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

S. Mook[a], H. Bonnefoi[b], G. Pruneri[c], D. Larsimont[d], J. Jaskiewicz[e], M.D. Sabadell[f], G. MacGrogan[g], L.J. van't Veer[a,h], F. Cardoso[d], E.J. Rutgers[a,*]

[a]Netherlands Cancer Institute – Antoni van Leeuwenhoek Hospital, Division of Pathology and Surgical Oncology, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands
[b]Geneva University Hospitals, Geneva, Switzerland
[c]European Institute of Oncology, University of Milan, Milan, Italy
[d]Institute Jules Bordet, Brussels, Belgium
[e]Medical University of Gdansk, Gdansk, Poland
[f]Vall d'Hebron University Hospital, Barcelona, Spain
[g]Institute Bergonié, Bordeaux, France
[h]Agendia B.V., Amsterdam, The Netherlands

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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 tumour 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 tumour samples were shipped on dry ice to Agendia B.V., where RNA was isolated and subsequently hybridised on the 70-gene prognosis-signature (MammaPrint™).

Results: Tumour samples were obtained in 60 of 64 patients. Among the 60 samples, 11 contained insufficient tumour cells (<50%) and three contained insufficient RNA quality. All 46 samples eligible for genomic profiling were successfully hybridised, 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.

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1. 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.[1–16] One of these gene expression

* *Corresponding author*: Tel.: +31 20 512 2551; fax: +31 20 512 2554.
E-mail address: e.rutgers@nki.nl (E.J. Rutgers).
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classifiers is the 70-gene prognosis-signature (MammaPrint™).[2,3] This 70-gene dichotomous classifier can accurately distinguish breast tumours with a high metastatic capacity from tumours with a low risk of developing distant metastases, by measuring the expression level of 70 genes in tumour tissue. Several retrospective validation studies have confirmed its prognostic value.[3,17–20]

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.[21] Moreover, RNA becomes heavily fragmented during storage of paraffin-embedded tissue.[22] In addition, slow freezing of samples promotes the formation of ice crystals, which can also provoke RNA damage.[23] Consequently, collection of snap-frozen tissue or fresh tissue preserved in RNA preservation fluid, such as RNARetain™ (Asuragen Inc., TX, USA),[24] is at present mandatory to obtain high-quality RNA and successful gene expression profiling. Recently, Bueno-de-Mesquita and colleagues[24] described the successful implementation of RNARetain™ tissue preservation and centralised MammaPrint™ testing for 16 community hospitals in the Netherlands for the prospective RASTER trial.[24] Evaluation of logistics of frozen tissue collection, centralised 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.[25–27] This trial will enrol 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 tumour samples. These local procedures, frozen sample shipment and sample analysis within a fixed timeframe entail complex logistics that requires a thorough organisation.

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 hybridised 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,[24] this pilot study provided crucial information for the feasibility of the MINDACT trial.

2. 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 tumour tissue to test the logistics for genomic profiling.

2.1. *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.

2.2. *On site training*

Before the start of the study, the study coordinator organised 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 summarised on provided pocket summaries, to support standardised procedures for tissue collection, freezing and shipment.

2.3. *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 (Fig. 1A) and a printed sticker sheet with a unique identification number (sample ID), suitable for freezing in liquid nitrogen and prolonged storage at –80 °C.

2.4. *Tumour sampling*

After surgical resection, tumour specimens were immediately transported from the operating room to the pathology department in a tumour container without fixatives, e.g. formalin. To ensure standardised tumour sampling, 6 mm biopsy punchers were provided (Fig. 1A). The pathologist obtained a tumour sample within 1 h of surgery, using this biopsy puncher. Samples were placed in an Eppendorf tube, labelled with a sample ID sticker. For tumours 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 tumour sample for gene expression profiling when he/she judged that there was a sufficient amount of tumour tissue.

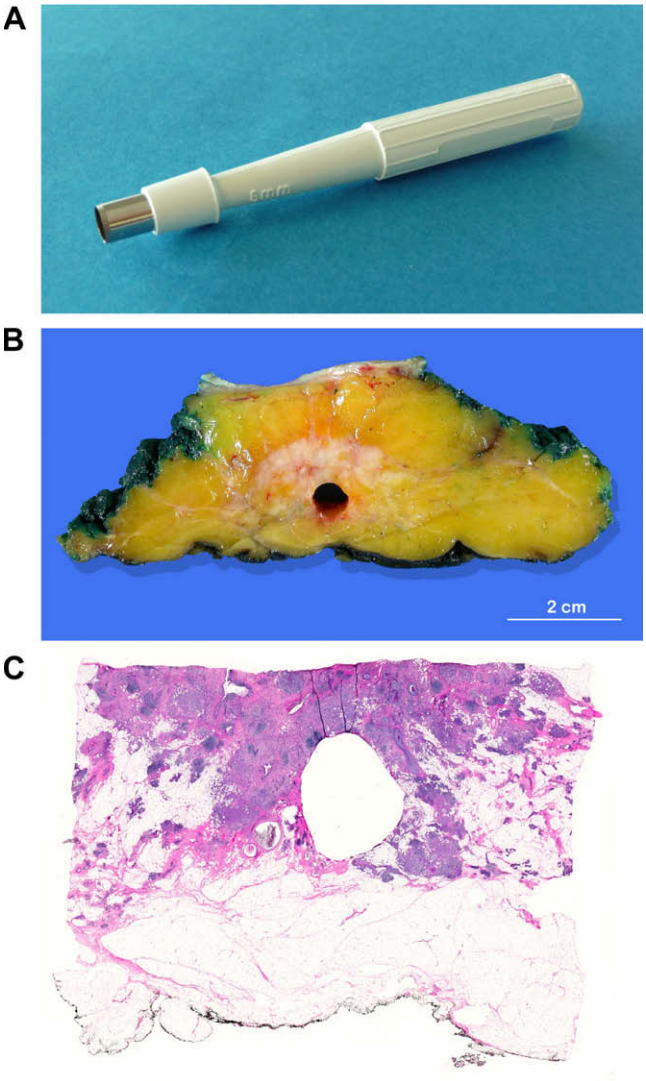

Fig. 1 – (A) Biopsy puncher for standardised tumour sampling. (B) Tumour specimen after sampling, using the 6mm biopsy puncher (by courtesy of J.F. Egger). (C) Haematoxylin and eosin (H&E) stained section of the tumour specimen (shown in B) After sampling, intact morphology is shown, and appropriate grading and staging of the tumour are allowed (by courtesy of J.F. Egger).

2.5. *Snap-freezing and storage*

Eppendorf tubes containing tumour 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 °C freezer until shipment.

2.6. *Shipment*

Frozen samples were shipped on dry ice by a contracted courier, specialised 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.

2.7. *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 haematoxylin and eosin (H&E), before and after cutting the sections for RNA isolation, to confirm the presence of tumour and to determine tumour cell percentage. If the mean tumour 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% tumour cells determined in duplicate were excluded from further analysis. RNA isolation, amplification and labelling were performed at Agendia Laboratories, as described previously.[2,28] RNA quality was assessed using the Agilent bioanalyzer. Samples without sample ID stickers or samples with damaged packaging material, less than 50% tumour cells or insufficient RNA quality (RIN < 7) were excluded from further processing.

A total of 200 ng of tumour RNA was co-hybridised with a standard reference to a custom-designed microarray (MammaPrint™), including eight identical subarrays, each containing oligonucleotide probes for the 70 genes in triplicate.[28] The standard reference sample consisted of pooled RNA of 105 primary breast tumours selected from patients of the retrospective validation series.[3] For this feasibility study, results were only presented as successful hybridisation 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 tumour tissue for gene expression profiling are shown in Fig. 3, left panel.

3. 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 tumour sample in 60 patients (94%): 55 samples were obtained using a

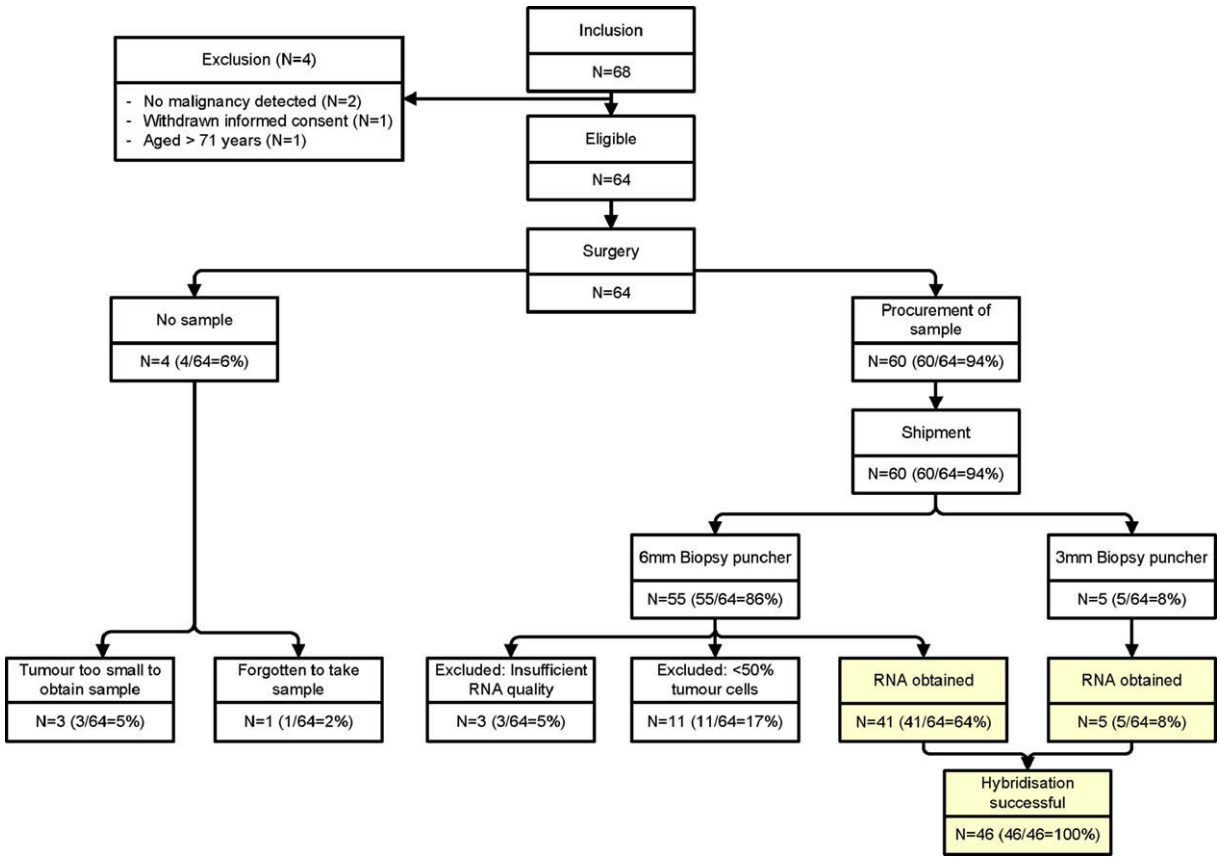

Fig. 2 – Flow diagram of patients enrolled and tumour samples.

6 mm biopsy puncher and five samples were obtained using a 3 mm biopsy puncher. Among the 60 tumour samples, 14 samples were inadequate (11 samples contained less than 50% tumour cells and three samples had insufficient RNA quality), whereas all 46 adequate samples were successfully hybridised 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 tumour sample was obtained; three patients had tumours that were too small to obtain a tumour sample and in one case the pathologist forgot to take a tumour sample. A summary is given in Fig. 2.

Remarkably, all 3 mm samples were representative and hybridised successfully. There was no significant differences in tumour size between the samples that were inadequate because of insufficient tumour cells and samples that were hybridised successfully (mean diameter 21 mm versus 19 mm; $p = 0.7$) (Table 1). The median time to freeze a tumour 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).

4. Discussion

Our study showed that collection and shipment of fresh frozen tumour 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 tumour sample, the success rate increased to 77% (46 out of 60). When RNA was obtained, all samples were successfully hybridised and a gene expression signature result was obtained in 100% (46 out of 46). The main reason for sample failure was a non-representative tumour sample; 18% (11 out of 60) of the samples contained <50% tumour cells. This proportion of non-representative samples is in agreement with the proportion reported by a previous feasibility study.[24]

The pathologist obtains a sample after macroscopic evaluation of the tumour specimen (Fig. 1B). As shown in Fig. 1C, tumour sampling does not alter morphology and allows appropriate grading and staging of the tumour. The best area for sampling is the periphery of the tumour, given that the central part is often sclerotic or necrotic and lack tumour cells. However, sampling in the periphery of the tumour could increase the amount of surrounding stroma in the sample. The balance between a sufficient amount of tumour 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% tumour 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 tumour cell percentage in the MINDACT trial has been lowered to 30%, and hence sample

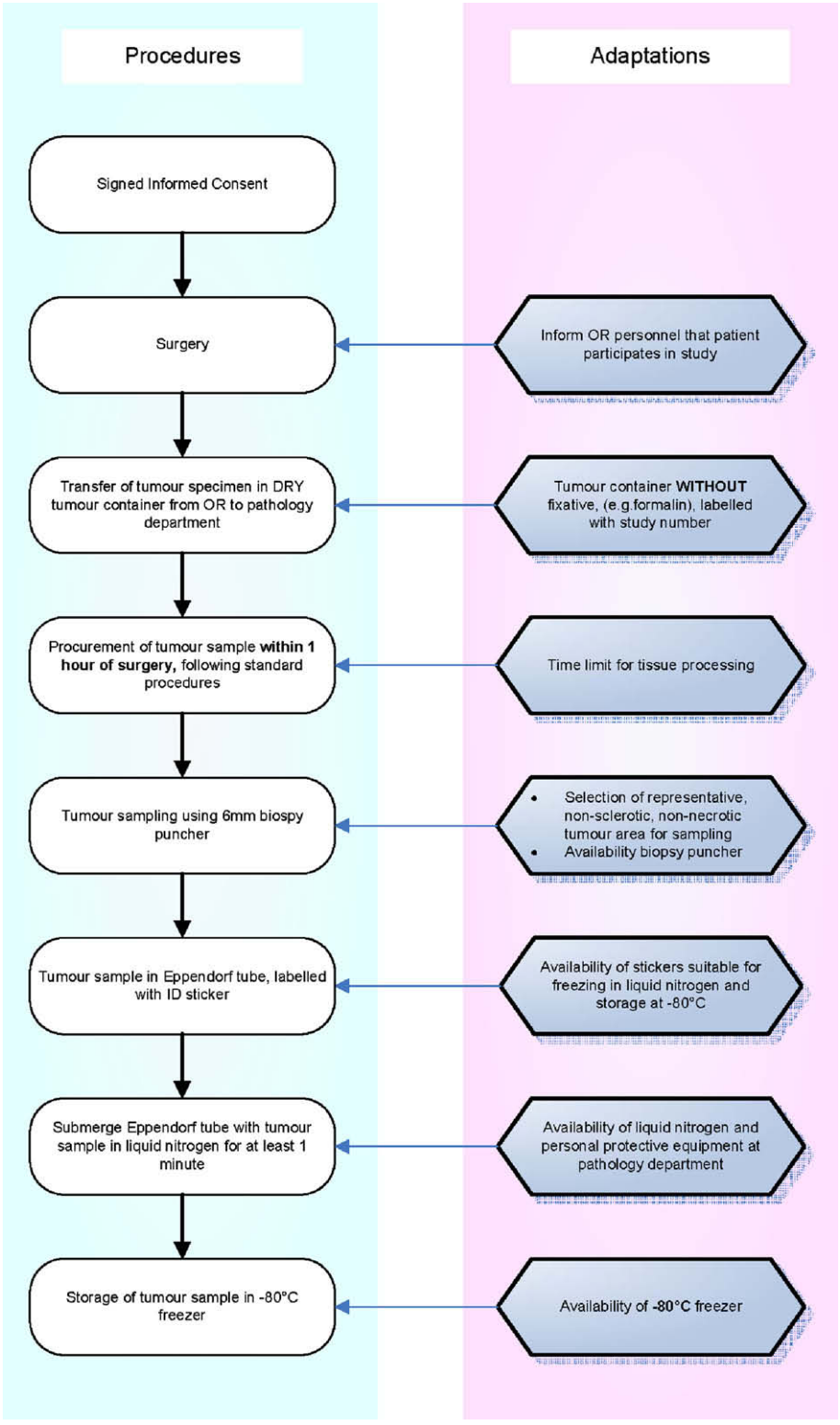

Fig. 3 – Flow chart of standardised procedures for obtaining good-quality fresh frozen tumour samples for microarray analyses (left panel) and adaptations to local standard procedures (right panel).

inclusion will be increased. In our study, inclusion of samples containing 30–50% tumour cells would have resulted in five additional hybridisations (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 tumour in itself can

Table 1 – Baseline characteristics.

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

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

be very heterogeneous.[29] To test if the biopsy sample was also representative for the tumour 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 oestrogen 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.[3,17–20]

Although gene expression profiling is becoming more and more standardised, operator and technical variability are well known to influence the measurement of gene expression levels.[30–32] 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 tumour 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 tumour 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 tumour tissue is feasible in community hospitals in the Netherlands.[24] In contrast to our study, tumour 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 tumour samples in RNARetain™ does not influence gene expression measurements,[33,34] 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 tumour 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 organisation with adjustments to local standard procedures. The major adjustments are shown in Fig. 3, right panel. These adjustments formed the basis of the standard operating procedures (SOPs) written for MINDACT.[35]

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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