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### Prognostic factors in breast cancer: one fits all?

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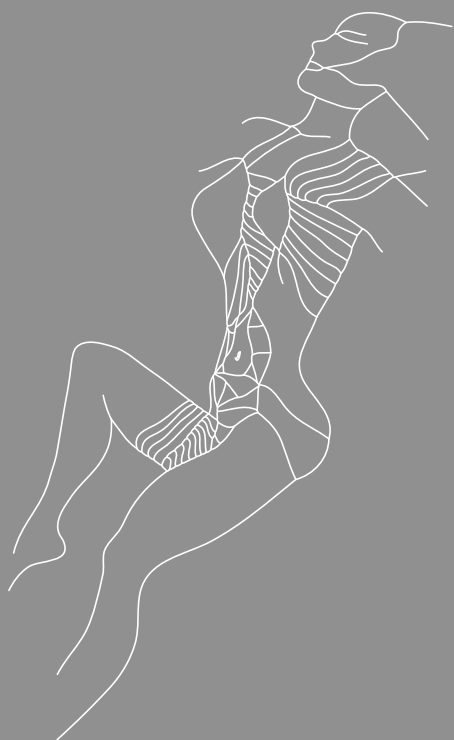
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# Chapter 3

## Daily clinical practice of fresh tumor tissue freezing and gene expression profiling; logistics pilot study preceding the MINDACT trial



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## **Abstract**

### **Purpose**

The 70-gene prognosis-signature is a prognostic tool for early breast cancer analysis. In addition to scientific evidence, implementation of the signature in clinical trials and daily practice requires logistical feasibility. The aim of our study was to test logistics for gene expression profiling on fresh frozen tumor tissue in the preparation for the prospective, multinational Microarray In Node-negative Disease may Avoid ChemoTherapy (MINDACT) trial.

### **Methods**

Sixty-four patients were included in six European hospitals. Fresh frozen tumor samples were shipped on dry ice to Agendia B.V., where RNA was isolated and subsequently hybridized on the 70-gene prognosis-signature (MammaPrint™).

### **Results**

Tumor samples were obtained in 60 of 64 patients. Among the 60 samples, 11 contained insufficient tumor cells (< 50%) and three contained insufficient RNA quality. All 46 samples eligible for genomic profiling were successfully hybridized, and the results were reported on average within 4-5 d.

### **Conclusion**

Gene expression profiling on fresh frozen tissue is feasible in daily clinical practice.

## Introduction

In the past 20 years, important advances have been made in the knowledge of the biology of breast cancer. Using new high-throughput techniques, such as microarray-based gene expression profiling, both prognostic and predictive profiles were established, and breast cancer was re-classified based on molecular characteristics.<sup>1-16</sup> One of these gene expression classifiers is the 70-gene prognosis-signature (MammaPrint™).<sup>2,3</sup> This 70-gene dichotomous classifier can accurately distinguish breast tumors with a high metastatic capacity from tumors with a low risk of developing distant metastases, by measuring the expression level of 70 genes in tumor tissue. Several retrospective validation studies have confirmed its prognostic value.<sup>3,17-20</sup>

Implementation of gene expression profiles requires logistical feasibility, in addition to scientific evidence provided by validation studies. An essential part of this logistics is the procurement of fresh frozen tissue as source of high-quality RNA. Traditional fixation of fresh tissue in formaldehyde results in degradation of RNA and cross-linking, which makes it unsuitable for comprehensive microarray analysis.<sup>21</sup> Moreover, RNA becomes heavily fragmented during storage of paraffin-embedded tissue.<sup>22</sup> In addition, slow freezing of samples promotes the formation of ice crystals, which can also provoke RNA damage.<sup>23</sup> Consequently, collection of snap-frozen tissue or fresh tissue preserved in RNA preservation fluid, such as RNARetain™ (Asuragen Inc., TX, USA),<sup>24</sup> is at present mandatory to obtain high-quality RNA and successful gene expression profiling. Recently, Bueno-de-Mesquita and colleagues described the successful implementation of RNARetain™ tissue preservation and centralized MammaPrint™ testing for 16 community hospitals in the Netherlands for the prospective RASTER trial.<sup>24</sup> Evaluation of logistics of frozen tissue collection, centralized microarray testing and swift reporting of results in the preparation of a multinational multicentre clinical trial is described here.

In 2007, the MINDACT trial (Microarray In Node-negative Disease may Avoid ChemoTherapy; EORTC 10041/ BIG 3-04) started to prospectively evaluate the 70-gene prognosis-signature as a risk assessment and decision-making tool.<sup>25-27</sup> This trial will enroll 6000 breast cancer patients throughout Europe, who will have their risk of disease recurrence assessed by both traditional clinicopathological criteria and the 70-gene prognosis-signature. Since decision-making for adjuvant treatment is based on both the risk assessments, the 70-gene prognosis-signature test result has to be available within a fixed timeframe suitable for daily clinical practice. Moreover, to avoid interlaboratory variability, which may artificially influence gene expression levels, all samples are obtained and frozen at local sites and shipped to Agendia B.V., Amsterdam, for RNA isolation and microarray analysis. Consequently, participation in the MINDACT trial demands personnel at local hospitals who will collect and freeze tumor samples. These local procedures, frozen sample shipment and sample analysis within a fixed timeframe entail complex logistics that requires a thorough organization.

In the preparation for the MINDACT trial, we conducted a pilot study to test the logistics for gene expression profiling in a multicentre and multinational setting. The first aim of this pilot study was to test and if necessary to improve the logistics to collect good-quality fresh frozen tissue at individual hospitals for microarray testing. The second aim was to determine the proportion of samples that was hybridized successfully. The last aim was to define Standard Operating Procedures (SOPs) for the tissue logistics in the MINDACT trial. Together with the Dutch RASTER trial,<sup>24</sup> this pilot study provided crucial information for the feasibility of the MINDACT trial.

## **Patients and Methods**

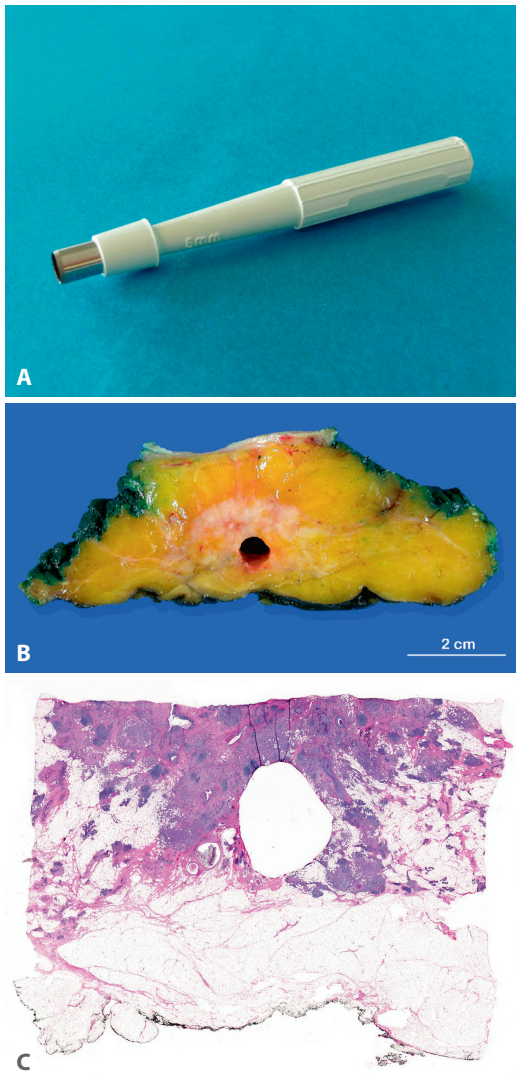
This logistics pilot study was coordinated by the Netherlands Cancer Institute (NKI) and was conducted in six European hospitals. The study was approved by the institutional ethical review board of each participating hospital, and all patients gave their written informed consent before surgery, for the donation of a piece of tumor tissue to test the logistics for genomic profiling.

### **Patients**

Women under the age of 71 years at diagnosis with a unifocal, unilateral pT1-pT2, invasive breast carcinoma and a clinically negative axillary lymph node status were eligible for inclusion. Patients with carcinoma in situ were eligible, provided that invasive cancer was present. Patients who received neoadjuvant therapy were not included. Each hospital included at least eight patients.

### **On-site training**

Before the start of the study, the study coordinator organized on-site instruction meetings in each participating hospital. These instruction meetings were attended by a multidisciplinary team, *i.e.* breast surgeons, medical oncologists, pathologists, data managers and research nurses. During this instruction visit, the logistical scheme was discussed and incorporated in the local standard procedures. Additionally, all study-specific standard procedures were explained in detail in a manual of operations and were summarized on provided pocket summaries, to support standardized procedures for tissue collection, freezing and shipment.



**Figure 1A.** Biopsy puncher for standardized tumor sampling.

**B.** Tumor specimen after sampling, using the 6 mm biopsy puncher (by courtesy of J.F. Egger).

**C.** Hematoxylin and eosin (H&E) stained section of the tumor specimen (shown in B). After sampling, intact morphology is shown, and appropriate grading and staging of the tumor are allowed (by courtesy of J.F. Egger).

### **Pre-assembled kits**

The study coordinator provided hospitals with pre-assembled sample kits for each patient, consisting of all case report forms (CRFs), a 6 mm biopsy puncher (*Figure 1A*) and a printed sticker sheet with a unique identification number (Sample ID), suitable for freezing in liquid nitrogen and prolonged storage at  $-80^{\circ}\text{C}$ .

### **Tumor sampling**

After surgical resection, tumor specimens were immediately transported from the operating room to the pathology department in a tumor container without fixatives, *e.g.* formalin. To ensure standardized tumor sampling, 6 mm biopsy punchers were provided (*Figure 1A*). The pathologist obtained a tumor sample within 1 h of surgery, using this biopsy puncher. Samples were placed in an Eppendorf tube, labeled with a sample ID sticker. For tumors smaller than 1 cm (at macroscopic examination), a 3 mm biopsy puncher was allowed to obtain a sample. Obviously, standard diagnostic pathology examination had priority over the procurement of a research sample, *i.e.* the pathologist only obtained a tumor sample for gene expression profiling when he/she judged that there was a sufficient amount of tumor tissue.

### **Snap-freezing and storage**

Eppendorf tubes containing tumor samples were snap-frozen by submerging the tubes in liquid nitrogen for at least 1 min. After snap-freezing of the sample, the total time from transportation of tissue to the pathology department till freezing of the samples was recorded. Samples were stored in a  $-80^{\circ}\text{C}$  freezer until shipment.

### **Shipment**

Frozen samples were shipped on dry ice by a contracted courier, specialized in transportation of frozen material. Samples were shipped as 'Biological Substance Category B UN 3373' (Exempt Human Specimen) in the applicable packaging material provided by the courier, *i.e.* an inner sealed plastic bag with absorbent material, an outer packaging and a polystyrene box with dry ice. Packaging and shipment complied with International Air Transport Association (IATA) criteria (<http://www.iata.org>). Samples were shipped as a batch of three samples or once in every 3 weeks, to reduce costs. Samples were shipped and delivered at Agendia B.V., Amsterdam, within 1 working day after collection at the local hospitals. Samples collected on Friday were delivered on Monday. The amount of dry ice was maintained during the weekend, to prevent thawing of the samples.

## Microarray analyses

Upon receipt of the samples at Agendia B.V., outer and inner packaging and Eppendorf tubes were checked for damage and for the presence of an appropriate sticker with unique sample ID. Samples were processed for microarray analysis, and the number of days required to generate a 70-gene prognosis-signature result was registered. Frozen sections were cut and stained with hematoxylin and eosin (H&E), before and after cutting the sections for RNA isolation, to confirm the presence of tumor and to determine tumor cell percentage. If the mean tumor cell percentage was < 50%, again two frozen sections were cut and stained with H&E before and after cutting the sections for RNA isolation. Samples with less than 50% tumor cells determined in duplicate were excluded from further analysis. RNA isolation, amplification and labeling were performed at Agendia Laboratories, as described previously.<sup>2,28</sup> RNA quality was assessed using the Agilent bioanalyzer. Samples without sample ID stickers or samples with damaged packaging material, less than 50% tumor cells or insufficient RNA quality (RIN < 7) were excluded from further processing.

A total of 200 ng of tumor RNA was co-hybridized with a standard reference to a custom-designed microarray (MammaPrint™), including eight identical subarrays, each containing oligonucleotide probes for the 70 genes in triplicate.<sup>28</sup> The standard reference sample consisted of pooled RNA of 105 primary breast tumors selected from patients of the retrospective validation series.<sup>3</sup> For this feasibility study, results were only presented as successful hybridization or exclusion, hence no good- or poor prognosis-signature was reported. Consequently, all patients included in this feasibility study were treated according to the standard national guidelines. The above-mentioned standard procedures for the collection of good-quality fresh frozen tumor tissue for gene expression profiling are shown in *Figure 3*, left panel.

## Results

Between November 2005 and November 2006, 68 patients were enrolled in six hospitals throughout Europe. Among the 68 patients, four were excluded (one patient had withdrawn informed consent, one patient was aged > 71 years and two had no detectable malignancy). All 64 eligible patients underwent the surgery. Baseline characteristics are shown in *Table 1*. The pathologist was able to obtain a tumor sample in 60 patients (94%): 55 samples were obtained using a 6 mm biopsy puncher and five samples were obtained using a 3 mm biopsy puncher. Among the 60 tumor samples, 14 samples were inadequate (11 samples contained less than 50% tumor cells and three samples had insufficient RNA quality), whereas all 46 adequate samples were successfully hybridized on the MammaPrint™. None of the samples were lost due to processing errors, such as initial storage in formalin. In 4 of the 64 eligible patients no tumor sample was obtained; three patients had tumors that

were too small to obtain a tumor sample and in one case the pathologist forgot to take a tumor sample. A summary is given in *Figure 2*.

Remarkably, all 3 mm samples were representative and hybridized successfully. There was no significant differences in tumor size between the samples that were inadequate because of insufficient tumor cells and samples that were hybridized successfully (mean diameter 21 mm *versus* 19 mm;  $p = 0.7$ ) (*Table 1*). The median time to freeze a tumor sample was 20 min (range 5-235 min). For three samples that had poor RNA quality, the freezing time was < 20 min. The median time to generate and report a 70-gene prognosis-signature result from the time of arrival at Agendia laboratories was 4 working days (range 3-14; mean 5.2).

**Table 1.** Baseline characteristics

	Successful hybridization		No hybridization		<i>p</i>
	N	%	N	%	
<b>Age</b>					ns
≤ 50 years	16	35	4	29	
51 - 60 years	14	30	3	21	
61 - 70 years	16	35	7	50	
<b>Tumor size</b>					ns
pT1 (≤ 20mm)	29	66	7	54	
pT2 (> 20mm)	15	34	6	46	
<b>Histology</b>					
Invasive ductal	38	83	8	57	
Others	6	13	5	36	
Missing	2	4	1	7	
<b>Grade</b>					ns
Grade 1	7	15	2	14	
Grade 2	22	48	7	50	
Grade 3	14	30	3	22	
Missing	3	7	2	14	
<b>Estrogen receptor status</b>					ns
Positive	36	78	9	64	
Negative	6	13	2	14	
Missing	4	9	3	22	
<b>Lymph node status</b>					ns
Positive	13	28	3	22	
Negative	31	68	10	71	
Missing	2	4	1	7	
<b>Total</b>	<b>46</b>	<b>100</b>	<b>14</b>	<b>100</b>	

Missing value were not used for calculation of *p*-values.

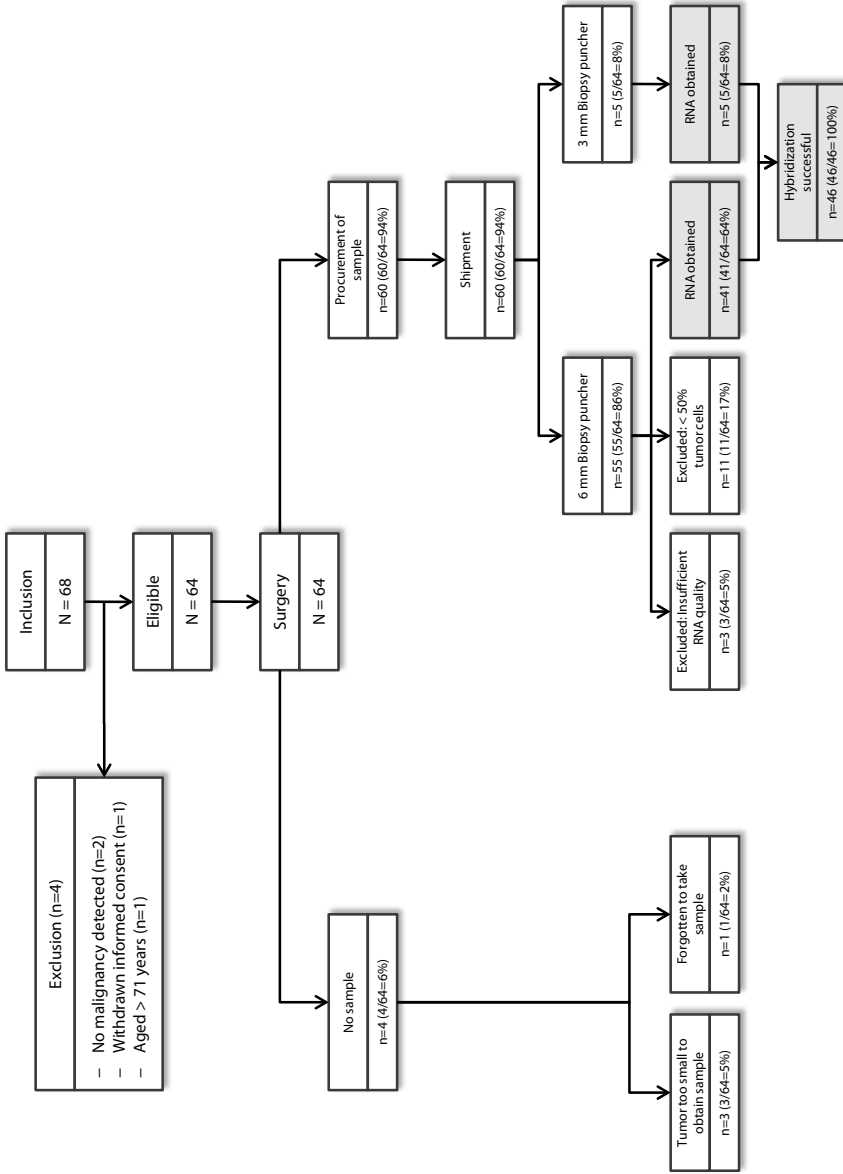
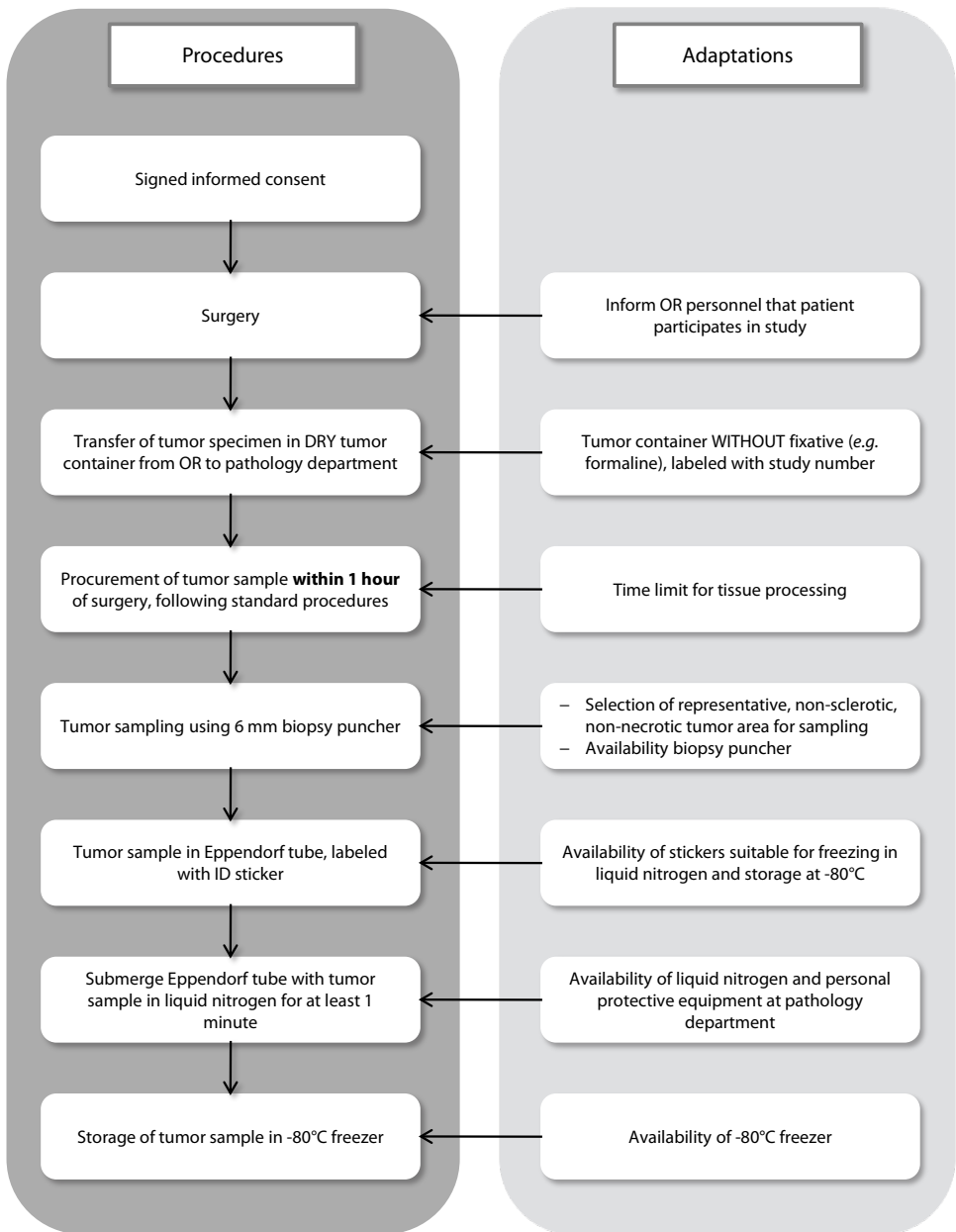


Figure 2. Flow diagram of patients enrolled and tumor samples.



**Figure 3.** Flow chart of standardized procedures for obtaining good-quality fresh frozen tumor samples for microarray analyses (left panel) and adaptations to local standard procedures (right panel).

## Discussion

Our study showed that collection and shipment of fresh frozen tumor tissue for gene expression profiling are feasible in a multicentre and multinational practice setting, with a success rate of 72% (46 out of 64). Provided that the pathologist was able to obtain a tumor sample, the success rate increased to 77% (46 out of 60). When RNA was obtained, all samples were successfully hybridized and a gene expression signature result was obtained in 100% (46 out of 46). The main reason for sample failure was a non-representative tumor sample; 18% (11 out of 60) of the samples contained < 50% tumor cells. This proportion of non-representative samples is in agreement with the proportion reported by a previous feasibility study.<sup>24</sup>

The pathologist obtains a sample after macroscopic evaluation of the tumor specimen (*Figure 1B*). As shown in *Figure 1C*, tumor sampling does not alter morphology and allows appropriate grading and staging of the tumor. The best area for sampling is the periphery of the tumor, given that the central part is often sclerotic or necrotic and lack tumor cells. However, sampling in the periphery of the tumor could increase the amount of surrounding stroma in the sample. The balance between a sufficient amount of tumor cells and a limited amount of stromal tissue can be improved in part by training and repetition. Additionally, recent research has shown that samples containing > 30% tumor cells are suitable for reliable 70-gene prognosis-signature read-out (Amendment 1, MINDACT trial; EORTC 10041/ BIG 3-04). As a consequence, the cut-off for tumor cell percentage in the MINDACT trial has been lowered to 30%, and hence sample inclusion will be increased. In our study, inclusion of samples containing 30-50% tumor cells would have resulted in five additional hybridizations (51 out of 60 = 85% success rate).

Although H&E stained sections of the material sampled for profiling were used to determine if the sample contained a certain amount of malignant tissue, a tumor in itself can be very heterogeneous.<sup>29</sup> To test if the biopsy sample was also representative for the tumor in its entirety, we compared the final pathology report with the genomic test result. In this pilot study, the profile was associated with grade and estrogen receptor status (ER) ( $p < 0.001$ ), which is in good agreement with previous validation studies, that have shown a strong association between the profile and grade, ER status and disease outcome.<sup>3,17-20</sup>

Although gene expression profiling is becoming more and more standardized, operator and technical variability are well known to influence the measurement of gene expression levels.<sup>30-32</sup> To avoid potential interlaboratory bias, all samples in the MINDACT will be shipped on dry ice to Agendia, Amsterdam, where quality controls, RNA isolation and gene expression analysis will be performed. Consequently, frozen tumor samples have to be shipped from all over the world to Amsterdam within a fixed timeframe. In this pilot study, samples were shipped once in every 3 weeks or as a batch, therefore time from sample arrival at Agendia till reporting of the genomic test was measured, instead of the interval between surgery and genomic test result. In our study, all tumor samples were delivered

within one working day. Furthermore, a 70-gene prognosis-signature result was available after a median of 4 d, thereby showing the feasibility of implementation of this signature in clinical trials and daily practice, with regard to the needed timeframe for clinical decision making.

Recently, Bueno de Mesquita and colleagues have shown that the collection of fresh tumor tissue is feasible in community hospitals in the Netherlands.<sup>24</sup> In contrast to our study, tumor samples were placed in a commercially available preservation fluid (RNARetain™) at room temperature, and were sent by conventional mail to the Netherlands Cancer Institute, where samples were subsequently frozen in liquid nitrogen. Although preservation of tumor samples in RNARetain™ does not influence gene expression measurements,<sup>33,34</sup> it is unclear whether it might influence levels of proteins. Since one of the aims of MINDACT is the establishment of a biological materials bank for future research, including proteomics, temporarily preservation of tissue in RNARetain™ as done by Bueno de Mesquita and colleagues is not suitable, and tumor tissue immediately frozen in liquid nitrogen was chosen. The complex logistics involved in the collection and shipment of fresh frozen tissue demands a thorough and detailed organization with adjustments to local standard procedures. The major adjustments are shown in *Figure 3*, right panel. These adjustments formed the basis of the standard operating procedures (SOPs) written for MINDACT.<sup>35</sup>

In conclusion, through detailed standard operating procedures, provision of necessary devices and close collaboration between surgeons, medical oncologists, pathologists, research nurses, data-managers and scientists, successful implementation of the logistics for gene expression profiling on fresh frozen tissue is feasible.

### **Conflicts of interest statement**

Laura J. Van 't Veer is a named inventor on a patent application for MammaPrint™ and reports holding equity in Agendia B.V.

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