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Anne PATSOURIS

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LE SCORE NPIS, UN NOUVEL OUTIL POUR PREDIRE LA RECHUTE EN SITUATION ADJUVANTE DANS LE CANCER DU SEIN RE POSITIF HER2 NEGATIF : A PROPOS DES RESULTATS D'UNE ETUDE RETROSPECTIVE, MULTICENTRIQUE.

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Directeur de thèse :

Monsieur le Professeur Mario CAMPONE

Membres du jury :

Professeur Pierre KERBRAT, Dr Agnès CHASSEVENT, Dr Véronique VERRIELE

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The NPIS score, a new tool to predict relapse in adjuvant setting in ER positive-HER2 negative early breast cancer: A retrospective study.

Background : ER+HER2- breast carcinomas are defined as luminal A and B by gene expression profiling and dysplays various outcomes, with a lack of benefit of adjuvant chemotherapy in the luminal A entitie. Distinction is mainly due to proliferative activity, associate with an accrual risk of distant and early relapse.

Purpose : To assess the significance prognostic value of S-phase fraction (SPF) evaluated by DNA flow cytometry associate with the Nottingham Prognosis Index (NPI) in a Estrogen Receptor (ER) positive and HER2 – early breast cancer population, compared as the Saint Gallen criteria.

Patients and Methods : We retrieved a serie of 1253 patients with early primary ER+HER2- BC, treated by surgery, radiotherapy ± systemic therapy, between early January, 2001 and late February, 2010 with an adequate DNA histogram. Cell preparation and histogram interpretation followed restrictive conditions. SPF classes (low, medium and high) were defined according to tertile thresholds after adjustment for ploidy. The primary end point was the distant disease free survival (DDFS), calculated using the non parametric Kaplan-Meier method and compared with the log-rank test.

Results : Median of age was 61.8 years. Chirurgical procedures consist in 70.4% and 29.4% of tumorectomy and mastectomy, and 22.3% and 74.1% of sentinel node biopsy and axillary dissection. 48.6% received adjuvant chemotherapy and 90.6% hormone therapy. Median of follow-up was 6.8 years. 117 patients experienced a distant relapse. The cumulative 5-year DDFS, DFS, OS and BCSS for all patients included were 94.1%, 86.9%, 92.7% and 94.1% respectively. Median time to metastasis was 4 years. High risk SPF group was significantly prognostic, as NPI components in a multivariate analysis for the DDFS. Medium and high-SPF group were regrouped as one group called high risk SPF group versus low risk SPF. SPF, NPI and Saint Gallen's criteria were independent in multivariate model. NPI and SPF were associated in combined score (NPIS score), resulting in four groups: score NPI 1 whatever the SPF value, score NPI 2 with low risk SPF, score NPI 2 with high risk SPF and score NPI 3 whatever the SPF value. This combined score was compared to the NPI score alone and St Gallen's criteria and was found as the most powerful with AIC for these three scores, respectively 1311.3, 1315.5 and 1364. The NPIS score was relevant in patients who did not receive chemotherapy and in node negative population where NPI 2 with low SPF risk group met the good prognosis of NPI 1 group while NPI 2 with high SPF risk remains poor prognosis.

Conclusion: In summary, this retrospective study assesses the prognostic value of SPF in a NPI 2 ER+ HER2- BC population and let to characterise luminal B patients for whom chemotherapy should be warranted. Interest of DNA flow cytometry is also to identify abnormal DNA content (aneuploidy), reflect of genomic instability. Another study is needed to confirm our present results.

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ANNEXE 1. Analyse ADN par cytométrie en flux et mesure de la phase S

Abréviations

A : aneuploidy

BC : Breast Cancer

BCSS : Breast Cancer Specific Survival

CT : chemotherapy

D : diploid

DDFS : Distant Disease Free survival

DFS : Disease Free survival

DI : DNA index

DNA : deoxyribonucleic acid

ER : estrogen receptors

ET : Endocrine therapy

FCM : Flow cytometry

NPI : Nottingham Prognostic Index

OS : Overall Survival

MA : Mitotic Account

PgR : progesterone receptors

pN : histological lymph node involvement

pT : Histological Tumor size

RFS : recurrence Free Survival

SPF : S-phase fraction

INTRODUCTION

Breast cancer is the most frequently occurring cancer in women in the developed world, with about 13 million women diagnosed each year, and the leading cause of cancer death in women (1). In Europe, the combination of mass mammographic screening programmes and improvement in adjuvant therapy has led to reduce breast cancer mortality (2). Estrogen receptor (ER) positive tumors represent about two-thirds of all breast cancers subdivided according to molecular classification in luminal A and B (3). ER positive breast carcinoma patients display various outcomes and the benefit of adjuvant chemotherapy in this subtype remains controversial (4). In addition, as the result of widespread use of screening mammography, the majority of breast cancers are now diagnosed at an early luminal stage. Thus, difficulty is to identify patients in whom chemotherapy can be safely avoided. Traditionally, clinicians have used an integrated assessment of the clinicopathological features of breast cancer to estimate the risk of relapse. Several tools, based on the combination of these factors, have been tailored to predict the prognosis of breast cancer including Adjuvant! Online (AOL) or NPI. AOL provides a 10 years survival, relapse probability and the benefit of chemotherapy. Nottingham prognostic Index (NPI) is derived from a retrospective multivariate regression analysis. It separates patients into good, moderate and poor prognostic group, based on tumor size, grade and lymph node involvement (5). NPI has been validated by further studies as a strongly prognostic factor (6,7). In parallel, current available expert consensus guidelines, such as Saint Gallen or NCCN criteria, are helpful, guiding in clinical decision. Gene expression profiling (GEP) as a great potential here to accurate outcomes prediction, with genomic test as Oncotype DX® or Endopredict® (8).

Tumor proliferation is a critical feature for tumor aggressiveness, represents the major component of gene expression signature and led to the distinction between luminal A and luminal B entities (9). This distinction can be done using the Ki67 or the grade in clinical practice, according the Saint Gallen's recommendations (10). Flow cytometry (FCM) provides information about proliferative activity as represented by the S-phase fraction (SPF) and DNA ploidy. SPF has been found to be a significant prognostic factor in early breast cancer and high level of SPF predict a worse DFS or RFS (11–14). Extensive use of FCM DNA measurement in clinical practice has been, for a long time, limited by the lack of standardized procedures to prepare and analyze samples and interpret histograms.

But several studies have shown, that it is possible to have consistent results, following restrictive conditions, according to the DNA Cytometry Consensus Conference (11,15,16).
The present study was designed to access the prognostic role of SPF in ER+ HER2- early breast cancer as compared as the Saint Gallen criteria. Specifically, the goals of this study were : (1) to validate the independant prognostic value of SPF ; (2) to accurate the prognostic value of NPI with SPF in a combined score; (3) To study the NPI-SPF score in the overall population and patients who did not received chemotherapy. The primary end-point was the Distant Disease Free Survival (DDFS).

PATIENTS AND METHODS

Patients

The study involved 1600 patients diagnosed and treated in six French cancer centers (Angers ICO, Clinical of Anjou, Angers; Clinical Saint joseph, Angers; Clinical of Saint Louis, Angers; CHR and Clinical Fardeau of Saumur) for an ER positive HER2 negative breast cancer, between early January, 2001 and late February, 2010. The primary inclusion criterion was an adequate DNA histogram obtained from a resected tumor sample (see below). Patients were included if they fulfilled the following criteria: (a) female primary unilateral invasive ER+ HER2 - breast carcinoma without previous or concomitant malignancies; (b) T_1T_2, N_0N_1 N_2 N_{3-}, M_0 staging according to UICC criteria; (c) older than 18 years old; and (d) surgical first-line treatment. The following information was recorded for each patient (Table 3): (a) age at diagnosis; (b) clinical stage; (c) pathological tumor size; (d) histological type and axillary lymph node involvement; (e) histological SBR grade; (f) steroid receptor status; (g) presence of lymphovascular invasion; (h) mitotic account; (i) type of surgery (tumorectomy or mastectomy and sentinel node or axillary dissection); (j) medical adjuvant treatment (chemotherapy and/or endocrine therapy) and (k) adjuvant radiotherapy.

Follow-up data were collected for all patients, including distant disease free survival (DDFS; time from diagnosis to distant recurrence of the disease), disease free survival (DFS; time from diagnosis to first recurrence of the disease or controlateral breast cancer or second primary other cancer), Breast cancer-specific survival (BCSS; time from diagnosis to cancer-related death), overall survival (time from diagnosis to death from any cause) and the site of first recurrence. Recurrences were defined as locoregional (breast, mammary region or regional lymph nodes) or metastatic (visceral or not).

Tumor characteristics and NPI subgroups

Pathological data were retrieved from the original pathology reports. Tumor size (pT) was measured on fresh resection specimens, as the maximum diameter (mm) of the tumor. Histological type was determined according to the WHO classification and histological grade according to the Elston and Ellis method (17). Histological grade was not determined in 30 resected tumors and data on pathological tumor size were not available for 15 resected tumors.

Lymph nodes were accessed on at least three sections stained with hematoxylin and eosin (HE). Number of lymph nodes involved was available for 1211 patients.

The Nottingham Prognostic Index (NPI) was calculated from the above mentioned characteristics: (0,2x size in cm) + lymph node stage (1-3) + grade (1-3). Lymph node staging was determined as follow: node negative as stage 1, 1-3 nodes positive as stage 2 and more than 3 nodes positive as stage 3. Patients were classed into three groups: good (or NPI 1 for a NPI < 3.4), moderate (or NPI 2 for a NPI between 3.4 and 5.4) and poor (or NPI 3 for a NPI > 5.4) prognostic groups (5).

Estrogen and progesterone receptor (PgR) status were accessed by immunochemistry on representative formalin-fixed tumors blocks at a 4-µm thickness, were determined as positive when ≥ 10 % cells staining positive cells. All patients where HER-2 negative, that means a immunostaining 1+, scored according to the HercepTest scoring system, or 2+ without HER-2 gene amplification investigated by florescence in situ. Lymphovascular invasion was determined by the presence of tumors cells in vessels afar the tumor. Mitotic index was defined by the number of mitoses on ten consecutives fields (x400). According to the diameter of the microscope used (0.60 mm), mitotic grade was classified in low (grade 1) when the number of mitoses was less than 10 and intermediate (grade 2) and high (grade 3), when it was, respectively, between 10 and 19, and equal or more than 20 mitoses.

Flow Cytometric DNA Analysis and SPF measurement.

All DNA analysis were performed in the laboratory of cytometry of Paul Papin center. The flow cytometer used was a FACScan (Becton Dickinson, San Jose, CA), equipped with Modfit 5.2 software (Verity Software House, Topsham, ME). Cell preparation fulfilled a standardized procedure already described (11). Briefly, tumor imprints were stained with May Grunwald Giemsa, and observed by a pathologist to control the presence of malignant cells. Fine-needle aspiration or mechanical were performed in order to obtain a tumor cell suspension; and DNA staining was performed according to Vindelov's method (18). FCM was performed as follows: the DNA diploid peak was located on DNA histograms according to an external standardization procedure using female normal human lymphocytes positioned at the fifth part of the red fluorescence scale. According to the tumor DNA-Index (DI), the number of peaks and the proportion of cells with an abnormal DNA content (aneuploidy fraction), seven ploidy subclasses were defined, regrouped in two classes: diploid and aneuploid (described in *table I*).

Calculation of SPF fulfilled restrictive conditions and was evaluated when the coefficient of variation (CV) of the G0-G1 peaks was lower than 5%, background was lower than 20% of total acquired events. A minimal aneuploidy fraction was required (*table I*). Rectangular option was chosen for SPF calculation, with a substraction of background. According to the DNA Cytometry Consensus Conference, SPF classes (low, medium and high) were defined according to tertile thresholds after adjustment for ploidy (*Table II*), based from a reference population in a previous study. In all subsequent analyses, a "low-SPF" group was formed by pooling the samples included in the first SPF tertile of the Diploid and Aneuploid groups and "medium-SPF" and "high- SPF" groups were respectively composed of the second and third tertiles of Diploid and Aneuploid groups.

Table I. DI Subclasses and conditions for SPF calculation

DI subclasses	DI data	No. of G0-G1 peaks	Minimal aneuploid fraction for SPF calculation
DNA diploid-near diploid			
DNA diploid	1	1	
Near-diploid	0.95 ≤ DI ≤ 1.1	2	> 70%
Aneuploid and hypodiploid			
Hypodiploid	< 0.95	2	> 70%
Aneuploid	1.1 ≤ DI ≤ 1.9	2	> 30 %
Tetraploid	1.9 ≤ DI ≤ 2.05	2	> 40 %
Hypertetraploid	DI > 2.05	2	> 30 %
Multiploid		> 2	> 60 %

Table II. SPF classes defined on the basis of S% tertiles and according to the DNA ploidy status

SPF Classes	Low	Medium	High
Diploid-Near Diploid	< 1,5	1,5 ≤ S% ≤ 2,5	> 2,5
Aneuploid	<3,5	3,5 ≤ S% ≤ 7,5	> 7,5

Subgroups definitions according to the Saint Gallen criteria

According to the Saint Gallen's recommendations, we defined two subgroups of patients: high risk and low risk, for which chemotherapy is respectively recommended or not (10). The high risk group includes luminal B breast cancer node negative and positive or luminal A breast cancer node positive with the involvement of more than three lymph nodes. The low risk group includes luminal A breast cancer with node negative or node positive with the involvement of less than three lymph nodes. Ki-67 was not available for most of patients, thus, according to the Saint Gallen's recommendations 2011, histological grade was used to distinct the two classes of luminal breast cancer (19). The luminal A patients where defined as ER and PgR positive breast carcinoma with a low or an intermediate grade. Luminal B patients were defined as ER positive and PgR negative or positive breast carcinoma and an high grade.

Statistical analysis

Tumor and clinical characteristics of patients are reported as frequencies, medians and ranges. The primary outcome was distant disease-free survival (DDFS) defined as the time from surgery to the date of distant recurrence or date of last news without evidence of distant recurrence. The secondary outcomes were DFS, BCSS and OS. Survival rates were calculated using the non parametric Kaplan-Meier method and compared between groups with the log-rank test. All tests were two-sided, and a *P* value of 0.05 was considered significant. Mutltivariate Cox models were used to verify independent the prognostic value of each variable, NPI classes and subgroups defined according to the Saint Gallen's criteria. Multivariate analysis was performed for the DDFS by using statistically significant variables as determined by univariate analysis ($p<0.15$) and known prognostic variables. A score was created by combining NPI and SPF. The Akaike's information criterion (AIC) was used to compare this score to the Saint Gallen criteria in the overall population and in the RE+ patients who did not received chemotherapy.

We applied the recommendations for MARKer prognosis studies (REMARK).

RESULTS

Patients and tumor characteristics

We retrieved 1600 patients with primary surgery for ER+ HER2- early invasive disease. Removing patients with exclusion criteria resulted in the inclusion of 1253 patients (*Figure 1*). Details of patient characteristics are found in *Table III*. The median age was 61.8 years (range, 29-96 years). The surgical procedure was tumorectomy in 70.4% of patients or modified radical mastectomy in 29.4% of patients with sentinel node procedure in 22.3% and axillary dissection in 74.1%. A median of 13 lymph nodes (range 1–45) were examined in each surgical axillary dissection specimen. In the overall population, 89.7% of patients had received radiotherapy after surgery. Adjuvant treatment consisted of chemotherapy in 48.6% of patients, hormone therapy in 90.6% of patients, and both in 48.1% of patients. Adjuvant chemotherapy was given to 32.2% of node-negative patients and to 75% of node-positive patients.

DNA ploidy and SPF

DNA ploidy was determined for all the tumors. The mean CV of the G0-G1 peak was 2.1% for diploid samples and 2.3% for aneuploid samples. The diploid and neardiploid (Diploid group) subclasses represented respectively 34.5 and 17.8% of tumors. The Aneuploid group (n = 606) included 6.5% of hypodiploid, 18.4% of aneuploid, 6.4% of tetraploid and 14.7% of multiploid tumors. Respectively 42.8% and 35.2% of ductal and lobular carcinoma were aneuploid. 1145 tumors could be evaluated for SPF, according to the restrictive conditions prespecified (94.3% of the Diploid group and 87.1% of the Aneuploid group). Missing SPF data was due to an insufficient aneuploidy fraction, multiploidy or high background.

Descriptive analysis

Median follow-up is 6.8 years. In total, 134 patients experienced a first relapse (117 distant metastasis and 17 locoregional relapses) and 24 patients a controlateral breast cancer. 41 patients experienced a second cancer (including 3 breast angiosarcoma and 5 endometrial carcinoma). We recorded 172 deaths, 63 breast cancer related, 60 non breast cancer related (including 11 due to a second cancer) and 49 due to unknown cause. The cumulative 5-year DDFS, DFS, OS and BCSS for all patients included in the study were 94.1%, 86.9%, 92.7% and 94.1% respectively. Median time to metastasis was 4 years.

Figure 1. CONSORT diagram of the study

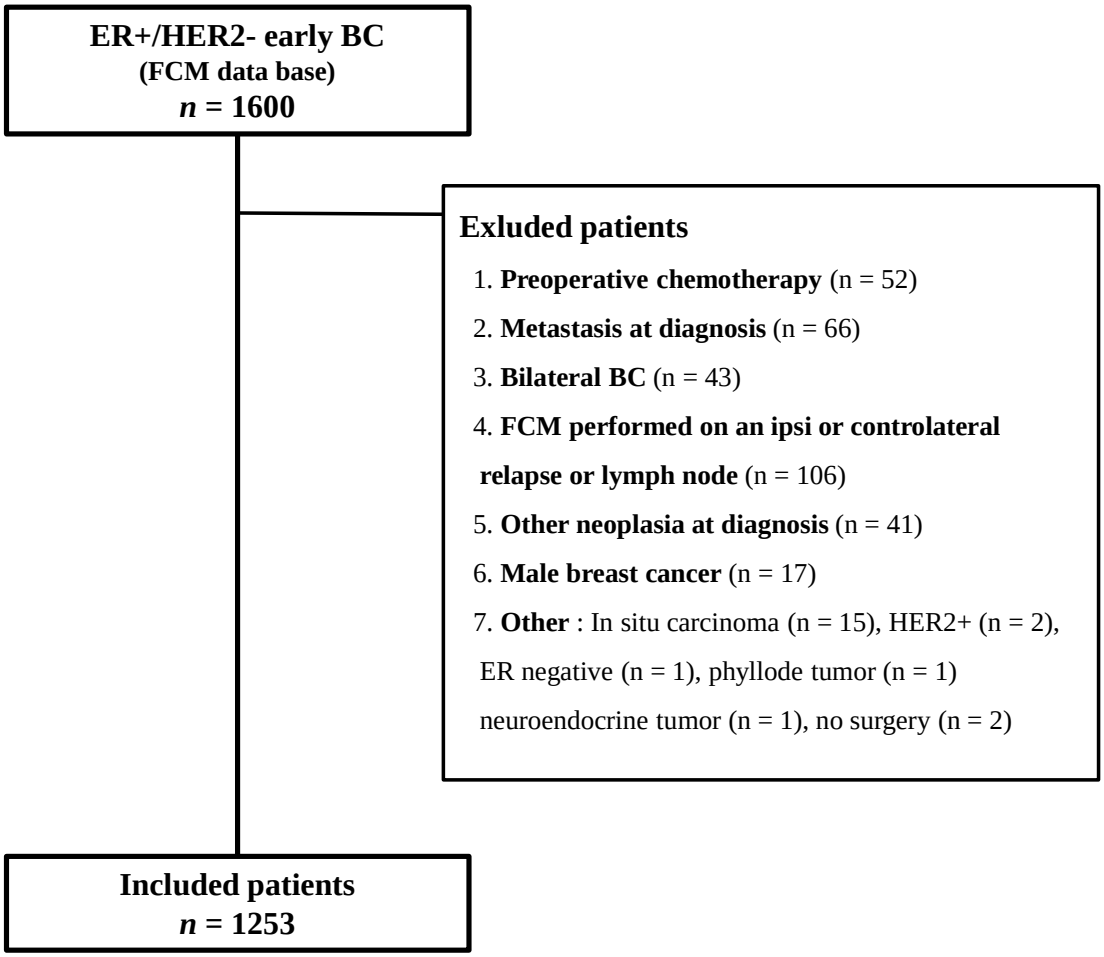


Table III. Baseline patients characteristics (n= 1253)

	n	*%*
All patients	1253	100%
Age (years) at diagnosis		
<35	18	1.4%
36-49	264	21.1%
50-75	773	61.7%
>75	198	15.8%
Median age [max-min]	61.8	[29-96]
Histology		
Ductal	1000	79.8%
Lobular	190	15.1%
Other	63	5%
pT (mm)		
T1a-b	179	14.3%
T1c	644	51,3%
T2	377	30%
T3	38	3%
pN		
0	693	55.3%
1-3	385	30.7%
4-10	106	8.4%
>10	27	2.1%
Grade		
1	350	27.9%
2	595	47.5%
3	278	22.2%
ER status (positive)	1253	100%
PgR status		
Positive	1087	86.7%
Negative	164	13.1%
Lymphovascular invasion		
Presence	284	22.6%
Unknown	395	31.5%
Mitotic Index		
1	546	43.5%
2	235	18.7%
3	144	11.5%
unknown	330	26.3%
Breast surgery		
Mastectomy	369	29.4%
Tumorectomy	884	70.4%

Axillary surgery			
	Axillary dissection	931	74.1%
	Sentinel node	280	22.3%
	None	40	3.2%
	Unknown	4	0.3%
Radiotherapy			
	Yes	1126	89.7%
	No	125	9.9%
	unknown	4	0.3%
Chemotherapy (CT)			
	None	638	50.9%
	CMF	2	0.2%
	Anthracyclin (A)	278	22.2%
	A + Taxane (T)	290	23.1%
	T only	33	2.6%
	unknown	14	1.1%
Endocrine therapy (ET)			
	Yes	1136	90.6%
	No	110	8.7%
	unknown	7	0.5%
CT + ET		603	48.1%
Ploidy			
	Diploidy or Near-D	638	51%
	Aneuploidy	615	49%
SPF			
	Low	476	37.9%
	Medium	397	31.7%
	High	263	21%
	Unknown	119	9.5%
NPI			
	0	237	18.9%
	1	369	29.4%
	2	479	38.2%
	3	132	10.5%
	unknown	38	3%
Saint Gallen			
	Low risk	725	57.8%
	High risk	464	37%
	unknown	62	4.9%

Univariate and Multivariate Cox Regression Analyzes for DDFS

Contribution of each covariable in the univariate analysis for the DDFS, DFS, OS and BCSS are presented in *Table IV*. All NPI components (pT, nodal status or pN, SBR grade) added prognostic information, as mitotic index and SPF for DDFS. NPI was a strong pronostic score wether for DDFS, DFS, OS or BCSS. Saint Gallen's criteria were statistically prognostic too.

The multivariate analyzes were conducted with the aboved mentioned variables (except the mitotic index which was available only for 925 patients) as CT, ET and PgR, on the 1053 patients with all available variables. High risk SPF group was significantly prognostic, as NPI components (*Table V*). We pursue the multivariate analyzes with SPF in two separate models including NPI or Saint Gallen's criteria in order to verify the independent prognostic information of SPF with respect to these scores (*Table VI*).

Table IV. Univariate models for DDFS, DFS, OS and BCSS [Hazard ratios (HR) and 95% confidence intervals (CI)]

	DDFS		DFS		OS	
	HR (IC)	*P* Value	HR (IC)	*P* Value	HR (IC)	*P* Value
Age at diagnosis	1.00 (0.989-1.019)	0.590	1.038 (1.027-1.048)	<0.001	1.07 (1.056-1.085)	<0.001
Tumor size (pT)						
T1c vs T1ab	5.73 (1.38-23.81)	0.016	1.77 (1.098-2.84)	0.019	2.76 (1.26-6.03)	0.011
T2 vs T1ab	21.76 (5.33-88.79)	<0.001	3.99 (2.485-6.39)	<0.001	7.49 (3.47-16.18)	<0.001
T3 vs T1ab	23.19 (4.82-111.69)	<0.001	4.67 (2.359-9.26)	<0.001	10.79 (4.24-27.41)	<0.001
Nodal status (pN)	1.00 (0.76-1.32)	0.990	1.79 (1.13-2.82)	0.013	1.27 (0.90-1.79)	0.174
1-3 vs none	2.15 (1.51-3.05)	<0.001	4.96 (3.01-8.17)	<0.001	2.37 (1.54-3.64)	0.000
4-10 vs none	3.75 (2.26-6.22)	<0.001	9.89 (5.24-18.67)	<0.001	2.72 (1.31-5.64)	0.007
>10 vs none						
SBR						
2 vs 1	2.74 (1.42-5.30)	0.003	1.14 (0.84-1.55)	0.386	1.72 (1.13-2.61)	0.010
3 vs 1	7.84 (4.12-14.92)	<0.001	1.96 (1.43-2.69)	<0.001	2.62 (1.70-4.04)	<0.001
PgR (Yes versus no)	1.14 (0.65-1.99)	0.650	0.99 (0.70-1.41)	0.976	1.00 (0.64-1.55)	0.989
Mitotic Index						
2 vs 1	2.99 (1.55-5.76)	0.001	1.43 (1.01-2.03)	0.044	1.58 (0.98-2.56)	0.062
3 vs 1	8.67 (4.74-15.86)	<0.001	2.58 (1.82-3.65)	0.000	3.68 (2.36-5.75)	<0.001
Ploidy (D vs A)	0.65(0.45-0.95)	0.025	0.92 (0.73-1.16)	0.491	1.72 (0.77-1.40)	0.798
SPF						
Medium vs low	2.62 (1.56-4.40)	<0.001	1.31 (0.98-1.75)	0.069	1.28 (0.89-1.84)	0.174
High vs low	3.80 (2.26-6.41)	<0.001	1.33 (0.97-1.83)	0.079	1.14 (0.76-1.72)	0.523
CT (yes versus no)	2.19 (1.47-3.24)	<0.001	0.85 (0.67-1.08)	0. 188	0.69 (0.51-0.93)	0.016
ET (yes versus no)	3.42 (1.09-10.77)	0.035	0.58 (0.41-0.83)	0.003	0.70 (0.43-1.12)	0. 137
NPI						
2 vs 1	7.37 (3.88-14.01)	<0.001	1.61 (1.23-2.11)	0.001	1.96(1.38-2.80)	<0.001
3 vs 1	20.19 (10.39-39.23)	<0.001	3.24 (2.33-4.51)	<0.001	3.71 (2.45-5.62)	<0.001
Saint Gallen (High vs low risk)	4.68 (3.07-7.13)	<0.001	1.69 (1.33-2.15)	<0.001	1.73 (1.28-2.34)	<0.001

Table V. Multivariate models for DDFS, n = 1053 [Hazard ratios (HR) and 95% confidence intervals (CI)]

	DDFS	
	HR (IC)	*P* Value
pT		
T1c vs T1ab	1.40 (.84-2.33)	0.004
T2 vs T1ab	2.74 (1.51-4.97)	0.001
T3 vs T1ab	4.86 (2.32-10.14)	<0.001
pN		
1-3 vs none	1.40 (0.84-2.33)	0.198
4-10 vs none	2.74 (1.51-4.97)	0.001
>10 vs none	4.86 (1.56-7.03)	<0.001
SBR		
2 vs 1	1.72 (.83-3.55)	0.146
3 vs 1	3.31 (1.56-7.03)	0.002
SPF		
medium vs low	1.66 (.942-2.93)	0.079
high vs low	2.05 (1.14-3.68)	0.016
CT (yes versus no)	0.95 (0.59-1.51)	0.816
ET (yes versus no)	1.02 (0.31-3.36)	0.035
PgR (Yes versus no)	1.48 (0. 79-2.76)	0.215

Table VI. Multivariate models for DDFS with NPI and St Gallen's subgroup, n = 1053 [Hazard ratios (HR) and 95% confidence intervals (CI)]

	DDFS	
	HR (IC)	*P* Value
NPI		
2 vs 1	6.39 (3.07-13.31)	<0.001
3 vs 1	14.5 (6.69-31.47)	<0.001
SPF		
medium vs low	1.67 (0.96-2.91)	0.069
high vs low	1.98 (1.13-3.47)	0.017
CT (yes versus no)	0.82 (0.52-1.29)	0.396
ET (yes versus no)	0.81 (0.24-2.68)	0.730
PgR (Yes versus no)	1.33 (0.72-2.45)	0.359
Saint Gallen (High vs low risk)	4.10 (3.07-6.70)	<0.001
SPF		
medium vs low	1.80 (1.02-3.16)	0.041
high vs low	2.17 (1.22-3.85)	0.008
CT (yes versus no)	1.08 (0.68-1.71)	0.735
ET (yes versus no)	1.24 (0.38-4.06)	0.722
PgR (Yes versus no)	2.46 (1.31-4.63)	0.005

Creation of the combined score NPI with SPF

After analyzing the survival curves according to SPF, we noticed that medium and high-SPF group were poorer prognosis compared to the low-SPF subgroup (*Figure 2*). Thus, we decided to combine these two classes in one, called high risk SPF group (versus low risk SPF) for the following analyzes. We studied the impact of SPF into each NPI group. SPF added prognosis information only for the NPI 2 subgroup (*Figure 2*). Therefore, NPI and SPF were associated in combined score, resulting in four groups defined as follow: score NPI 1 whatever the SPF value, score NPI 2 with low risk SPF, score NPI 2 with high risk SPF and score NPI 3 whatever the SPF value. Survival curves according to this four groups are presented in the *Figure 3*. Analyzes for the combined score are donned in 1120 patients (recovering the 67 patients for whom the SPF value were missing in NPI groups 1 and 3). This results in two distinct curves for NPI 2 group. This NPI + SPF combined score was compared to the NPI score alone and St Gallen criteria and was found as the most powerful with AIC for these three scores, respectively 1311.3, 1315.5 and 1364. The NPI+ SPF score was relevant in patients who did not receive chemotherapy where NPI 2 with low SPF risk group met the good prognosis of NPI 1 group while NPI 2 with high SPF risk remains poor prognosis. The combined score has also let to separate NPI 2 group in node negative population whereas it is not the case if patients where node positive (*Figure 3*).

Figure 2. Kaplan Meier plots of DDFS according to **A**. NPI Subgroups, **B**. SPF groups (adjusted on the ploidy), **C**. the risk level of SPF in the NPI 1, **D**. in the NPI 2, **E**. NPI 3 subgroups.

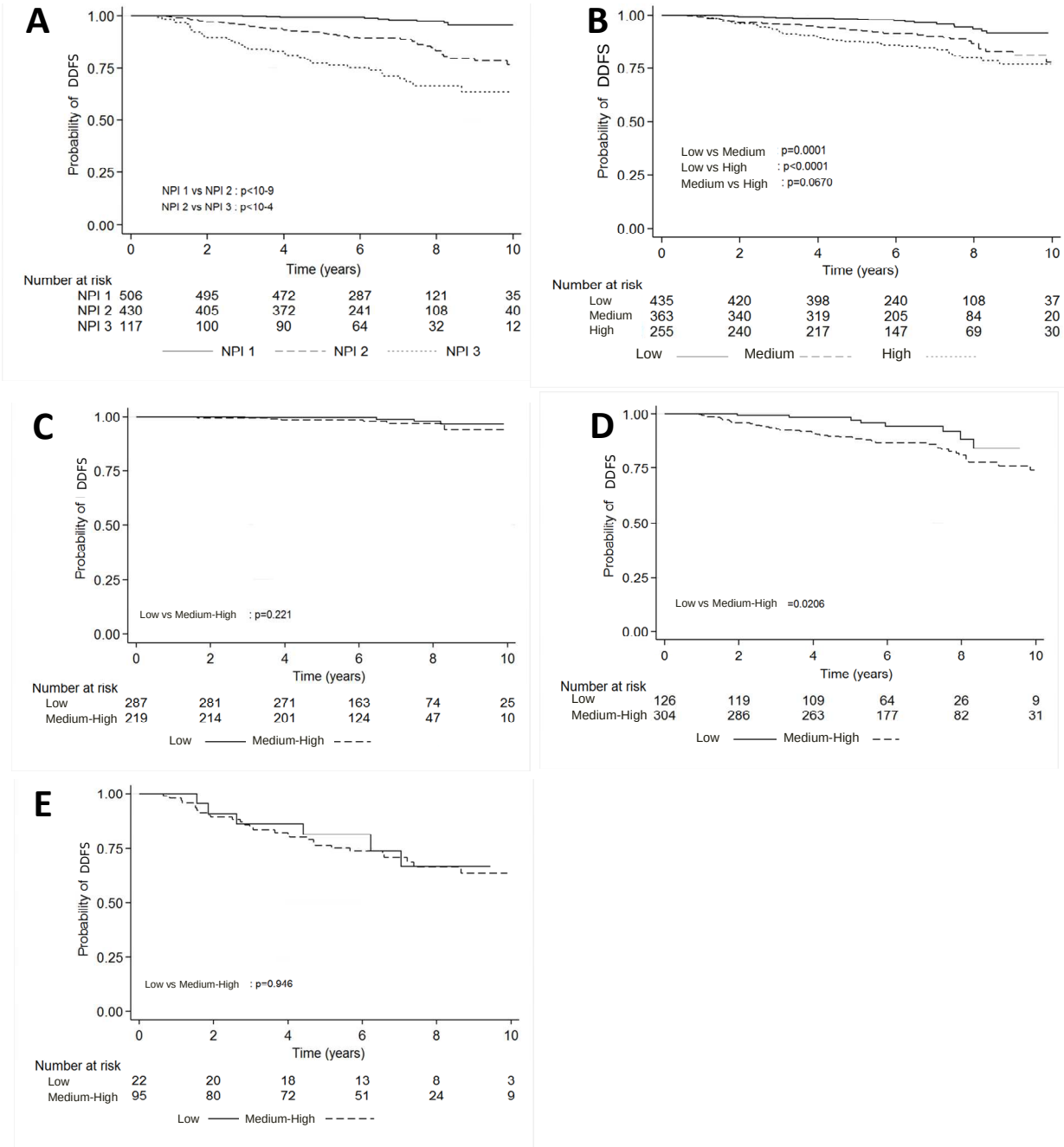
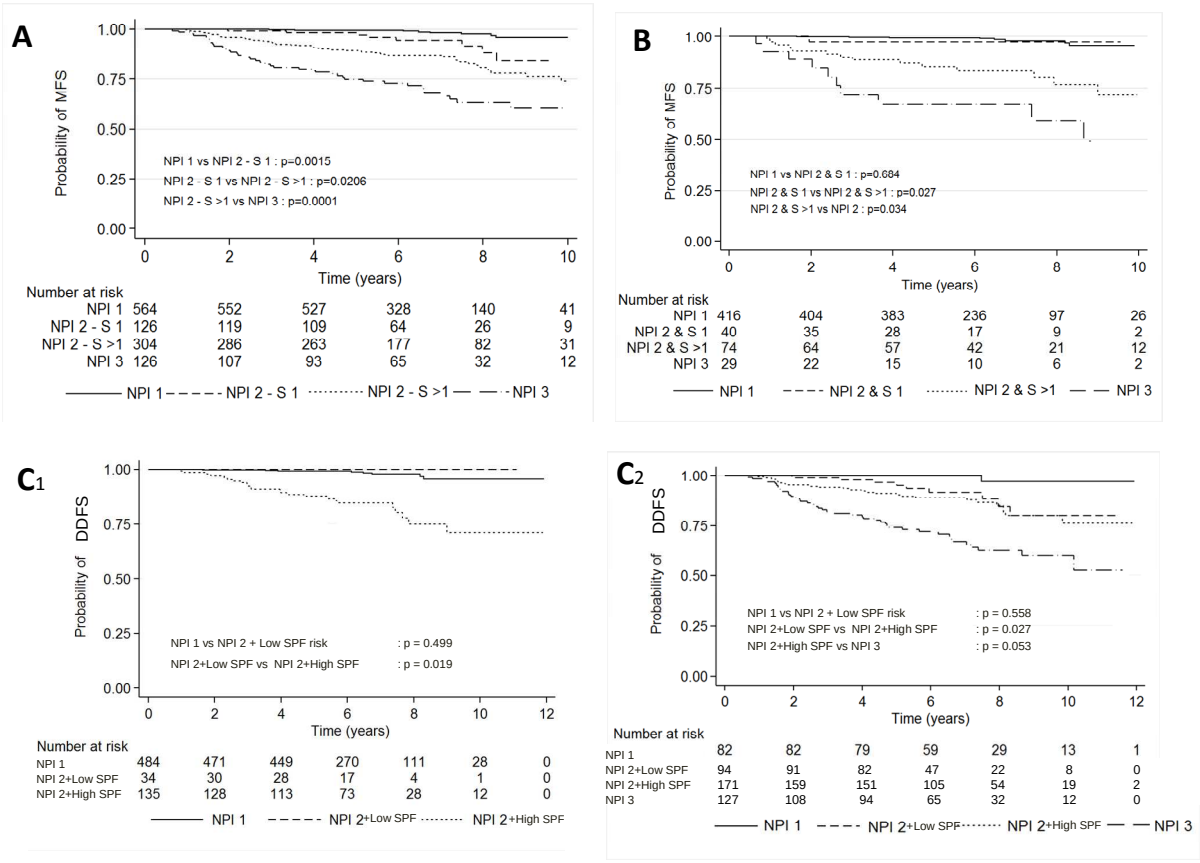


Figure 3. Kaplan Meier plots of DDFS according to the combined score NPI + SPF in the A. Overall population (n=1120), B. patients who did not received chemotherapy; C_1. N0 patients (n=693); C_2. Node positive patients (n = 518)



DISCUSSION

This retrospective cohort study assesses the prognosis significance of proliferation as represented by S-phase fraction associate with NPI in ER+ HER2- early breast cancer. The combined score has let to distinguish 2 subgroups of patients in the NPI 2 class with different outcomes. It was particularly relevant in patients who did not receive chemotherapy or in node-negative patients where the NPI 2 with a low risk SPF sub-group meet the good prognosis of NPI 1 group.

Five years of tamoxifen in ER+ HER2- early breast cancer (BC) reduce the risk of relapse at 10 years of 14,3% (20). Adjuvant chemotherapy reduces by a third the risk of recurrence and improves survival in both estrogen receptor negative and positive cases. Nevertheless, this risk reduction depends on the absolute risk without chemotherapy and with, in ER+ breast cancer, an appropriate endocrine therapy (4). ER+ HER2- breast carcinoma displays various outcomes and can be classed as luminal A and luminal B according to molecular classification (3). There is evidence from retrospective analysis, that chemotherapy does not add benefit when compared with endocrine therapy alone in luminal A breast carcinoma defined with high expression of ER and low proliferation assessed by Ki67 (21). In addition, the widespread use of screening mammography has caused a shift toward diagnosis of early stage disease as stage I, known to have a good prognosis, with a 10 years relapse free survival (RFS) rates more than 90% without adjuvant systemic therapy (22). Hanrahan *and al*, report in 29, 625 ER+ T1a,bN0M0 breast carcinoma a ten year probability of death from BC of 4% with 21% of probability of death from other cause (23). If we consider the risk reductions described by the ECBCTG, with a benefit of 4% with 5 years of tamoxifen in terms of 10-year RFS, the absolute benefit of chemotherapy would be approximately of 2% (24). Thus, there is clearly a need to defined patients for whom chemotherapy could be safely avoided. Gene-expression profiling has great potential to improve BC management by providing a better prediction of clinical outcomes than traditional histopathological factors. Further gene signatures as Oncotype DX® or Mammaprint® have been showed prognostic and predictive and are commercialized but remain expensive. Furthermore, results of the ongoing prospective trials (TAILORx, RxPONDER, MINDACT) are awaiting to provide the level I evidence about the relevance of applying gene expression predictors in clinical practice (8).

Amoung clinicopathological factors, tumor size, lymph node involvement and histological grade remains constantly prognostic (23,25–28). However, diagnosis at an early stage may

reduce the discriminative value of time dependant prognostic features as size or lymph node involvement. In this study, most patients are classified as NPI 1 or 2 (respectively 45.4 and 38%) and NPI appears to be clearly prognostic with respectively a 6.39 and 14.5 accrual risk of distant relapse in multivariate analysis for NPI 2 and 3 versus NPI 1. Nonetheless, this prognosis index needs to be improved, notably to accurate the risk stratification of the intermediate NPI 2 group. Rhaka *and al*, created an NPI+ score based on the combination of NPI with 10 biomarkers determined by IHC resulting in seven classes including three luminal classes tumor characterized by high luminal Ck7/8 and hormone receptor, with clinical relevance (27,29). Level of PgR is yet known as a prognostic factor and is part of the definition of distinction of luminal A and B BC with a cut-off of 20% according to the Saint Gallen criteria (10). It is for this reason that this variable has been maintained in the multivariate model despite his lack of prognostic significance in the univariate analysis. Proliferation and invasion are two critical features for tumor aggressiveness. Invasion could be accessed in BC, by proteolytic factors uPA and PAI-1 (30) and lymphovascular invasion (23). Lymphovascular invasion, in our study, has not been included in univariate analysis because it was available only for 858 patients. Proliferation markers have been extensively evaluated as prognostic tools in breast cancer (31,32). Prognostic abilities of gene signature are mostly due to the detection of proliferation activity, which is associated with an increased risk of metastatic relapse. Proliferation is the common denominator of most of them (9). Desmedt and al, have shown, in a meta-analysis, that in the ER+ HER2- population, only the proliferation module and the histological grade were significantly associated with clinical outcomes (33). Saint Gallen defined luminal entities according to proliferation activity accessed by the Ki67 with a cut off of 20%, or grade if KI67 is not available (10,19). In the present study, proliferation represented by S-phase fraction has let to identify 40% of patients in the NPI 2 subgroup with a low SPF risk who show a significant better prognosis than the high SPF risk subgroup where the DDFS rate at 10 years was approximatively 75%. SPF was particularly relevant in patients who did not receive chemotherapy, where the NPI 2 with low SPF risk had similar outcomes compared to NPI 1 subgroup, whereas the high SPF risk subgroup had a 10 years risk of distant relapse superior to 25%, and who have been candidate to an adjuvant warranted chemotherapy. The combined score has let also to identify 20% of the 169 N0 BC patients classed as NPI 2 with a low SPF risk, who present a risk of distant relapse inferior to the NPI 1 subgroup. The use of S phase fraction for treatment decision making in BC patients has not been recommended by the Tumor

Marker Panel of the American Society of Clinical Oncology, although high SPF values are clearly linked, in the literature, with an increased risk of recurrence and death in early BC (13). It was due to a lack of standardized procedure to prepare, analyze samples and interpret histograms (31). Several groups attempt to standardize procedure for cell preparation and precise rules for software use and data interpretation according to DNA Cytometry Consensus Conference (11,15,34,35). Chassevent *and al* show in 2001, in a multicenter study of 633 BC, that interlaboratory reproducibility was possible by following restrictive conditions as previously described (11). One of the recommendations was to interpret SPF value according to tertile thresholds after adjustment for ploidy. Previous studies have shown that only one cut-off was insufficient (14). Recent studies using similar rules have found consistent results, notably in N0 BC population (12,36). In clinical practice, proliferation can be also assessed by the nuclear antigen Ki67 whose prognostic value is clearly established (31,37). One of the limits was the variation in analytical practice, but recommendations have been made in order to increase interlaboratory reproducibility and harmonized interpretation (38). It is, nonetheless, a procedure time-consuming for anatomopathologists. One of another proliferation prognosis factor is the mitotic account (MA), who is part of histological grade constantly associated with an accrual risk of relapse (32,39). In our study, MA was significant in the univariate analysis but has not been included in the multivariate model because was only available for 925 patients and found to be correlated to the SPF. Question remains if MA would be a more affordable way to approach proliferation aspect than SPF. Interest of DNA histogram analysis is not only to provide SPF value but also information about ploidy. Indeed, aneuploidy reflects genetic instability and subsequent predisposition to metastasis (16,40).
Finally, the median of follow-up is quite short (6.8 years) and, even if proliferation is associate with early relapse, ER+ BC population is known to have risk a late recurrence and we must expect results 10-year follow.

CONCLUSION:

In summary, this retrospective study assesses the prognostic value of SPF in a NPI 2 ER+ HER2- BC population and let to characterise luminal B patients for whom chemotherapy should be warranted. Interest of DNA flow cytometry is also to identify abnormal DNA content (aneuploidy), reflect of genomic instability. Another study is needed to confirm our present results.

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ANNEXE 1. Analyse ADN par cytométrie en flux et mesure de la phase S

1. Rappel et phase préanalytique

1.1. Rappels

La cytométrie en flux est la mesure des propriétés optiques de cellules transportées par un liquide vecteur (flux) jusqu'à une source d'excitation lumineuse (souvent un laser). Elle permet une analyse automatisée de suspensions cellulaires monodispersées.

Le prélèvement à analyser est injecté au centre d'une veine liquide qui aligne les cellules, entraînées par le flux de manière à défiler une à une devant un faisceau laser. Le fluorochrome contenu dans chaque cellule va alors être excité et émettre un signal fluorescent qui va être filtré par l'intermédiaire de miroirs sur des capteurs appelés photomultiplicateurs. Ces signaux seront ensuite digitalisés par le convertisseur analogique. Sera mesurée une intensité de fluorescence directement proportionnelle à la quantité d'ADN dans chacune des noyaux.

Peuvent être analysées :

- toute suspension cellulaire (prélèvement sanguin, de moelle osseuse, LCR, brossage -bronchique, cytoponction)
- des fragments tissulaires après dissociation tissulaire mécanique ou cytoponction
- des coupes épaisses (70-100 µ) en paraffine, après dissociation à l'aide de pepsine en milieu acide, qui permettent de quantifier l'index d'ADN mais qui rendent peu fiable le calcul de la phase S.

1.2. Préparation des échantillons

1.2.1. Prélèvements :

Après exérèse chirurgicale, les prélèvements sont réalisés sur la pièce fraiche au laboratoire d'anatomie pathologique dans un délai qui ne doit pas excéder 1 heure. Si la lésion mesure plus d'un cm, un cube de 5 mm3 est prélevé dans le foyer tumoral et est déposé à sec dans un tube à congélation. L'échantillon peut être traité extemporanément ou de manière différée après congélation dans l'azote liquide. Le fragment tumoral fait par la suite l'objet d'une cytoponction et d'une dissociation mécanique à l'aide d'un bistouri afin d'obtenir une suspension cellulaire.

Pour les tumeurs de taille inférieure à 1 cm, un grattage est réalisé sur la pièce tumorale fraîche au moyen d'une lame neuve de microtome, alors rincée dans un tampon contenu dans un tube à congélation.
Les prélèvements tumoraux réalisé hors du centre Paul Papin, étaient directement congelés dans de l'azote liquide mise à disposition des préleveurs.

1.2.2. Préparation des échantillons prélevés

Elle suit le protocole de Vindelov ou marquage de l'ADN sur noyaux isolés (1).
Les suspensions cellulaires sont successivement incubées dans 3 solutions contenant :

- de la trypsine
- un inhibiteur de la trypsine et de la RNase
- de l'iodure de propidium, un fluorochrome intercalant, incorporé dans l'ADN de manière stœchiométrique.

2. Mesure de l'index d'ADN et de la Phase S

Le cytomètre utilisé était un FACScans (Becton Dickinson, San Jose, CA). La calibration du cytomètre se fait à l'aide de billes fluorescentes, dont les différents signaux de fluorescence sont stables et connus. L'étalonnage s'effectue à l'aide d'érythrocytes de truite dont la quantité d'ADN est égal à 80% de celles de lymphocytes humains normaux diploïdes issus d'un témoin féminin.

Le logiciel d'analyse du cycle cellulaire utilisé au laboratoire d'Angers est le logiciel ModFit 5.2 (Verity Software House, Topsham, ME). Un fenêtrage particulier est nécessaire pour éliminer des doublets de cellules, liés à des agrégats cellulaires, pouvant augmenter artificiellement le pourcentage de cellules en G2-M.

2.1. INDEX ADN

L'ADN-ploïdie est quantifiée par l'index d'ADN de la population tumorale. Cet index d'ADN est défini par le rapport du contenu d'ADN d'une cellule tumorale sur celui d'une cellule diploïde normale.

Un index d'ADN (ID) égal à 1 caractérise une population ADN-diploïde.

Une valeur inférieure à 0,95 ou supérieure à 1,1 caractérise une population au contenu anormal en ADN ou aneuploïde qui regroupe les populations(figure 1) :

- Hypodiploide quand l'ID est inférieur à 0,95
- Aneuploïde quand l'ID est situé entre 1,1 et 1,9
- tétraploïde quand l'ID est est situé entre 1,91 et 2,05
- hypertetraploïdes quand l'ID est supérieur à 2,05

On conclut à une fraction aneuploïde quand les pourcentages de cellules tumorales classées aneuploïdes dépassent un seuil défini selon les sous-groupes (tableau X) respectivement 10, 5, 10% pour les groupes hypodiploïdes, aneuploïdes et tétraploïdes. La multiploïdie est définie par la coexistence de plusieurs index d'ADN anormaux. Les proches diploïdes représentent une population dont l'ID est situé entre 0,95 et 1.1, et du fait de caractéristiques phénotypiques (histologique et clinique) similaire aux tumeurs diploïdes, elles sont regroupées en une seule classe. Les tumeurs au contenu anormal en ADN (hypodiploïde, aneuploïde, tétraploïde, hypertétraploïde et multiploïde) sont également regroupées en une seule classe dite « Aneuploïde »

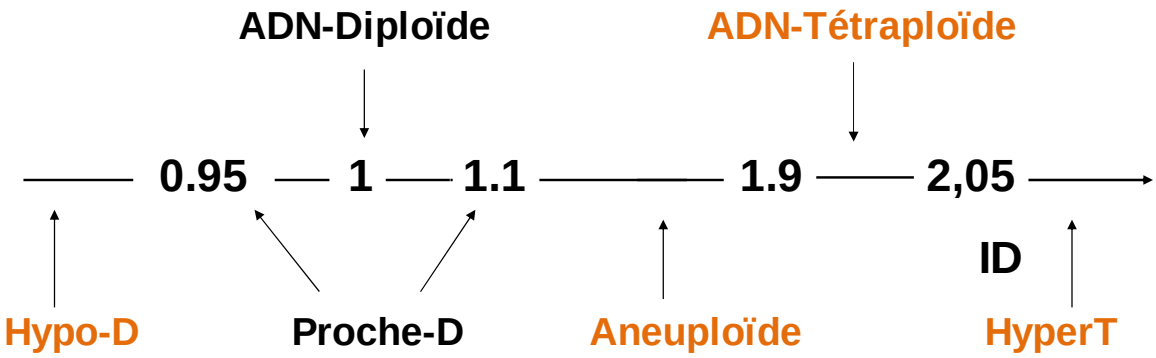

Figure 1. Sous-classes définies par l'ADN ploïdie.

2.2. CYCLE CELLULAIRE ET PHASE S

L'histogramme d'ADN obtenu par CMF est analysé par le logiciel ModFit 5.2 qui modélise la courbe expérimentale, dans sa représentation en 256 canaux (Figure 2).

La méthode des rectangles est appliquée avec une option permettant de soustraire le bruit de fond :

- la phase S est assimilée à un rectangle limité latéralement par les pics de G0-G1 et de G2-M
- les pics G1 et G2 sont assimilés à des gaussiennes
- les débris (ou bruit de fond) sont modélisés suivant le type single-cut

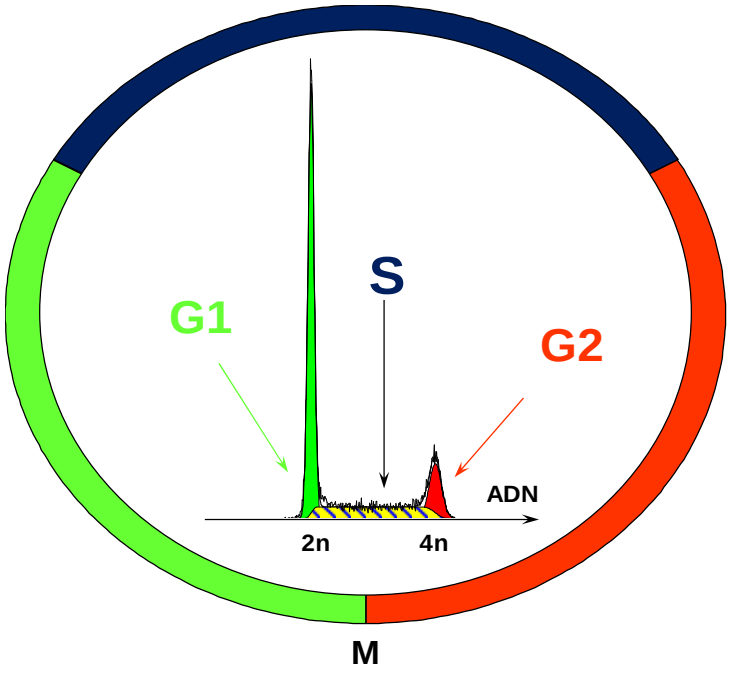

Figure 2. Histogramme d'ADN

La largeur du pic G1, ou sa dispersion défini par le coefficient de variation (CV% = 100.[écart-type/moyenne]) est une estimation de la précision des mesures.

Une conférence de consensus en 1992 a permis la réalisation de recommandations concernant la phase pré-analytique et l'interprétation des histogrammes d'ADN (2). Ces recommandations étaient cependant insuffisantes pour assurer une reproductibilité interlaboratoire, représentant l'une des limites de l'utilisation de la phase S en pratique clinique. Ce défaut de reproductibilité est plus lié à l'interprétation des histogrammes qu'aux performances techniques de l'analyse(3). Plusieurs travaux ont montré qu'en appliquant des règles strictes portant sur la concentration cellulaire tumorale, le nombre de cellules par cycle, le coefficient de variation, les débris, on augmentait le taux de concordance entre les différents laboratoires. Enfin, le choix prédéfini des options (notamment de résolution) liés au logiciel d'analyse du cycle cellulaire peut permettre de limiter les écarts (4).

Dans cette étude les résultats de mesure de SPF sont pris en compte quand :

- le pourcentage de débris (ou bruit de fond de fluorescence) est inférieur à 20%
- Le pic G1 représente un coefficient de variation (CV) inférieur à 5%
- Les populations comptent plus de 10 000 cellules par cycle
- En l'absence de multiploïdie
- Avec une proportion de cellules tumorales dont le seuil dépend de la ploïdie (Tableau I).

DI subclasses	DI data	No. of G_0-G_1 peaks	Minimal aneuploid fraction for SPF calculation
DNA diploid-near diploid			
DNA diploid	1	1	
Near-diploid	0.95 ≤ DI ≤ 1.1	2	> 70%
Aneuploid and hypodiploid			
Hypodiploid	< 0.95	2	> 70%
Aneuploid	1.1 ≤ DI ≤ 1.9	2	> 30 %
Tetraploid	1.9 ≤ DI ≤ 2.05	2	> 40 %
Hypertetraploid	DI > 2.05	2	> 30 %
Multiploid		> 2	> 60 %

Tableau I. Classes en fonction de l'ID (Index d'ADN) et conditions de calcul de la phase S (SPF)

Quand un histogramme unimodal est obtenu, l'option diploïde du logiciel est utilisée. Dans le cas d'un contenu anormal en ADN, seule la fraction aneuploïde est prise en compte (fraction diploïde alors non calculée).

2.3. INTERPRETATION DES HISTOGRAMMES

L'interprétation de S% est très dépendante de l'appartenance au sous-groupe de ploïdie. La conférence de consensus (1992) préconise que chaque laboratoire définisse sa propre distribution de valeurs de SPF afin d'établir une classification en 3 groupes (faible, moyen, élevé) ajusté en tertile selon le statut diploïde et aneuploïde (Tableau II).Les valeurs cut-offs définissant les tertiles de cette étude sont basée sur une étude antérieure réalisée à l'ICO Paul Papin sur une population de référence (5).

SPF Classes	Low	Medium	High
Diploid-Near Diploid	< 1,5	1,5 ≤ S% ≤ 2,5	> 2,5
Aneuploid	<3,5	3,5 ≤ S% ≤ 7,5	> 7,5

Tableau II. Classes de SPF en tertiles ajusté selon la ploidïe.

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PERMIS D'IMPRIMER

THÈSE DE Madame Anne PATSOURIS

Vu, le Directeur de thèse

Pr Mario CAMPONE
PU-PH de cancérologie
ICO Paul Papin – Angers

Vu, le Président du jury de thèse

Le L. Bourgeaux

Vu, le Doyen de la Faculté de Médecine d'ANGERS

FACULTE DE MEDECINE - ANGERS

Professeur I. RICHARD

Vu et permis d'imprimer