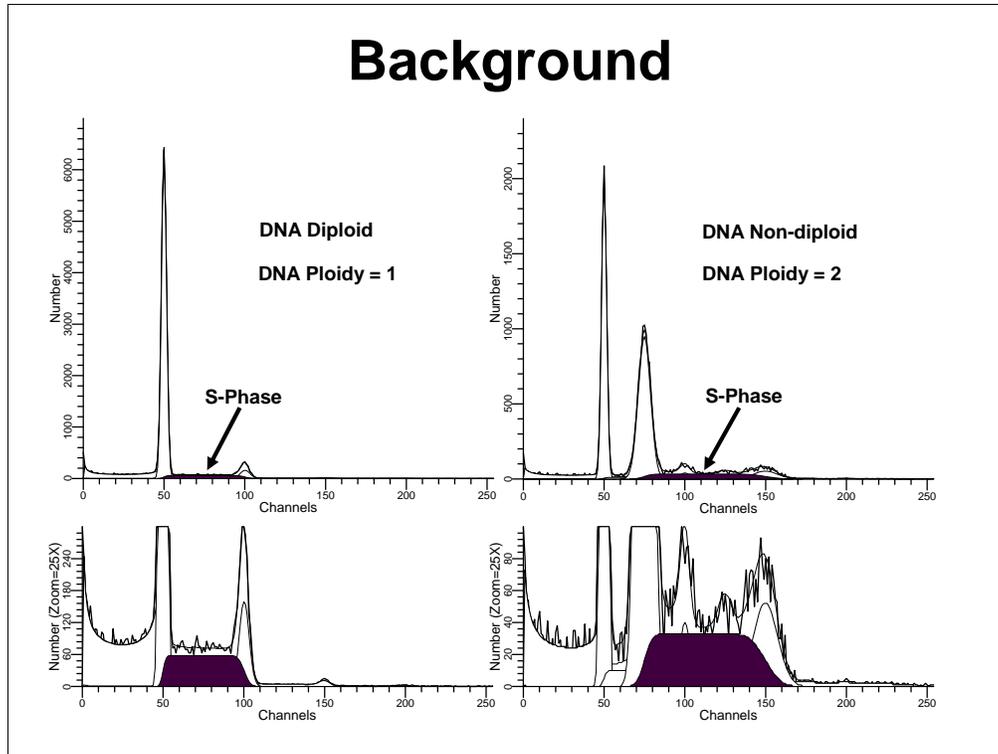
Is it possible to obtain a small cell sample from a primary tumor and, with the appropriate methodologies and mathematics, make some predictions about a tumor's virulence? Is it possible to create a breast cancer prognostic procedure and model that works well for any laboratory in the world? Can DNA histogram analysis be complemented with other prognostic markers to better separate high and low risk patients? The major purpose of this lecture is to answer all these questions with data from several large clinical studies.

Approach

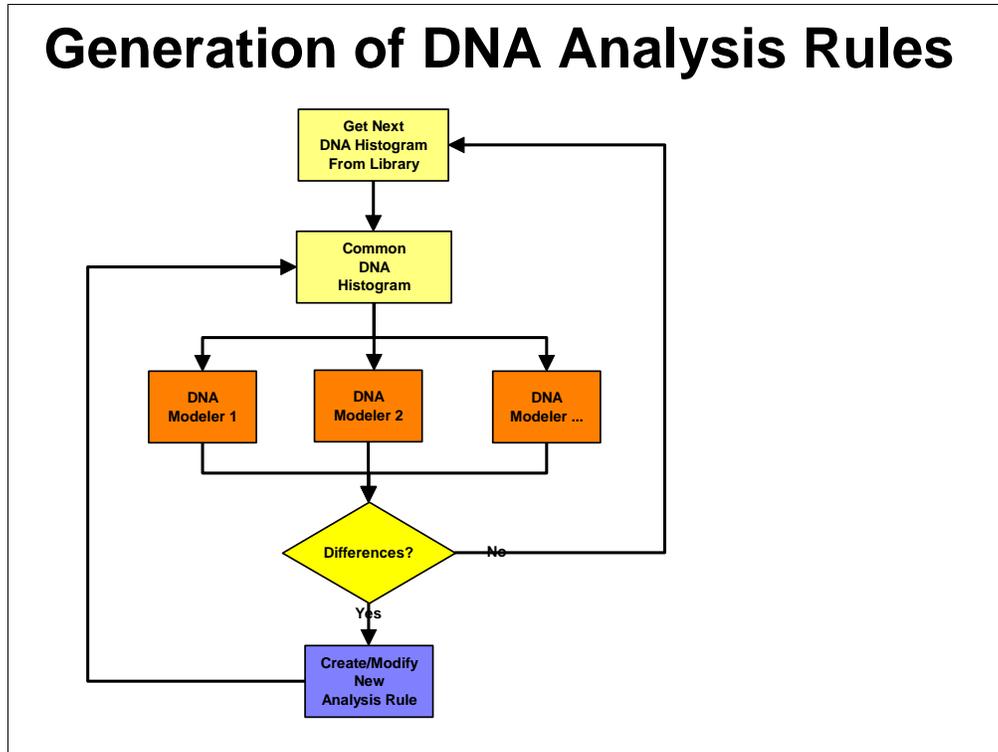
- **Develop a set of DNA analysis rules by minimizing differences between parallel analyses of common DNA histograms.**
- **Apply these rules to the analysis of a large primary database of DNA histograms with appropriate clinical follow-up.**
- **Develop a set of adjustments to DNA ploidy and S-Phase Fraction (SPF) estimates that minimize potential variability and maximize the model's prognostic strength.**
- **Evaluate the prognostic model's ability to stratify patients in the primary database and then apply the same model and procedures to two other large databases and compare patient stratifications.**

A set of analysis rules was developed to insure that all cell cycle analyses were reproducible. These rules were applied to both a large primary database (Baylor, n=992 DNA histograms) and a confirming database (Sweden, n=210) with clinical follow-up data. In laboratory we will examine how these rules are implemented in some detail.

A detailed statistical analysis of the primary database revealed a number of necessary SPF adjustments and ploidy reclassifications that minimized spurious correlations between SPF and ploidy and maximized a Cox proportional hazard's model's prognostic strength. The procedures and prognostic model developed from the primary database were then applied to large confirming databases and the patient stratifications were compared.



Flow cytometry DNA Ploidy and S-Phase prognostic variables. DNA Ploidy is a binary variable, 1 for DNA diploids and 2 for non-diploids. S-Phase for DNA diploid histograms is the fraction of nuclei or cells in S-Phase compared to other phases of the cell cycle. S-Phase for DNA non-diploid histograms is the fraction of nuclei or cells in the aneuploid S-Phase compared to the corresponding aneuploid phase of the cell cycle. For DNA multiploids, the S-Phase is calculated as the total number of events in all aneuploid S-Phases divided by the sum of all aneuploid events, expressed as a percent.



In order to obtain consistent DNA analysis results, it is very important that the DNA histogram modeler follow very specific rules. At this stage of the analysis, the rules are designed so that different modelers will obtain the same exact data.

The above flow chart demonstrates how the DNA analysis rules were initially generated. A common set of DNA histograms was used in the process. If independent operators arrived at different answers, a rule was found to eliminate the differences. Iterating through this process for hundreds of histograms generated a set of rules that if followed, allowed operators to achieve reproducible results. General model type was found to be the most important difference to minimize. Most of these rules are targeted at guiding operators to choose the same kind of DNA model. The rules also cover more subtle differences such as range positioning strategies.

Note. Over the last 20 years there have been numerous discussions and arguments on the best way of analyzing DNA histograms. The approach taken here was to let the above algorithm generate the rules and not to introduce personal biases into the decision.

DNA Analysis Rules

A: Model Selection Check

The most important step in analyzing DNA histograms in a consistent manner is checking the correct ploidy model for a particular DNA histogram. In some cases this process may require several analyses to achieve the correct and optimal fit, i.e. the RCS value should be as low as possible (< 3.0). Use the rules below to help guide you through this process.

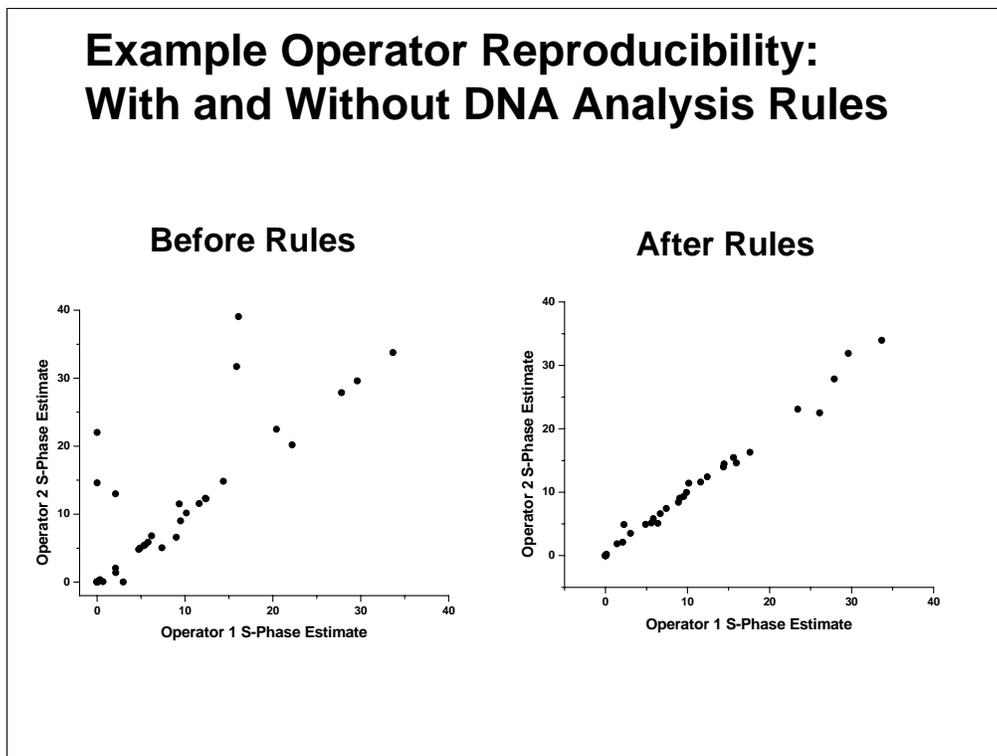
1. General Considerations

- a) If two model components are of similar shape and are highly overlapped (>75%) it may be necessary to add additional constraints to the model or, in the worse case, disable the model component of lesser importance.
- b) If a G2M peak is clearly visible and well-defined, allow its mean to be fitted (float).
- c) Always model S-Phase as a single, broadened rectangle.
- d) After the appropriate model is selected, optimize the linearity settings in the cell-cycle analysis software to the data.
- e) (ModFitLT only) Try to standardize the configuration, peak finder and autoanalysis settings.
- f) When choosing between two very similar models, select the one that gives consistent results with slightly different range settings.

Example
Rule
Fragment...

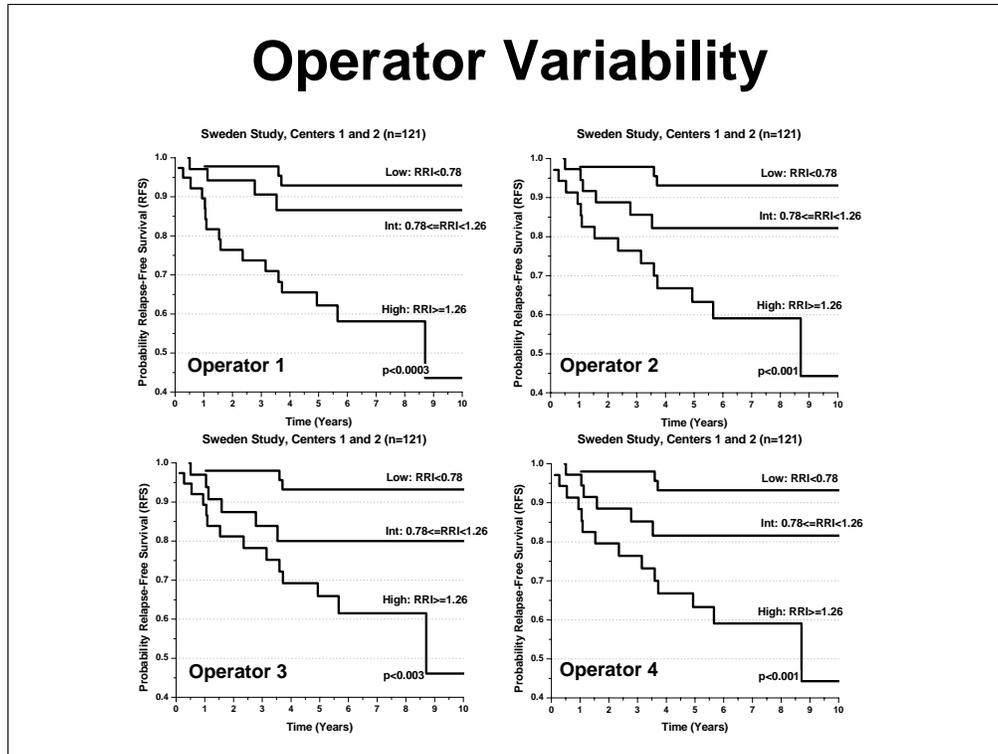
An example of this rule might be when trying to use an aneuploid model with a near-tetraploid type of histogram. If the aneuploid model only works with very specific range settings, choose the tetraploid model instead.

Small example fragment of the final rule set. See notebook for full printout of the rules.



Correlation of %SPF estimates before and after use of the developed rules on a set of common DNA histograms. Convergence of operator estimates occurs after a series of training exercises on defined sets of DNA histograms.

Note. When doing this kind of reproducibility study it is very important not only to show that one has reproducibility between independent users, but also that the data generated is relevant (see next slide).



The above slide shows four Relapse-free survival curves from data independently generated by four operators. The operators followed the DNA analysis rules described earlier. The primary data is from the Sweden Study, Centers 1 and 2 (n=121). The Chi-square probabilities for these analyses were: operator 1, $p < 0.0003$; operator 2, $p < 0.001$; operator 3, $p < 0.003$ and operator 4, $p < 0.001$.

These data demonstrate that the rules not only create reproducible data but the data is clinically relevant.

Approach

- Develop a set of DNA analysis rules by tolerating no or minimal differences between parallel analyses of common DNA histograms.
- • **Apply these rules to the analysis of a large primary database of DNA histograms with appropriate clinical follow-up.**
- Develop a set of adjustments to DNA ploidy and SPF estimates that minimize potential variability and maximize the model's prognostic strength.
- Evaluate the prognostic model's ability to stratify patients in the primary database and then apply the same model and procedures to another large database and compare patient stratifications.

Cell cycle analysis was performed on the primary database following the rules described in the preceding section.

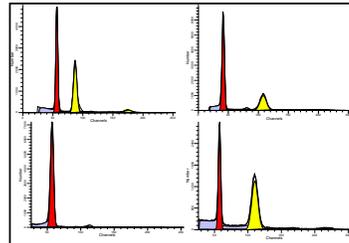
Primary Flow Cytometry Database (Baylor Study) Overview

Preparation

Baylor*
**(Clark,
Nichols)**

Type: Frozen-Pulverized (>200mg)
Fluor: PI
Method: Krishan
Cytometer: EPICS 753
Cases: 992

Example DNA Histograms



Baylor Study

A total of 961 node-negative cases was initially considered for analysis. Most analyses were done on 935 cases having acceptable DNA histograms, %Diploid CV's of less than or equal to 7.0 and total modeled events of greater or equal to 15,000. Median follow-up for those patients alive with no recurrence is 6.6 years. Recurrence-free survival is defined as the time interval between diagnosis and distant metastatic recurrence of tumor. Median age is 61 years. Adjuvant chemotherapy treatment breakdown is 775 (80.6%) none, 3 (0.3%) single alkylator, 145 (15.1%) combined alkylators, 6 (0.6%) not specified and 32 (3.3%) unknown. Endocrine treatment breakdown is 640 (66.6%) none, 272 (28.3%) single additive hormone, 6 (0.6%) single ablative hormone, 9 (0.9%) multiple and 34 (3.5%) unknown. The radiation therapy breakdown is not known. Preparative technique for DNA Histograms is pulverized frozen tissue stained with propidium iodide.

The primary database originates from primary breast cancer samples which were frozen and then pulverized and subsequently stained with PI following the Krishan method. A EPICS 753 acquired the data and stored it in a listmode format.

A very aggressive approach was followed in modeling the 992 DNA histograms in the primary dataset. All peaks were modeled regardless of size. Skewed and very near diploid peaks were modeled as aneuploid populations. This aggressive approach made it possible to create low risk ploidy reclassification rules that were based entirely on statistical analysis of patient outcome data and not on any other subjective criteria or operator bias.

Confirmatory Studies

Sweden Study

Three Swedish laboratories provided data from a total number of 210 node-negative patients: center 1, Lund (42); center 2, Linköping (79); and center 3, Stockholm (89). The median follow-up for patients alive with no recurrence is 5.9 years. Recurrence-free survival is defined as the time interval between diagnosis and distant metastatic recurrence of tumor. Node-negative adjuvant therapy breakdown is 24 (11%) unknown, 116 (55%) no adjuvant therapy, 70 (33%) tamoxifen and 0 (0%) cytostatic chemotherapy. No radiation therapy was given to any patients. Preparative technique for DNA histograms is frozen tissue stained with propidium iodide.

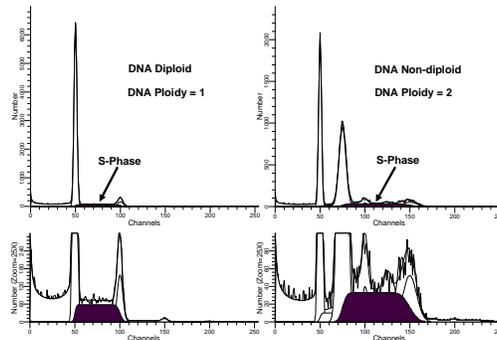
French Study

Four French institutions provided data from a total number of 220 node-negative patients: Angers (24), Marseille (57), Saint Cloud (69) and Tours (70) receiving no chemo or hormonal adjuvant therapy. The radiation therapy breakdown is 46 (20.9%) none, 174 (79.1%) treated. The median follow-up for patients alive with no recurrence is 8.5 years. Recurrence-free survival is defined as the time interval between diagnosis and occurrence of distant metastatic disease. The median age is 58 years. Preparative technique for DNA histograms is frozen tissue stained with propidium iodide.

A very important element of this study design is that the rules developed on the Baylor database will be tested on two other studies to confirm the validity of the approach.

Is Raw DNA Ploidy a Prognostic Factor?

1: Diploid, 2: Non-diploid



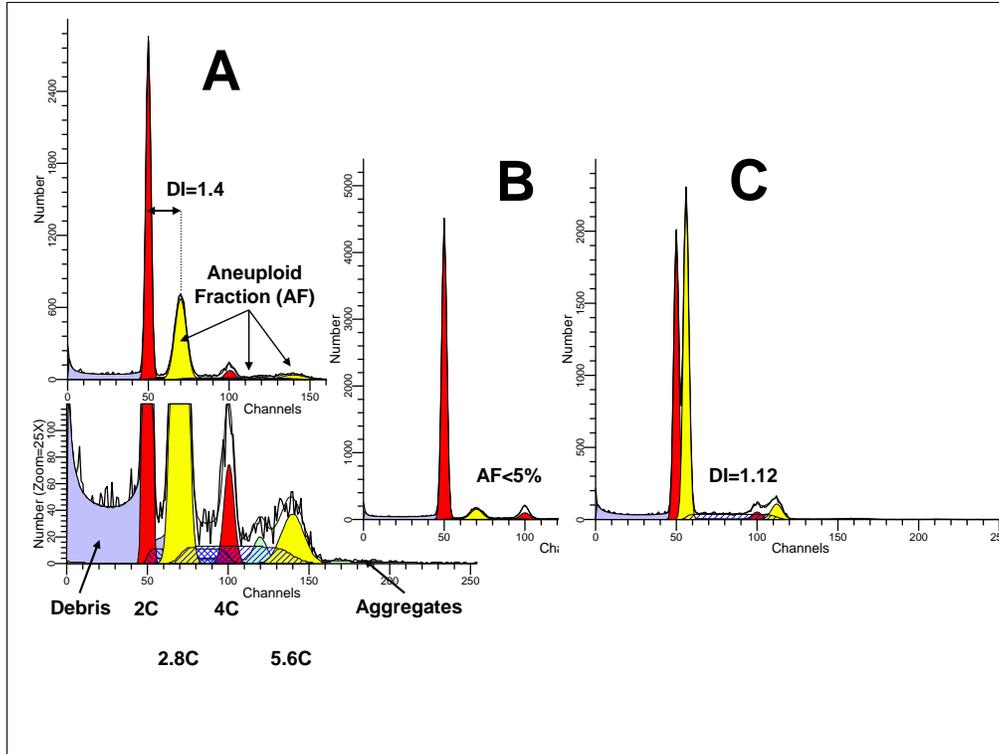
Ploidy

Study	n	Beta	StdDev	p-value
Baylor	988	0.227	0.145	0.112

Before showing you the necessary adjustments to DNA ploidy and S-phase, let's look at the situation without any adjustments. Suppose we go back to the Baylor Study and test to see if raw ploidy (1=DNA Diploid, 2=Non-Diploid) is a statistically significant prognostic variable.

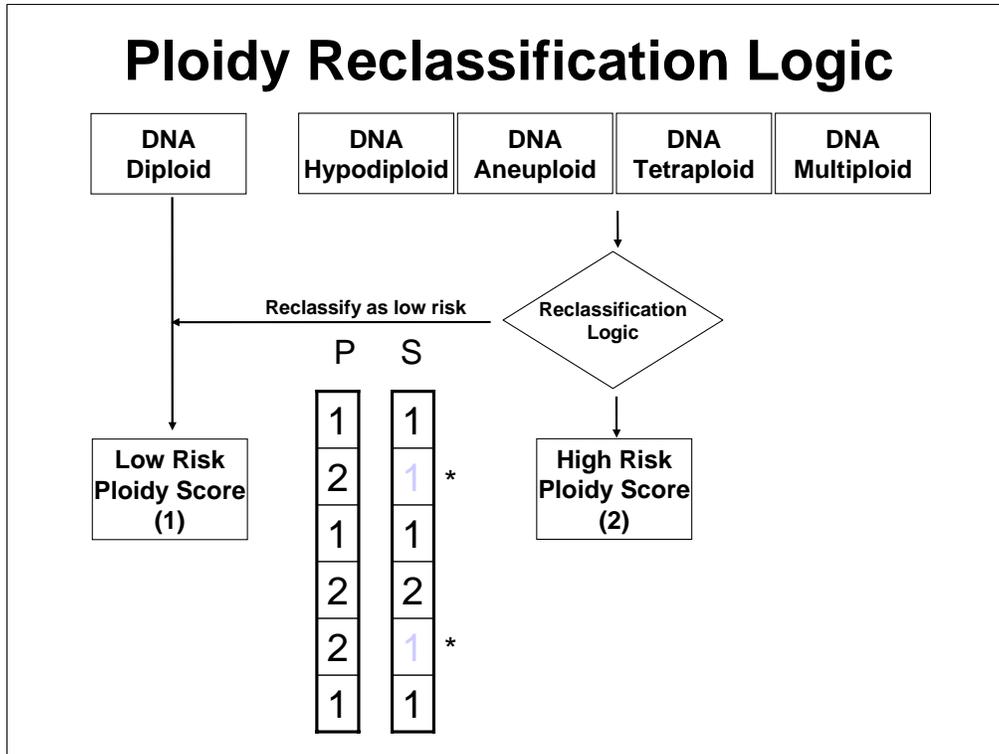
The results shown in this table should surprise you. The Baylor Study shows absolutely no significance for DNA ploidy. Many investigators probably ran into this observation and either published negative results for DNA ploidy or decided not to publish. When the Sweden Study was analyzed in the same exact manner as the Baylor Study, its DNA ploidy variable was significant at $p < 0.01$ (not shown).

Why would DNA ploidy be significant for the Sweden Study but not for the Baylor Study? Why would there be this kind of variability with relatively large studies? We believe the variability in findings between these two databases is indicative of what has been happening in the flow cytometry literature. As you soon will see, the answer to this dilemma is found in the details. Oddly enough, those investigators who were very careful in their analysis were the least likely to see significance. Let's see why.



In the picture above, we see some typical DNA histograms. A classic DNA aneuploid histogram is shown in Panel A, demonstrating DNA Index (DI) and Aneuploid Fraction (AF) calculations. In the 25X Y-zoom graph immediately below Panel A are labels identifying debris, aggregates and some important peak locations. Panel B shows a DNA histogram with a very low AF (<5%) and Panel C, a near diploid G1 population with a DI of 1.12.

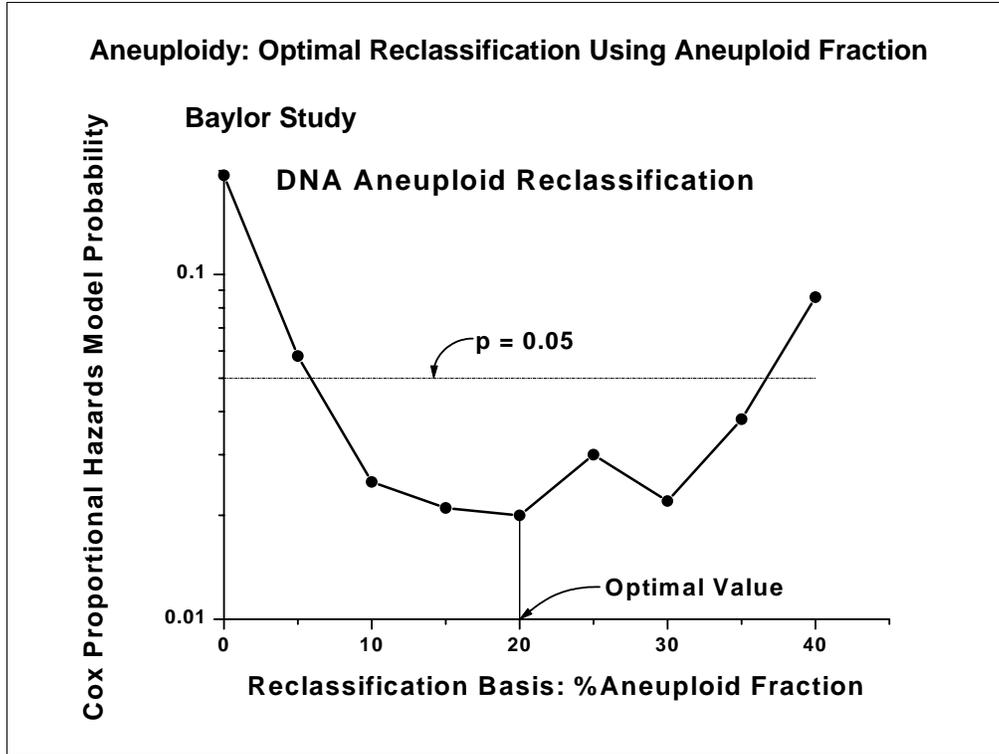
As mentioned earlier, a very aggressive DNA analysis approach was undertaken for the DNA histograms in the Baylor Study. If a small aberrant population was found (see panel B for a typical example), it was modeled as a DNA aneuploid population. Also, if a diploid G0G1 peak was split, we assumed that there was a near-diploid aneuploid population present and appropriately modeled it (see panel C). The question to ask at this point is whether there is a better way of determining when an aberrant peak is important from a prognostic point-of-view. In other words, can we find some objective rules to follow to know when and when not to model aberrant peaks and/or populations.



With a large database like the Baylor Study, we can determine the optimal values of a number of decision points such as the size of an aberrant peak or the closeness of a near-diploid population.

The way this was done is shown in the above slide. If a DNA histogram was classified as a simple DNA diploid, it was given a low risk ploidy score of 1. If the DNA histogram had some other kind of ploidy pattern (DNA hypodiploid, aneuploid, tetraploid, or multiploid), we used some yet-to-be-determined reclassification logic to either classify this case as low risk or high risk. In other words, we made the assumption that within the apparent DNA aneuploid ploidy patterns, there were some patterns that had a low prognostic risk.

The above reclassification is very simple, but it allows us to determine some very important criteria that have traditionally been a "seat-of-the-pants" type of decision. Let's go through a few examples to see how this works before summarizing all the reclassification rules.



The above graph is only looking at simple DNA aneuploids, excluding DNA tetraploids, hypodiploids and multiploids. The data shown in the graph answers the question, when is it "best" to consider an unusual bump in a DNA histogram as a high risk ploidy pattern.

On the X-axis we have %aneuploid fraction, which is essentially the size of this "extra" population. On the Y-axis we have the Cox Proportional Hazards Model p-value. Lower p-values indicate that there is a significant difference between the low risk and high risk ploidy patterns for the relevant DNA histograms.

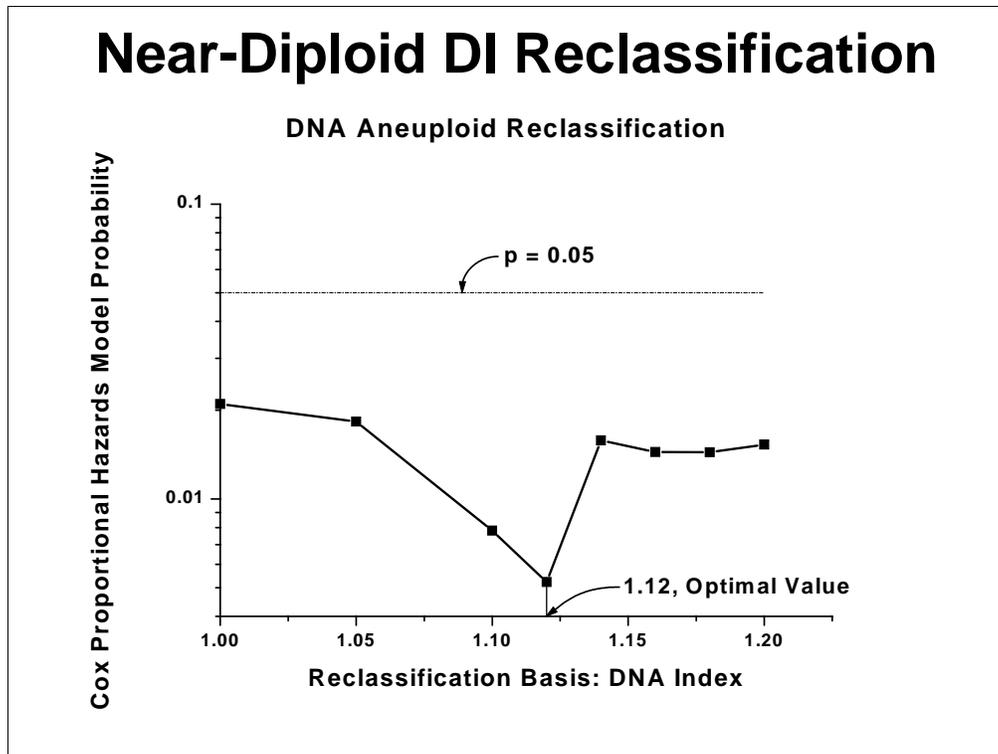
Let's start at the first point (upper-left). This p-value is what you would find if there were no reclassification scheme (e.g. traditional ploidy analysis), no significance in this case (see slide 11).

The second p-value is obtained when those DNA aneuploid histograms that have a %Aneuploid Fraction of less than or equal to 5% are reclassified as low risk (the example in panel B on slide 13 is about 5% so it would fall into this category). All other aneuploid histograms are considered high risk.

If we proceed with this analysis with successively higher Aneuploid Fraction% thresholds for this reclassification, we note that there is a definite minimum p-value, 0.02, at an aneuploid fraction of 20%.

Thus, DNA aneuploid histograms that have $\leq 20\%$ aneuploid fraction are best reclassified as a low risk ploidy pattern, which is our first reclassification rule.

A few points need to be emphasized at this point. The first is that it is very important to have a large study like the Baylor Study to do this type of analysis, especially for some of the rules that follow. Second, these rules can sometimes be verified in other studies. For example, the Sweden Study shows the exact same minimum value for DNA aneuploids as the Baylor Study. Third, there is no subjectivity in this analysis. We are simply letting the data determine these criteria.



This slide demonstrates when a shoulder or split peak should be considered a high risk ploidy pattern.

The aneuploid fraction reclassification discussed in the previous slide is in effect for this analysis. Note that the preceding slide demonstrated a minimum at 0.02 which is where this analysis starts (left most point).

The X-axis is now DNA index. The second point shows the p-value associated with reclassifying DNA aneuploid histograms with a DI of less than or equal to 1.05.

Again, there is a definite minimum p-value at a DI or 1.12.

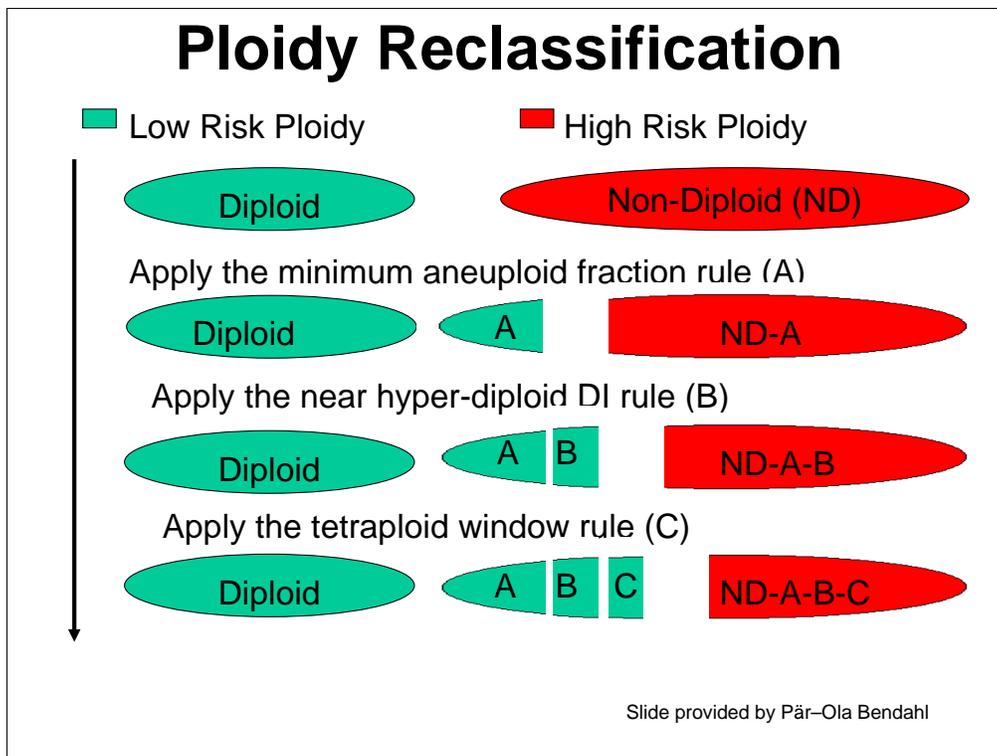
This observation should come as some relief to those of us involved in interpreting DNA histograms since the high risk near-diploid peaks are quite separate with typical %CV's.

Also note that this observation may partially explain why low resolution technologies such as image analysis have historically been successful in showing the prognostic significance of DNA ploidy. Now, you can understand my point about why careful investigators were likely not to find prognostic significance for DNA ploidy. By attempting to model small peaks and skewed/split peaks, they were stacking the deck against their finding significance. No wonder our literature is filled with contradictory results for this important prognostic factor.

Summary of Adjustments

Notation	Target	Description
DNA Ploidy Adjustments (ADJP)		
PH1	Hypodiploid	$\leq 15\%$ AF reclassified as low risk.
PH2	Hypodiploid	$0.95 \leq DI < 1.0$ reclassified as low risk.
PA1	Aneuploid	$\leq 20\%$ AF reclassified as low risk.
PA2	Aneuploid	$1.0 < DI \leq 1.13$ reclassified as low risk.
PT1	Tetraploid	$\leq 20\%$ AF reclassified as low risk.
PT2	Tetraploid	$(\text{Mean DI} - 0.04) \leq DI \leq (\text{Mean DI} + 0.04)$ reclassified as low risk.
PM1	Multiploid	$\leq 25\%$ AF reclassified as low risk.
S-Phase Adjustments (ADJS)		
SD1	Diploid S-Phase	DS/AF to adjust for diploid dilution effect.
SA1	Aneuploid S-Phase	$\%AS - b1 \cdot (100 - \%AF) / \%AF$ to compensate for aneuploid fraction effect.
SA2	Aneuploid S-Phase	Substitution of the average SA1 adjusted AS with $\%AF$'s less or equal to 5%.

The first seven rows in the above chart show all the DNA ploidy reclassification rules. Note PA1 and PA2 are the ones we just showed in the examples. The others were arrived at in the same way.



Here is another way of looking at what we're doing. The shapes in green are low-risk and those in red are high-risk. As we come up with the reclassification rules we are moving DNA histograms from the high-risk category to low-risk.

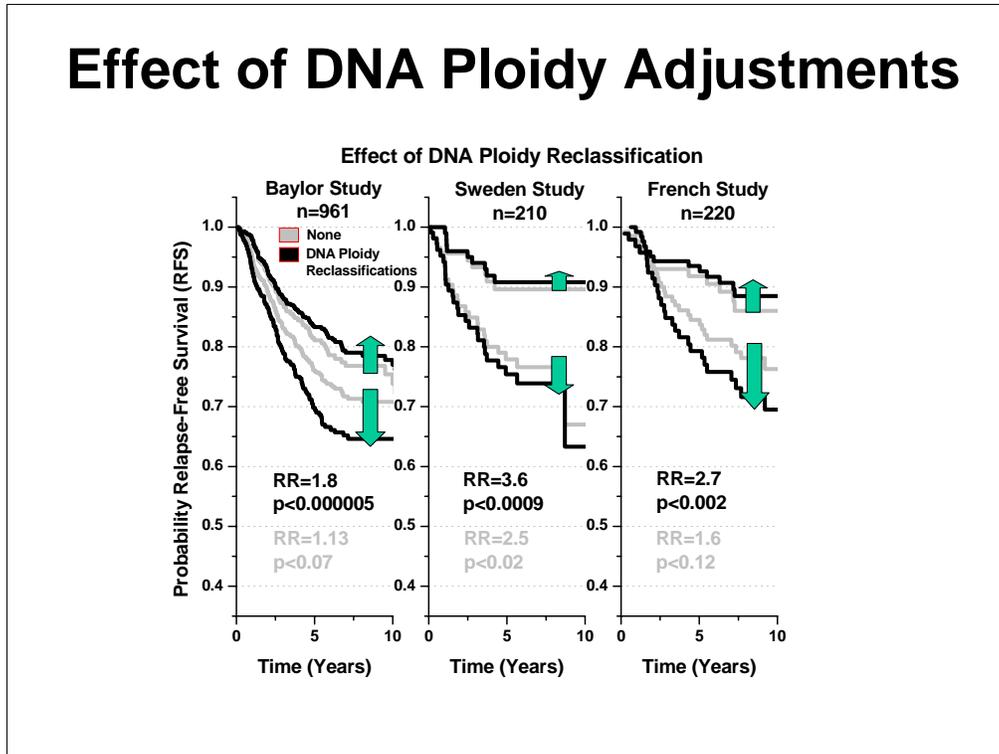
Importance of DNA Ploidy Reclassification

Cox Proportional Hazards Analysis

Reclassification	Global Chi-Square	Beta	Std Error	p
No	2.38	0.224	0.147	0.12
Yes	16.3	0.548	0.135	0.000049

n=961 Cases

Let's go back to our original analysis and add all the previously discussed DNA Ploidy reclassifications. As can be seen in the above table, by applying these reclassifications our DNA Ploidy variable becomes highly significant.



The above graph is another way of appreciating how the reclassification enhances the prognostic effect of DNA Ploidy. It also shows how this approach works for the other two confirming databases (Sweden and French studies). The graphs represent relapse-free survivals (RFS) with (black) and without (light gray) DNA Ploidy reclassification for Baylor Study (left), Sweden Study (center) and French Study (right). Low-risk stratifications correspond to DNA diploid (gray) or DNA diploid plus low-risk non-diploids (black). High-risk stratifications correspond to DNA non-diploid (gray) or non-diploid minus low-risk non-diploids (black). Arrows indicate the improved separation of patient stratifications with DNA Ploidy reclassification.

DNA S-Phase

As you might imagine, there are similar types of adjustments to make with DNA S-Phase.

Aneuploid Fraction and SPF Estimation

- **Adjust non-diploid SPF's.**

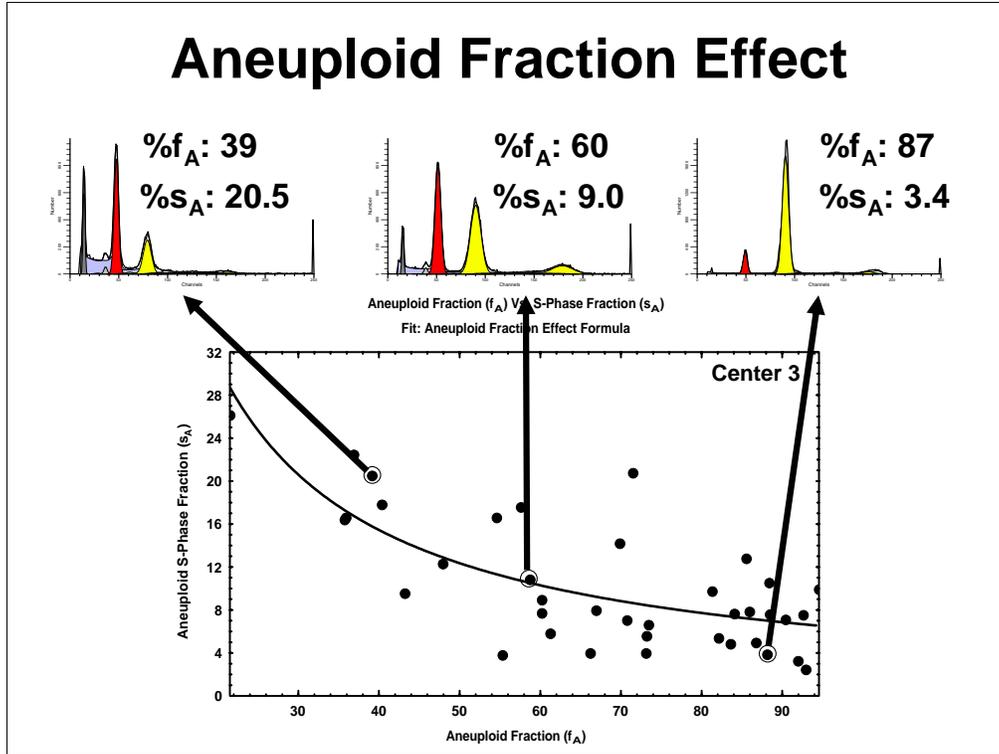
- Improves prognostic strength of SPF.
 - Reduces "technical" correlation between high SPF and aneuploidy.
 - Reduces laboratory variability due to preparation differences.

- **Adjust diploid SPF's.**

- Scale diploid SPF in order to pool with aneuploid SPF's.

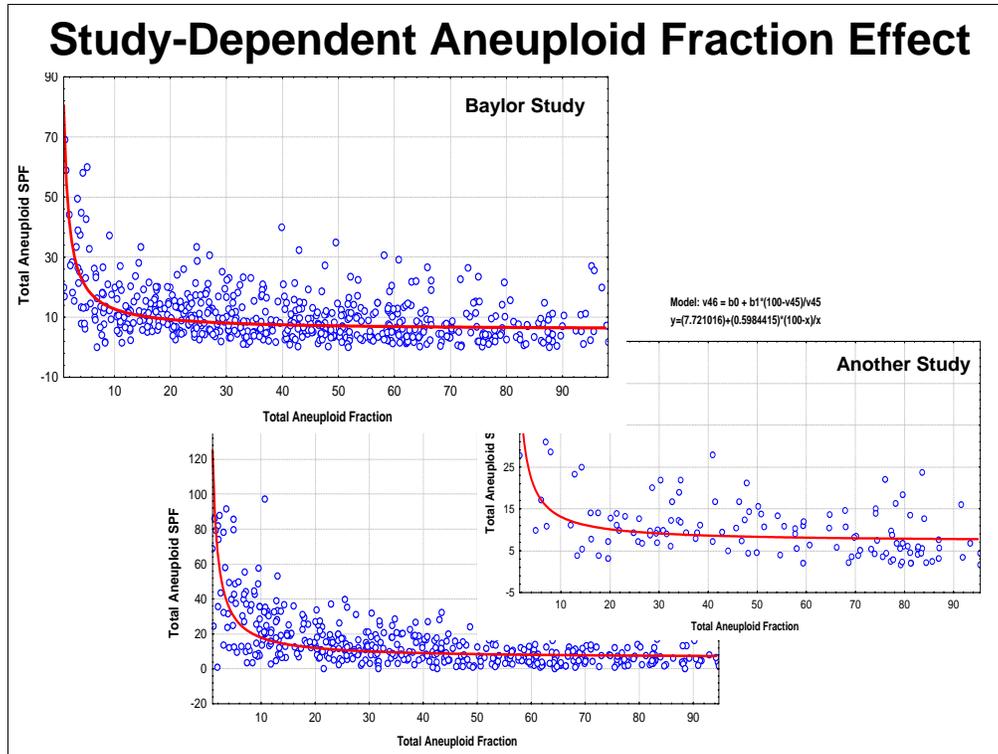
In many published studies, there is a significant correlation between S-Phase estimates and DNA Ploidy, making it difficult to reliably demonstrate independence of these prognostic variables. DNA diploid histograms generally have low S-Phase estimates and aneuploid histograms have higher estimates. Although there is evidence that aneuploid cells do in fact have slightly higher S-Phases than diploid cells, the difference is quite modest and does not adequately explain the high correlation generally found between these two variables.

The data that will be shown in the next few slides demonstrate that the principle reasons for the correlation between DNA Ploidy and SPF are technical in nature and can easily be compensated for by an appropriate set of adjustments.



As the aneuploid fraction, f_A , approaches zero there is a strong tendency for the modeling software to over-estimate aneuploid %SPF's (s_A). Presumably this tendency is because of an inability of modeling software to distinguish very low levels of S-Phase from background debris and aggregates originating primarily from the non-aneuploid cells. The three graphs at the top show three points in this database (Sweden, Center 3).

Notice the regression curve for this data. As the Aneuploid Fraction approaches zero there seems to be a high S-phase bias in our estimates. If we assume that the reason for this bias is due to a signal-to-noise type of explanation, we can derive this function as: $\%S_A = b_0 + b_1 \cdot (100 - \%f_A) / \%f_A$. We can use this function to fit the data and then use the variables to adjust for this effect ($\%S_A - b_1 \cdot (100 - \%f_A) / \%f_A$).

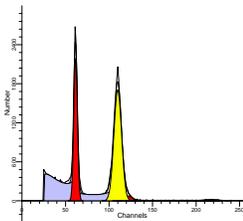


The above graphics show this "aneuploid fraction effect" from three other studies.

We have found that the exact coefficients for these regressions depend on the source of the DNA histograms and thus are probably influenced by the sample preparation and instrument; therefore, each study must be independently adjusted for this effect. Please note that you don't need clinical follow-up data to perform this adjustment.

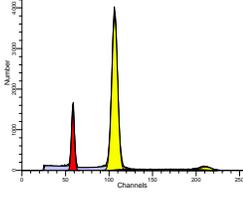
Rescaling Diploid %S

First, let's examine a few DNA aneuploid histograms...



Total %S: 1.62
Anp Fraction: 0.62
Anp %S: 2.61

Note that...
 $1.62/0.62 = 2.61$



Total %S: 5.95
Anp Fraction: 0.84
Anp %S: 7.08

$5.95/0.84 = 7.08$

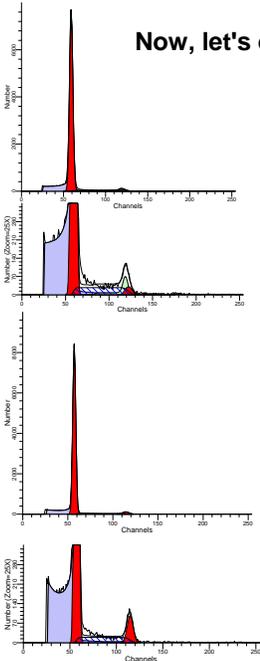
-Total %S is less than the Aneuploid %S because of a diploid dilutional effect.
-Total %S can be rescaled to equal the Aneuploid %S by dividing by the aneuploid fraction*.

*: Assumes diploid S is zero

The other spurious correlation between SPF and ploidy is due to the difficulty in pooling diploid and aneuploid SPF's. In order to better understand how the diploid SPF's are scaled so they can be properly pooled with the aneuploid SPF's, we first examine some aneuploid histograms. Notice that the total %S (or average %S) is always less than the aneuploid %S because of a diploid (non-tumor) dilutional effect. Also note that the total %S can be rescaled to equal the aneuploid %S by dividing by the aneuploid or tumor fraction.

Rescaling Diploid %S

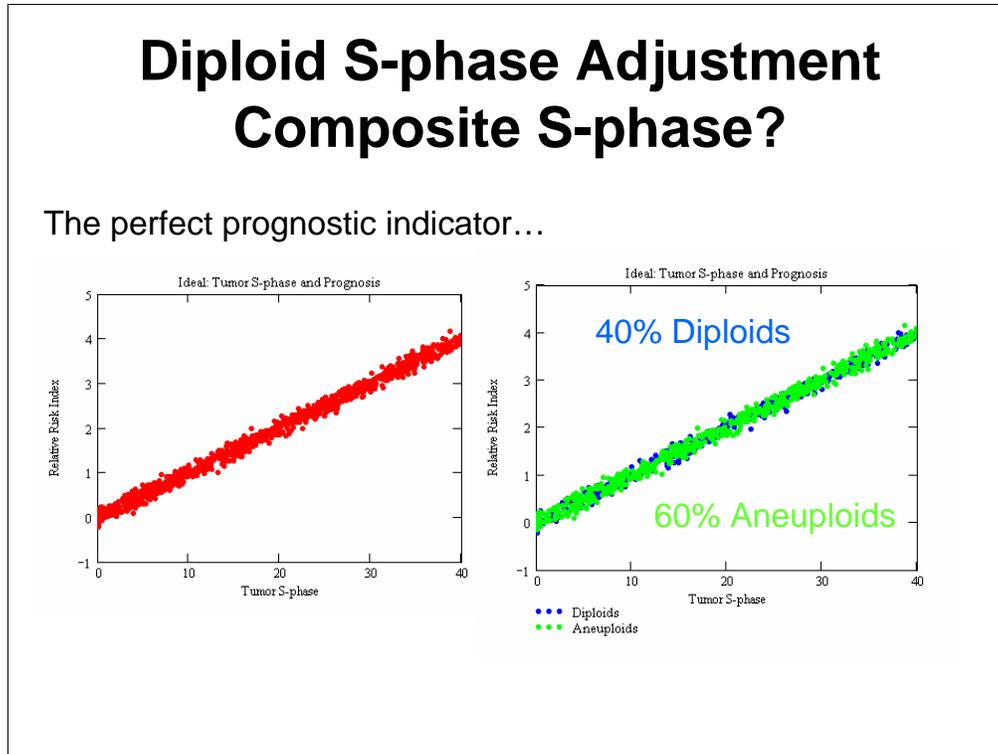
Now, let's examine a few DNA diploid histograms...



Diploid %S or Total %S: 4.50
Tumor Fraction: ???
Approximate Tumor Fraction with Aneuploid Fraction Mean (e.g. 0.50)
Rescaled %S: $4.50/0.50 = 9.0$

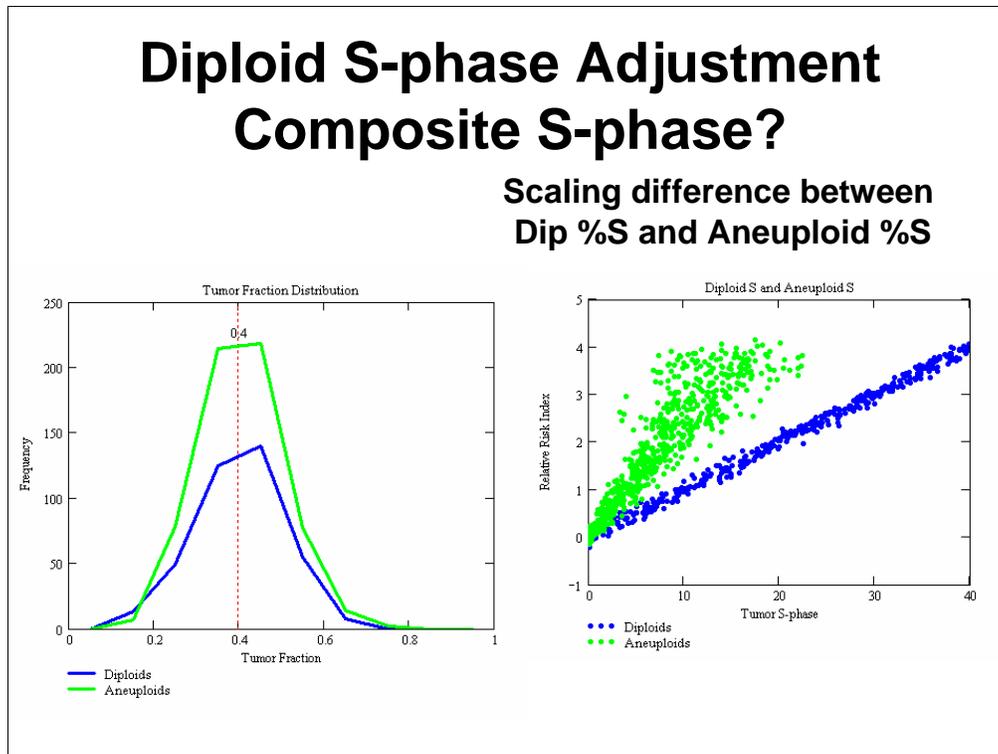
Diploid %S or Total %S: 3.11
Tumor Fraction: ???
Approximate Tumor Fraction with Aneuploid Fraction Mean (e.g. 0.50)
Rescaled %S: $3.11/0.50 = 6.22$

In DNA Diploid histograms, the diploid %S is equivalent to total or average %S by definition. Therefore to convert the diploid %S to a scale that is equivalent to aneuploid %S, we divide the %S by the tumor fraction for the sample. If this value is not known, it can be crudely approximated as the average aneuploid fraction value. The above example shows the calculation assuming the average aneuploid fraction was observed to be 0.5.



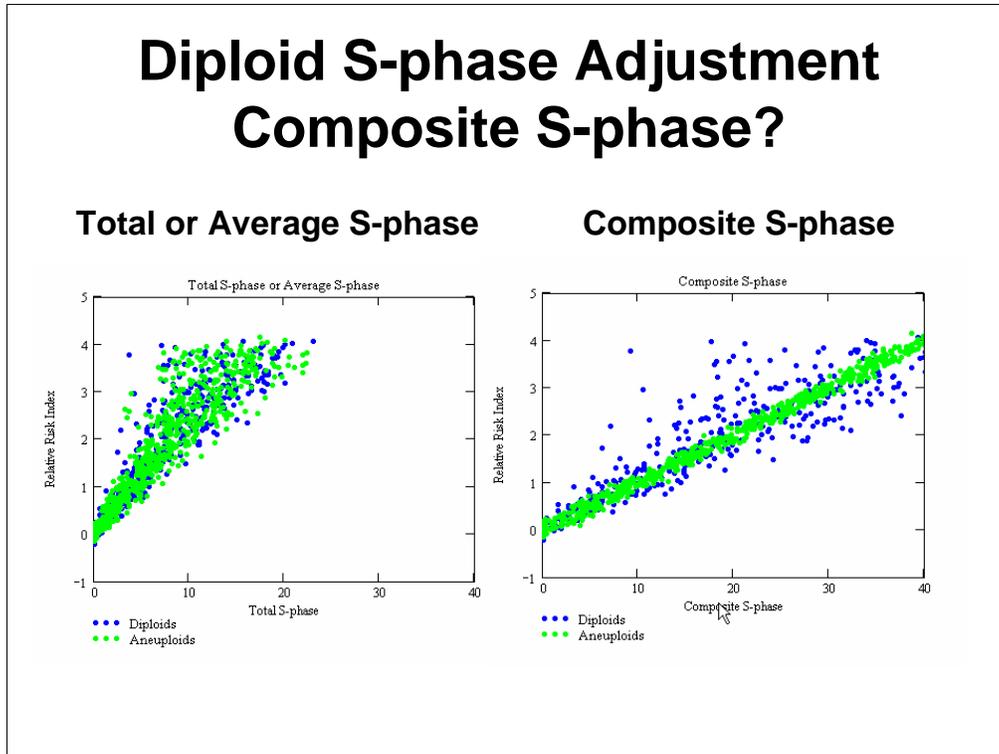
The next few slides demonstrate the advantages of scaling the diploid S-phase (Composite S-phase). The left panel shows some simulated data where tumor S-phase is almost a perfect prognostic indicator. There is practically a linear relationship between the magnitude of the tumor S-phase and an index of the relative risk of relapse.

The right panel shows the same cases that have been randomly assigned to either a DNA diploid state (40%) or DNA aneuploid state (60%).



The left panel shows the distribution of tumor fractions for DNA aneuploids and diploids. The distribution for the DNA aneuploids is measurable, but the DNA diploids is not, at least not without an additional tumor marker. The average tumor fraction is about the same for the DNA diploids and aneuploids, 0.4.

The right panel shows what happens to Dip %S for the DNA diploids as compared to the Aneuploid %S. Since the S-phase calculation in DNA diploids is lowered by the presence of non-tumor cells and the aneuploid S-phase is not, these two S-phases are at two different scales and should not be combined.



The left panel shows the total or average S-phases for this population. As can be seen the variance due to the tumor fraction variance affects both the diploid and aneuploid S-phases. By rescaling S-phase (divide by average aneuploid fraction), one effectively adds the same amount of variance in the population for the diploid S-phases, but the aneuploid S-phases are not affected. This is the reason that the Composite S-phase is recommended as the prognostic indicator.

Importance of SPF Adjustments

Adjustments	r*	Chi-Square**	p-value**
Raw***	0.42	3.78	0.039
Adjusted S****	0.07	10.27	0.0006

*: Correlation coefficient between ploidy (0=diploid, 1=non-diploid) and SPF. The 0.42 is significant at $p < 0.05$.

**: Cox Proportional Hazards Parameter p-value, n=961 cases.

***: DS for DNA diploid cases, AS for non-diploid cases.

****: Adjusted DS and AS values (SD1, SN1 and SN2).

How important are these S-Phase adjustments?

If the S-Phases are not adjusted for both the aneuploid fraction and the diploid normal dilution effects, there is a significant correlation between DNA ploidy and SPF, 0.42, and a rather modest p-value. Statisticians would normally either drop the S-Phase or the DNA Ploidy in the analysis due to this very high correlation (usually the DNA Ploidy was dropped).

If we do both adjustments, the correlation between DNA Ploidy and S-Phase is reduced and the p-value becomes more significant.

One last comment before leaving this slide. The diploid S-Phase rescaling does not infer that there is no measurable biologic difference in S-Phases between DNA diploids and aneuploids. It has long been known that S-Phases from diploid tumors are normally lower than aneuploid tumors. It is merely a mechanism to allow us to appropriately pool these S-Phases in order that we may construct a prognostic model from both DNA Ploidy and S-Phase.

DNA S-Phase – Ploidy (S-P) Prognostic Model

	N	Chi-Square	P value	RR	95%CL
Model	935	27.0	0.000002	3.50	2.1-5.7

Parameter	Beta	SE	P value	Mean	r
ADJP	0.700	0.169	0.00004	1.40	
AJDS	0.0295	0.00953	0.002	7.44	-0.033

Index	33rd %	67th %	Formula
SPI	-	-	$0.700*(ADJP-1.40) + 0.0295*(ADJS-7.44)$
RRI	0.73	1.33	Exp(SPI)

The above table summarizes a Cox proportional hazards analysis of the previously described adjusted S-phase (ADJS) and DNA Ploidy (ADJP) for the Baylor Study (n=935). The model P value, 0.000002, and the ADJP and ADJS parameter P values, 0.00004 and 0.002, are highly significant. The ADJP and ADJS beta coefficients, 0.700 and 0.0295 along with their respective means are used to create a composite S-phase and DNA Ploidy Index (SPI). The Exp(SPI) or RRI is a prognostic model that assigns a patient a relative risk given specific values of ADJP and ADJS. RRI is an easily understood means of conveying the prognostic importance of both DNA Ploidy and S-phase and can be easily extended to include other important prognostic variables (we'll see this in just a moment).

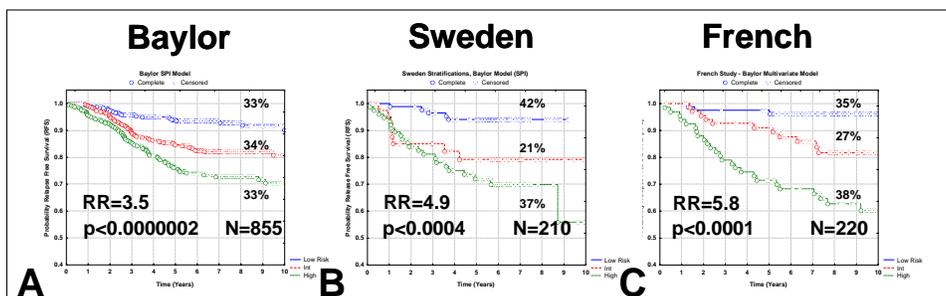
Approach

- Develop a set of DNA analysis rules by tolerating no or minimal differences between parallel analyses of common DNA histograms.
- Apply these rules to the analysis of a large primary database of DNA histograms with appropriate clinical follow-up.
- Develop a set of adjustments to DNA ploidy and SPF estimates that minimize potential variability and maximize the model's prognostic strength.
- • **Evaluate the prognostic model's ability to stratify patients in the primary database and then apply the same model and procedures to another large database and compare patient stratifications.**

In order to make sure the procedures developed described earlier are not unique for the primary database, we apply the same adjustments and prognostic model to independent and confirming databases. If the patients stratify in a similar manner as the Baylor Study we confirm our approach.

S-P Model Results

How well do the Baylor RRI's stratify patients into low, intermediate and high risk categories? Can the same RRI model be used to stratify Sweden and French study patients into similar risk categories?



Panel A shows how well the Baylor prognostic model stratifies the 935 patients into low, intermediate and high risk categories. Also shown on the graphs are the percentages in each risk category and RR, a measure of overall relative risk. When the same model was applied to other large studies such as the Sweden, Panel B, and French, Panel C, studies, similar highly significant patient stratifications were observed.

How does SPI compare with other prognostic factors such as primary size, menopausal status, estrogen receptor, progesterone receptor and histologic grade?

Study	Prognostic Variable	Beta	SE	P Value
Baylor n=935	S-phase/DNA ploidy Index	0.837	0.198	0.00003
	Primary Size	0.515	0.181	0.005
	Menopausal Status	-0.316	0.182	0.09
	Progesterone Receptor	0.075	0.190	0.7
	Estrogen Receptor	-0.047	0.208	0.9
Sweden n=210	S-phase/DNA ploidy Index	1.472	0.483	0.003
	Menopausal Status	-1.010	0.347	0.004
	Estrogen Receptor	-0.967	0.383	0.01
	Primary Size	0.406	0.504	0.5
French n=220	Menopausal Status	-1.242	0.364	0.0007
	S-phase/DNA ploidy Index	1.383	0.466	0.003
	Primary Size	0.915	0.339	0.007
	Progesterone Receptor	-0.357	0.436	0.5
	SBR (Histologic Grade)	-0.240	0.318	0.5
	Estrogen Receptor	0.101	0.464	0.9

How does our S-Phase/DNA Ploidy prognostic model stack up against other well-known prognostic markers?

The above table summarizes a Cox proportional hazards multivariate analysis of all the recorded prognostic variables for the Baylor, Sweden and French Studies. The prognostic variables are ranked according to their P values within each study. S-phase/DNA ploidy index (SPI) was a very strong prognostic factor in all the studies. A stepwise elimination of variables did not change the order of importance of any of the prognostic variables.

Can we improve the prognostic strength of the SPI model by adding primary size and menopausal status as additional prognostic variables?

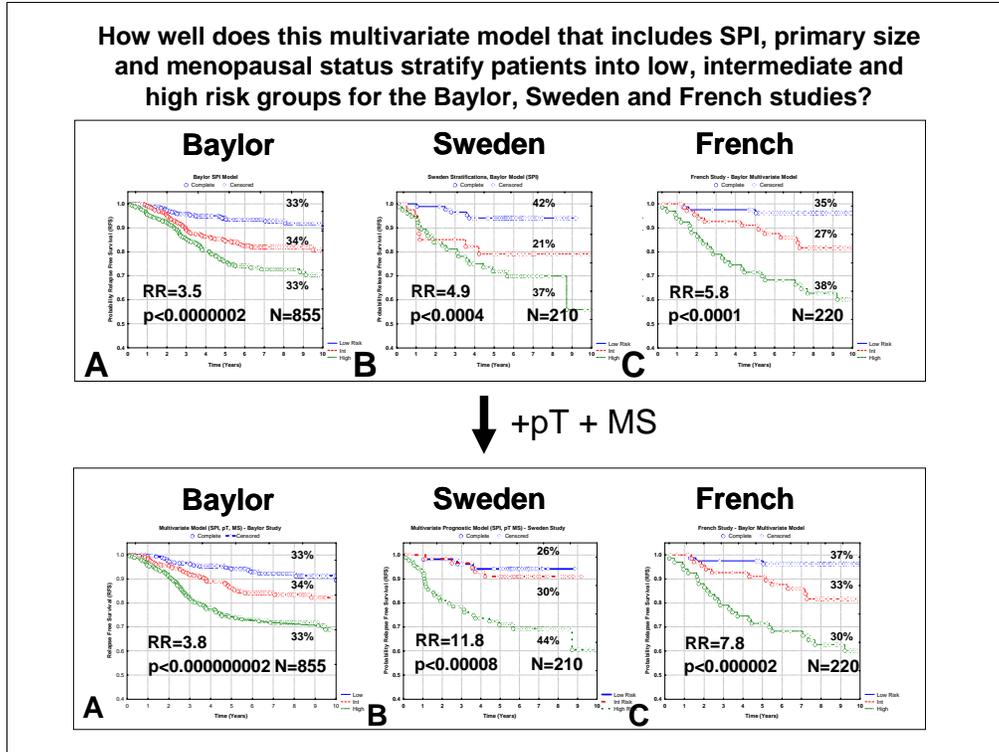
	N	Chi-Square	P value	RR	95%CL
Model	935	39.39	0.00000002	3.8	2.4-6.1

Parameter	Beta	SE	P value	Mean	SPI	pT	MS
SPI	0.869	0.191	0.000005	-0.00136	1		
pT	0.525	0.180	0.004	0.514	-0.139	1	
MS	-0.318	0.182	0.08	0.742	0.126	0.055	1

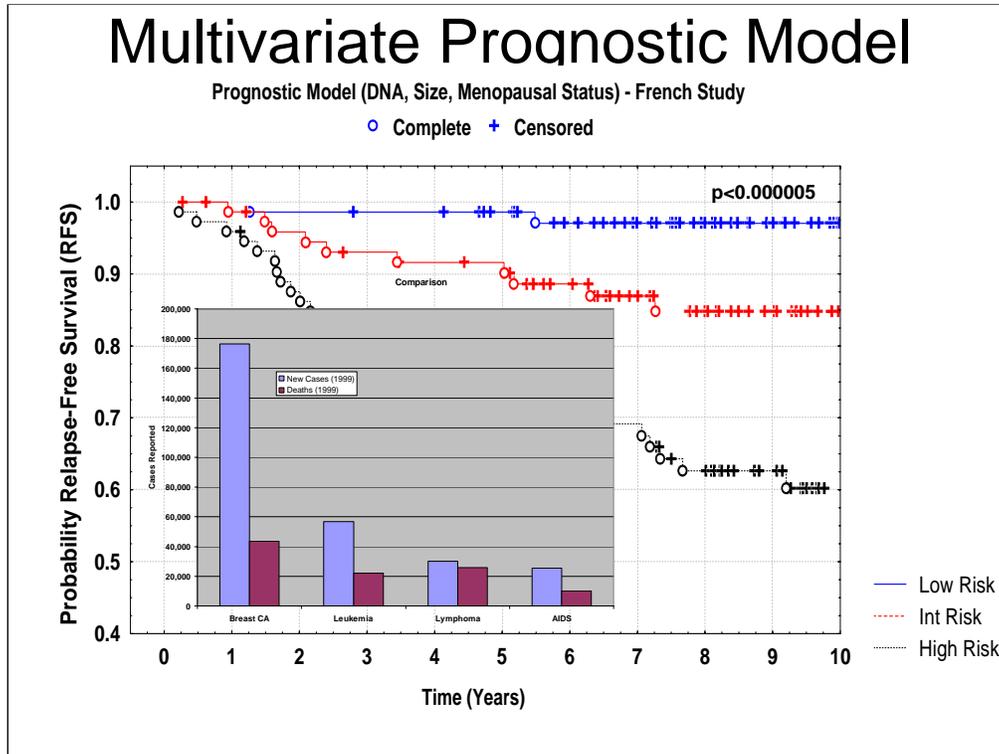
Risk Index	33.0th %	67th %	Formula
RRIM	0.82	1.24	$\text{Exp}(0.869*(\text{SPI}+0.00136) + 0.525*(\text{pT}-0.514)-0.318*(\text{MS}-0.742))$

Since the primary tumor size (pT) and menopausal status (MS) are easy prognostic variables to obtain, we have used them to further augment the Breast Cancer Prognostic model.

The above table summarizes a Cox proportional hazards multivariate analysis of S-phase/Ploidy Index (SPI), primary size (pT) and menopausal status (MS) for the Baylor Study (n=935). The model is highly significant with a relative risk (RR) of 3.8. Very little correlation is observed between the three prognostic variables. The multivariate relative risk index (RRIM) is formed from the beta coefficients and means of each of the prognostic variables. The 33rd and 67th tertile boundaries of all the RRIM's are 0.82 and 1.24 respectively.



The above graphs show this model enhancement using relapse-free survival curves. The top set of three panels shows just the stratification power of using S-Phase and DNA Ploidy. When primary size (pT) and menopausal status (MS) is added, the stratifications improve. Pay particular attention to the Chi-square probability values that appear in the lower-left part of each panel.



How important is the ability to stratify node-negative patients?

Giving oncologists such clear cut patient stratifications for node-negative breast cancer patients can only improve the overall management of this disease. The low-risk group, for example, perhaps should not receive adjuvant chemotherapy and should instead be followed carefully. The high-risk group, which is almost identical to the RFS curve of node-positive women, should perhaps receive a more aggressive therapy.

How important is this test to cytometry? Consider the above graph of incidences. In 1999 over 170,000 women were diagnosed with breast cancer and over 40,000 died in the same interval of time. According to the National Breast Cancer Foundation, every 2 minutes a woman is diagnosed with breast cancer in America. In 2003 it is estimated that 212,600 new cases of breast cancer will occur with over 40,000 deaths attributed to breast cancer (American Cancer Society).

This incidence was greater than leukemia, lymphoma and AIDS combined for the same year. Most patients when initially diagnosed with breast cancer are node-negative and it is quite likely that this will continue due to earlier diagnosis of the disease.

Conclusions

➤ **Are S-phase and DNA Ploidy strong independent prognostic variables?**

Yes, when properly adjusted, both S-phase and DNA Ploidy become highly significant independent prognostic variables.

➤ **When optimally combined, can they effectively stratify patients in other large studies?**

Yes, the relative risk index (RRI) stratifies patients in both the French and Sweden studies into highly significant risk groups.

➤ **Can the analysis of DNA histograms be standardized?**

Yes, by following a strict set of rules, DNA analysis operators can expect to obtain consistent S-phase values.

➤ **How does SPI compare to other prognostic factors such as primary size, estrogen receptor, progesterone receptor, menopausal status and histologic grade?**

SPI was consistently one of the stronger prognostic factors for all the tested studies. Both primary size and menopausal status were also important prognostic factors.

➤ **Can the SPI prognostic model be improved by adding primary size and menopausal status prognostic variables?**

Yes, the prognostic strength of the model for all studies improved when primary size and menopausal status were added to the model.