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Determination of Oestrogen Receptors by Enzyme Immunoassay. Technical Differences Between Laboratories and Their Consequences

S. Romain, J.L. Formento, O. Guirou, M. Francoual, G. Milano and P.M. Martin

When multicentre breast cancer trials are performed, receptor analyses must be comparable both over time and in the participating laboratories. However, we show for the first time a high variability for the distribution of oestradiol receptor (ER) values measured by enzyme immunoassay (EIA) from 1987 to 1991. This variability could be explained by calibration changes in the immunoassay kits. We have also analysed the influence on ER-EIA levels of technical differences between laboratories apart from the assay itself. Many steps emerged as being critical, i.e. homogenisation buffer, homogenisation procedure and cytosol dilution. Finally, we show that addition of 4-monohydroxytamoxifen increases the apparent ER content measured by EIA in 92% of cytosols. Thus, many factors must be controlled to ensure high precision with ER-EIA assays. We have to be particularly cautious with the conformational changes that could occur during cytosol preparation and that could also pre-exist in the tumour samples. Quality controls of cytosol preparation are essential.
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INTRODUCTION

THE CLINICAL usefulness of oestradiol receptor (ER) assays in breast cancer specimens has been clearly established by correlations with patients' response to endocrine therapies and prognosis. For many years, ER determinations have been routinely performed in many breast cancer centres [1]. Until recently, ER was quantified exclusively by radioligand binding assay (RLA). The Abbott (Abbott Laboratories, Chicago, U.S.A.) enzyme immunoassay (EIA) using monoclonal antibodies now provides an alternative approach for ER assays in breast cancer [2–4]. When multicentre therapeutic trials are performed, receptor analyses must be comparable both over time and in the participating laboratories. Thus, the putative major advantage of EIA is the complete standardisation of the assay itself. A multicentre study conducted in 1986 showed an excellent correlation between EIA and RLA in most laboratories [5]. However, high variability over time has since been reported for this correlation [6, 7]. In addition, important variations in the results of EIA as well as RLA are observed in international quality control programs. EIA variability could be due to calibration problems in the immunoassay kits. Other possible sources of variation could be the technical procedures apart from the assay itself which are used for routine analyses instead of the recommended Abbott protocol.

We present a comparison of results obtained for EIA routine measurements in the period 1987–1991 by the same technical team. In addition, an interlaboratory study enabled us to distinguish between the composition of homogenisation buffer, the homogenisation procedure, the conditions of cytosols storage and the conditions of cytosol dilution as sources of variations in ER-EIA measurement.

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MATERIALS AND METHODS

Tumours and cytosols

Breast tumours used in this study were obtained through pathologists after confirmation of the neoplastic nature of the specimens. Tumours were obtained immediately after surgery and stored in liquid nitrogen until use. For cytosol preparation, all procedures were carried out at 0–2°C. Tumours were homogenised using a microdismembrator or a polytron in 10 volumes of Tris homogenisation buffer, unless indicated otherwise [10 mmol/l Tris–HCl, 0.5 mmol/l dithiothreitol (DTT), 1.5 mmol/l disodium EDTA, 10 mmol/l sodium molybdate, 10% glycerol, pH 7.4]. Cytosol was obtained by centrifugation of the homogenate for 60 min at 105 000 *g*. Cytosol protein concentrations were determined by the Bradford's method [8].

RLA dextran-coated charcoal (DCC)

RLA ER assays were performed using the DCC method and Scatchard plot assay [9–14]. Briefly, 100 µl of cytosol were incubated with [^{3}H]oestradiol (0.15–5 nmol/l) either separately or with 100-fold diethylstilboestrol. Incubations were performed overnight at 4°C and were stopped by adding 500 µl of DCC supension (0.7% w/v charcoal Sigma, 0.07% w/v dextran T70 Pharmacia) to the reaction mixtures. After 30 min, the charcoal was pelleted by centrifugation at 800 *g* for 10 min and the supernatant was then counted for radioactivity in a liquid scintillation counter. The EORTC and French Receptor Study Group interlaboratory quality controls were systematically assayed.

Table 1. Distribution of ER-EIA levels from 1987 to 1991

	n	ER-EIA (fmol/mg protein) Q(0.25)	Q(0.50)	Q(0.75)	Mean	S.D.	P value
Overall population							
Cytosols A	864	8.0	77.0	262.5	156.4	185.8	
Cytosols B	1166	10.0	97.0	305.0	194.4	299.5	0.009
Cytosols C	1191	19.0	111.0	300.0	202.9	319.8	<0.001
Cytosols D	922	19.0	122.5	315.0	197.3	246.3	<0.001
Premonopausal							
Cytosols A	225	4.0	30.0	80.0	58.4	79.6	
Cytosols B	301	8.0	47.0	111.5	93.4	183.4	0.006
Cytosols C	298	9.0	58.0	139.0	96.0	123.7	<0.001
Cytosols D	224	8.5	49.5	133.5	92.7	120.0	<0.001
Postmenopausal							
Cytosols A	579	15.0	133.0	341.7	197.6	201.3	
Cytosols B	728	19.0	176.0	364.5	241.7	335.2	0.038
Cytosols C	714	28.0	183.5	345.0	251.5	378.3	0.012
Cytosols D	630	29.0	182.5	351.0	235.6	272.5	0.015

Cytosols A: June 1987–May 1988, B: June 1988–May 1989, C: June 1989–May 1990, D: June 1990–May 1991. *n*, number of cases; Q(0.25): 25th percentile; Q(0.50): median value; Q(0.75): 75th percentile; S.D.: standard deviation. *P* value by Mann–Whitney U test, as compared to cytosols A.

Abbott EIA

The immunoassays were performed with the Abbott ER-EIA kit.

Statistical methods

The Mann–Whitney U test was used to test whether the distribution of a specified variable of one group is the same as its distribution in another group. The Passing–Bablock linear regression procedure was used to compare results of two assays [6, 15, 16]. *P* values less than 0.05 were considered to be statistically significant.

RESULTS

Distribution of ER-EIA levels from 1987 to 1991

From June 1987 to May 1991, 4143 breast tumours were collected in the same laboratory (Marseille) for routine assay of ER. These were homogenised in Tris homogenisation buffer. Assays were performed by the same technicians. Table 1 shows the distribution of ER-EIA levels for the overall population, the premenopausal patients and the postmenopausal patients. Data from each individual year are presented. Samples for which the EIA result exceeded the standard range and could not be analysed in dilution were not included. In the overall population, ER-EIA levels significantly increased over time. For each individual year, for example, median values were chronologically 77.0, 97.0, 111.0 and 122.5 (Table 1). Significant differences were also observed for premenopausal and postmenopausal patients. From 1987 to 1991, a significant increase was also observed for the frequency of ER-EIA-positive tumours (67.6 versus 70.1 versus 74.9 versus 74.8%, respectively) ($P = 0.0003$).

Distribution of ER-EIA levels and ER-RLA levels

From 1988 to 1991, 768 breast tumours were collected in the same laboratory (Nice) for routine assay of ER. These were homogenised in Tris homogenisation buffer. In 1988 and at the beginning of 1989, ER determinations were performed using the RLA assay. At the end of 1989 and until the end of 1991, assays were performed using the EIA. Table 2 shows the distribution of ER levels for RLA and EIA. The ER median value was 24.0 for RLA and 58.0 for EIA. Thus, the absolute ER levels measured by EIA were higher than ER levels previously measured by RLA. Results of this retrospective analysis were confirmed by a recent Abbott survey made in 1992 in French centres (data not published).

Effect of short-term storage of cytosols in liquid nitrogen on ER-EIA assay

ER-EIA levels were measured both in fresh cytosols and in cytosols stored for 3 days in liquid nitrogen ($n = 24$). Short-

Table 2. Distribution of ER levels measured by RLA and EIA

	n	ER (fmol/mg protein) Q(0.25)	Q(0.50)	Q(0.75)	Mean	S.D.	≥100	≥10
RLA	322	7	24	90	94	175	23.3%	69.0%
EIA	446	13	58	194	131	159	38.6%	78.0%

RLA: January 1988–September 1989; EIA: October 1989–December 1991. *n*: number of cases; Q(0.25): 25th percentile; Q(0.50): median value; Q(0.75): 75th percentile; S.D.: standard deviation.

term storage of cytosols in liquid nitrogen had little effect on ER-EIA levels. The ER-EIA mean values of the fresh cytosols and the stored cytosols were, respectively, 241.4 and 239.5. The Passing–Bablock regression curve between ER-EIA levels of fresh cytosols (x) and cytosols stored 3 days in liquid nitrogen (y) was $y = 1.035 + 0.964\ x$.

Effect of short storage of cytosols at −80°C on ER-EIA assay

ER-EIA levels were measured both in cytosols stored for 3 days in liquid nitrogen and in cytosols stored for 10 days thereafter at −80°C ($n = 85$). The ER-EIA mean values of the fresh cytosols and the stored cytosols were 185.3 and 196.9, respectively. The Passing–Bablock regression curve between ER-EIA levels of cytosols stored 3 days in liquid nitrogen (x) and cytosols stored 10 days thereafter at −80°C (y) was $y = 0.980 + 1.010\ x$.

Effect of homogenisation buffer components on ER-EIA assay

As shown by a recent Abbott survey made in French centres, many differences between laboratories still exist for the homogenisation buffer's composition despite efforts to standardise it by receptor study groups. We thus investigated the importance of the composition to ER-EIA measurements. Eleven breast tumours were homogenised with a buffer containing only Tris (10 mmol/l Tris–HCl, pH 7.4, 4°C). Five 1:2 dilutions were prepared for each cytosol (protein concentration approximately 2–3 mg/ml). In the first dilution, the final concentrations were the same as the Tris homogenisation buffer (see Materials and Methods). The other four dilutions were prepared in the same way but without, respectively, sodium molydate, glycerol, EDTA and DTT. ER levels were measured by EIA in parallel for the five dilutions of each cytosol. The highest levels were found in the presence of all components of the Tris homogenisation buffer (Figure 1). Variations in percentages found in the absence of DTT ($P = 0.06$) and disodium EDTA ($P = 0.02$) were approximately 25%, even for ER levels representative of the 'cut-off value' area, when expressed per mg protein (cytosols 6 and 7). However, the absence of the other components had a less marked influence on ER-EIA levels.

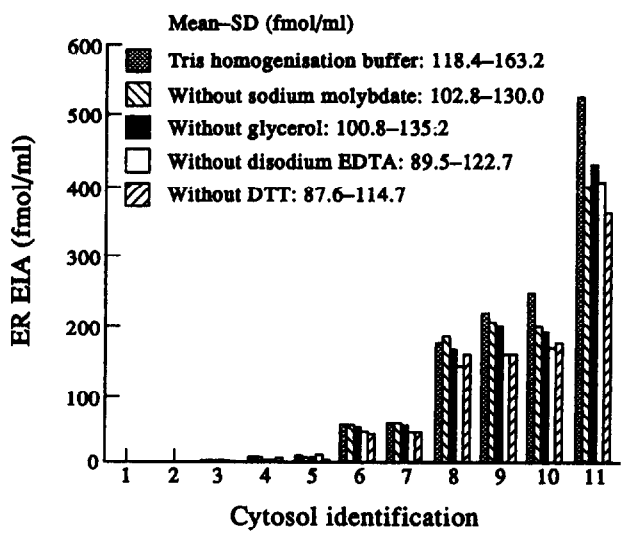

Figure 1. Comparison of ER-EIA levels measured in the presence of all components of the Tris homogenisation buffer (10 mmol/l Tris–HCl, 0.5 mmol/l DTT, 1.5 mmol/l sodium EDTA, 10 mmol/l sodium molybdate, 10% glycerol, pH 7.4) and in the absence of each component.

Effect of homogenisation procedure on ER-EIA levels

Mixed fragmented tissue preparations were obtained from 41 breast cancer tumours stored at −80°C. An aliquot of each fragmented tissue preparation was distributed to two laboratories (Marseille and Nice) routinely engaged in performing steroid receptor assays. The fragmented tissue preparations were stored and transported at −80°C. Samples were all homogenised in Tris homogenisation buffer but with a microdismembrator in Marseille and a polytron in Nice. ER-EIA levels were measured in Marseille by only one technician. The Passing–Bablock regression curve between ER-EIA levels of cytosols prepared with a microdimembrator (x) and with a polytron (y) was $y = -0.971 + 1.486\ x$. Mean values were 95.9 and 118.9, respectively.

Interlaboratory variability of ER-EIA assay

ER-EIA levels were measured both in Nice and in Marseille for 41 cytosols. An excellent correlation was found. The mean values of ER-EIA levels measured in Marseille and Nice were 95.9 and 117.8, respectively. The Passing–Bablock regression curve between ER-EIA levels obtained by the two laboratories was $y = 0.703 + 1.074\ x$.

Effect of dilution on ER-EIA levels of kit controls

Five kit controls were reconstitued in the homogenisation buffer. Four dilutions (1:2, 1:4, 1:8, 1:16) were performed in the kit's specimen diluent and in the Tris homogenisation buffer. ER was measured by EIA in parallel for all dilutions. The ER-EIA levels found for the dilutions in the kit's specimen diluent were in excellent agreement with the expected value. In contrast, dilution of the kit control in the homogenisation buffer enhanced ER levels measured by EIA (Figure 2). Reconstitution in Abbott reconstitution buffer and dilution in 0 standard as recommended in the package insert was not tested.

Effect of dilution on ER-EIA levels of cytosols

ER-EIA levels were measured in parallel in undiluted cytosols, in cytosols diluted 1:4 in the Tris homogenisation buffer and in cytosols diluted 1:4 in the kit's specimen diluent ($n = 26$). The Passing–Bablock regression curve between ER-EIA levels of undiluted cytosols (x) and diluted cytosols (y) was $y = 9.785 + 0.883\ x$ for Tris homogenisation buffer and $y = -9.560 + 0.743\ x$ for the kit diluent. Of 26 ER-EIA levels determined for dilutions in the kit diluent, 24 were lower than the values obtained without dilution.

In 7 out of 19 samples, serial dilutions (1:2; 1:4; 1:8; 1:16) were performed in the Tris homogenisation buffer and in the kit diluent. ER-EIA levels were measured in parallel in undiluted and diluted cytosols. For each specimen, the result of each dilution assayed was plotted as a function of protein concentration (Figure 3). Very bad linearity was found, both for dilutions in the Tris homogenisation buffer and in the kit diluent, with a dramatic ER underestimation in the cytosols with the highest levels.

Effect of treatment of cytosols with 4-monohydroxytamoxifen on ER-EIA levels

It has been previously shown that treatment of human breast cancer cytosols with tamoxifen or 4-monohydroxytamoxifen (MHT) enhances the immunoreactivity of the ER toward mono-

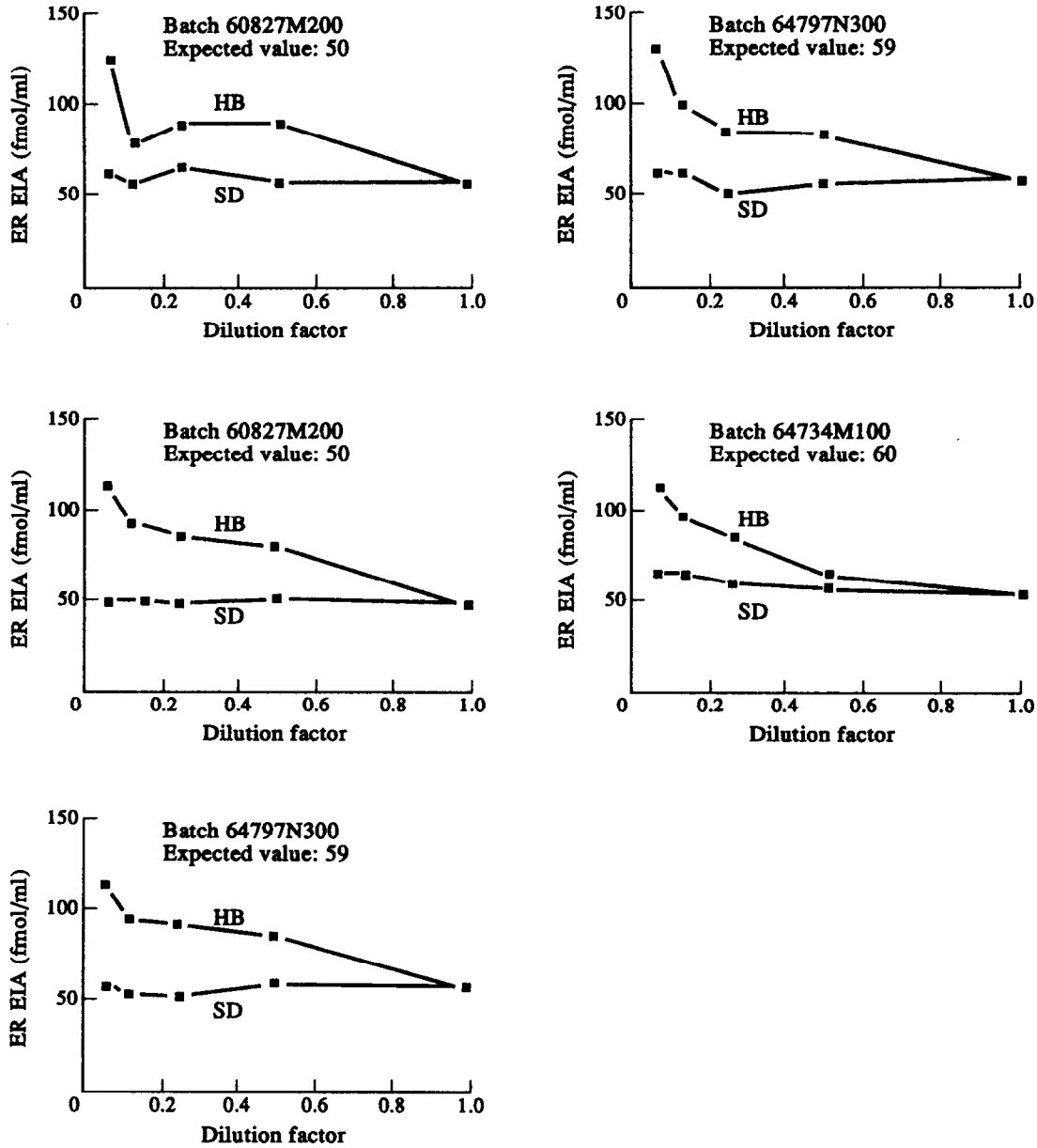

Figure 2. Effect of dilution in the homogenisation buffer and in the kit's specimen diluent on ER-EIA levels of kit controls (n = 5). Serial dilutions (1:2; 1:4; 1:8; 1:16) were performed in the Tris homogenisation buffer (HB) and in the kit's specimen diluent (SD). ER-EIA levels were measured in parallel in undiluted and diluted controls. For each specimen, the result of each dilution was plotted as a function of the dilution factor.

colonal antibody H222 [17]. In order to study the distribution of this effect in a large series of patients, 200 cytosols were diluted in the diluent buffer KCl 0.8 M, bovine serum albumin (BSA) 5 mg/ml, Na_2HPO_4 mmol/l, bacitracin 70 mg/ml, sodium molybdate 40 mmol/l in the presence or absence of 50 mmol/l MHT and assayed by ER-EIA. The ratio between mean ER-EIA levels measured in the presence and in the absence of 50 mM MHT was 1.3. The median value was also 1.3. The minimal and the maximal values were 0.37 and 4.00, respectively. The addition of MHT increased the apparent ER content measured by EIA in 92% (184/200) of cytosols (Figure 4).

DISCUSSION

Our study examined assays performed under routine conditions by the same team over a 4-year period and revealed a high variation in distribution of EIA levels over time. Variability could be explained by calibration problems in the immunoassay kits. In fact, the reliability of the EIA technique depends on the manufacturer's successive calibrations and the stability of the kits. Non-significant variations at each calibration can result in a significant total drift if all differences are in the same direction. Based on these and other results, Abbott ER-EIA standardisation had been recalibrated by the manufacturer and has been in kits since early 1993. Laboratories using the EIA must, therefore, validate this assay against RLA-DCC assays in their own setting and routinely perform quality controls.

The EORTC Receptor Study Group has been involved in the standardisation of steroid receptor assays in breast cancer tissues. However, as shown by a recent Abbott survey made in French centres, many differences between laboratories still exist for cytosol preparation and cytosol dilution prior to EIA measurement. The majority of the studies on ER-EIA reliability have

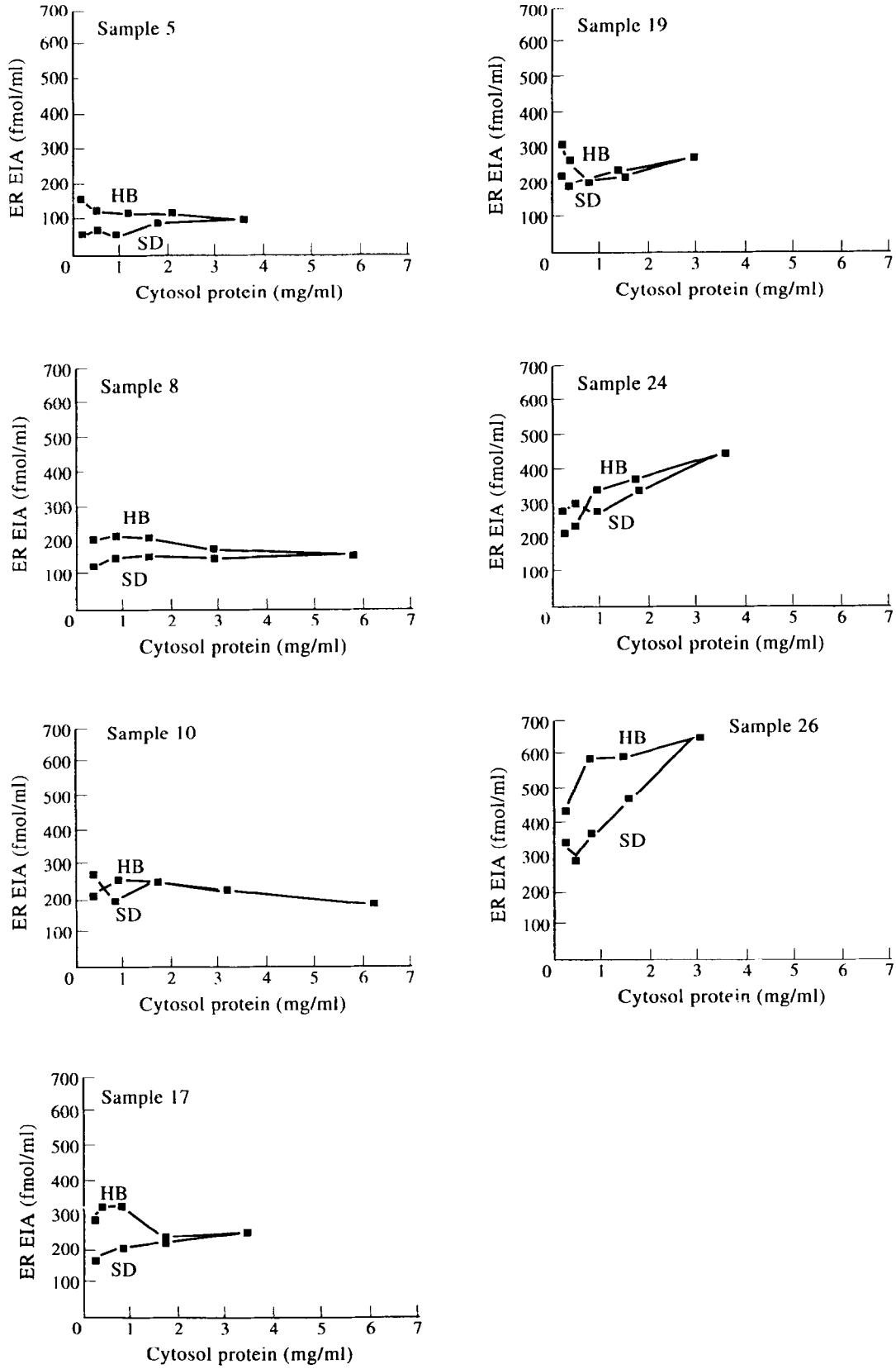

Figure 3. Effect of dilution in the homogenisation buffer and in the kit's specimen diluent on ER-EIA levels of cytosols (n = 7). Serial dilutions (1:2; 1:4; 1:8; 1:16) were performed in the Tris homogenisation buffer (HB) and in the kit's specimen diluent (SD). ER-EIA levels were measured in parallel in undiluted and diluted cytosols. For each specimen, the result of each dilution assayed was plotted as a function of protein concentration.

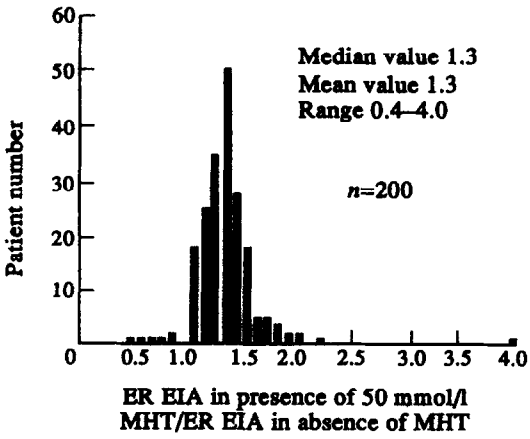

Figure 4. Distribution of the ratio between ER-EIA levels measured in cytosols in the presence and in the absence of 50 mmol/l MHT (n = 200).

been concerned with correlation between EIA and RLA-DCC assays [5, 18–29]. The work presented here is of particular interest in that the influence on ER-EIA of technical differences between laboratories has been analysed. We found a low variability for the ER-EIA assay itself between the two laboratorics in the present study. Short-term storage of cytosol in liquid nitrogen or at −80°C appeared to have little effect on ER-EIA levels, in agreement with results obtained previously over longer time periods [21]. Cytosol preparation was found to be the major source of variability in this study. We showed that the absence of certain components of the Tris homogenisation buffer lead directly to insufficient measurements. Another possible cause of ER-EIA assay variability could stem from the use of a polytron or a microdismembrator for cytosol preparation. Pulverisation by microdismembrator is the method recommended by the EORTC to avoid denaturation of the heat-labile receptors. However, cytosols prepared in the same conditions as the EIA standards are likely to be more accurately evaluated. The conditions of cytosol dilution could be another major source of variation.

It has been shown previously that treatment of human breast cancer cytosol with tamoxifen or its metabolite MHT enhances the immunoreactivity of the ER toward monocolonal antibody H222 [17]. These results indicate that reaction with anti-oestrogen causes a change in the receptor molecule, which is probably conformational, and reveals occult antigenic determinant recognised solely by H222. This effect was seen in most tumours in this study, but to various extents. Thus, patients having ER levels while on tamoxifen therapy could have falsely elevated ER-EIA levels. The correlation between the direct effect of MHT on ER-EIA levels of primary tumours and the response of patients to hormonal therapy needs to be investigated.

In conclusion, many factors must be controlled to ensure high precision with the ER-EIA assay. These affect both the proportion of tumours that are classified as ER positive and the detected ER levels. Classification of patients as ER positive is the element of receptor assays that is most widely used in clinical practice. However, the accuracy of ER levels is also important. Postmenopausal patients with the highest ER levels experience the greatest benefit from hormonal therapy. In addition, the highest ER levels predispose for poorer prognosis among postmenopausal patients not treated with adjuvant therapy [30]. We thus have to be particularly cautious with the conformational changes that could occur during all the steps of cytosol preparation and that also pre-exist in the tumour samples. The recommended protocol for cytosol preparation should, therefore, be strictly followed. It would perhaps be useful to include necessary reagents in the ER-EIA kit. In addition, interlaboratory quality controls of cytosol preparation are essential.

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Local Control of Soft Tissue Sarcoma of the Extremity: The Experience of a Multidisciplinary Sarcoma Group With Definitive Surgery and Radiotherapy

A.N. Wilson, A. Davis, R.S. Bell, B. O'Sullivan, C. Catton, F. Madadi, R. Kandel and V.L. Fornasier

Data gathered on 62 patients with soft tissue sarcoma of an extremity, treated in entirety by an experienced multidisciplinary sarcoma group, were analysed. With a philosophy of emphasising attainment of histologically negative margins at carefully planned limb sparing surgery, combined with either pre-operative or postoperative radiation therapy, a crude local control rate of 95% (59 of 62 patients) at a minimum of 24 months follow-up was obtained. Of 9 patients with microscopically positive margins after definitive surgery, 8 had undergone maximal resection compatible with preservation of function. One of these 9 failed locally, indicating that radiation therapy is effective in eradicating microscopic disease in this tumour. The excellent local control obtained with limb-sparing surgery in this series justifies early referral of patients with these uncommon cancers to an experienced multidisciplinary unit. 26 patients (42%) failed systemically at a minimum of 24 months follow-up, and 19 (30.6%) died of their disease, confirming the need for effective systemic therapy in soft tissue sarcoma. Tumours greater than 10 cm in diameter had a greater risk of systemic relapse.

Key words: sarcoma, extremity, radiotherapy, surgery
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INTRODUCTION

IN THE past two decades, the management of patients with soft tissue sarcoma of the extremity has undergone a considerable evolution. The recognition that sarcoma cells tend to spread widely within the fascial barriers that form the compartments of a limb led to the advocacy of radical resection of the entire involved muscle compartment or, alternatively, amputation [1]. Standard nomenclature was developed that defined wide resection as excision of the tumour in a complete layer of normal tissue, marginal resection as excision of the tumour through its