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Breast Cancer Occuring in Ashkenazi Women: A Historical
Cohort Approach

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13. ABSTRACT (Maximum 200 Words) Using a historical approach, we studied the clinicopathological features of hereditary breast cancer (BC) and related this to the outcome following the BC diagnosis. We ascertained all self-reporting Ashkenazi Jewish (AJ) women diagnosed with primary invasive BC from 1980 to 1995. Diagnostic, treatment and follow-up information was extracted from the medical chart and the pathology blocks (PB) were re-examined by one pathologist. Slides were prepared for immunohistochemistry (IHC) and DNA extracted from the PB was used to look for the recurrent AJ mutations [185delAG, 5382insC (BRCA1) and 6174delT (BRCA2)]. Of the 202 PB, 32 carried a mutation in BRCA1 (24) or BRCA2 (8). BRCA1 was associated with high grade, ER- BC ($P < .001$). BRCA2+ BC were usually ER+. Neither BRCA1 nor BRCA2 were correlated with tumor size. BRCA1/2+ status was also correlated with p53 over-expression and p27 ^{Kip1} under-expression by IHC, but not with HER2 status. A positive BRCA1/2 status was an independent poor prognostic marker in BC. In conclusion, BRCA1/2 mutations confer a specific phenotype on the BC that occur and when combined, are an independent adverse prognostic marker for breast cancer in AJ women. Larger studies in other populations will be required to confirm our findings.				
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The influence of BRCA1 and BRCA2 mutations on prognosis in breast cancer occurring in Ashkenazi Jewish women: a historical cohort approach

William D Foulkes

Idea Grant: DAMD17-98-1-8112

Introduction

In this study, we were funded to use an innovative, pathological specimen-based historical cohort approach to determine whether the prognosis for hereditary breast cancer differs from its sporadic counterpart. We also plan to study established and novel prognostic factors and determine, in a multivariate model, the relative contribution of mutation status to clinical end-points. Our key resources include an unusually well-defined population where mutations in breast cancer susceptibility genes are more common than in other North American populations; a single hospital where many members of this population will be diagnosed and treated for breast cancer; and a multi-disciplinary team who are conducting this research. The methodological approach we are using should eliminate biases that may have rendered previous studies unreliable. The research is clearly of most relevance to Ashkenazi Jewish women with breast cancer, both with and without mutations, but the results, and more especially the methods, may be applicable to other populations of women with hereditary breast cancer.

Body of text

In this section, I will show how we have managed each task.

Task 1. Case identification and data abstraction

- Identify all Ashkenazi Jewish women with breast cancer diagnosed at age <65 in the years 1985-1995 (and if possible to 1980)
- Extract all relevant clinicopathological data
- Data entry

We have completed this task and have written short reports, letters and 2 manuscripts describing this work (references 1-3 and appendix 1). We have now identified over 300 women who are eligible for this study.

Task 2. Pathological examination

- Locate pathology blocks
- Review pathology
- Perform immunohistochemistry
- Anonymize blocks

As stated for task 1, two manuscripts have been published (or are in press, appendix 1), so this task has been met. Immunohistochemical studies of ER and P53 have been completed, as described in the enclosed manuscript (appendix 1). Immunostaining for p27^{Kip1} has also been carried out, and this is almost complete.

Task 3. Laboratory Component (runs concurrently with task 2)

- Cut sections from tumour blocks
- DNA extraction
- Mutation analysis

This work is now complete (1,2).

Task 4. Statistical Aspects

- Construct survival curves
- Perform stratified analysis
- Develop Cox (proportional hazards) model

We have now finished the first sets of analyses (1-3) (see appendix 1). Our first publication during the tenure of this proposal showed that small *BRCA1*+ tumors had an unexpectedly poor outcome (1). The Cox model shows that *BRCA* status is an independent prognostic marker for overall survival in lymph node negative cancer (2) and for disease-specific survival if all cases are included (3).

Task 5. Manuscript preparation

- Report major findings

We have reported these (see references 1-3 and appendix 1), and are in the process of completing the final phase of this study, which will lead to further publications. We have published a review of all relevant studies in our field of study (4).

Key Research Accomplishments

- Establishment of database with full clinicopathological correlates from over 200 Ashkenazi Jewish women with breast cancer. The information we have collected includes: year of birth, year of diagnosis, tumour type, size, grade, nodal status, ER/PR, p53, p27^{Kip1}, HER2 status, *BRCA1*/*BRCA2* mutation status, surgery, adjuvant treatment, first, second, third recurrence (if any), death (if applicable), last date of follow-up.
- *BRCA1* positivity is commonly seen in high grade, ER negative breast cancers. *BRCA2* mutations are less commonly associated with grade 3 cancers, and they are more usually ER+. Neither *BRCA1* nor *BRCA2* are correlated with size or tumour type. *BRCA1* positive status is correlated with p53 over-expression and p27^{Kip1} under-expression by immunohistochemistry, but not with HER2 status.
- *BRCA1* is an adverse prognostic factor in Ashkenazi Jewish women with lymph node negative primary breast cancer. When combined with *BRCA2*, *BRCA* status is an independent prognostic factor for disease free survival in breast cancer patients. *BRCA1* status is associated with under-expression of p27^{Kip1} and both contribute independently to a poor outcome following breast cancer.
- We have published our work widely. The letter in the *Lancet* was the first published statement that the prognosis for lymph node negative hereditary breast cancer was worse than expected. This was followed up by a full paper showing this and relating it to the p53 status of the tumors. We have gone on to study p27^{Kip1} and have shown for the first time that these two factors are associated and independently predict outcome. This paper is now in press in *J Clin Oncol*. We have also published the first comprehensive review of

familial and hereditary factors in breast cancer, which would not have been possible without the insight we have gained from our own studies. We were chosen to give oral presentations of our work at two prestigious meetings: the American Society of Human Genetics (October 1998, Denver) and NCIC/CBCRI Reasons for Hope (June 1999, Toronto) and in collaboration with Memorial Sloan-Kettering Cancer Center, we have submitted a manuscript of penetrance of *BRCA1* and *BRCA2*.

Reportable outcomes

1. Published work

NB The preliminary reports did not include any acknowledgements

Foulkes WD, Wong N, Brunet J-S and Narod SA: *BRCA* mutations and survival in breast cancer. *J Clin Oncol*, **16**: 3206-7, 1998 (letter)

Foulkes WD, Wong N, Rozen F, Brunet J-S and Narod SA: Breast cancer survival in *BRCA1* carriers. *Lancet* **351**: 1359, 1998 (letter)

Foulkes WD. *BRCA1* and *BRCA2*: penetrating the clinical arena. *Lancet*, **352**: 1325-1326, 1998 (Invited editorial)

Chappuis PO, Rosenblatt J and Foulkes WD: The influence of familial and hereditary factors on the prognosis of breast cancer. *Ann Oncol* **10**: 1163-1170, 1999.

Foulkes WD, Chappuis PO, Wong N, Brunet J-S, Vesprini D, Rozen F, Yuan ZQ, Pollak MN, Kuperstein G, Narod SA and Bégin LR. Primary node negative breast cancer in *BRCA1* mutation carriers has a poor outcome. *Ann Oncol* **11**: 307-313, 2000.

Chappuis PO, Deschênes J and Foulkes WD. Long-term outcome of invasive breast cancer. *Lancet*, **355**: 1551, 2000 (letter).

Chappuis PO, Kapusta L, Bégin LR, Wong N, Brunet J-S, Narod SA, Slingerland J and Foulkes WD: Low p27^{Kip1} expression and *BRCA1/2* germ-line mutations are associated and contribute to a poor outcome after breast cancer *J Clin Oncol* (in press).

2. Abstracts published and accepted

Wong N, Brunet J-S, Bégin LR, Vesprini D, Rozen F, Yuan ZQ, Abrahamson J, Karp SE, Pollak M, Narod SA and Foulkes WD: Axillary node negative primary breast cancer in Ashkenazi Jewish *BRCA1* mutation carriers is associated with P53 over-expression and has a poor prognosis. *Am J Hum Genet* **63S**: A47, 248, 1998

Foulkes WD, Wong N, Brunet J-S, Vesprini D, Rozen F, Yuan ZQ, Pollak M, Narod SA, Trudel M and Bégin LR. Node negative breast cancer in Ashkenazi Jewish women has a very good prognosis if the tumor is both HER2 and *BRCA1* germ-line mutation negative Reasons for Hope: NCIC/CBCRI, A39, 1999.

Foulkes WD, Wong N, Brunet J-S, Trudel M, Paradis A-J, Kuperstein G, Chappuis PO, Kapusta L, Slingerland J, Narod SA and Bégin LR. Germ-line BRCA mutation is an adverse prognostic factor in Ashkenazi Jewish women with breast cancer: Survival disadvantage depends on nodal status. *Am J Hum Genet* 65S: A62, 322, 1999

Robson ME, Roberge D, Chappuis PO, Offit K and Foulkes WD. Risk of Ipsilateral Recurrence (IBTR) and Metachronous Contralateral Breast Cancer (CBC) after Breast Conservation Therapy (BCT) in Women with Germline *BRCA1* or *BRCA2* Mutations. Slide presentation at the 23rd Annual San Antonio Breast Cancer Symposium. To be presented in General Session IV, December 8, 2000.

3. Manuscripts submitted

Satagopan JM, Offit K, Foulkes W, Robson ME, Wacholder S, Eng CM, Karp SE, Begg CB. The lifetime risk of breast cancer in Ashkenazi Jewish carriers of *BRCA1* and *BRCA2* mutations.

4. Presentations including the work described here

September 15, 1998

Title: *Overview of studies of prognosis in familial and hereditary breast cancer*
Breast Cancer Linkage Consortium,
Dublin, Ireland.

September 17, 1998

Title: *Genetics of Breast Cancer*
Division of Investigative Sciences,
Imperial College of Science and Medicine,
Hammersmith Hospital, London

May 26, 1999

Title: *The influence of familial and hereditary factors on the clinicopathological features and prognosis of breast cancer*

Department of Epidemiology
Fred Hutchinson Cancer Research Center
Seattle, WA, USA

February 24, 1999

Title: *Genetics of Breast Cancer: some observations from the study of founder populations in Quebec*
Division of Cancer Biology Research Seminar,
Sunnybrook and Women's College Hospital Health Sciences Centre,
Toronto, ON, Canada

May 19, 1999

Title: *Genetics of Breast and Ovarian Cancer*

"New Developments in prenatal diagnosis and medical genetics"

University of Toronto CME course

Toronto, ON, Canada

June 20, 1999

Title: *Node negative breast cancer in Ashkenazi Jewish women has a very good prognosis if the tumor is both HER2 and BRCA1 germ-line mutation negative*

Reasons for Hope: NCIC/CBCRI conference

Toronto, ON, Canada

May 29, 1999

Title: *Hereditary predisposition to breast and ovarian cancer*

Annual Congress of the Quebec Obstetrics and Gynecology Association (AOGQ)

Hotel Delta Sherbrooke,

Sherbrooke, Quebec

January 12, 2000

Title: *Genetic predisposition and outcome from cancer*

Montreal Cancer Research Group,

McGill Cancer Centre,

Montreal, Quebec.

Conclusions

We have attained all the goals we set ourselves in this project. In the second and final year of funding we have completed a major piece of work on the roles of both the *BRCA* genes in determining the prognosis for Ashkenazi Jewish women with breast cancer. We have an excellent database with which to probe related questions such as the role of other prognostic markers in hereditary breast cancer.

So what does this all mean? Firstly, our database will allow us to determine who is most likely to carry *BRCA* mutations on the basis of clinicopathological data. This will be of use to oncologists, when they are deciding who to offer *BRCA* genetic testing to, particularly when the family history is truncated. Secondly, and most importantly, determining the prognosis for women with hereditary breast cancer is of crucial importance if we are to be able to evaluate the role of prevention and early diagnosis in the management of women at high risk of breast cancer. We believe that this study is one of the few studies worldwide that can answer this crucial question. While our work was initially highly controversial, two recent studies have achieved the same results that we have shown.

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3. Chappuis PO, Kapusta L, Bégin LR, Wong N, Brunet J-S, Narod SA, Slingerland J and Foulkes WD: Low p27^{Kip1} expression and *BRC A1/2* germ-line mutations are associated and contribute to a poor outcome after breast cancer *J Clin Oncol* (in press).
4. Chappuis PO, Rosenblatt J and Foulkes WD: The influence of familial and hereditary factors on the prognosis of breast cancer. *Ann Oncol* **10**: 1163-1170, 1999

Appendix 1:

Foulkes WD, Wong N, Brunet J-S and Narod SA: *BRCA* mutations and survival in breast cancer. *J. Clin. Oncol.* **16**: 3206-7, 1998.

Foulkes WD, Wong N, Rozen F, Brunet J-S and Narod SA: Breast cancer survival in *BRCA1* carriers. *Lancet* **351**: 1359, 1998.

Foulkes WD: *BRCA1* and *BRCA2*: penetrating the clinical arena: *Lancet* **352**: 1325-1326, 1998.

Chappuis PO, Rosenblatt J and Foulkes WD: The influence of familial and hereditary factors on the prognosis of breast cancer. *Ann. Oncol.* **10**: 1163-1170, 1999.

Foulkes WD, Chappuis PO, Wong N, Brunet J-S, Vesprini D, Rozen F, Yuan ZQ, Pollak MN, Kuperstein G, Narod SA and Bégin LR: Primary node negative breast cancer in *BRCA1* mutation carriers has a poor outcome. *Ann. Oncol.* **11**: 307-310, 2000.

Chappuis PO, Deschênes J and Foulkes WD: long-term outcome of invasive breast cancer. *Lancet* **355**: 1151, 2000 (letter).

Chappuis PO, Kapusta L, Bégin LR, Wong N, Brunet J-S, Narod SA, Slingerland J and Foulkes WD: Low p27^{Kip1} expression and *BRCA1/2* germ-line mutations are associated and contribute to a poor outcome after breast cancer. *J. Clin. Oncol.* (in press).

for these micrometastases at this time is surgical; thus, at present, most centers recommend evaluation of the status of regional lymph nodes in patients with intermediate-thickness melanoma by sentinel lymph node assessment. This technique is effective in identifying and removing involved lymph nodes with minimal morbidity.² Most importantly, in numerous studies, the overall lymph node recurrence rate in the group previously treated with sentinel lymph node biopsy is less than 5%²⁻⁴; this is substantially less than the lymph node recurrence rates reported in either arm of this trial. Thus, present standard surgical care provides a superior result to a 1-year course of interferon, as used in this trial, avoids long-term therapy, and spares patients who do not have involved lymph node any adjuvant therapy at all.

The question then remains, does the interferon regimen tested by Pehamberger et al¹ impact distant recurrence rates? The data, in fact, do not answer this question. Only eight patients in the observation arm developed visceral metastases during the follow-up period of their trial; only four on the treated arm did. This difference does not support a conclusion. When skin recurrences are added to these numbers (the investigators do not stipulate whether these are local or distant metastases), the number of non-lymph node recurrences remains small in both arms. It is noteworthy that recent randomized clinical trial data from World Health Organization (WHO) Trial 14 suggest that patients may benefit from early removal of involved lymph nodes.⁵ Thus, one cannot even say that the rate of visceral and skin recurrence observed in this trial reflects expected rates of such events in a population of patients who have been assessed by sentinel lymph node biopsy.

In summary, the results of this trial do not apply to the surgically staged melanoma patient population treated in the United States at this time. Present data suggest that patients with intermediate-thickness melanomas who are pathologically node-negative suffer a very low recurrence rate, and at present, there are no data to suggest that adjuvant therapy of any kind benefits this group. Current standard practice remains sentinel assessment of the lymph nodes, and treatment with high-dose adjuvant interferon⁶ for pathologically node-positive patients.

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BRCA Mutations and Survival in Breast Cancer

To the Editor: The prognosis for women with breast cancer who carry mutations in breast cancer susceptibility genes is controversial.¹⁻⁴ Recent studies have given conflicting results with one study that showed a significantly worse prognosis for *BRCA1* mutation carriers than non-carriers,¹ another that showed a nonsignificantly worse outcome,³ and two other studies that showed no difference.^{2,4} The prognosis for *BRCA* mutation carriers who develop breast cancer is important, because this knowledge may influence the management of women at risk.

The study in the *Journal of Clinical Oncology* by Robson et al⁴ discusses some of the difficulties of estimating survival from clinic-based breast cancer cases. For example, from Fig 3, there seems to be at least one individual who was followed up for 160 months, and at least one individual was entered into the study 36.5 years after diagnosis. However, only patients alive between January 1992 and December 1995 were included in the study; thus, patients who were diagnosed and who died before 1992 were not ascertained. As the investigators acknowledge, survival analyses that include prevalent cases are subject to question. Women diagnosed 30 years or more ago at the time of ascertainment, by definition, are long-term survivors.

Surprisingly, no specific information on death is provided. One would expect some deaths in this cohort of 91 women. The cumulative probability of dying of breast cancer within 5 years of diagnosis is 20% for white American women diagnosed between the ages of 30 to 39 and 26% for those diagnosed under 30 years of age.² Presumably, breast cancer-related deaths have been included in the event-free survival (Figs 3 and 5), but specific mortality rates are not given.

Just more than one quarter of all *BRCA* mutation carriers were diagnosed at state I, and 57% were diagnosed at stage II. For noncarriers, the percentages were 46% and 37%, respectively. These results suggest that *BRCA* mutation carriers presented with breast cancer at a more advanced stage and would be expected to have a poorer survival. Given these differences, adjustment for stage in a proportional hazard model may have obscured the overall effect of *BRCA* mutations. The inclusion of both *BRCA1* and *BRCA2* mutation carriers in the analysis also has made it difficult to determine the specific influence of each of these two genes. Were there any differences? We and others have observed that *BRCA2*-related tumors may differ from those that occur in *BRCA1* mutation carriers.^{6,7} Finally, the generalizability of the findings of Robson et al⁴ may be somewhat limited, as in our series of Ashkenazi Jewish women with breast cancer diagnosed in Montreal, in which only 32% of all *BRCA1* mutation carriers who developed breast cancer did so before the age of 42 years. It is possible that the prognosis for *BRCA1* mutation carriers is worse than expected for those diagnosed after the age of 42 years.

Published studies that address prognosis in hereditary breast cancer have been limited by sample size¹ or ascertainment.²⁻⁴ The actual prognosis for hereditary breast cancer will not be precisely determined until large prospective or retrospective cohort studies are completed.

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In Reply: Despite the observation that *BRCA*-associated breast cancers are of generally higher histologic grade than sporadic tumors and more frequently are estrogen-receptor negative, initial reports have not documented a more aggressive clinical course.¹⁻⁶ As suggested by Foulkes et al, and as we acknowledged in the discussion of our results,⁵ survival bias cannot be entirely excluded in retrospective studies of a prognostic factor when all members of the population of interest are not tested for the presence or absence of the factor. In our series, in contrast to studies in which mutation carriers are compared with matched population controls, women with and without mutations were ascertained according to the same criteria before mutation analysis was performed. The only criterion for entry was a diagnosis of early onset breast cancer. Women who subsequently were shown to lack mutations were subject to the same survival ascertainment bias as those with mutations. For this reason, preferential ascertainment of mutation carriers who are long-term survivors is unlikely to explain the absence of a significant difference between the groups. However, the 5-year overall survival after initial diagnosis of invasive cancer was better than would be expected in both groups (95.8% for those with mutations v 97.1% for those without; log-rank, $P = .50$). Thus, it remains possible that the presence of a *BRCA* mutation may confer an adverse short-term prognosis that would not be detected in our series. To address this possibility, we separately analyzed those women who donated a blood sample within 2 years of their initial breast cancer diagnosis. Although the power of the analysis is limited by a small sample size, there were no differences in relapse-free or overall survival between women with or without mutations. There also were no detectable differences in outcome between women with *BRCA1* and *BRCA2* mutations, which makes it unlikely that a negative prognostic influence of *BRCA1* mutations was obscured by the presence of *BRCA2* heterozygotes in the study group.

The correlation of histologic appearance with clinical behavior is imperfect. For example, medullary breast cancer clearly does not have a worse prognosis than the more typical infiltrating ductal carcinoma, despite being very poorly differentiated.⁷ This particularly is interesting in view of the excess of medullary cancers described in *BRCA* heterozygotes with breast cancer.^{1,8} The apparent lack of influence of

histology on prognosis in *BRCA*-associated breast cancer may be because of the distribution of other prognostic factors. For example, we and others have reported a relatively low prevalence of *HER2/neu* overexpression in *BRCA*-associated cancers.^{2,3} *BRCA*-associated tumors also may be uniquely susceptible to DNA damage induced by adjuvant chemotherapy, as suggested by the recent description of chemosensitivity in a human pancreatic cancer-cell line that lacked *BRCA2* function.⁹

We agree that the effect of germline *BRCA* mutations on prognosis will best be determined by large prospective studies or properly designed retrospective cohort analyses. It will be important to ensure that such studies have an adequate sample size, because the simple dichotomous analysis of small subgroups may suggest effects through the operation of chance, particularly if few patients are at risk at the time points reported. Focusing attention on younger women to increase the prevalence of mutations in the study group is one approach to maximize the power of such studies. Concerns about the ability to generalize results from younger cohorts to a broader population of breast cancer patients are unfounded, provided that the distribution of other prognostic factors is taken into account. Maldistribution of such factors in small series may lead to unexpected results, and could account for the observation in a recent publication of a 95.7% 5-year survival among Ashkenazi breast cancer patients without *BRCA1* mutations.¹⁰ Multivariate analyses will be necessary to determine whether germline mutations have an independent prognostic effect. Rather than obscuring an effect of *BRCA* mutations on outcome, such analyses will be mandatory to eliminate the potential confounding influence of established prognostic factors, such as age, stage, hormone receptor status, and grade.

Mark Robson

Kenneth Offit

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age adjustment leave adequate statistical power for the main contrasts in the analysis?

(8) Were the endpoint/outcome measures (particularly arthritic and rheumatic findings) formally validated before the fieldwork began—or before the definitive analysis plan was adopted?

(9) Members of a follow-up cohort are classified and then assigned or recruited by exposure status at “zero time”, before the specified outcome becomes manifest. They are then followed up. Documents and correspondence obtained by Freedom of Information mechanisms from the coordinating office at the NCI suggest that recruitment of women was done extensively in plaintiffs’ lawyers’ practices and among advocacy groups of women with adverse events (ie, women who considered themselves already affected by an outcome of interest). How will the investigators contain major biases introduced, almost certainly, by such recruitment methods?

(10) Have there been any formal protocol revisions? If so, how were they reviewed and approved?

The NCI Follow-up Study of Women with Augmentation Mammoplasty is considered by some to be very objective because it is governmentally funded. But the absence of sponsorship by plastic surgeons, implant manufacturers, and other interest groups does not guarantee lack of bias on methodological grounds.

After five years and several million dollars of public funds the NCI study has the potential to bring important new evidence to bear on the controversies surrounding breast implants and women’s health. However, unless the above questions are answered—and none, surprisingly are resolvable via the protocol—the findings may be uninterpretable.

WOS has consulted with one defendant in breast-implants litigation. None of the other authors have had any involvement with any aspect of the controversy.

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Survival of patients with breast cancer and *BRCA1* mutations

Sir—L C Verhoog and co-workers (Jan 31, p 316)¹ found that 5-year survival rates for women with breast cancer and *BRCA1* mutations were similar to those for patients with sporadic disease. Their results are surprising, given that *BRCA1*-associated breast cancers are more likely than sporadic cancers to be high grade,² oestrogen-receptor negative,³ and p53 positive.³

Accurate estimates of survival for patients with hereditary cancer based on clinic records is difficult, and the results of all such studies must be questioned. Difficulties arise because living affected women are preferentially referred to the clinic and offered genetic testing. Once a mutation is known in a family it may be possible to assess the mutation status of deceased cases with stored histological samples, but this technique does not always succeed and is not usually offered when there is no living affected case.

Verhoog and colleagues analysed the dataset twice: first with all 49 patients and then after the exclusion of 13 probands. As expected, these exclusions negatively affected the observed survival rate. However, exclusion of the proband is insufficient to correct for ascertainment bias in clinic-based genetic studies. This fact is often overlooked; for example, if each living woman with familial breast cancer in Holland were equally likely to be referred to a cancer clinic for assessment, then a family with three living affected women would be three times more likely to be included in a clinic-based study than a family with only one living affected woman. So, the differences in survival between patients with or without the *BRCA1* mutation in the Dutch breast cancer cohort may be even greater than that reported.

An unbiased way to estimate relative survival is to ascertain *BRCA1*-mutation status on an unselected sample of pathology breast specimens in a hospital tumour bank, and to compare survival for women with and without mutations. We analysed 187 tumour blocks from unselected Ashkenazi Jewish women with breast cancer, aged 28–65 years, diagnosed between 1986 and 1996. We reviewed the medical records of each case to determine tumour stage and grade and survival.

36 women had died by the end of 1997. The median length of follow-up was 4.5 years. 25 (13%) women were carriers of *BRCA1* mutation. Death from breast cancer was more common

in the *BRCA1*-mutation carriers than in controls. Eight of the nine deaths in carriers were attributed to breast cancer. Kaplan-Meier actuarial survival methods showed that the 5-year survival rate was worse for carriers than for non-carriers (70.8% vs 85.9%, log-rank test $p=0.05$). The difference was especially striking for node-negative patients. Only 58.3% of node-negative carriers survived for 5 years, compared with 94.1% of the node-negative controls ($p=0.0001$). The *BRCA1*-mutation carriers were younger on average, but restricting the analysis to premenopausal cancer had little effect on the survival differences. The result was equally strong for the node-negative cases diagnosed before age 50 (37.5% survival for carriers vs 87.5% non-carriers, $p=0.0013$). Positive-node status was an adverse prognostic factor among the *BRCA1*-negative cases (94.1% of node-negative cases survived 5 years vs 73.9% of node-positive cases, $p=0.003$). By contrast, *BRCA1*-mutation carriers had poor survival, independent of nodal status.

We believe that previous investigators may have come to different conclusions because of the biases inherent in studying survival historically, in clinic-based populations. Although our data are preliminary and our sample size is small, our findings call into question the practice of relying on lymph-node status to grade early-stage breast cancer in *BRCA1* carriers.

We thank L Bégin, M Pollak, P Tonin, and S Karp for assistance.

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Authors' reply

Sir—William Foulkes and colleagues’ explanation of ascertainment bias in our study resulting in selection for longevity is too simple. The assumption that we selected families with high proportions of surviving patients is incorrect. This assumption might only hold if all

probands were living breast-cancer patients. But this is not the case since healthy women also consult our family cancer clinic, and these families were also included in our study. At our clinic, we offer DNA-analysis to families without a living affected case with histological samples stored in paraffin of deceased patients or blood from living relatives who have a 50% risk of carrying a mutation.

We agree with Faulkes that a population-based study of patients with breast cancer is the best way to calculate relative survival. Nevertheless, we think that their selection procedure did not exclude the risk of any bias, chance, and inappropriate comparisons within their own and other studies. In their initial hospital-based study¹ Foulkes and colleagues investigated mainly Jewish patients, excluded patients older than 65 years, and those with long-term follow-up and proven long-term survival. They selected patients with a recent diagnosis of breast cancer (initially 1990–96,¹ later 1986–96), and most patients (about 94%) were treated with breast-conserving surgery. Their control group had an excellent survival rate, suggesting a specific reference pattern to their hospital, which is not representative and could indicate increased awareness of breast cancer. In their initial study¹ in 12 *BRCA1*-positive and 100 *BRCA*-negative tumours, Foulkes and co-workers showed a significant difference in tumour size in favour of the control group, but this difference disappeared after the addition of 75 other patients (13 *BRCA1* positive). After that, the tumour size in the *BRCA1*-positive group dropped from 2.41 cm to 2.07 cm and increased from 1.71 cm to 1.99 cm in the controls. The between-group difference in breast-cancer-specific death rate dropped from 31.4% ($p=0.002$) in their initial study¹—to 15.1% ($p=0.05$) in their present extended study. This difference reflects the impact of potential bias by small sample size and the selection of time for years since breast cancer diagnosis.

The small mutation spectrum in their study differs from the broad spectrum in our study and might per se explain the difference in conflicting results of the two studies. The site of both *BRCA1* mutations (185 delAG, 5382 insC) specific for the Ashkenazi Jewish population have been associated with highly proliferating undifferentiated hereditary breast cancer.² However, there are no major differences in death rates between the *BRCA1*-positive patients in their and our study whereas there is a great between-study difference between the control groups (14.1% *vs*

29%). This difference could result from Faulkes and colleagues' selection procedure and small subgroups that were not matched adequately for age, which is an independent prognostic factor. In addition, the results of two recent age-matched studies^{3,4} confirm our findings.

We conclude that there was no ascertainment bias towards lengthy survival in our study, but differences in populations, small sample size, and selection criteria are responsible for the discrepant results. The findings of Faulkes and co-workers in the node-negative subgroup and their hypothesis that *BRCA1*-mutation carriers may be especially prone to develop distant metastases without evidence of axillary lymph-node involvement are intriguing, but their subgroups and number of events are too small to allow definitive conclusions.

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Prenatal exposure to famine and health in later life

Sir—A C J Ravelli and colleagues' (Jan 17, p 173)¹ observations on adults born around the time of the Dutch famine add further support to the notion that prenatal conditions might contribute to metabolic programming. Insulin resistance might be the main determinant of the resulting adverse metabolic profile,^{1,2} but Ravelli and co-worker's insulin data raise questions about their conclusion regarding the critical timing of prenatal exposure to famine. On the basis of their 2-h glucose observations, these investigators have implicated late gestational exposure in higher insulin resistance later in life. However early gestation

could be the critical time window, as the unadjusted data on fasting insulin, fasting 32–33 proinsulin, and 2-h insulin suggest (Ravelli's table 2). We are informed that insulin and proinsulin concentrations, controlled for sex and body-mass index, did not differ significantly according to the timing of famine exposure, but it may not be appropriate to control for an obesity variable that could be intermediate in the causal pathway. It would be of interest to examine insulin concentrations according to time of exposure and strata of body-mass index. Similarly, data on insulin concentrations stratified by birthweight and according to time of exposure might shed further light.

Ravelli's data also suggest that there may have been reduced survivorship in men exposed to famine during early or mid gestation. There were 70 men and 93 women among those exposed to famine during early or mid gestation, whereas on the basis of the sex distribution of unexposed individuals, one would have expected about 83 men and 80 women ($p=0.07$). The sex distribution among those exposed in late gestation (56 men and 60 women) was what might have been expected ($p=0.69$).

Insulin resistance is a predictor of coronary heart disease, the illness that largely accounts for the lower survivorship among men than women in middle age.⁴ Thus, if our interpretation is correct regarding the reduced male representation among famine-exposed individuals in Ravelli and colleagues' study, the reduced survivorship in men may also point to early or mid gestation (rather than late gestation) as the critical period for determining insulin resistance in later life. Consistent with this view is the higher rate of obesity noted earlier among young men exposed to the Dutch famine during the first half of pregnancy.⁵

More evidence about the mechanism(s) by which prenatal circumstances may determine later metabolism and health is needed. At the same time, we must attempt to clarify the gestational time window (late *vs* early), and the exact nature (eg, nutritional deprivation *vs* stress hormones) of the implicated prenatal exposure

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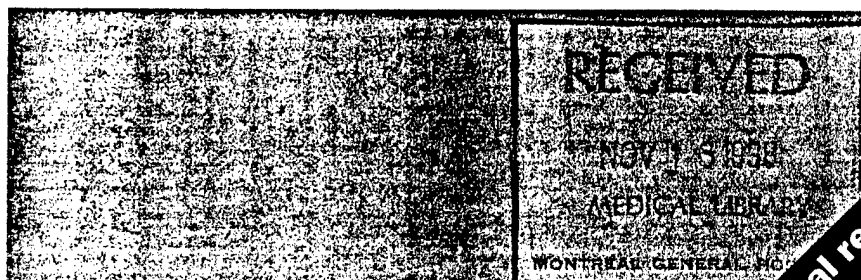
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interest in the subject, as well as clinical caution. An expansion of this style of study should be encouraged, to include all crashes and injury and death from other forms of mobility, such as walking and cycling. Prospective studies are also needed, perhaps as perimarketing clinical trials.⁷ In the interim, clinicians who prescribe benzodiazepines need to recognise that most adult patients are drivers or potential drivers. Active consideration should be given to whether the illness is likely to affect driving skills and whether the patient has a history of crashes. The patient should be advised not to drive if he or she cannot abstain from alcohol while on treatment with benzodiazepines. Most importantly, prescribers should query whether the patient really needs a benzodiazepine, and if so, whether it needs to be long acting.

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BRCA1 and BRCA2: penetrating the clinical arena

See page 1337

The identification of *BRCA1* in 1994¹ and *BRCA2* in 1995² were landmarks in the investigation of the genetics of breast cancer. Even before the genes were identified, it was clear that management issues following on from gene identification would need to be addressed quickly if the impetus gained from these discoveries was not to be lost. So what are the key questions facing clinicians who care for women with *BRCA* mutations? One is that of penetrance—that is, what is the likelihood that a carrier of a mutated allele of a breast-cancer-susceptibility gene will develop breast cancer by a given age? Another important question is, when advising individuals at risk, whether counsellors should give more weight to the mutation itself (the gene affected and the type of mutation), the position of the mutation along the gene, or the family history? A third question is whether the prognosis of *BRCA1*-related breast cancer differs from that of *BRCA1*-negative breast cancer. Finally, and most importantly, there is the question of the choice of medical and surgical preventive measures.

Initial estimates of the penetrance of *BRCA1* and *BRCA2* used large pedigrees with many cases of cancer as the sample on which to make the calculation. For both genes, the point estimate for the penetrance of all disease-associated alleles seemed to be 70–90% for breast cancer by age 70,^{3,4} but the risk of breast cancer by age 50 may be lower for *BRCA2* mutations.^{4,5} A large population-based study of Ashkenazi Jewish volunteers

suggested a lower penetrance of 56% by age 70 for the three founder mutations in the *BRCA* genes (185delAG, 5382insC, and 6147delT), a founder mutation being one that has been shown, by haplotype analysis, to have arisen in a single common ancestor of individuals now carrying the mutations. These three founder mutations are common among the Ashkenazi Jews, and there was no evidence for differences between them in penetrance.⁶

In today's *Lancet*, Steinunn Thorlacius and colleagues have used the same method in another reproductively isolated population, the Icelanders, to estimate the penetrance of the *BRCA2* mutation, 999del5. The investigators studied all 34 male breast-cancer patients diagnosed in Iceland between 1955 and 1996, and a sample (n=541) of female breast-cancer cases. Again, the penetrance (17% by age 50 and 37% by age 70) was lower than expected from pedigree-based studies. The same investigators had previously shown that 25 of 61 carriers of this mutation had no first-degree or second-degree family history of breast cancer, so the penetrance of this mutation was likely to be low.⁷ Interestingly, the 999del5 mutation accounts for virtually all hereditary breast cancer in Iceland, so the finding of a low penetrance will be relevant to the 0.6% of Icelanders who are likely to carry this mutation.⁷

The reasons for differences in penetrance observed between studies could be methodological (pedigree-based studies by definition contain many affected individuals), stochastic (due to the play of chance within families and populations), biological (due to modifying genes in families with many cases of breast and/or ovarian cancer), or environmental (due to diet, smoking, and lifestyle). Risk-modifying genes may be present.^{8,9} Such genes could be having an influence in Iceland, but as yet their existence is entirely speculative. To resolve these issues, it will be important to conduct a meta-analysis of all available penetrance studies to disentangle the methodological differences from the others.

In the assessment of survival in hereditary breast cancer, although the prognosis is better for cancers in women with, than in those without, a family history,¹⁰ recent data^{11,12} and a review of published work indicate that the prognosis of *BRCA1*-related breast cancer (and for that matter, *BRCA1*-related ovarian cancer) is similar to or worse than that of age-matched tumours in women without *BRCA1* mutations. This conclusion is in complete agreement with histopathological studies that show that *BRCA1*-related breast cancers are high grade, oestrogen-receptor negative, and P53 positive, all features normally associated with an adverse outcome.¹³ Similar histopathological studies of ovarian cancer are now underway under the aegis of the Breast Cancer Linkage Consortium (BCLC).

Studies that aim to provide answers to pressing clinical questions that will narrow the gap between gene identification and effective prevention and treatment must recruit large numbers of *BRCA*-mutation carriers. At the BCLC meeting in Dublin, Ireland, on Sept 14–16, data provided from all over Europe showed the presence of founder effects in different European countries. These are important findings, because in outbred populations not more than 4% of unselected women with breast cancer diagnosed under the age of 50 will carry *BRCA1* or *BRCA2* mutations, and these mutations will be distributed throughout both genes. If

founder populations can be identified, the cost of mutation identification can be lowered considerably, as can the cost of studies that address crucial questions such as the role of tamoxifen chemoprevention, oral-contraceptive use, and preventive surgery, whether conducted prospectively or retrospectively. Intermediate endpoints (mammographic density is a good candidate) may prove to be very useful. Although large trials done previously in outbred populations may provide ready outcome information, the only way to study the effect of mutations on various endpoints is to analyse archived specimens to find all the mutations, but mutation detection in tissue blocks is technically challenging and very time-consuming. Mutation carriers must be unambiguously identified, and the inability to select the correct control group has bedevilled other *BRCA*-related studies, such as that of the prognosis of hereditary breast cancer.

Although penetrance has been the focus of research in the past few years, whether an accurate estimate of the "real" penetrance of *BRCA* alleles is possible remains an issue. Even so, *BRCA* mutations have been confirmed to be the most significant breast-cancer risk factor for hundreds of thousands of women, and full attention must now be focused on the crucial management questions that so urgently need answers. In the 4 years since *BRCA1* was identified, the issues have become more not less, complex. Clinical cancer genetics specialists now have a clear role in both the management of individuals at high risk and in training a new generation of specialists.

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What makes DOT work?

See page 1340

"Supervised swallowing" sums up directly observed therapy (DOT), a strategy by which health workers or trained volunteers watch patients take their treatment for tuberculosis. The aim is to improve the patient's adherence to the therapeutic regimen, and DOT is one of the five key elements in the WHO Global Tuberculosis Programme control strategy. It is a main component of the "breakthrough" in tuberculosis control announced by the WHO last year, and it is widely promoted globally.¹⁻³ In today's *Lancet* Merrick Zwarenstein and colleagues question current dogma in a trial comparing DOT with self-administered therapy. The investigators conclude that the two approaches result in equivalent outcomes. They comment that direct observation is authoritarian, alienates the patient, and decreases the responsibility for self-care. This study is conducted carefully in a service setting, and is a broadside against the current consensus. How do the findings fit with the global picture?

The researchers randomly assigned patients to direct observation or self-treatment. Staggering as it may seem in view of the current level of investment in DOT strategies, this work is the first published randomised trial evaluating the specific strategy of direct observation to improve adherence.⁴ To date, policies have been based on observational studies in which DOT is a label for multifaceted programme inputs, ranging through strengthened laboratory services, investment in drug supply, jail for recalcitrant patients, certificates of completion, cash for compliance, advice on finding housing, and birthday parties.⁵⁻⁷ All these factors are important practical interventions that represent a variety of provider approaches, and they clearly work in the circumstances in which they are implemented. Managers need to know the right ingredients that make their own programmes work, otherwise the results will not be easy to replicate. Some are obvious, such as a good supply of drugs, but others rely on good research. This is the nub of the debate around the effectiveness of DOT: what exactly are the inputs in the observational studies reported that make up "directly observed" therapy? Is it direct observation of patients that results in better adherence, or other factors that accompany these initiatives? This question is one that randomised trials can help to answer.

Ironically, this trial is also the first randomised comparison in specific antituberculosis adherence-promoting strategies that does not show an obvious benefit of the experimental intervention. A systematic review has shown that all strategies across five trials had a positive effect on adherence. The strategies included cash incentives, reminders to patients, and supervision by health workers.⁴ Still, a null effect of DOT cannot be concluded from this trial, since only 60% completed treatment in the self-treatment arm, and a smaller proportion did so in the DOT group. Thus no conclusion can be drawn as to whether the equivalence is a failure of DOT per se to improve adherence, or a general management and resource problem resulting in difficulties in delivering care in either form. Good qualitative research could help explain the findings of the trial, and its absence in this instance provides a good reminder for its judicious use in future implementation trials.

Yet these results cannot be ignored. One of the

Review

The influence of familial and hereditary factors on the prognosis of breast cancer

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Summary

Background: Family history is a well recognized risk factor for breast cancer, but its impact in terms of breast cancer survival is uncertain. The recent identification of breast cancer predisposing genes has provided new clinical insights in this field.

Design: English literature identified through Medline between 1976 and February 1999 was reviewed including search terms: breast cancer, survival, prognosis, family history, genetics, BRCA1, BRCA2, and related articles.

Results: Publications were divided into three categories. **Family history-based studies:** eighteen articles were reviewed. Four studies showed a statistically significant better survival in patients with a family history of breast cancer, and two studies demonstrated a significantly worse prognosis in this context. The remaining articles showed no significant difference. **Linkage studies:** Two studies based on linkage to BRCA1 found that overall survival was better in linked families. A third one concluded to a worse outcome in BRCA2-linked tumors. **Mutation-based studies:** 10 studies looking at the association

between germ-line mutations in BRCA1/BRCA2 and clinical outcomes were reviewed. Eight articles reported no significant difference in outcome, whereas two studies showed a worse outcome in patients with mutations.

Conclusions: Conflicting data exist as to whether the prognosis of familial or hereditary breast cancer differs from that of sporadic cases. Some of the discrepancies may be explained by methodological differences or biases. However, no studies showed a survival advantage for BRCA1 mutation carriers. This seems to indicate that BRCA1-related breast cancer is not associated with a survival advantage, and that in fact, certain BRCA1 germline mutations confer a worse prognosis. However, to adequately answer this question, more efficient molecular tools to identify all the genetic changes responsible for breast cancer predisposition, and large cohort studies to evaluate their clinical consequences, are needed.

Key words: BRCA1, BRCA2, breast cancer, family history, survival

Introduction

Family history of breast cancer is an established risk factor for the development of the disease [1]. Five to ten percent of breast cancer cases are hereditary and germ-line mutations in the breast cancer predisposing genes BRCA1 and BRCA2 may account for 80% of the hereditary cases [2]. It is unclear whether the prognosis of hereditary breast cancer differs from that of sporadic cases. In hereditary non polyposis colorectal cancer, patients with constitutional mutations in the MLH1 or MSH2 genes have been found to have a better prognosis than those without mutations [3, 4]. Whether or not the same survival advantage is true for hereditary breast cancer is unclear. Pathological features suggest that there may be underlying differences in familial/hereditary breast cancer compared to sporadic cases. BRCA1-tumors are more often poorly differentiated, highly proliferating tumors, with a high frequency of estrogen receptor negativity, and a higher rate of p53 mutations [5-7]. Nevertheless, BRCA1 associated tumors also demonstrate intratumoral infiltrating lymphocytes, an increased proportion of medullary histological type, less

frequent node involvement, a relatively low HER2/c-erbB-2/neu overexpression, and a decreased angiogenesis [8-10]. Distinct somatic genetic changes have been preliminary reported in hereditary cases compared to sporadic breast carcinoma [11]. Somatic mutations in BRCA1 or BRCA2 are extremely rare in sporadic breast cancer, but the expression of BRCA1 may be decreased in some cases [12].

Survival studies may reveal further information on the biological differences between hereditary and sporadic tumors. Survival information is essential for the elaboration of preventive and therapeutic strategies, and for counseling women at increased risk of breast cancer.

Studies looking at differences in survival between familial/hereditary and sporadic cases of breast cancer can be grouped into three study categories: family history-based, linkage-based, and mutation-based. Family history-based studies were particularly useful prior to the localization of the BRCA1 and BRCA2 genes. However, the definition of hereditary breast cancer has not been clearly established. Moreover, not all women with a family history of breast cancer have BRCA1/BRCA2 mutations, and not all BRCA1/BRCA2 affected carriers

Table 1. Family history-based studies.

Study	Inclusion criteria	FH of BC: definition	Cases	Controls	Median follow-up	Results (cases vs. controls)	P
<i>A. Early-onset BC, non-population based</i>							
Greenberg et al., 1985 [14]	BC < 50 years	n/s	54	528	n/s	5 years OS: n/s	NS
Chabner et al., 1998 [15]	Stage I–II BC < 36 years; breast conservation treatment	FDR < 50 years or OC at any age	29	172	11 years	5 years OS: 92% vs. 80% 10 years OS: 78% vs. 67%	0.21
Mohammed et al., 1998 [16]	BC < 45 years	F or S DR	95	329 Age, years of diagnosis-matched	7.8 years (cases) 8.6 years (controls)	5 years OS: 92% vs. 70% 10 years OS: 87% vs. 54%	< 0.001
<i>B. Population-based</i>							
Ruder et al., 1988 [17]	1. BC aged 25–54 years 2. Consecutive BC aged 21–60 years Manitoba Cancer Registry, Canada	F or S DR on maternal side	1. 165 2. 145	462 Age-matched	End of 1985 (cohort study)	Cancer-specific S: 66.1% vs. 59.1%	NS
Slattery et al., 1993 [18]	Utah Population Database and Cancer Registry, USA	any R with BC	2800	778	n/s	OS: RR of dying with FH+ in FDR < 50 years: 1.54 (0.98–2.41) > 50 years: 0.85 (0.70–1.03)	N/A
Malone et al., 1996 [19]	BC aged 21–44 years Metropolitan Seattle area, USA	F or S DR	118 with FDR, 154 with SDR	461 Stage, age, years of diagnosis-matched	6.4 years	RR (adjusted) of dying for cases with FDR+: 0.5 (95% CI: 0.3–0.9)	N/A
<i>C. Referral and/or cancer clinics</i>							
Langlands et al., 1976 [20]	n/s Department of Radiotherapy, Edinburgh, Scotland	FDR	165	2833 Negative FH	n/s	5 years OS: 69.6% vs. 54.5% 10 years OS: 52.3% vs. 40.2%	NS NS
Albano et al., 1982 [21]	HBC syndrome Creighton University, Omaha, USA	'Large number of BC per family': putative dominant inheritance	106 (18 fam.)	24136	9 years	5 years DFS: 67% vs. 46.5% (age-adjusted)	< 0.05
Herrmann et al., 1985 [22]	n/s Cleveland Clinic, Cleveland, USA	F or S DR	271	1322	7.3 years	5 years OS: n/s	NS
Anderson et al., 1986 [23]	n/s M.D. Anderson Hospital, Houston, USA	F, S or T DR	556; 293 staged	4551 (1946–1976)	n/s	5 years OS: 66% vs. 67% 10 years OS: 47% vs. 49%	NS NS
Wobbes et al., 1987 [24]	n/s St. Radboud University Hospital, Nijmegen, The Netherlands	F, S or T DR	96	380	n/s	5 years OS: 75% vs. 73% 10 years OS: 65% vs. 50%	NS NS
Lees et al., 1989 [25]	n/s Northern Alberta BC Registry, Canada	Any R with BC	216	586	10 years	10 years OS: 50% vs. 55%	NS ^a
Fukutomi et al., 1992 [26]	n/s National Cancer Center Hospital, Tokyo, Japan	F or S DR	394	3969	n/s	5 years OS: 86.4% vs. 80.1% 10 years OS: 79.3% vs. 67.2%	< 0.05 < 0.01
Israeli et al., 1994 [27]	n/s Mont Sinai Medical Center, New York, USA	F, S or T DR	179	569	n/s	5 years DFS: 52% vs. 85%	NS
Peterson et al., 1995 [28]	Stage I–II BC University of Pennsylvania, Philadelphia, USA	F or S DR	264	517	5 years	5 years OS: 91% vs. 90% 10 years OS: 85% vs. 82%	NS
Chen et al., 1996 [29]	Breast conservation treatment University of Chicago Hospital, USA	FDR	134	660	3.8 years	5 years OS for > 50 years: 84.4% vs. 92% 5 years OS for < 50 years: 84.4% vs. 89.6%	0.017 NS
Schooten et al., 1997 [30]	Stage I–III BC < 71 years University of Maastricht and Utrecht, The Netherlands	FDR	123	723	7.4 years	Cancer-specific S: RR = 0.93 (95% CI: 0.66–1.30)	NS
Haas et al., 1998 [31]	Stage I–II BC; breast conservation treatment University of Pennsylvania, Philadelphia, USA	F or S DR	312	638	6.1 years	5 years OS: 90% vs. 90% 10 years OS: 81% vs. 83%	NS NS

Abbreviations: CI = confidence interval; HBC = hereditary breast cancer; FH = family history; F = first; S = second; T = third; DR = degree relative; m = months; N/A = not available; NS = not significant (at 5% level); n/s = not stated; O = ovarian; OS = overall survival; DFS = disease-free survival; RR = relative risk.

^a Log-rank analysis stratifying by stage, worse prognosis with a positive FH ($P = 0.007$).

Table 2. Linkage-based studies.

Study	Selection criteria (time frame)	BC cases	Controls	Median follow-up	Results (cases vs. controls)	P
Porter et al., 1994 [32]	<i>BRCA1</i> -linked fam. (1942-1992)	35; 8 <i>BRCA1</i> -linked fam.	910 age-matched	n/s	5 years OS: 83% vs. 61%	<0.05
Marcus et al., 1996 [33]	HBC fam. ≥ 3 BC (1966-1991)	157 <i>BRCA1</i> -related BC from 26 fam. 115 <i>BRCA2</i> /other-related BC from 26 fam.	187	1. <i>BRCA1</i> -related: 3.6 years 2. <i>BRCA2</i> /other-related: 5 years 3. controls: 8.3 years	5 years OS: <i>BRCA1</i> -rel.: 67% vs. 59% <i>BRCA2</i> /other-rel.: 63% vs. 59%	0.051 0.15
Sigurdsson et al., 1996 [34]	Icelandic HBC fam. (n/s)	42; 5 <i>BRCA2</i> -linked fam.	115 age-matched	n/s	10 years OS: 45% vs. 65% 10 years DFS: 40% vs. 55%	<0.05 0.009

Abbreviations: DFS = disease-free survival; fam. = families; HBC = hereditary breast cancer; n/s = not stated; OS = overall survival.

have a family history of breast cancer. It is known that familial clustering of postmenopausal breast cancer occurs, which has not been attributed to a genetic syndrome. Therefore, studies based on family history have the disadvantage of grouping true hereditary cases with those of familial clustering. With the identification of the chromosomal region where *BRCA1* and *BRCA2* genes were located, linkage studies became possible. Finally, once the *BRCA1* and *BRCA2* genes were cloned [review in 13], studies comparing the survival in mutation carriers compared to controls were started. Results of these studies have been conflicting. This paper offers a review of the literature in an attempt to answer the question of whether hereditary factors influence prognosis in breast cancer.

Design

English literature identified through Medline between 1976 and February 1999 was reviewed including the following search terms: breast cancer, survival, prognosis, family history, genetics, *BRCA1*, *BRCA2*, and related articles. We included thirty-one studies providing information on the association between either family history of breast cancer, linkage to *BRCA1/BRCA2*, or germline mutations in *BRCA1/BRCA2*, and the prognosis of breast cancer.

Results

Family history-based studies (Table 1)

In the absence of an established epidemiological definition of familial or hereditary breast cancer, the studies can be separated in early-onset breast cancer analyses, case-control population-based studies and studies undertaken in the context of referral or cancer clinics.

Three studies evaluated the influence of a family history of breast cancer in early-onset cases [14-16]. All three have less than one hundred cases and only one [16] demonstrated a better survival for the affected women with a positive family history. The latter study included nine cases from breast/ovarian cancer families and

there was significantly more node negative breast cancer in the cases than in the control group.

Three studies can be considered as case-control population-based [17-19]. In a large study based on the population database and cancer registry of the Utah state, a worse prognosis was significantly associated with family history, but only in the sub-group of premenopausal women [18]. Malone et al. demonstrated a better prognosis for early onset cases with a positive family history [19].

Twelve studies investigated the prognostic impact of family history in the context of breast cancer seen in referral or cancer clinics [20-31]. Among these, only five described selection criteria for cases [21, 28-31]. Two studies found a better prognosis in association with a positive family history [21, 26]. Notably, the study by Albano et al. [21] was restricted to hereditary breast cancer syndrome. A worse outcome, in post-menopausal patients only, was associated with first degree relatives affected by breast cancer in one study [29]. In this study, family history was an independent prognostic factor in multivariate analysis despite a short follow-up and a sixteen year period of time over which cases were collected. Lees et al. [25] concluded, after stratification by stage, that the presence of a positive family history (defined as any relative affected by breast cancer) was associated with a worse outcome, but family history was not a significant prognostic factor in multivariate analysis. The eight other studies with often larger number of cases concluded to the absence of a significant impact on the prognosis of the family history [20, 22-24, 27, 28, 30, 31].

The worse prognosis usually associated with very early-onset breast cancer (≤ 35 years) seems not to be influenced by family history.

Linkage-based studies (Table 2)

In two of the three linkage-based studies, linked cases were found to have a better prognosis than did controls [32, 33]. Porter et al. [32] studied thirty-five breast cancer patients from eight *BRCA1*-linked families, and showed an 83% five year survival. The survival for age matched controls was 61%. Similarly, Marcus et al. [33] reported a five-year survival of 67% in *BRCA1*-linked cases and

Table 3. Mutation-based studies.

Study	Population: type, source (time frame)	Cases (mutations screened)	Controls	Median follow-up	Results (cases vs. controls)	P
Foulkes et al., 1997 [35]	Consecutive BC <65 years in AJ women Jewish General Hospital, Montreal, Canada (1990-1995)	12 AJ <i>BRCA1</i> mut. carriers (185delAG, 5382insC)	100 No AJ <i>BRCA1/2</i> mut.	3.3 years	5 years BC-specific S: 64.3% vs. 95.7%	0.0023
Agnarsson et al., 1998 [36]	BC families University Hospital of Reykjavik, Iceland (1937-1994)	40 <i>BRCA2</i> mut. carriers (999del5)	n/s Age, years of diagnosis-matched	n/s	10 years OS: n/s	NS
Ansquer et al., 1998 [37]	BC <36 years Institut Curie, Paris, France (1990-1995)	15 <i>BRCA1</i> mut. carriers	108 No <i>BRCA1</i> mut.	3.6 years	5 years OS: 69% vs. 83% 5 years DFS: n/s	<0.04 NS
Gaffney et al., 1998 [38]	<i>BRCA1/2</i> mut. carriers with BC State of Utah, USA (1957-1994)	30 <i>BRCA1</i> mut. carriers 7 families, 6 mut. + 1 linkage 20 <i>BRCA2</i> mut. carriers 5 families; 3 mut. + 2 linkage	17396 Age, years of diagnosis, tumor size-matched	9.8 years (<i>BRCA1</i>) 7.5 years (<i>BRCA2</i>)	5 years actuarial S: 75% for <i>BRCA1</i> , 73% for <i>BRCA2</i> , 70% for controls	NS
Garcia-Patino et al., 1998 [39]	Living women with BC, negative FH for B/OC Clinica Puerta de Hierro, Madrid, Spain (before 1995)	9 <i>BRCA1</i> mut. carriers	96 No <i>BRCA1</i> mut.	n/s	Median S: 51 months vs. 24 months	NS
Johansson et al., 1998 [40]	BC families University Hospital of Lund, Sweden (1958-1995)	40 <i>BRCA1</i> mut. carriers 21 families; 14 mut.	112 Age, years of diagnosis, stage-matched	n/s	5 years OS: 64% vs. 80% ^a	NS
Robson et al., 1998 [41]	Living AJ women with DCIS and/or BC <42 years Memorial Sloan-Kettering Cancer Center, New York, USA (1992-1995)	30 AJ <i>BRCA</i> mut. carriers (<i>BRCA1</i> 185delAG, 5382insC; <i>BRCA2</i> 6174delT)	61 NO AJ <i>BRCA1/2</i> mut.	5.2 years	5 years OS: n/s 5 years DFS: 65% vs. 69%	NS NS
Verhoog et al., 1998 [42]	BC families University of Rotterdam, The Netherlands (1969-1995)	48 <i>BRCA1</i> mut. carriers, 1 <i>BRCA1</i> -linked patient 19 families; 13 mut.	196 Age, years of diagnosis, stage-matched; FH negative for B/OC	n/s	5 years OS: 63% vs. 69% ^a 5 years DFS: 49% vs. 51%	NS NS
Wagner et al., 1998 [43]	2 FDR with BC <50 years or OC, or ≥3 F or SDR with ≥2 BC <60 years, or 1 BC <30 years, or male and female with BC within the same lineage University of Vienna, Austria (n/s)	34 <i>BRCA1</i> mut. carriers 17 families; 17 mut.	328 Lymph node negative BC; negative FH for B/OC	7.5 years (cases) 6 years (controls)	Actuarial S: 74% vs. 87% (node neg. BC) DFS: n/s	NS NS
Lee et al., 1999 [44]	AJ community-based Washington, DC; National Cancer Institute, Bethesda, USA (n/s)	58 BC affected FDR of 50 AJ <i>BRCA</i> mut. carriers (<i>BRCA1</i> 185delAG, 5382insC; <i>BRCA2</i> 6174delT)	979 BC affected FDR of 907 non-AJ <i>BRCA</i> mut. carriers Age and years of diagnosis-matched	n/s	5 years OS: 74% vs. 78% ^a 20 years OS: 41% vs. 46% ^a	NS NS

Abbreviations: AJ – Ashkenazi Jewish; BC – breast cancer; DCIS – ductal carcinoma *in situ*; FH – family history; F – first; S – second; DR – degree relative; mut. – mutation; NS – not significant (at 5% level); n/s – not stated; O – ovarian; OS – overall survival; DFS – disease-free survival.

^a 56% vs. 71% when probands excluded ($P = 0.21$).

63% in the *BRCA2*/other gene-linked group, compared to 59% in controls. However, after correcting for age and stage, the adjusted crude death hazards ratio for *BRCA1*- and *BRCA2*/other gene-linked cases was 1.65 ($P = 0.12$) and 1.43 ($P = 0.18$), respectively. The third linkage based study looked at forty-two *BRCA2*-linked breast cancer patients from five families in Iceland [34]. The 10 year overall survival was 45% in cases, compared to 65% in controls ($P < 0.05$).

Mutation-based studies (Table 3)

There are 10 mutation-based studies [35–44]. These can be divided into four categories based on the study population selected. Three papers reported studies from a broad population of women with *BRCA1* mutations [38, 40, 42], three studies looked at specific founder *BRCA1/BRCA2* mutations in Ashkenazi Jewish women [35, 41, 44], two studies reflected the experience of referral cancer clinics [39, 43], one study selected *BRCA1* germ-

line mutation carriers with early onset-breast cancer [37], and one study reported results from the single *BRCA2* germline mutation identified in Iceland [36]. Of the three studies screening for specific Ashkenazi Jewish *BRCA1/BRCA2* germline mutations, one showed a significantly worse survival in mutation carriers [35] whereas the other two studies showed no survival difference between carriers and non-carriers [41, 44]. When early onset-breast cancer patients with *BRCA1* mutations were selected as the cases, a worse five-year overall survival was seen in the germline mutation carrier group [37]. No significant difference in survival between cases and controls was shown in the six other studies [36, 38–40, 42, 43].

Discussion

Family history-based studies

Family history is an instructive source of information in medicine. The validity of the familial history in oncology, either reported by the patients themselves or obtained through a population-based cancer registry, has been well recognized [45, 46]. Positive family history is a function of the number of relatives, the background risk and the etiologic heterogeneity of the disease, and the age distribution of relatives. In population-based case-control studies, no adjustment are usually made for family size (number of relatives) or characteristics of the relative (mean age, sex, age-specific risks). Therefore, a family history report cannot give stable or consistent estimations from one study to another because of variations in familial variables (size and age distribution from one population to another). This is responsible of bias in using family history as a risk factor in case-control studies [47]. Constructing two historical cohorts of the relatives of cases and the relatives of controls, and comparing cumulative incidence of cancer in these two cohorts can avoid some of these problems [44, 48].

Positive first degree family history is recorded in 10%–20% of the affected women with breast cancer and family history of breast cancer has been used to identify women at high cancer risk for genetic studies. In the absence of a strict definition of familial breast cancer and hereditary breast cancer syndromes, these family history-based studies are heterogeneous studies according to their criteria for a positive family history. So family history-based studies have the disadvantage of grouping true hereditary cases with those of familial clustering. These studies also markedly vary by how (in-person interview, chart review) and when they have assessed it. Family history noted at time of diagnosis for early-onset cases cannot be easily compared to family history collected in an age-unrestricted study, simply because, by definition, their relatives will be younger.

Population-based studies would give an *a priori* more precise appraisal of the outcome in a defined population,

compared to the evaluation of cases recruited through referral centers.

A crucial issue is the definition of an adequate control group. Ideally, cases should be at least matched for age, stage and year of diagnosis to allow some comparisons. As some studies spanned over some years or collected cases in the 1960s, control groups have to be appropriate, otherwise it would not take in account potential differences in diagnosis and treatment of breast cancer over time. In other respects, lead-time bias may result from the knowledge of family history, resulting in a diagnosis of breast cancer at an earlier stage because of screening procedures actively followed. None of the studies mentioned the importance of ethnicity-matching for controls, which may be relevant as some publications strongly suggested an outcome difference linked to ethnic origins [49, 50]. These numerous methodological biases encountered in family history-based studies are probably responsible for the discrepancies noted in the impact of family history as a prognostic factor.

Linkage-based studies

The interpretation of linkage-based studies is problematic. There are sources of bias inherent in the study design, and additional confounders exist in each study. In the study by Porter et al. [32], differences in stage were not taken into account. Secondly, cases and controls were not matched for date of diagnosis, cases being diagnosed from 1942–1992, while controls were diagnosed between 1971–1973. As such, treatment may have differed. Support this possibility, the five-year survival rate in the control group was 59% which is lower than one would expect now. Also, four families in the study have a probability of linkage of less than 95%. A sporadic early-onset breast cancer case in a family investigated by linkage analysis can result in a negative lod score. In fact, some families with negative lod scores at the *BRCA1* locus, actually do carry a *BRCA1* germline mutation [51]. In the study of Marcus et al. [33], only 51% of cases were evaluated for survival and they were diagnosed at a younger age than were controls (average age of 42.8 years in cases *versus* 62.9 years in controls). In addition, there were more stage I and II tumors in the linked groups. It is therefore of interest that after adjusting for age and stage, there was a non significant trend toward worse survival in the linked groups ($P = 0.12$ for *BRCA1*-linked, $P = 0.18$ for *BRCA2*/other gene-linked). In a preliminary report from Icelandic women with breast cancer, *BRCA2*-linked cases had a worse survival than did controls [34]. Linkage to *BRCA2* was an independent prognostic variable in multivariate analysis. However, this study is based on a small number of individuals from a population with only one common *BRCA2* mutation (999del5), and therefore results may not be generalisable. Moreover, a more recent study of this Icelandic *BRCA2* mutation in the same population demonstrated no difference in survival, when the control group was matched for age and year of diagnosis [36].

This probably reflects the impact of the improvement in the management and diagnosis of breast cancer during the last decades.

Difficulties in linkage-based studies include the fact that they generally contain rather small numbers of living individuals. Families included are those in which several individuals have breast cancer, raising awareness, and potentially leading to screening and lead time bias. Ascertainment bias is also an issue, as inevitably, living cases are preferentially included in the studies. Interestingly, an increased risk for breast cancer associated with recent birth cohort in *BRCA1* mutation carriers has been reported [52]. Therefore, results implying improved survival in the linked group must be interpreted with caution.

Mutation-based studies

Several sources of bias exist in mutation-based studies. Ascertainment bias is an issue in many, as living affected women are preferentially offered testing [41]. Verhoog et al. [42] attempted to correct for this by analyzing the data with the exclusion of the nine affected probands. This resulted in a non-significant trend toward a higher death and recurrence rate in *BRCA1*-mutation carriers. However, exclusion of the proband does not adequately correct for ascertainment bias [53]. The likelihood for a patient affected with breast cancer to be ascertained also depends of the structure of the pedigree (e.g., small families, predominance of males, deceased relatives), and the knowledge of the family history. Foulkes et al. [35] eliminated survivor bias with their study design, as mutation status was studied from paraffin blocks regardless of whether or not the patient was living. In this study, a worse survival was seen in *BRCA1*-mutation carriers (five-year survival: cases 64.3%, controls 97.5%; $P = 0.002$). However, in this study, as in the study by Robson et al. [41], only Ashkenazi Jewish women were selected. It may be that different *BRCA1* mutations confer a different prognosis, and the results demonstrated in founder populations as seen in the South Sweden [40], in the Ashkenazim [35, 41, 44], or the Icelandic population [36] may not apply to other populations.

The absence of prognostic significance of *BRCA* mutations in a Ashkenazi Jewish community-based survey has been recently reported [44]. This interesting study evaluated the survival of breast cancer-affected first degree relatives of Ashkenazi Jewish mutation carriers compared to breast cancer cases diagnosed in first degree relatives of non-carriers. Even with an adjustment for age and period of diagnosis, this study has limitations because of the absence of ascertainment of cases or cause of death, of adjustment for stage of the disease, and of mutation screening in cases and controls.

Determining the overall survival of the *BRCA*-affected carriers is not an accurate measure of their survival from breast cancer, as they could die from other *BRCA* related tumors. In Johansson study [40] four of the patients with both breast and ovarian cancer died of ovarian cancer.

Non-exclusion of patients with *in situ* breast carcinoma might influence the survival evaluation [41].

Another point to be noted in several of the mutation studies is the inclusion of patients with missense mutations [39, 43]. Many of these mutations are of unknown biological significance, and including them in the case group may have confounded the results. In addition, in most of the studies, the *BRCA1/BRCA2* genes were not sequenced in the control group. As such, the presence of mutations in this group cannot be ruled out. However, in the studies by Foulkes et al. [35] and by Robson et al. [41], the cases and controls were all tested for the same three mutations commonly found in the Ashkenazi Jewish population. Finally, all of the mutation-based studies have a small sample size, making the play of chance more likely to be a problem than in larger studies, and the control group may not be appropriate in all studies, e.g., not adequately staged-matched. The study designed of Ansquer et al. [37] is attractive, as it was based on a prospective follow-up with the same mutation screening in cases and controls, but for reliable results, this kind of approach requires a multicentre recruitment of cases and many years of follow-up. A retrospective cohort design using populations with founder mutations, and mutation analysis of archived tissue, may be able to achieve similar results in a shorter time.

Perspectives and conclusion

Some of the discrepancies in the outcome attributed to familial or hereditary breast cancer noted through these heterogeneous studies may be explained by methodological issues. It is not clear that further prognostic studies based solely on family history will be able to completely resolve these problems. Moreover, as molecular analysis of *BRCA1* and *BRCA2* is now available, using family history as a surrogate for mutation status in case-control population-based studies of breast cancer is no longer justified. Linkage studies, because of their inherent biases, should be restricted to use as a research tool to confirm or exclude chromosomal regions to be investigated, or interpreted with extreme caution.

A strongly positive family history of breast cancer is not always associated with mutations in *BRCA1* or *BRCA2*. This is partly attributable to technical limitations, but also because of the assumed existence of other susceptibility gene(s). The reverse situation is also true. Among the one hundred twenty carriers of any of the three founder *BRCA1/BRCA2* Ashkenazi Jewish mutations identified by Struwing et al. in a population-based designed study [54], thirty-one (26%) did not report a family history of breast or ovarian cancer among first- or second-degree relatives. In a population-based study of breast cancer occurring before age forty years [55], 7 out of 10 (70%) of the *BRCA1* mutation carriers and 4 out of 9 (44%) of the *BRCA2* mutation carriers, had no history of breast cancer in a first- or second-degree relative. Thus, family history is not a very sensitive

parameter of the existence of constitutional *BRCA1/BRCA2* mutations.

Because of imperfect molecular assays, small number of patients studied and an insufficient follow-up time, we cannot yet conclude on the precise impact of the breast cancer predisposing genes on the outcome of affected women. Nevertheless, no studies have showed a survival advantage for mutation carriers. This seems to indicate that *BRCA1*-related breast cancer is not associated with a survival advantage, and that in fact, certain *BRCA1* germline mutations confer a worse prognosis. However, to adequately answer this question, we need more efficient molecular tools to identify all the genetic changes responsible for breast cancer predisposition, and large prospective studies or well designed retrospective analyses, to evaluate their clinical consequences. These future studies will also provide essential insights into this heterogeneous disease, such as a better understanding of genotype-phenotype correlations, the identification of modifier genes and relevant environmental factors, and a more complete appreciation of the tumorigenic process involved in familial and hereditary breast cancer [56]. The absence of somatic mutations of *BRCA1/BRCA2* in the majority of sporadic breast cancer favors the hypothesis of a different carcinogenic mechanism in hereditary cases, which may ultimately be evident in the outcome evaluation.

An accurate appraisal of the survival according to the familial or genetic status is essential for counseling at risk individuals or breast cancer gene carriers. The prognosis for *BRCA* mutation-related tumors is important, because this knowledge may influence the management of women at risk, by predicting the overall benefit of preventive measures [57, 58]. Finally, a thorough understanding of the biological functions of *BRCA1* and *BRCA2*, and their respective influence on the response to radiation or chemotherapy, may also help in the design of the optimal treatment of breast cancer developing in *BRCA* mutation carriers.

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Original article

Primary node negative breast cancer in *BRCA1* mutation carriers has a poor outcome

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Summary

Background: The association between *BRCA1* germ-line mutations and breast cancer prognosis is controversial. A historical cohort study was designed to determine the prognosis for women with axillary lymph node negative hereditary breast cancer.

Patients and methods: We tested pathology blocks from 118 Ashkenazi Jewish women with axillary lymph node negative breast cancer for the presence of the two common *BRCA1* founder mutations, 185delAG and 5382insC. Patients were followed up for a median of 76 months. Somatic *TP53* mutations were screened for by immunohistochemistry, and direct sequencing was performed in the *BRCA1*-positive tumours.

Results: Sixteen breast cancer blocks (13.6%) carried a *BRCA1* mutation. Young age of onset, high nuclear grade, negative estrogen receptor status and over-expression of p53 were highly associated with *BRCA1*-positive status (*P*-values all < 0.01). *BRCA1* mutation carriers had a higher mortality than non-carriers (five-year overall survival, 50% and 89.6%, respectively, *P* = 0.0001). Young age of onset, estrogen receptor

negative status, nuclear grade 3, and over-expression of p53 also predicted a poor outcome. Cox multivariate analyses showed that only germ-line *BRCA1* mutation status was an independent prognostic factor for overall survival (*P* = 0.01). Among nuclear grade 3 tumours, the *BRCA1* mutation carrier status was a significant prognostic factor of death (risk ratio 5.8, 95% confidence interval: 1.5–22, *P* = 0.009). Sequencing of *BRCA1*-related breast cancers revealed one *TP53* missense mutation not previously reported in breast cancer.

Conclusions: Using a historical cohort approach, we have identified *BRCA1* mutation status as an independent prognostic factor for node negative breast cancer among the Ashkenazi Jewish women. Those managing women carrying a *BRCA1* mutation may need take these findings into consideration. Additionally, our preliminary results, taken together with the work of others suggest a different carcinogenic pathway in *BRCA1*-related breast cancer, compared to non-hereditary cases.

Key words: *BRCA1*, breast cancer, p53, survival

Introduction

Most women who present with primary breast cancer have axillary lymph nodes that are free from cancer. There have been numerous studies of factors that influence prognosis in lymph node negative breast cancer [1]. The most important factor is tumour size, followed by grade, histological type, proliferation status and to a lesser extent, estrogen receptor (ER) status. More recently, other possibly independent prognostic variables such as p53, p27^{Kip1}, ERBB2, microvascular density, and cathepsin D have been identified [2]. Age is a predictor of adverse disease-free survival (DFS) and overall survival (OS), but is probably accounted for by the presence of poor prognostic factor profiles, rather than age itself [3].

Conflicting data exist as to whether the prognosis of familial or hereditary breast cancer differs from that of sporadic cases [4, 5]. Families with multiple cases of early-onset breast and ovarian cancer often carry muta-

tions in tumour suppressor genes, *BRCA1* or *BRCA2* [6]. Nine studies comparing DFS or OS in *BRCA1*-positive and *BRCA1*-negative women with breast cancer have been carried out [7–15] with inconsistent results. In our preliminary study, we noted a striking survival disadvantage for *BRCA1* mutation carriers with small, lymph node negative breast cancers [9].

The carcinogenic pathway which links a constitutional *BRCA1* mutation in histologically normal mammary cells to invasive breast adenocarcinoma harboring particular anatomopathological characteristics is still unknown. Some indirect data support the hypothesis of a distinct molecular pathway in hereditary breast cancer cases compared to their sporadic counterparts. For example, the accumulation of somatic genetic alterations in *BRCA1*-associated breast cancer differs from that of sporadic breast cancer [16]. Recently, it has been observed that breast cancers arising in *BRCA1* mutation carriers are more likely to over-express p53 than their

sporadic counterparts [17]. *In vitro* and *in vivo* experiments have demonstrated the biochemical and functional interactions between the *BRCA1* and *TP53* genes products [18]. For example, in a heterozygous *BRCA1*-deficient mouse model, an increased incidence of mammary tumours was only observed in the context of a *TP53* null background [19]. Data suggest that loss of p53 checkpoint control is an early and important event in the carcinogenic pathway of cancer arising in *BRCA1* mutation carriers [20]. It has been postulated that the genetic instability associated with the loss of *BRCA1* functions triggers mutation of *TP53*, which in turn facilitates tumour formation. A particular spectrum of *TP53* mutations has been reported in *BRCA1* and *BRCA2*-associated breast cancer [21–24], but this has not been confirmed by others [25].

To address the question of the effect of a *BRCA1* germ-line mutation on prognosis in lymph node negative breast cancer, we studied 118 unselected Ashkenazi Jewish women with lymph node negative breast cancer diagnosed at a single institution between 1986 and 1995. We chose to study the Ashkenazi Jewish population because the majority of hereditary breast cancer can be attributed to two mutations in *BRCA1* (185delAG and 5382insC) and one mutation in *BRCA2* (6174delT) [26]. These three mutations are founder mutations and have attained a high frequency in the Ashkenazi Jewish population [27]. We have excluded *BRCA2*-positive tumours in this study. Only three *BRCA2* 6174delT mutations (2.5%) have been identified in this series of 118 women by a combination of various assays, and moreover, some studies showed clinicopathological differences between *BRCA1*- and *BRCA2*-associated tumours [24, 28, 29]. We determined *BRCA1* mutation and p53 expression status by polymerase chain reaction-based assays and immunohistochemistry (IHC), respectively. *BRCA1*-positive cases were directly sequenced for somatic *TP53* mutations in exons 5–9. The *BRCA1* status was then related to clinical outcome.

Patients and methods

Cases

Study subjects were identified in the medical records department of the Sir Mortimer B. Davis-Jewish General Hospital (SMBD-JGH), Montreal, Quebec and included all 118 women who reported themselves as being Ashkenazi Jewish by birth. All women were diagnosed with invasive breast cancer below 65 years of age between 1 January 1986 and 1 November 1995 and had undergone axillary node dissection. The routine base-line evaluation of the patients included laboratory analyses, chest radiography, mammography, liver ultrasonography and bone scanning. We selected the age of 65 as the upper age limit. Interestingly, it appears that the likelihood of observing a *BRCA1* mutation in a woman with breast cancer diagnosed after age 65 is about the same as that in the general Ashkenazi Jewish population [30]. The study was approved by the Research Ethics Committee of the SMBD-JGH. Breast cancer blocks were identified from each of these eligible women. Clinicopathological and follow-up information was obtained from chart review. The median age of patients at the time of diagnosis was 54.3 years (range 27.6–64.9). Various regimens of adjuvant chemotherapy were administered to 41 (37.3%) patients. In

general, patients at the SMBD-JGH were seen on a regular basis every six months for five years, then after, on a yearly basis. The follow-up evaluation consisted of a clinical evaluation, a physical examination, radiology and serum chemistry tests. The median follow-up duration was 76 months (range 10–133). Specimens were reviewed by one pathologist (L. R. Bégin). Tumour size was less than 20 mm in 79 cases (67%). Histological tumour type and nuclear grade were determined by specimen and chart review. Tumours were pure histological variants of invasive breast carcinomas comprising 74.1% ductal, 9.3% lobular, 5.5% tubular and 11.1% other types. The specimens were then coded and DNA was extracted from the paraffin wax embedded blocks using standard techniques. Clinical, pathological and molecular data from the 118 samples were collected in a mutually blinded fashion. OS rates were calculated as the number of months from the date of primary surgery until the date of death. Eleven (9.7%) women were lost to follow-up within three years of diagnosis. Four of these women had had a breast cancer-related event and were subsequently lost to follow-up and therefore the event was included in the survival analyses and the patients were censored after this event. One of the eleven women lost to follow-up had a *BRCA1* mutation. Two *BRCA1* mutation carriers developed an ovarian cancer and were censored after this event for the breast cancer-specific survival evaluation. Nine (7.6%) patients developed an ipsilateral recurrence and ten (8.5%) a contralateral tumour. One person had both an ipsi- and a contra-lateral tumour and so was recorded once in each group. Information on family history was not available.

ER status

In 109 (95.6%) cases ER nuclear protein expression was detected using a standard streptavidin-biotin-peroxidase complex immunohistochemical technique. Positivity implies >10% of tumour cell nuclei showing immunoreactivity. In five cases (4.4%), the conventional radioimmunoassay (RIA) assays were used to determine ER nuclear protein status. A positive ER score was taken as >10 fmol/mg protein. Details of these assays have been previously described [31].

BRCA1 and *BRCA2* mutation status

Mutation analysis for the recurrent Ashkenazi Jewish *BRCA1/2* mutations (*BRCA1*: 185delAG, 5382insC; *BRCA2*: 6174delT) was carried out as described previously [9]. Briefly, the extracted DNA was amplified using the polymerase chain reaction using oligonucleotide primers specific to *BRCA1* exons 2 (185delAG) and 20 (5382insC). The products, with suitable positive controls, were electrophoresed overnight in denaturing polyacrylamide gels. After autoradiography, the sizes of the fragments were compared with the positive controls. PCR-RFLP endonuclease digestion assays, specific for the 185delAG or the 5382insC mutations were also used for all cases. Haplotype analysis was also used to confirm 5382insC mutations. We used breast cancer tissue as our source of DNA as no somatic mutations have been reported in *BRCA1* and therefore the mutations observed were assumed to be germ-line in origin. We also looked for the common Ashkenazi *BRCA2* mutation (6174delT) by single-strand conformation analysis, by a mutation-specific PCR-RFLP endonuclease digestion analysis and by direct sequencing. *BRCA2*-positive tumours (three) were excluded in the further analysis.

p53 IHC

p53 protein accumulation was detected as previously described [32], using a standard streptavidin-biotin peroxidase immunohistochemical technique with an anti-p53 (DO-7) monoclonal antibody (Dako Corp., Carpinteria, California).

TP53 sequencing

DNA isolated from paraffin-embedded tumour tissue from 14 individuals who carried *BRCA1* mutations and whose tissue was available

Table 1. Association of *BRCA1* status and clinicopathological variables.

Clinicopathological variable (number of patients)	All subjects (n = 115)	<i>BRCA1</i> -negative (n = 99)	<i>BRCA1</i> -positive (n = 16)	Odds ratio	95% CI	P-value
Age at diagnosis (years)						
Median	53.9	53.4	46.1	—	—	0.006
Range	27.6–64.9	27.6–64.9	33.2–62.1			
Tumour size (mm)						
Median	1.30	1.30	1.50	—	—	0.24
Range	0.15–5.00	0.30–5.00	0.15–5.00			
Nuclear grade (115)						
1	32	32	0	1.0		
2	45	43	2	3.7	0.2–80.5	0.51
3	38	24	14	38.5	2.2–677	<0.0001
Estrogen receptor status (114)						
Positive	68	67	1	1.0		
Negative	46	31	15	32.2	7.7–100	<0.0001
p53 status (IHC assay, 109)						
Negative	84	78	6			
Positive	25	16	9	7.3	2.5–21.2	<0.001
Adjuvant chemotherapy (108)						
No	67	60	7			
Yes	41	32	9	2.4	0.8–6.9	0.17

for analysis was screened for mutations in *TP53*. Intronic primers were designed for amplifying exons 5–9 of *TP53*. Cycle sequencing was performed directly on PCR products using both the forward and reverse primers in separate reactions using the ³³P-dideoxy-labelled terminators kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The products of cycle sequencing were electrophoresed on glycerol tolerant 6% acrylamide gels. Samples that harbored a mutation were reamplified from the original genomic DNA sample, and the mutation was confirmed by DNA cycle sequencing.

Statistical analyses

All statistical tests were two-sided. Clopper–Pearson exact 95% confidence intervals (95% CI) were calculated for the proportion of *BRCA1* mutation carriers in the cohort. *P*-values were calculated for categorical variables using Fisher's exact test. For continuous variables, the non-parametric Wilcoxon's two-sample test was used. Five year and median survival rates were estimated using an actuarial approach. Significance was assessed using a log-rank test. A Cox proportional hazards model was developed for the risk of death at the median follow-up.

Results

Paraffin blocks from 118 breast cancer cases diagnosed among Ashkenazi Jewish women younger than 65 years were analyzed for the presence of two *BRCA1* mutations and one *BRCA2* mutation. Sixteen *BRCA1* mutations were identified (13.6%, 95% CI: 7.9%–21%). Eleven mutations were 185delAG and five were 5382insC. Nine of twenty-eight (32.1%) women diagnosed under the age of 45 and seven of ninety (7.7%) diagnosed between the ages of 45 and 64 carried a *BRCA1* mutation. Three individuals with the 6174delT *BRCA2* mutation (2.5%) were identified, and excluded from subsequent analyses. The histological types of *BRCA1*-related tumours were ductal invasive (14), papillary (1), and medullary (1).

Clinicopathological characteristics of the studied population and association with *BRCA1* status are summarized in Table 1. *BRCA1* mutation carrier status was significantly associated with young age, high nuclear grade, ER negativity, p53 over-expression, but not with tumour size. One hundred nine tumour blocks were available for p53 IHC studies using antibody DO-7. Twenty-five (22.9%) of the breast cancers were positive by IHC. Among the 15 *BRCA1* mutation carriers who were studied for p53 over-expression, the frequency was 60% (9 of 15) compared with 17% (16 of 94) in non-carriers of *BRCA1/2* mutations (OR: 7.3, 95% CI: 2.5–21, *P* = 0.0009). A strong correlation between detection of p53 expression with IHC and the presence of somatic *TP53* mutations in *BRCA1* mutation carriers was demonstrated by direct sequencing of *TP53* exons 5–9 (Table 2). One breast cancer specimen did not show p53 over-expression by IHC, but was found to have a frame-shift mutation in exon 7. Two specimens were positive by IHC but no mutations could be detected in exons 5–9. It is possible that a mutation exists in other exons or non-coding regions of *TP53* for these two cases, or that p53 over-expression is occurring in the absence of a *TP53* mutation. All except one (missense A760G; Ile254Val) of the somatic *TP53* mutations identified among the *BRCA1*-positive tumours have been previously reported in breast cancer (IARC *TP53* database [33]).

Women with *BRCA1* germ-line mutations experienced a poor survival. Women who carried *BRCA1* mutations were more likely to relapse and to die of breast cancer in the first five years after diagnosis than women who were *BRCA1*-negative (five-year OS: 50% vs. 89.6%, *P* = 0.0001; five-year breast cancer specific survival (BCSS): 57.1% vs. 90.5%, *P* = 0.0001) (Figure 1). *BRCA1* mutation carriers also developed contralateral tumours significantly more frequently than non-mutation carriers

Table 2. *TP53* mutations (exons 5-9) in *BRCA1* mutation carriers.

Individual	<i>TP53</i> status by direct sequencing (exons 5-9)				p53 status by IHC ^a
	Exon	Codon	Mutation	Effect	
1	8	273	G818A	Arg273His	+
2	8	270	T809G	Phe270Cys	+
3	7	242	T724A	Cys242Ser	+
4			-		-
5			-		+
6	7	254	A760G	Ile254Val	+
7			-		-
8			-		-
9			-		+
10	5	145	T434C	Leu145Pro	+
11	7	248	G743A	Arg248Gln	+
12	7	248	G743A	Arg248Gln	+
13	7	241	722delC	Frameshift (stop 246)	-
14			Not done		-

^a Abbreviation: IHC = immunohistochemistry. Two *BRCA1*+ individuals were not studied for *TP53* by IHC or by sequencing.

Table 3. Cox proportional hazards model for overall survival.

Variable	Univariate analysis		Multivariate analysis	
	RR (95% CI)	P-value	RR (95% CI)	P-value
Age				
≥ 50 years	1.0		1.0	
< 50 years	2.7 (1.1-7.2)	0.04	1.6 (0.6-4.5)	0.34
Tumour size				
< 20 mm	1.0		1.0	
≥ 20 mm	1.5 (0.6-3.9)	0.43	0.9 (0.3-2.4)	0.80
Nuclear grade				
1, 2, 3 (discrete)	1.0		1.0	
	3.3 (1.5-7.3)	0.003	1.6 (0.6-4.3)	0.36
Estrogen receptor				
Positive	1.0		1.0	
Negative	4.0 (1.4-11)	0.009	1.3 (0.3-5.6)	0.72
<i>BRCA1</i> mutation carrier				
Negative	1.0		1.0	
Positive	8.2 (3.2-21)	0.0001	3.8 (1.3-11)	0.01
p53 expression				
Negative	1.0		1.0	
Positive	2.5 (1.0-6.7)	0.06	1.0 (0.3-3.0)	0.98

Abbreviations: RR = relative risk; 95% CI = 95% confidence interval.

(incidence at five years 19.3% vs. 2.0%, $P = 0.002$). By contrast, there was no significant difference in the rate of ipsilateral tumours in *BRCA1* mutation carriers compared with non-carriers (incidence at five years 4.2% vs. 12.9%, $P = 0.10$) (data not shown). Univariate analysis of OS showed that young age of onset, high nuclear grade, ER negativity, p53 over-expression, and germline *BRCA1* mutation all had prognostic value (Table 3). Similar results were seen for BCSS. Multivariate analysis showed that only *BRCA1* mutation carrier status was an independent prognostic factor (Table 3). To study this effect more specifically, we evaluated the *BRCA1* status in less favorable subgroups. Among the subpopulation of nuclear grade 3 tumours ($n = 38$), 8 out 14 *BRCA1*

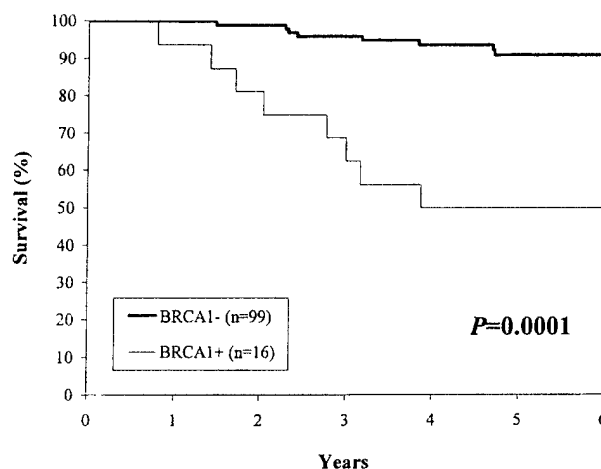


Figure 1. Kaplan-Meier survival curves with regard to survival until death are shown for *BRCA1* carriers (thin line) and *BRCA1* non-carriers (bold line).

mutations carriers died compared with 3 out of 24 sporadic cancer cases (RR: 5.8, 95% CI: 1.5-22, $P = 0.009$). Similarly, 7 deaths occurred in the 15 ER negative *BRCA1*-positive cases compared to 5 deaths in the 31 ER negative sporadic cases (RR: 3.4; 95% CI: 1.1-11, $P = 0.038$). Only one *BRCA1* mutation carrier was ER positive: this person did not die of breast cancer. The tumour size was not significantly different between the *BRCA1*-positive and *BRCA1*-negative subgroups, but *BRCA1* mutation carrier status was a strong outcome predictor in the 79 tumours with a size < 20 mm (RR: 12.5, 95% CI: 3.8-42, $P = 0.0001$). A restricted number of larger tumours did not permit analysis of the role of the *BRCA1* status in this subgroup. Interestingly, the adverse outcome for *BRCA1* mutation carriers was independent of age at diagnosis (< 50 years: $P = 0.003$; ≥ 50 years: $P = 0.04$). Women whose tumours over-expressed p53 had a significantly reduced five-year OS (71.8% vs. 88.4%, $P = 0.03$). However, *BRCA1* mutation status did not predict outcome for women with tumours that over-expressed p53 ($P = 0.17$). Overall, these observations suggest that *BRCA1* status is a stronger determinant of survival than *TP53* status.

Discussion

Nowadays, two-thirds of women with breast cancer have no evidence of axillary lymph node involvement at diagnosis [1]. On average, two-thirds of these women will be alive 10 years later [34]. The very good survival rate in our cohort of *BRCA1*-negative women (mostly affected by tumours of small size) is in agreement with recent studies among node negative patients [35, 36]. Numerous factors are associated with an increased risk of distant relapse. Our results suggest that in Ashkenazi Jewish women with negative axillary lymph nodes at diagnosis, the presence of a *BRCA1* mutation is an adverse prognostic factor. At 5 years follow-up, 8 of 16

BRCA1 mutation carriers (50%) had died, in contrast to 9 of 99 *BRCA1* non-carriers (9.1%). This difference was highly significant ($P = 0.0001$) (Figure 1). As previously described [28, 37, 38], we confirmed the very strong association between *BRCA1* mutation carrier status and adverse clinicopathological features of breast cancers, such as young age of onset, high nuclear grade, ER negative status, and somatic *TP53* mutations. All these characteristics are recognized as being indicators of worse prognosis in breast cancer. In Cox multivariate analyses, only germ-line *BRCA1* mutation status had an independent prognostic value for OS. Among the ER negative, nuclear grade 3 and tumour <20 mm sub-population, the *BRCA1*-positive status still confers a significant worse prognosis. As these data were obtained from relatively small subsets of the studied population, they need to be confirmed in larger series and should be regarded as hypothesis-generating at this point.

Previous studies have shown that p53 positivity (as demonstrated by IHC) or somatic *TP53* mutations are frequent in hereditary breast cancer [39] and particularly so in *BRCA1*-related breast cancer [17, 21, 22]. It has been suggested that *BRCA1*-related breast tumorigenesis requires a somatic *TP53* mutation [40, 41]. Here, the *TP53* mutation screening in the *BRCA1*-positive cases was performed by IHC and DNA direct sequencing, with a good correlation between the two methodologies. We identified one new missense mutation (A760G) not previously reported in the IARC breast cancer *TP53* database [33]. Another missense mutation we observed (G818A) has been already reported in a *BRCA2*-related breast cancer [25]. The other *TP53* mutations we identified were already described in the IARC *TP53* database [33]. We confirm that *TP53* mutations are significantly more common in *BRCA1*-related hereditary breast cancer than in non-*BRCA1*, non-*BRCA2*-related breast cancer ($P = 0.0009$). However, we do not find evidence for a worse prognosis for *BRCA1* mutation carriers whose tumours over-express p53 compared with those carriers whose tumours do not overexpress p53.

This study has demonstrated a survival disadvantage for node-negative women with germ-line *BRCA1* mutations. This present study includes 71 individuals from our previous study [9], where we reported that *BRCA1*-related breast cancers in Ashkenazi Jewish women were associated with a worse outcome. By identifying more *BRCA1* mutation carriers and almost doubling the median follow-up time, we have been able to generate a multivariate Cox model for survival. The key observation that the presence of a *BRCA1* mutation is an independent poor prognostic factor for lymph node negative breast cancer has extended our preliminary results.

Several previous survival studies in hereditary breast cancer have indicated that there is no clear survival disadvantage associated with a *BRCA1* mutation [7, 8, 10, 12, 14, 42, 43]. However, one study of women diagnosed with breast cancer at less than 36 years of age did find a significantly worse survival for *BRCA1* mutation carriers [11]. No studies have specifically studied lymph

node negative women. The conflicting findings in these studies require further explanation. There are several sources of selection bias in clinic or pedigree-based studies, all of which favorably influence prognosis [4, 5]. This is because the biases in linkage or clinic-based studies all result in the preferential inclusion of living women (as compared to deceased women) in the study cohort. In general, to test for *BRCA1* mutation status, it is necessary to have a source of constitutional DNA. Mutation analysis is most readily performed using DNA extracted from peripheral blood as it is relatively straightforward to perform mutation analysis with this source of DNA. If the patient is deceased, it may be possible to obtain DNA from a preserved tumour specimen. However, it is generally not possible to search the entire coding region of *BRCA1* and *BRCA2* for mutations using DNA from paraffin-embedded specimens. An additional problem is that if the proband has had breast cancer, then including her in the study will result in a spurious elevation in the survival estimates. Even if the proband is excluded from the study of survivorship, as was recently carried out [8], the problem of ascertainment bias is not eliminated, because not all families with hereditary breast cancer in a population will be ascertained. Ideally, one would ascertain incident cases of breast cancer in a population, obtain information on *BRCA1* status and other prognostic factors, and follow these women forward in time for survival [11]. However, to give robust estimates, this method requires 10 or more years of follow-up. An alternate method is to use historical cases and archival specimens from ethnic groups with founder mutations, such as the Ashkenazi Jewish [44, 45] French Canadian [46] and Icelandic [47] populations. Interestingly, a recent large community-based study of breast cancer limited to the Ashkenazim [15] found no survival difference between carriers and non-carriers of *BRCA1/2* mutations. Even this study was not free of interpretative difficulties, as the retrospective cohort design did not permit the confirmation of diagnosis of breast cancer or the cause of death in the cohort. More seriously, the mutation status of all affected individuals was inferred from the tested index case. Thus, large prospective or retrospective cohort studies where all individuals in the cohort are tested for disease-associated mutations are required. In this regard, Robson et al. [48] very recently reported the results of a retrospective ethnically restricted hospital-based study similar in design to this and our previous study [9]. Among 305 breast cancer patients of Ashkenazi Jewish descent who underwent conservative treatment, 28 were identified as carriers of a founder *BRCA1* or *BRCA2* mutation. Women with mutations were more likely than non-mutation carriers to develop contralateral breast cancer ($P = 0.002$). In univariate analysis the 5- and 10-year distant disease-free survival and BCSS were significantly worse among the *BRCA1/2* mutation carriers compared with patients with mutation-negative breast cancers. In multivariate analysis, only tumour stage and nodal status retained prognostic significance, but

positive *BRCA1/2* mutation status was associated with a statistically non significant trend towards a worse outcome ($P = 0.14$). As results were not dichotomized between *BRCA1* and *BRCA2* mutation carriers or according to the nodal status, it is difficult to exactly compare the two data sets, but clearly the results point in the same direction as those reported here.

Finally, a possible explanation for the worse survival encountered by this hereditary breast cancer subgroup is that the *BRCA1* germ-line mutations found in the Ashkenazi Jewish population are in fact associated with a particular unfavorable prognosis compared to other *BRCA1* mutations. To date, no clearly documented gene modifiers or specific somatic genetic abnormalities have been reported in this population.

Other studies will be required to confirm our findings. However, we believe the methods we have employed are robust and as free from bias as is possible in follow-up studies. If our observation that small, lymph node negative breast cancers occurring in *BRCA1*-positive women have a poor prognosis is confirmed, then different detection and treatment regimens may be required for this particular subgroup of patients.

Acknowledgements

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CORRESPONDENCE

Long-term outcome of invasive breast cancer

Sir—László Tabár and colleagues (Feb 5, p 429)¹ report striking results about a novel prognostic factor in breast cancers of small size.¹ Currently, the proportion of small breast cancers diagnosed is increasing as a result of large-scale mammographic screening. However, the selection of women who will benefit from adjuvant treatment remains a challenge. Also, prognostic factors for breast-cancer outcome, such as involvement of axillary lymph nodes or high-grade tumours, have not been consistently reported as useful for small tumours.²

In their prospective study of invasive breast cancers of less than 15 mm diameter, Tabár and colleagues showed that casting-type calcification, when present on a diagnostic mammogram, was associated with a significantly worse survival. Comedocarcinoma results in casting-type mammographic calcifications and is associated with residual microscopic disease, local recurrence, and high-grade invasive cancer.^{3,4} All these features could negatively influence outcome. By contrast, little is known about the influence of host factors on outcome.⁵ To address this question, we did an ethnically restricted retrospective study. We studied 85 consecutive pathology blocks from Ashkenazi Jewish women under the age of 65 years, who had been diagnosed between 1986 and 1995 with breast cancers of less than 15 mm diameter. All except one (99%) patient were treated by breast conservative surgery and 29 (34%) patients received adjuvant chemotherapy. After recording histopathological variables, DNA was extracted from tumours and tested for the presence of the three common founder mutations present in Ashkenazi Jewish women in the breast-cancer predisposing genes *BRCA1* and *BRCA2*. Ten (11.8%) *BRCA1* and two (2.4%) *BRCA2* mutation carriers were

identified. Breast-cancer-specific survival was assessed after a median follow-up of 88 months. Seven breast-cancer deaths were recorded. As reported by Tabár and colleagues, axillary lymph-node status was not identified as a prognostic factor ($p=0.8$) and nuclear grade was of borderline significance ($p=0.08$). The strongest outcome predictor was the *BRCA1/2* mutation carrier status. At the median follow-up, breast-cancer-specific survival for *BRCA1/2* mutation carriers was 60% versus 95% for women without *BRCA1/2* mutations ($p<0.0001$).

The numbers are small, but these findings suggest that *BRCA1/2* mutation status, which is a risk for breast cancer that is present at birth, has a significant impact on outcome, even when tumours are very small. It is uncertain whether or not *BRCA1/2*-associated breast cancer is associated with ductal carcinoma in situ (DCIS). In our series, none of the 12 *BRCA1/2*-associated breast cancers was associated with comedo-type or had an important component of DCIS, compared with 17 of 73 non-*BRCA1/2* tumours. Our findings suggest that prevention will be particularly important for *BRCA1/2* mutation carriers because mammography is unlikely to detect serious preinvasive disease and the outcome following small invasive breast cancers is surprisingly poor.

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- 1 Tabár L, Chen H-H, Duffy SW, et al. A novel method for prediction of long-term outcome of women with T1a, T1b, and 10–14 mm invasive breast cancers: a prospective study. *Lancet* 2000; 355: 429–33 (published errata appear in *Lancet* 2000; 355: 850 and 1372).
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Sir—László Tabár and colleagues¹ suggest that mammographic classification is an important prognostic indicator in early breast cancer. However, there are several limitations to their data. Theirs was a very small study based on only 35 deaths resulting from breast cancer. As the supplementary tables listed on *The Lancet* website (www.thelancet.com) show, there were 128 deaths from other causes and 180 women still alive at the time of analysis from a total of 343 women. Thus, almost 90% of the observations included in their survival curves come from censored data.

The investigators state that they “assessed significance with proportional hazards regression”, but their statement that the “effectiveness of mammographic findings to predict survival was significant ($p<0.001$), which remained significant after adjustment for tumour size, . . . node status, and malignancy grade” is rather vague. Did they test significance with mammographic findings alone as a variable in a proportional hazards model with death from breast cancer as the outcome? The p value for such a model would have little relevance since confounding would not be addressed. And how were mammographic findings categorised in the model? Did they adjust for the other variables by adding them to the multivariate model? If so, what was the exact model used, and what was the p value? Did the investigators test for appropriateness of the

proportional hazards model? They apparently did not consider the possibility of overfitting, which is a serious concern since the number of events (35) is less than ten times the number of variables modelled (4).² The investigators do not even mention hormone-receptor status, which is much too important a prognostic variable to be ignored.³ Also, it is inappropriate to model long-term survival without including age-at-diagnosis as a variable.⁴ Yet, including two additional variables would result in a hopelessly overfitted proportional hazards model.

At best, Tabár and colleagues present an interesting hypothesis-generating study. A much larger study with information on hormone-receptor status and age is needed to test their hypothesis.

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- 1 Tabár L, Chen H-H, Duffy SW, et al. A novel method for prediction of long-term outcome of women with T1a, T1b, and 10–14 mm invasive breast cancers: a prospective study. *Lancet* 2000; 355: 429–33 (published errata appear in *Lancet* 2000; 355: 850 and 1372).
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Sir—The paper by László Tabár and colleagues¹ is a good illustration of a YAPI (Yet Another Prognostic Indicator). Calcification as a method of prognosis is not as accurate as other methods and has no practical clinical use.

I draw attention to two publications from my own unit. The first, by Kollias and colleagues,² was a substantially larger study of 789 small breast cancers (319 ≤1 cm). This study showed that small, as with larger, tumours are well stratified into groups with significantly differing prognoses by the Nottingham Prognostic Index (NPI), an integration of size, grade, and lymph-node stage. As with larger tumours the patient's NPI is very important in giving individual advice on adjuvant systemic therapies to women with small tumours. Grade and NPI are well validated prospectively.

Clearly, calcification in a tumour is not a primary determinant of tumour behaviour. High-grade invasive tumours were shown in the second study, by Evans and colleagues,³ to frequently have a surround of high-grade DCIS with calcification. Thus calcification is associated with grade, which is strongly associated with prognosis. Calcification is qualitative and of no clinical use. Grade is quantifiable and of considerable clinical importance.

The paper by Tabár and colleagues also suffers from being a retrospective study and therefore has no validation and seems not to have any correction for multiple comparisons.

R W Blamey

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- 1 Tabár L, Chen H-H, Duffy SW, et al. A novel method for prediction of long-term outcome of women with T1a, T1b, and 10–14 mm invasive breast cancers: a prospective study. *Lancet* 2000; 355: 429–33 (published errata appear in *Lancet* 2000; 355: 850 and 1372).
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Sir—The article by László Tabár and colleagues¹ ignores the extreme variations in growth rates. Therefore, length bias sampling and a lead time bias influence the conclusions drawn and negate the significance of the conclusions that earlier diagnosis improves survivorship. The investigators provide no data on the proliferative indices nor other biological characteristics of the cancers.

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- 1 Tabár L, Chen H-H, Duffy SW, et al. A novel method for prediction of long-term outcome of women with T1a, T1b, and 10–14 mm invasive breast cancers: a prospective study. *Lancet* 2000; 355: 429–33 (published errata appear in *Lancet* 2000; 355: 850 and 1372).

Authors' reply

Sir—The findings of Pierre Chappuis and colleagues are very interesting and this work is to be encouraged. In our study the presence of *BRCA1/2* mutations could have accounted for the few patients who died from their breast cancers but did not have

casting-type calcifications on their mammograms. However, it seems unlikely that the *BRCA1/2* mutation is responsible for our results, because there was no important intraductal component in the *BRCA1/2*-positive tumours.

Carl Atkins is mistaken. His comment that our study is a small one is quite clearly in error. Ours is a large series of invasive tumours of size 1–14 mm. Because of the small tumour size, the death rate from breast cancer is low. We encourage other researchers with similar data to try to confirm our results. His criticism that our results are inadequately adjusted or a product of overfitting is not justified. We assessed the effect of our mammographic prognostic features on survival alone and adjusted for grade, node status, and size in the Cox regression model. The effect on survival was substantial and significant. Atkins' assertion that the number of variables in the model should be less than one tenth of the number of events is arbitrary. Also, Atkins' belief in oestrogen-receptor status as the fundamental prognostic indicator in these small tumours is likely to be misconceived. Reed and colleagues¹ found oestrogen-receptor status to be unrelated to survival in node-negative breast cancers, which form the majority of the tumours in our study. However, we believe that future research should take place to establish other biomarkers that correlate with casting-type calcifications. The findings by Padmore and colleagues² seem to provide confirmation of our observations.

We share R W Blamey's belief in the accuracy of prediction of prognosis in tumours larger than 15 mm diameter with the first-generation prognostic factors: tumour size, malignancy grade, and node status. It would make life simpler for all of us if this were universally the case. However, these factors were not adequate prognostic indicators in the tumours we studied (1–14 mm diameter). We found that the four mammographic prognostic features were significantly predictive of long-term outcome. We are astonished by his assertion that our finding is a product of retrospective study and multiple comparisons: it is neither. Ours was a prospective study and the tables A–D (published by *The Lancet* in electronic form at www.thelancet.com) clearly show that most of the patients who died from their breast cancer and who had casting-type calcifications had no

positive axillary nodes, and that the invasive component had an intermediate histological malignancy grade in most of the cases, especially in 1-9 mm tumours. The very point of our article is that these first-generation histological prognostic variables would not have predicted the high rate of fatality, nor would the NPI.

Our article did not deal with calcification overall as a method of prognosis, although there is an obvious positive correlation between the histological and mammographic image of different subtypes of DCIS.¹ Instead, we pointed out the prognostic value of the presence of a very specific type of calcification, the so-called casting type, which is strongly correlated with grade 3 DCIS. The substantial prognostic significance of casting-type calcification is shown by our data. Also, Blamey assumes that the associated T1a and T1b invasive carcinoma is frequently poorly differentiated. In the patients in our study these associated invasive tumours more frequently had intermediate malignancy grade, especially the 1-9 mm tumours. The prerequisite for the poor outcome is the presence of extensive grade 3 DCIS and not the tiny invasive carcinoma.² There is no reason to believe that it is the tiny invasive carcinoma that leads to a poor outcome, since women with unifocal, grade 2 cancers of similar size (ie, solitary stellate lesions on the mammogram) had an excellent outcome.

In response to John Spratt's comments, tumours with casting-type calcifications are associated with increased fatality. If lead time or length bias were an issue here, which we doubt, they would be expected to dilute the observed association. Thus the effect of this radiological marker might be even stronger than we observed.

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Pertussis transmission in England and Wales

Sir—We were surprised by the statement made by Pejman Rohani and colleagues (Jan 22, p 285)¹ that pertussis vaccination is not thought to prevent transmission. In the review of pertussis epidemiology² which we did and which was cited by Rohani and colleagues, we concluded that "whole-cell pertussis vaccines can give good protection against both clinical disease and transmission of infection". We showed the impact vaccination has on transmission of infection by investigating the incidence of notified disease in infants aged 0-2 months (figure, with data updated to 1999). These infants are too young to be protected directly by vaccination, so changes in their incidence of disease reflect changes in the level of pertussis transmission in the population.² This is a far more direct measure of the level of pertussis transmission than are changes in the spatio-temporal pattern of pertussis epidemics. Rohani's observation of the increased frequency of fade-outs of notifications after vaccination does not necessarily indicate any reduction in transmission, but may simply be the consequence of a reduction in disease.

Although it is clear that pertussis vaccination does reduce transmission

of infection, data from recent years suggest that there has been an increase in undetected transmission. In the 1990s the overall notification rate for all ages has continued to show a downward trend, but no such trend is evident in the notification rate in infants younger than 3 months. Significant transmission between infants of this age is unlikely, and the most probable source of these infant infections would seem to be undiagnosed infections in older vaccinated people.

*Nigel J Gay, Elizabeth Miller

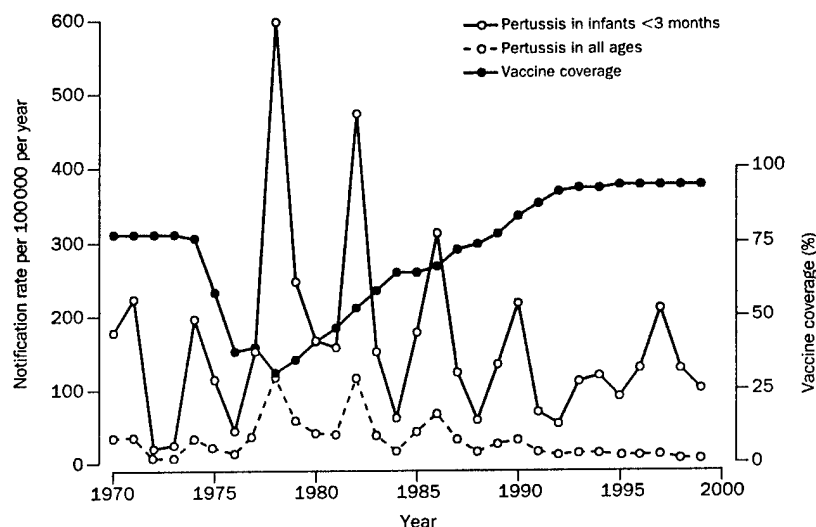
Immunisation Division, PHLS Communicable Disease Surveillance Centre, London NW9 5EQ, UK

- 1 Rohani P, Earn DJD, Grenfell BT. Impact of immunisation on pertussis transmission in England and Wales. *Lancet* 2000; 355: 285-86.

- 2 Miller E, Gay N. Epidemiological determinants of pertussis. *Dev Biol Stand* 1997; 89: 15-23.

Authors' reply

Sir—Nigel Gay and Elizabeth Miller raise several important issues. First, they correctly point out that pertussis vaccination does reduce the incidence and transmission of infection.¹ This conclusion is indeed supported by our study and we are pleased to have the opportunity to emphasise this aspect of their paper. However, we seem to be in the minority—as we stress in our paper, most other investigators have concluded that pertussis vaccination is not very effective in preventing transmission.²⁻⁴ It is this conventional wisdom that we, and Miller and Gay are questioning, from different perspectives.



Pertussis notification rate in infants aged less than 3 months and for all ages, and pertussis vaccine coverage at age 2 years in England and Wales, 1970-99

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July 17, 2000

William D. Foulkes, M.D.

Sir Mortimer B. Davis-Jewish General Hospital

3755 Cote Ste Catherine

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Montreal, Quebec

Canada H3T 1E2

Dear Dr. Foulkes:

I am pleased to inform you that your manuscript, "Low p27kip Expression and BRCA1/2 Germ-Line Mutations Are Associated and Contribute to a Poor Outcome After Breast Cancer" (MS# 5-200002104R), has been accepted for publication in the *Journal of Clinical Oncology*.

For your paper to be published, the abstract should be shortened to less than 250 words, and figure legend(s) to less than 45 words each, per *Journal* style. One copy of your manuscript is being returned for your convenience. Your double-spaced shortened abstract and figure legends should be sent directly to the Editorial Office at the address shown on this letterhead. If we do not receive your shortened abstract and figure legend(s) we may edit these in-house, the shortened versions of which will be on the galley proofs of your manuscript for your approval.

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Thank you for giving us the opportunity to publish your paper in the *Journal of Clinical Oncology*.

Sincerely,



Stephen A. Cannistra, M.D.
Associate Editor

enclosures

**Low p27^{Kip1} expression and *BRCA1/2* germ-line mutations
are associated and contribute to a poor outcome after
breast cancer**

Pierre O. Chappuis, Linda Kapusta, Louis R. Bégin, Nora Wong,
Jean-Sébastien Brunet, Steven A. Narod, Joyce Slingerland
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Running title: p27^{Kip1}, *BRCA1/2* and breast cancer survival

ABSTRACT

PURPOSE: Decreased levels of the cyclin-dependent kinase inhibitor p27^{Kip1} in breast cancer are associated with a poor outcome. The prognostic significance of *BRCA1/2* mutations is less clear, and the relationship between *BRCA1/2* mutation status, p27^{Kip1} protein levels and outcome has not been studied.

PATIENTS AND METHODS: Pathology blocks from 202 consecutive Ashkenazi Jewish women with primary invasive breast cancer were studied. Tumor DNA was tested for the three common *BRCA1/2* founder mutations present in Ashkenazi Jews and p27^{Kip1} expression was evaluated by immunohistochemistry. The median follow-up was 6.4 years.

RESULTS: Thirty-two (16%) tumors were positive for a *BRCA1/2* mutation. Low p27^{Kip1} expression was seen in 110 (63%) tumors and was significantly associated with *BRCA1/2* mutations (odds ratio: 4.0, 95% confidence interval (CI): 1.4-11.1; $P = 0.009$). *BRCA1/2* mutation carriers had a significantly worse 5 year distant disease-free survival (DDFS) compared with women without *BRCA1/2* mutations (58% vs. 82%; $P = 0.003$). Similar results were seen for women whose tumors expressed low levels of p27^{Kip1}, compared with those with high levels (5 year DDFS: 68% vs. 93%; $P < 0.0001$). In a multivariate analysis, both *BRCA1/2* mutation and low p27^{Kip1} expression were associated with a shorter DDFS (relative risk (RR): 2.1, 95% CI: 1.0-4.3; $P = 0.05$ and RR: 3.9, 95% CI: 1.4-11.1; $P = 0.01$, respectively).

CONCLUSION: In this study, we showed that *BRCA1/2* mutations were associated with low levels of p27^{Kip1} in breast cancer. Both *BRCA1/2* and p27^{Kip1} status were identified as independent prognostic factors.

INTRODUCTION

Breast cancer affects one out of nine women in the western world, where it is a leading cause of cancer mortality in women. Adjuvant therapy is widely used to prevent relapse, but the heterogeneous nature of the disease makes selection of patients who will benefit from it a major clinical challenge. This is particularly true for node negative patients, a majority of whom have little or nothing to gain from adjuvant therapy¹. Beside classical prognostic factors like tumor size, involvement of axillary lymph nodes, histological type and grade, a plethora of new biological and molecular markers have been proposed as predictors of aggressive clinical behavior or response to adjuvant treatment². None of them has definitively demonstrated its clinical usefulness in the management of women with breast cancer³.

The putative tumor suppressor gene *CDKN1B/p27/Kip1* is a member of the CDKI gene family. Its product, p27^{Kip1}, inactivates G1 cyclin-Cdk complexes in response to growth inhibitory cytokines, such as TGF- β and IL-6^{4,5}. Specific mutations in *CDKN1B/p27/Kip1* have only been rarely reported in human cancers⁶. Post-translational control via the ubiquitin-proteasome pathway is thought to be the main process involved in the decrease of p27^{Kip1} expression observed in a broad spectrum of cancers⁷. Other posttranscriptional mechanisms regulate p27^{Kip1} abundance, such as translational control, phosphorylation, and subcellular compartmentalization⁸. Numerous studies showed an association between low amounts of p27^{Kip1} and worse outcome in various human cancer types, including breast cancer⁹. Using p27^{Kip1} immunohistochemical assays and multivariate analyses,

several breast cancer studies have demonstrated that a low level of p27^{Kip1} is a significant predictor of reduced survival¹⁰⁻¹⁵.

Families with multiple cases of early-onset breast and ovarian cancer often carry germ-line mutations in tumor suppressor genes, *BRCA1* or *BRCA2*¹⁶. The pathological features of hereditary breast cancers, particularly the tumors which occur in *BRCA1* mutation carriers, are typically associated with poor prognosis, i.e. high grade and proliferation rates, aneuploidy and lack of estrogen receptor (ER)^{17,18}. *BRCA2*-related breast cancers are also higher grade tumors compared to non-hereditary cases, although less significantly so than *BRCA1*-associated tumors. *BRCA2*-related tumors are more frequently of lobular subtype and exhibit substantially less tubule formation compared with sporadic cases¹⁹. The carcinogenic pathway which links a constitutional *BRCA1/2* mutation in histologically normal mammary cells to invasive breast adenocarcinoma harboring particular anatomopathological characteristics is still unknown. Some indirect data support the hypothesis of a distinct molecular pathway in hereditary breast cancer cases compared to their sporadic counterparts. For example, novel somatic *TP53* mutations are seen in *BRCA1/2*-associated breast cancer²⁰. Despite the clear evidence for an association between poor prognostic factors and *BRCA1/2* mutations, conflicting data exist as to whether the prognosis of familial or hereditary breast cancer differs from that of sporadic cases. So far, a dozen studies comparing outcome of *BRCA1*-positive and *BRCA1*-negative women with breast cancer have been carried out with inconsistent results²¹⁻²⁴. However, studies which have ascertained breast cancer cases irrespective of vital status have shown a worse outcome in univariate analysis^{23,25}. Fewer studies have investigated the outcome of *BRCA2*-associated breast cancers, and no definitive conclusions can be reached^{26,27}.

To study the relationship between inherited *BRCA1/2* mutations and p27^{Kip1} expression, and to measure the impact these markers have on survival, we studied 202 unselected Ashkenazi Jewish women with primary invasive breast cancer diagnosed at a single institution between 1986 and 1995. We followed up the patients for local and distant recurrences and death. We chose to study the Ashkenazi Jewish population because the majority of hereditary breast cancer can be attributed to two mutations in *BRCA1* (185delAG and 5382insC) and one mutation in *BRCA2* (6174delT)²⁸. These three mutations are founder mutations and have attained a high frequency in the Ashkenazi Jewish population²⁹.

PATIENTS AND METHODS

Case identification and clinicopathological review

The study design is an ethnically restricted single hospital-based retrospective cohort study. Study subjects were identified in the medical records department of the Sir Mortimer B. Davis Jewish General Hospital (SMBD-JGH) and included all 202 women who reported themselves as being Ashkenazi Jewish by birth. We used the medical chart to review names at birth and reported religious affiliation, and therefore it is very unlikely that non-Ashkenazi Jewish women would have been included in this study. The study was approved by the Research Ethics Committee of the SMBD-JGH. All women were diagnosed with primary invasive breast cancer between January 1, 1986 and November 1, 1995. We selected the age of 65 as the upper age limit because from our clinical experience, we suspected that few breast cancers associated with *BRCA1/2* germ-line mutations would be diagnosed in women > 65 years of age.

Interestingly, it appears that the likelihood of finding a *BRCA1/2* mutation in a woman with breast cancer diagnosed after age 65 is about the same as that in the general Ashkenazi Jewish population³⁰. The routine base-line evaluation of the patients included laboratory analyses, chest radiography, mammography, liver ultrasonography and bone scanning. One hundred eighty-nine (94%) patients underwent breast conservative treatment and 13 (6%) patients were treated by total or modified radical mastectomy. Breast cancer blocks were identified from each of these eligible women. Clinicopathological and follow-up information was obtained from chart review. The median age of patients at the time of diagnosis was 53.3 years (range, 26.5-64.7). Various regimens of adjuvant chemotherapy were administered to 95 (49%) patients. In general, patients at the SMBD-JGH were seen on a regular basis every 6 months for 5 years, then after on a yearly basis. The follow-up evaluation consisted of a clinical evaluation, a physical examination, radiology and serum chemistry tests. The median follow-up duration was 6.4 years (range, 0.5-13.3). The histopathology of all the specimens was reviewed by one pathologist (L.R.B). Tumors were pure histological variants of invasive breast carcinomas comprising 80% ductal, 7% lobular, 5% tubular and 8% other types. The specimens were then coded and DNA was extracted from the paraffin wax embedded blocks using standard techniques. Clinical, pathological and molecular data from the 202 samples were collected in a mutually blinded fashion. Distant disease-free survival (DDFS) rate was calculated as the number of months from the date of primary surgery until the date of diagnosis of first distant failure. Overall survival (OS) was the time interval between the date of primary surgery and the date of death. At 5 years follow-up, 42 (21%) patients developed distant relapse and 37 (18%) died. Thirteen (6%) patients developed an ipsilateral recurrence and 5 (2%) a contralateral tumor at 5 years. Ipsilateral recurrences and contralateral breast

tumors were not considered in the DDFS evaluation. Three (1.5%) women were lost to follow-up within three years of diagnosis. None of these women had had a breast cancer-related event. One of the 3 women lost to follow-up had a *BRCA2* mutation. Information on family history was not available.

Mutation and immunohistochemical analysis

Mutation analysis for the recurrent Ashkenazi Jewish *BRCA1/2* mutations (*BRCA1*: 185delAG, 5382insC; *BRCA2*: 6174delT) was carried out as described previously²⁵. ER status was determined using standard techniques³¹. Expression of p27^{Kip1} was determined immunohistochemically. Paraffin sections of tumor blocks were deparaffinized with xylene, rehydrated, and microwaved for 3 min at full pressure in 10 mM citrate buffer (pH 6.0) using a microwaveable pressure cooker. Sections were blocked with 5% hydrogen peroxide in Tris buffer followed by normal goat serum and then incubated 1 hour at room temperature with anti-p27 monoclonal antibody (Transduction Laboratories, Lexington, KY) diluted 1:1400 (0.18 µg/ml). Slides were then reacted with biotin-labeled anti-mouse IgG and incubated with Streptavidin horseradish peroxidase (Zymed Laboratories, San Francisco, CA). Diaminobenzidine (DAB) substrate (Sigma, St-Louis, MO) was then added in the presence of hydrogen peroxide. Sections were counterstained with hematoxylin, dehydrated, and mounted. Negative controls substituting buffer for the primary antibody showed no staining. Strong p27^{Kip1} immunostaining in lymphocytes provided an internal positive control. The immunostaining pattern detected with the Transduction Lab. antibody was similar to that observed with a polyclonal rabbit antibody. The p27^{Kip1} stain was abolished by pre-incubation of the antibody with blocking peptide. The degree of p27^{Kip1} staining was scored independently by two pathologists (LK and IMP). Between 15 and 20

high-power fields of tumor were scored for the percentage of nuclei showing positive p27^{Kip1} staining. Protein levels were classified as low (staining in $\leq 50\%$ of cells) and high (staining in $> 50\%$ of cells) as previously reported¹¹⁻¹⁴.

Statistical analysis

All statistical tests were two-sided. Exact Clopper-Pearson 95% confidence intervals were calculated for the proportion of *BRCA1/2* mutation carriers in the cohort. *P*-values were calculated for categorical variables using Fisher's exact test. For continuous variables, the non-parametric Wilcoxon's two-sample test was used. Five year survival rates were estimated using the actuarial method (Kaplan-Meier) and survival rates are also presented for the median follow-up time of 6.4 years.

Significance was assessed using the log-rank test. A Cox proportional hazards model was developed for the risk of distant relapse at 5 years follow-up. All the results found to be statistically significant at 5 years were confirmed at the median follow-up time (6.4 years). We noted no significant differences in survival trends or association with p27^{Kip1} expression between *BRCA1* and *BRCA2* mutation carriers and because the *BRCA2* positive subgroup was small, we grouped *BRCA1*- and *BRCA2*-related breast cancers in order to increase the power of the study. In fact, no difference in outcome has been demonstrated between *BRCA1* and *BRCA2* mutation carriers²¹. Patients with missing information regarding p27^{Kip1} expression (n=28, insufficient material to make satisfactory sections or absence of internal positive control staining) were only excluded from the multivariate analysis.

RESULTS

Paraffin blocks from 202 breast cancer cases diagnosed among Ashkenazi Jewish women younger than 65 years were analyzed for the presence of two *BRCA1* mutations and one *BRCA2* mutation. Thirty-two *BRCA1/2* mutations were identified (16%, 95% CI: 11-22%). Fifteen mutations were 185delAG (*BRCA1*), 9 were 5382insC (*BRCA1*), and 8 6174delT (*BRCA2*). Eighteen of 81 (22%, 95% CI: 14-33%) women diagnosed under the age of 50 and 14 of 121 (12%, 95% CI: 7-19%) diagnosed between the ages of 50 and 65 carried a *BRCA1* or a *BRCA2* mutation. The histological types of *BRCA1/2*-related tumors were ductal invasive (n=28; 90%), medullary (n=1; 3%) and others (n=2; 7%). Clinicopathological characteristics of the studied population and association with *BRCA1/2* status are summarized in Table 1. *BRCA1/2* mutation carrier status was significantly associated with younger age ($P = 0.05$), high nuclear grade ($P < 0.0001$), ER negativity ($P < 0.001$), but not with tumor size or axillary nodal status. One hundred seventy-four tumor blocks (86%) were available for p27^{Kip1} immunohistochemical studies. Low p27^{Kip1} expression (p27^{Kip1} staining in less than 50% of tumor nuclei) was seen in 110 (63%) of these breast cancers. The median age of diagnosis did not differ between women with low and high levels of p27^{Kip1} expression ($P = 0.19$). Among the 27 *BRCA1/2* mutation carriers who were studied for p27^{Kip1} expression, the frequency of low level of p27^{Kip1} expression was 85% (23 of 27) compared with 59% (87 of 147) in non-carriers of *BRCA1/2* mutations (OR: 4.0, 95% CI: 1.4-11.1; $P = 0.009$). When *BRCA1*- and *BRCA2*-related breast cancers were analyzed separately, a low p27^{Kip1} expression level was present in all of the 7 (100%) breast cancers in *BRCA2* mutation carriers and in 16 of 20 (80%) breast cancers in *BRCA1* mutation carriers. Low p27^{Kip1} expression level was also

associated with larger tumor size ($P = 0.002$), high nuclear grade ($P < 0.0001$) and ER negativity ($P < 0.0001$), but not with nodal status ($P = 0.24$).

The 5 year DDFS and OS were 78% and 81% respectively, for the studied population. Women carrying a *BRCA1/2* germ-line mutation or whose tumors had a low level of p27^{Kip1} experienced a poor survival. Women who carried *BRCA1/2* mutations were more likely to relapse in the first six years after diagnosis than women who were *BRCA1/2* mutation negative (5 year DDFS: 58% vs. 82%; $P = 0.003$; Figure 1). Differences in adjuvant therapy did not explain the differences in survival rates, as *BRCA1/2* mutation carriers were more likely to have received adjuvant chemotherapy ($P = 0.03$). The difference in OS between *BRCA1* and *BRCA2* mutation carriers was not significant (62% vs. 57%; $P = 0.79$). At 5 years, DDFS among women whose tumors expressed low level of p27^{Kip1} was 68% compared with 93% for women whose tumors expressed high level of p27^{Kip1} ($P < 0.0001$; Figure 2). *BRCA1/2* mutation carriers also developed contralateral tumors significantly more frequently than non-mutation carriers (incidence at 5 years: 10% vs. 2%; $P = 0.02$). By contrast, there was no significant difference in the rate of ipsilateral tumors in conservatively treated *BRCA1/2* mutation carriers compared with non-carriers (incidence at 5 years: 6% vs. 7%; $P = 0.93$). It is not clear why ipsilateral breast tumors would be less common than contralateral tumors in *BRCA1/2* mutation carriers. In particular, the intriguing possibility of a protective effect of radiotherapy that exists only among mutation carriers cannot be excluded at this time.

Univariate analysis of DDFS showed that young age of onset, tumor size, nodal status, nuclear grade, ER negativity, germ-line *BRCA1/2* mutations and low p27^{Kip1} expression all had prognostic value (Table 2). Multivariate analyses for the

major adverse factors identified in univariate analysis are shown in Table 3. *BRCA1/2* mutation carrier status and low p27^{Kip1} expression were both independent prognostic factors for DDFS (relative risk (RR): 2.1; $P = 0.05$ and RR: 3.9; $P = 0.01$, respectively), as were tumor size (RR: 3.2; $P = 0.002$). When *BRCA1* and *BRCA2* mutation status was analyzed separately, significant prognostic impact was detected in univariate analysis for each gene (*BRCA1*, RR: 2.3; $P = 0.04$; *BRCA2*, RR: 4.2; $P = 0.008$). When OS was considered, age ($P = 0.02$), tumor size ($P = 0.004$), grade ($P = 0.0001$), ER status ($P = 0.0001$), *BRCA1/2* status ($P = 0.001$) and p27^{Kip1} expression ($P = 0.001$) were all prognostic factors in univariate analysis. However, only p27^{Kip1} expression and nuclear grade retained significance in multivariate analysis (RR: 6.4; $P = 0.01$ and RR: 2.0; $P = 0.05$, respectively). When *BRCA1/2* germ-line mutation carriers were excluded, only tumor size (RR: 4.0; $P = 0.008$) and p27^{Kip1} expression (RR: 3.5; $P = 0.05$) were independent prognostic factors for DDFS.

In node negative patients, age of diagnosis (RR: 5.7; $P = 0.003$) and low p27^{Kip1} expression (RR: 10.0; $P = 0.03$) were the only independent prognostic factors for DDFS. A borderline trend was noted for *BRCA1/2* mutation carrier status (RR: 2.8; $P = 0.09$). In node positive patients, only tumor size (RR: 8.7; $P = 0.007$) and *BRCA1/2* mutation status (RR: 3.5; $P = 0.03$) were independent factors for DDFS.

Finally, we evaluated the effect of the *BRCA1/2* status combined with the level of p27^{Kip1} expression. Among the 60 *BRCA1/2* negative women with high p27^{Kip1} there were only 3 relapses, compared with 11 among 23 *BRCA1/2* positive women with low p27^{Kip1} ($P < 0.0001$; Figure 3). Of note, the four women who were

BRCA1/2 positive and whose breast cancers expressed high levels of p27^{Kip1} were all alive at the median follow-up (6.4 years).

DISCUSSION

We have demonstrated here for the first time a clear association between low p27^{Kip1} expression and the *BRCA1/2* mutation carrier status (OR: 4.0, 95% CI: 1.4-11.1; $P = 0.009$). The association was stronger for the *BRCA2*- than for the *BRCA1*-related cancers, but the number of *BRCA2*-related tumors is small. We also showed that both the *BRCA1/2* mutation status and p27^{Kip1} expression strongly influenced the prognosis in univariate analysis and that they are independent prognostic factors for DDFS in multivariate analysis. Interestingly, in combination these 2 parameters increased their effect. The 5 year DDFS for a *BRCA1* or *BRCA2* mutation carrier whose breast tumor had a low level of p27^{Kip1} was 45% compared to 95% for a non-*BRCA1/2* mutation carrier with a high p27^{Kip1} level in the tumor ($P < 0.0001$; Figure 3). In previous breast cancer studies, low p27^{Kip1} expression demonstrated by immunohistochemistry was associated with high grade, ER negative tumors, but not with size or axillary lymph node status^{11-13,32,33}. The prognostic impact of p27^{Kip1} in breast cancer has been previously reported¹⁰⁻¹⁵ and our findings are consistent with these observations. With regard to ER status, we found that all the *BRCA2*-associated breast cancers that were evaluated ($n = 7$) had low levels of p27^{Kip1} expression, despite the fact that 6 of these 7 tumors were ER positive. This suggests that the association between low p27^{Kip1} expression and *BRCA1/2* germ-line mutations

in hereditary breast cancer does not necessarily result from alteration of the ER pathway.

Numerous factors are associated with an increased risk of distant relapse, but until recently, host factors have not been studied in detail. Our results suggest that in Ashkenazi Jewish women with primary invasive breast cancer, the presence of a *BRCA1/2* germ-line mutation is an adverse prognostic factor. At 5 years follow-up, 12 of 32 (38%) *BRCA1/2* mutation carriers developed metastatic disease, compared with 30 of 170 (18%) *BRCA1/2* non-carriers. This difference was highly significant ($P = 0.003$). In Cox multivariate analyses, germ-line *BRCA1/2* mutation status had an independent prognostic value for DDFS in the whole cohort and in the node positive patients. A borderline trend in the node negative sub-group was noted. Thus, in our study population, we have demonstrated a survival disadvantage for women with germ-line *BRCA1/2* mutations. This study extends our previous observation from a smaller series of patients ($n=112$) who are included in this study²⁵. Several previous survival studies in hereditary breast cancer have indicated that there is no clear survival disadvantage associated with a *BRCA1/2* mutation, although a trend was discernible in some papers²¹⁻²⁴. However, several sources of ascertainment and selection bias may exist in clinic or pedigree-based studies, all of which favorably influence prognosis^{21,22}. Using historical cases and archival specimens from an ethnic group with founder mutations, and testing all individuals in the cohort irrespective of vital status, has enabled us to eliminate most of these biases. Robson *et al.* recently reported the results of a similarly designed study with 28 *BRCA1/2* mutation carriers among 305 breast cancer patients of Ashkenazi Jewish descent who underwent conservative treatment²³. In univariate analysis, the 5 and 10 year DDFS and breast cancer specific survival were significantly worse among the *BRCA1/2* mutation

carriers compared with patients with *BRCA1/2*-negative breast cancers. In multivariate analysis, only tumor stage and nodal status retained prognostic significance, but positive *BRCA1/2* mutation status was associated with a statistically non significant trend towards a worse outcome ($P = 0.14$). No somatic genetic abnormalities or differential expression of proteins were described in their study. One prospective study of early-onset breast cancers had previously shown a significantly worse survival for *BRCA1* mutation carriers³⁴. In an extension of this work, Stoppa-Lyonnet *et al.* showed a worse 5 year OS ($P = 0.002$) in a series of 40 *BRCA1* mutation carriers compared to 143 familial breast cancer cases without germ-line *BRCA1* mutations. *BRCA1* status was an independent prognostic factor for both DDFS and OS (D. Stoppa-Lyonnet, personal communication). In addition, Loman *et al.* reported a worse breast cancer specific survival ($P = 0.003$) among 54 *BRCA2* mutation carriers compared to 214 age- and date of diagnosis-matched controls, but this difference did not retain significance in a multivariate analysis when clinical stage was included (N. Loman, personal communication).

There is extensive evidence that fine-tuning of the cell cycle is important, and *a fortiori*, for its deregulation in the carcinogenic process. Deregulation of CDK and CDKI, which normally regulate the progression through the cell cycle, are frequently seen in human cancers. Normally, p27^{Kip1} is strongly expressed in non-proliferating cells and plays important roles in the regulation of both quiescence and G1 phase of the cell cycle. p27^{Kip1}, as a cyclin-dependent kinase inhibitor, is a negative regulator of this delicate balance. Only one study has reported p27^{Kip1} expression in the context of hereditary breast cancer³⁵. Among 58 breast cancers diagnosed <42 years in Ashkenazi Jewish women, no difference in the level of p27^{Kip1} expression was

detected between 17 *BRCA1/2* mutation carriers and the remaining sporadic cases. Of note, in that study³⁵, the percentage of tumors with a positive immunostaining for p27^{Kip1} was higher than that in any other published report, and the p27^{Kip1} scoring system was not described. The frequency of low p27^{Kip1} expression we observed in this study is in very good agreement with results previously reported in unselected breast cancer^{11,14,33}.

Links between CDK or CDKI and the *BRCA1* gene product have been previously described. For example, BRCA1 can transcriptionally induce p21 expression, one of the putative mechanisms of action for growth inhibition of BRCA1³⁶. There is currently no evidence that BRCA1 or BRCA2 actually directly affect p27^{Kip1} regulation. Instead, germ-line mutations in *BRCA1* and *BRCA2* could simply “set the scene” for a lower expression of p27^{Kip1}, or could favor the selection of clones with decreased levels of p27^{Kip1}. Interestingly, low p27^{Kip1} expression was detected in both *in situ* and invasive components of breast carcinoma, suggesting that deregulation of p27^{Kip1} may be an early event in the carcinogenic process^{11,37}. As the tumor suppressor genes *BRCA1* and *BRCA2* have also been involved in the maintenance of genome integrity, they have also been described as caretakers³⁸. Studies have showed that disruption of BRCA1/2 functions causes genetic instability^{39,40}. In this context, the loss of BRCA1/2 function might interfere with the normal regulation of p27^{Kip1}. Some potential regulators have been identified, such as cyclin E-Cdk2 complex itself, Ras, and c-Myc^{4,41}. p27^{Kip1} haplo-insufficiency for tumor suppression has been demonstrated in a murine model, as carcinogen-induced tumors in p27^{+/-} mice retain a functional allele⁴². If this phenomenon is present in human mammary carcinogenesis, one could hypothesize that p27^{Kip1} haploinsufficient

phenotypes are enhanced by *BRCA1/2* mutations. The association between *BRCA1/2* mutation status and p27^{Kip1} expression described in this work could open a new avenue of investigation for these multifunctional proteins.

While our study is robust in its design, it has some weaknesses. Despite a long follow-up, the population studied is relatively small. In addition, the study contained a limited number of node positive patients (38%) and a high proportion of small tumors (57% smaller than 2 cm). A correlation between size and involvement of axillary lymph nodes is well described⁴³. Therefore, it is perhaps not surprising that lymph node status was not found to be an independent prognostic factor in this series. In fact, only 19 (9%) patients presented with metastasis to 4 or more axillary lymph nodes. Interestingly, survival, irrespective of the tumor size, is only dramatically different when patients with ≥ 4 nodes are compared to N0 or to patients with 1-3 nodes, particularly when the follow-up is limited to 5 years⁴³. Thus, the relatively small proportion of lymph node positive patients and the high proportion of tumors with a small size may have influenced the results. As samples were rendered anonymous, we were not able to correlate our findings with family history. Nevertheless, this study design has allowed an unbiased ascertainment, which is a crucial issue in evaluating most of the previously reported studies of breast cancer survival in the context of *BRCA1* or *BRCA2* germ-line mutation^{21,22,24}.

Using a historical cohort approach, we have shown here that Ashkenazi Jewish women who carry a *BRCA1* or a *BRCA2* mutation are likely to develop a breast cancer that has low levels of p27^{Kip1} protein. The presence of a *BRCA1* or *BRCA2* mutation and the low level of p27^{Kip1} expression contribute independently to a worse outcome. As yet, there is no known biological connection between these proteins and the relationship is associative, rather than causal. Therefore, experiments

are needed to elucidate whether a mechanistic link exists between p27^{Kip1} levels and a *BRCA1/2* mutation. From the clinical perspective, unlike other prognostic indicators, knowledge of *BRCA1/2* status by genetic testing is available before diagnosis; hence our findings will be of great interest to women and their physicians considering management options.

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Figure legends

Figure 1. Kaplan-Meier DDFS curves are shown for *BRCA1/2* carriers and *BRCA1/2* non-carriers. The curves have been truncated at the median follow-up. At 5 years, there were 12 patients at risk with *BRCA1/2* mutations and 107 patients at risk without *BRCA1/2* mutations.

Figure 2. Kaplan-Meier DDFS curves are shown for tumors with low p27^{Kip1} and high p27^{Kip1} expression. At 5 years, there were 53 patients at risk whose tumors expressed low p27^{Kip1} levels and 48 patients at risk whose tumors had high p27^{Kip1} levels.

Figure 3. Kaplan-Meier DDFS curves are shown for combination of *BRCA1/2* germline mutation status and p27^{Kip1} expression. Significant differences (log rank test) were noted between each survival curve: P -value = 0.001 between the two-upper curves and P = 0.006 between the two lower curves.

Table 1. Association of *BRCA1/2* mutation status with clinico-pathological features.

Variable (number of patients*, %)	<i>BRCA1/2</i> -negative [number (%)]	<i>BRCA1/2</i> -positive [number (%)]	<i>P</i> -value
Age at diagnosis			
<50 yrs (81; 40.1)	63 (37)	18 (56)	
≥50 yrs (121; 59.9)	107 (63)	14 (44)	0.05
median (range)	53.7 (26.5-64.9)	48.0 (33.2-65.7)	0.05
Tumor size			
<2 cm (115; 56.9)	98 (58)	17 (53)	
≥2 cm (87; 43.1)	72 (42)	15 (47)	0.70
median (range)	1.5 (0.3-10.5)	1.7 (0.2-5.0)	0.41
Axillary lymph node status			
N0 (118; 62.4)	99 (62)	19 (63)	
N+ (71; 37.6)	60 (38)	11 (37)	0.99
Nuclear grade			
1 (53; 26.2)	52 (31)	1 (3)	
2 (76; 37.6)	67 (39)	9 (28)	
3 (73; 36.2)	51 (30)	22 (69)	< 0.0001
Estrogen receptor			
positive (126; 62.7)	115 (68)	11 (34)	
negative (75; 37.3)	54 (32)	21 (66)	< 0.001
p27 ^{Kip1} expression			
positive (64; 36.8)	60 (41)	4 (15)	
negative (110; 63.2)	87 (59)	23 (85)	0.009
Adjuvant chemotherapy			
no (97; 50.5)	87 (54)	10 (32)	
yes (95; 49.5)	74 (46)	21 (68)	0.03

* Where the total falls below 202, information on the remaining patients was not available.

Table 2. Univariate Cox proportional hazards model for distant disease-free survival (n = 202 pts)

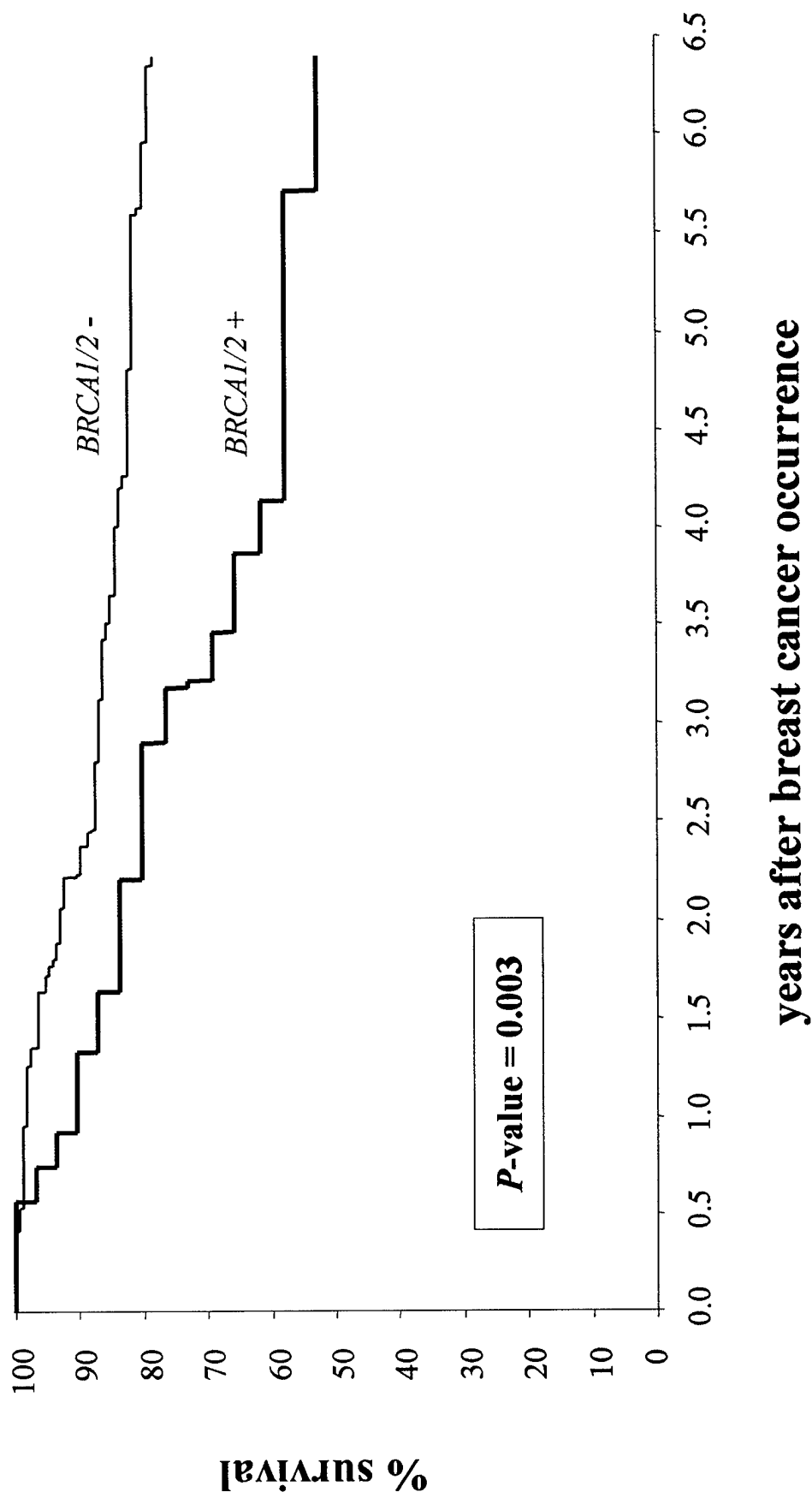
Variable	RR [95% CI]	P-value
Age		
≥50 years	1.0	0.01
<50 years	2.2 [1.2-4.1]	
Tumor size		
<2 cm	1.0	0.0001
≥2 cm	3.9 [2.0-7.7]	
Axillary lymph node status		
N0	1.0	0.019
N+	2.1 [1.1-3.9]	
Nuclear grade		
1, 2, 3 (discrete)	2.9 [1.8-4.8]	0.0001
Estrogen receptor		
positive	1.0	0.0006
negative	2.9 [1.6-5.6]	
BRCA1/2 mutation status		
negative	1.0	0.004
positive	2.7 [1.4-5.2]	
p27 ^{Kip1} expression		
high	1.0	0.0008
low	5.9 [2.1-16.7]	

Table 3. Multivariate Cox proportional hazards model for distant disease-free survival (n = 174 pts)*

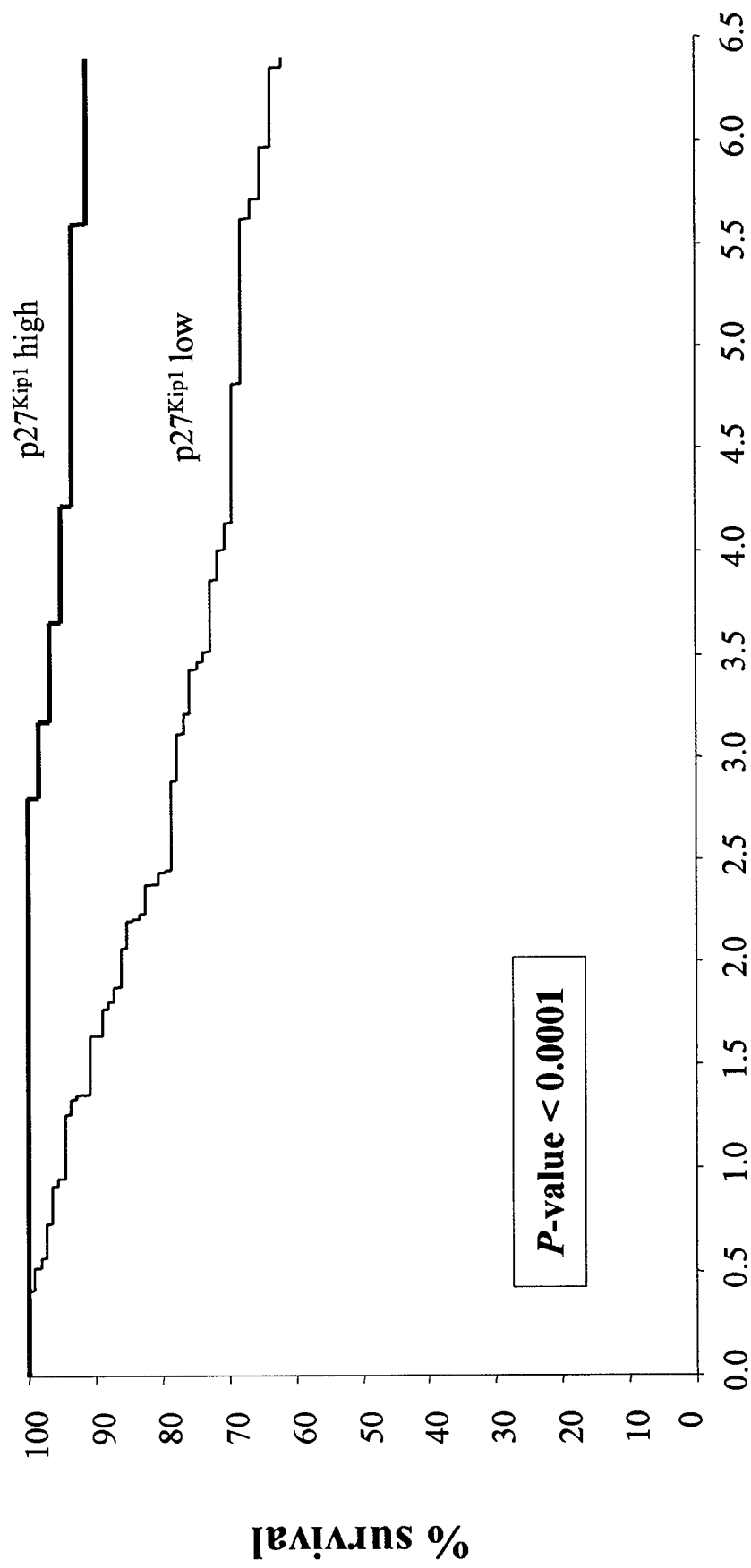
Variable	RR [95% CI]	P-value
Age		
≥50 years	1.0	
<50 years	1.6 [0.8-3.1]	0.15
Tumor size		
<2 cm	1.0	
≥2 cm	3.2 [1.6-6.5]	0.002
Nuclear grade		
1, 2, 3 (discrete)	1.7 [0.9-3.2]	0.07
Estrogen receptor		
positive	1.0	
negative	1.3 [0.6-2.9]	0.45
<i>BRCA1/2</i> mutation status		
negative	1.0	
positive	2.1 [1.0-4.3]	0.05
p27 ^{Kip1} expression		
high	1.0	
low	3.9 [1.4-11.1]	0.01

*Axillary lymph node status was excluded from the multivariate analysis. This was because among the 13 women for whom we had missing lymph node status, 11 had p27^{Kip1} expression results and 2 were *BRCA1/2* mutation carriers. Moreover, when lymph node status was tested in the Cox multivariate model, it was not an independent prognostic factor ($P = 0.54$), whereas the RRs and P values were not significantly changed for *BRCA1/2* mutation status (RR: 2.0; $P = 0.07$) or for p27^{Kip1} low expression level (RR: 3.5; $P = 0.02$).

BRCA1/2 mutation status and DDFS

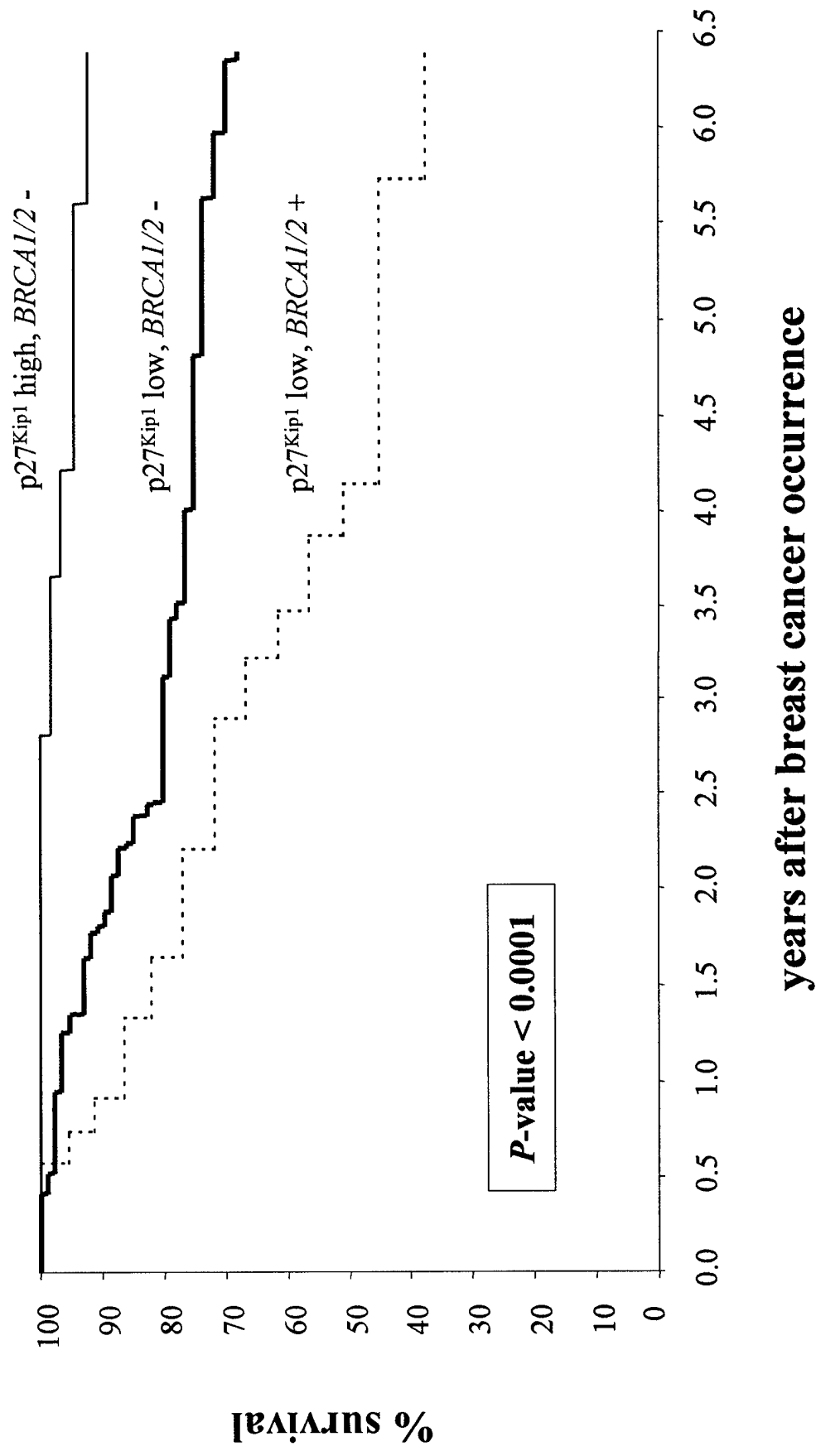


p27^{Kip1} expression and DDFS



years after breast cancer occurrence

BRCA1/2 mutation status, p27^{Kip1} expression and DDFS



Appendix 2:

PERSONNEL RECEIVING PAY FROM THIS RESEARCH EFFORT

1. Anne-Josée PARADIS
2. Antonio SABETTI
3. Nora WONG