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Original article

Simplified phenotyping of CYP2D6 for tamoxifen treatment using the N-desmethyl-tamoxifen/ endoxifen ratio

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ABSTRACT

Introduction: CYP2D6 protein activity can be inferred from the ratio of N-desmethyl-tamoxifen (NDMT) to endoxifen (E). CYP2D6 polymorphisms are common and can affect CYP2D6 protein activity and E level. Some retrospective studies indicate that E < 16 nM may relate to worse outcome.

Materials and methods: A target NDMT/E ratio was defined as associated with an E level of 15 nM in the 161 patient Test cohort of tamoxifen-treated patients, dichotomizing them into 'Normal' (NM) and 'Slow' (SM) CYP2D6 metabolizer groups. This ratio was then tested on a validation cohort of 52 patients. Patients were phenotyped based on the standard method (ultrarapid/extensive, intermediate or poor metabolizers; UM/EM, IM, PM) or a simplified system based on whether any variant allele (V) vs wildtype (wt) was present (wt/wt, wt/V, V/V). Comprehensive CYP2D6 genotyping was undertaken on germline DNA.

Results: A target NDMT/E ratio of 35 correlated with the 15 nM E level, dichotomizing patients into NM (<35; N = 117) and SM (>35; N = 44) groups. The ratio was independently validated by a validation cohort. The simplified system was better in predicting patients without slow metabolism, with specificity and sensitivity of 96% and 44% respectively, compared with the standard method - sensitivity 81% and specificity 83%.

Conclusions: The simplified classification system based on whether any variant was present better identified patients who were truly not CYP2D6 slow metabolizers more accurately than the current system. However, as CYP2D6 genotype is not the only determinant of endoxifen level, we recommend that direct measurement of endoxifen should also be considered.

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1. Introduction

Tamoxifen is an important endocrine therapy for hormone sensitive breast cancer [1]. It is a selective estrogen receptor modulator and a pro-drug that is metabolised extensively by cytochrome P450 enzymes to produce active metabolites. Pharmacogenetic variability of Cytochrome P450 2D6 (CYP2D6)[2] is one of the key factors in the metabolism of tamoxifen, resulting in variability of active metabolite levels [3]. Of these, 4-OH-N-desmethyltamoxifen (endoxifen) and 4OH-tamoxifen are the most antioestrogenic. Endoxifen is the most abundant [2] and is largely responsible for tamoxifen's effect on outcome [4,5]. Two retrospective datasets have shown that patients whose endoxifen levels were <15 nM or <16 nM respectively, were associated with worse cancer-related outcomes [6,7], suggesting a critical threshold for tamoxifen effect. In addition to CYP2D6 activity, a number of other factors are involved in determining endoxifen level, such as CYP3A4, CYP2C9/19, UDP-glucuronosyltransferases and sulfotransferase activity, as well as compliance, use of food supplements [8], BMI [9,10], menopausal status [11] and concomitant medications that inhibit CYP2D6 enzyme activity [4].

Single nucleotide polymorphisms of germline [12] *CYP2D6* are an important determinant of CYP2D6 activity and influence the variability of many drugs including tamoxifen [13]. *CYP2D6* is highly polymorphic with over 100 known allelic variants [13] and significant interethnic differences exist [14,15]. Guidelines have been made which provide recommendations on tamoxifen dose based on *CYP2D6* genotype [16]. Alleles that are routinely examined include *1, *2, *1 x N or *2 xN, *3, *4, *5, *6, *10, *17, *41, with *1 and *2 representing wild type normal function and with the others associated with varying degrees of impaired function. The diplotype of these is used to categorize a phenotype of CYP2D6 activity [17] which was originally based on codeine metabolism and extrapolated to tamoxifen [16]. This system defines four metabolic activity categories, ranging from poor to ultrarapid. However, with regards to tamoxifen, clinicians are ultimately concerned about only identifying slow metabolizers (which imply low endoxifen level) and it can be argued that such a complex, granular system is unnecessary.

In this study, we assessed a simplified CYP2D6 phenotype system in tamoxifen-treated patients to determine whether a diplotype comprising *any* genetic variant could predict slow metabolizer status and compared this with the standard, complex classification. We directly assessed CYP2D6 activity by using the ratio of tamoxifen metabolites to define patients with slow metabolism.

2. Material and methods

Informed consent was obtained from all participants and the study was conducted with approval from the local Human Research Ethics Committee, Westmead Hospital, Sydney, Australia.

A total of 159 patients were studied from Australia and 54 from the Netherlands and were divided into test and validation cohorts, with ultimately 161 patients in the test cohort and 52 patients in the validation cohort. Germline CYP2D6 SNPs were measured in each patient. All patients were women who were treated with tamoxifen endocrine therapy for treatment of invasive hormone sensitive breast cancer. Patients who were treated with concomitant CYP2D6 inhibitors were excluded from this study.

In each cohort, patients were CYP2D6 genotyped from genomic DNA isolated from blood using a high-salt method [18]. Briefly, all DNA samples (5 ng/μl) were transferred into 384 well PCR plates for genotyping. The genotyping analysis was performed as recommended by the manufacturer with reagents included in the iPLEX ADME PGx Pro genotyping kit (Agena) and the software and equipment provided with the MassARRAY platform (Agena). In brief, DNA samples were PCR amplified according manufacture protocol. Subsequently, PCR products were treated with 0.5 U shrimp alkaline phosphatase to neutralize unincorporated dNTPs. Extension reaction was carried out with iPLEX Pro extension before purification using SpectroCLEAN resins. The cleaned extension products were dispensed onto a 384 SpectroCHIP array using an RS1000 Nanodispenser. Finally, the array was introduced into a MassARRAY Compact mass spectrometer. Spectra were acquired using SpectroAcquire software, and data analysis, including automated allele calling, was done using MassARRAY Typer software, version 4.0.5.

The following CYP2D6 SNPs were measured: *1, *2 A; *31; *51,*2 L; *35; *71, *3, *4, *4 M, *6, *7, *8, *9, *10; *36; *37; *47; *49; *52; *54; *57; *65; *72, *11, *12, *14 A, *14 B, *15, *17, *18, *19, *20, *21 A, *21 B, *30, *40, *41, *42, *44, *56 A, *56 B, *58, *64, *69. CYP2D6 phenotype was described using the clinical pharmacogenetics classification system based on codeine metabolism [17]. These are in order of highest function to lowest: ultrarapid metabolizers (UM) and extensive metabolizers (EM), which we classified together as EM, intermediate metabolizers (IM) and poor metabolizers (PM). We also categorized each patient's haplotype using a simplified system defined by whether they were wild type (wt; defined as *1, *2 or duplication) or had any variant (V) present. Alleles other than *1 or *2 were classified as "Variant" alleles. Diplotypes were then categorized as homozygous wt/wt, homozygous V/V or heterozygous wt/V. Additionally, *9 and *15 which are not classified by the current codeine based system were considered to be partially functioning and non-functioning variants, respectively [19]. Hence *9/*9 and *15/*15 diplotypes were considered IM and PM respectively, using the Crews categorization [17], and V/V using our simplified system.

Each patient had tamoxifen and metabolites assessed on plasma, after a minimum of eight weeks of daily dosing of tamoxifen to ensure endoxifen steady state [20] had been reached. Endoxifen and n-desmethyl hydroxy tamoxifen (NDMT) and other tamoxifen metabolite levels were measured according to methods as previously described in the Australian cohort [21] and in the Dutch [22]. The tamoxifen metabolite results for the Australian and Dutch patients were cross-validated. Endoxifen is produced by CYP2D6 metabolism of NDMT and so CYP2D6 activity can be described using the ratio of NDMT to endoxifen (NDMT/E). So we could define the ratio associated with slow metabolism, we first determined a NDMT/E associated with a low endoxifen level then classified patients as normal metabolizers (NM) if below the cutoff NDMT/E ratio and slow metabolizers (SM) if above the cutoff NDMT/E ratio.

An E level of 15 nM[6,7] was used to calculate the cut-off NDMT/E ratio. The NDMT/E ratio vs E curve was best fitted with the exponential equation:

NDMT/E ratio $= Ae^{-k*EDF}$ where A & k were constants determined by obtaining the least sum of the differences between the measured data and the fitted curve with the above described equation using the solver function of Microsoft Excel spreadsheet (Excel 2013, Microsoft, USA).

We compared this ratio to individual *CYP2D6* genotypes to define phenotypes associated with slow CYP2D6 activity using the standard categorization method as well as a simplified system based on the presence of absence of *any CYP2D6* variant. We hypothesized that the simplified system may more accurately categorize slow CYP2D6 metabolizer phenotype compared to the standard method in tamoxifen-treated patients. We acknowledge

that genotyping analysis of CYP2D6 is complicated and that there are many potential ways of defining phenotypes. We felt that our method of distinguishing between variant and wildtype alleles simplified the analysis and performed at least as well as the other phenotyping methods based on genotype.

3. Results

One hundred and sixty-one patients were prospectively enrolled in the test cohort, comprising three quarters Australian and one quarter Dutch patients. The validation cohort comprised 52 patients, comprising three quarters Australian and one quarter Dutch patients, who were also prospectively enrolled. In both the test (161 patients) and validation cohorts (52 patients), there was wide variability of alleles. The distribution of genotypes in both test and validation cohorts was similar.

An NDMT/E ratio of 35 (95% CI 21.29–55.89) corresponded to an endoxifen level of 15 nM in the test cohort. We thus categorized patients with NDMT/E 35 and greater (corresponding to endoxifen level of below 15 nM) as slow CYP2D6 metabolism (SM), whereas patients with NDMT/E less than 35 were categorized as normal CYP2D6 metabolism (NM). This threshold was validated by the validation cohort.

We then examined NDMT/E in single alleles *1 and *2, which are traditionally considered 'wildtype' using codeine as a substrate to measure CYP2D6 activity. In the test cohort, there were 114 alleles that were *1. In these 114 alleles, the mean NDMT/E was 17.5 (range 0.12–150.33) and only 7 (6%) had a ratio >35. In the test cohort, 74 alleles were *2 wildtype. The mean NDMT/E ratio for these single *2 wildtype alleles was 23.5 (range 0.17–156.30) and 10 (13.5%) had a ratio >35.

In the validation cohort, 38 single alleles were *1 with a mean NDMT/E of 17.2 (range 0.17–87.3) and 2 (5%) had a ratio >35. Twenty-one were *2 with a mean NDMT/E of 21.79 (range 19.33–54.63) and one (5%) with a NDMT/E > 35.

Diplotype stratification according to NDMT/E is shown in Table 1 and Fig. 1 for the test cohort and Supplement Table 1 and Supplement Figure 1 for the validation cohorts. These data

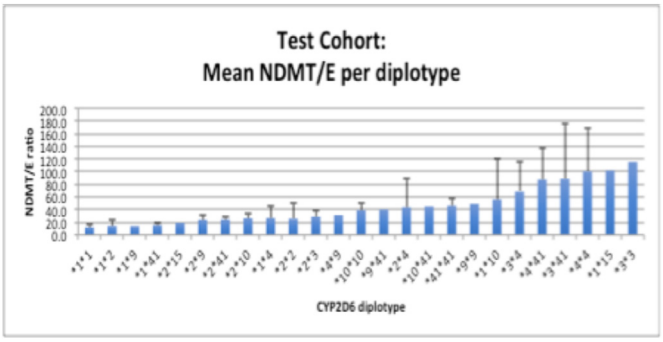

Fig. 1. Test cohort: Mean NDMT/E per diplotype.

demonstrate that diplotype describes NDMT/E as expected, with wt/wt diplotypes and wt/V diplotypes tending to have low NDMT/E ratio (higher CYP2D6 activity) and V/V diplotypes tending to have the highest NDMT/E ratio (lower CYP2D6 activity).

We then compared the NDMT/E ratio with genotype using the complex standard classification system. As seen in Fig. 2, when 117 patients with normal metabolizer status (NDMT/E < 35) were categorized using the current phenotype system, three were PM (3%), two were IM (2%) and 112 were EM (96%). The standard system was therefore good at identifying true normal metabolizers. However, in 44 patients with slow CYP2D6 metabolism (NDMT/E > 35), using the standard system 15 were PM (34%), six were IM (14%) and 23 were EM (52%). Thus, a minority of patients who would be predicted to have ineffective CYP2D6 activity using the standard system had slow metabolism. Conversely, less than half the EM patients had normal CYP2D6 metabolism (likely to have effective endoxifen level).

Using our simplified system, where wt/wt is assumed to result in normal metabolism, and wt/V and V/V remain uncertain, only 3 of 44 patients (7%) with low CYP2D6 activity as measured by NDMT/E > 35, were misclassified as potentially normal metabolizers (wt/wt; Fig. 3).

The accuracy of prediction of slow CYP2D6 metabolism was then

Table 1
Test Cohort stratified per diplotype.

Diplotype	Frequency	Mean NDMT/E	Range	Crews Phenotype	Simplified Phenotype	# pts ratio >35	% pts ratio >35
*1*1	28	12.2	7.5–23.9	EM	wt/wt	0	0%
*1*2	29	13.5	0.12–38.6	EM	wt/wt	1	4%
*1*9	1	13.5	13.5	EM	wt/V	0	0%
*1*41	4	15.1	11.54–18.3	EM	wt/V	0	0%
*2*15	1	18.6	18.6	EM	wt/V	0	0%
*2*9	4	24.1	15.82–32.3	EM	wt/V	0	0%
*2*41	4	24.5	19.02–29.7	EM	wt/V	0	0%
*2*10	2	26.3	20.61–31.909	EM	wt/V	0	0%
*1*4	20	26.7	0.16–96.64	EM	wt/V	3	15%
*2*2	11	28.2	0.17–81	EM	wt/wt	2	20%
*2*3	3	28.8	21.9–39.7	EM	wt/V	1	33%
*4*9	1	31.1	31.1	IM	V/V	0	0%
*10*10	7	39.0	24.83–60.28	EM	V/V	4	57%
*9*41	1	39.9	39.9	IM	V/V	1	100%
*2*4	10	43.8	8.4–156.3	EM	wt/V	4	40%
*10*41	1	44.9	44.9	EM	V/V	1	100%
*41*41	5	46.2	32.26–56.52	EM	V/V	4	80%
*9*9	1	49.1	49.1	IM	V/V	1	100%
*1*10	4	56.1	14.42–150.32	EM	wt/V	2	50%
*3*4	3	68.6	23.20–118.9	PM	V/V	2	67%
*4*41	3	87.7	53.03–144.2	IM	V/V	3	100%
*3*41	2	88.7	26.98–150.43	IM	V/V	1	50%
*4*4	14	100.5	10.41–291.42	PM	V/V	12	86%
*1*15	1	102.0	102.0	EM	wt/V	1	100%
*3*3	1	115.5	115.5	PM	V/V	1	100%

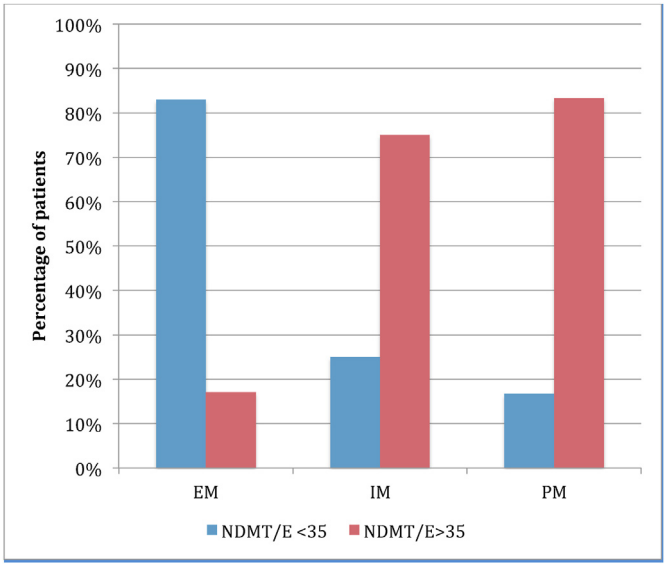

Fig. 2. Test Cohort Stratification by Crews categorization i.e. EM, IM and PM by NDMT/E ratio <35 and >35. *<35 is normal metabolizer; ratio >35 is slow metabolizer.

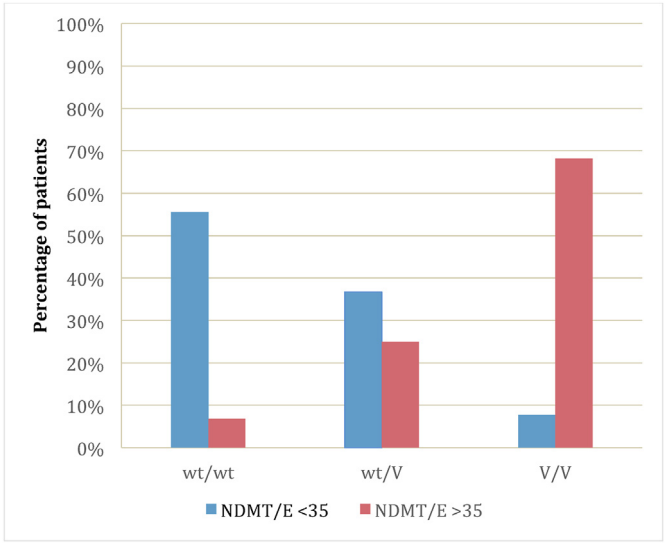

Fig. 3. Test cohort: Stratification of wt/wt, wt/V and V/V by NDMT/E ratio*. *<35 is normal metabolizer; ratio >35 is slow metabolizer.

compared between the standard and simplified systems. The simplified system had higher specificity (96%) but lower sensitivity (44%) compared with the standard method which had a sensitivity of 81% and specificity of 83%. (Table 2). Therefore, the simplified method more accurately identified patients who were not slow metabolizers. That is, the wt/wt patients in the simplified system were less likely to actually have slow metabolism than those categorized as EM by the standard phenotype system.

These results were validated in the validation cohort. In the test cohort, when stratified by the standard classification system, slow metabolizer status was found in 17% of EM, 75% of IM, and 83% of PM patients (Table 2). Using the simplified classification system, the likelihood of having slow CYP2D6 metabolism was 4% of wt/wt, 20% of wt/V, and 77% of V/V patients.

4. Discussion

The overarching aim of our study was to find the optimal method of CYP2D6 phenotyping in tamoxifen treated patients to predict slow CYP2D6 activity and hence imply low endoxifen level. Our study tested the standard phenotyping method and a proposed simplified method for categorizing *CYP2D6* variants. CYP2D6 metabolizes N-desmethyl-tamoxifen (NDMT) to endoxifen (E) and we used the ratio of these two metabolites to measure CYP2D6 enzyme activity. In a test cohort, we found that an NDMT/E ratio of above 35 correlated with a low endoxifen level and thus slow CYP2D6 metabolism. This ratio was subsequently validated in our validation cohort.

An endoxifen threshold of 15 nM was used to derive the ratio, a level shown in two retrospective studies to correlate with worse breast cancer outcome in tamoxifen treated patients [6,7]. We acknowledge that this threshold was not reproduced in two other recent studies [23,24], although we suggest that these findings be interpreted with caution [25,26]. The adjuvant CYPTAM study [27] examined clinical outcome at three years, too brief an interval to assess relapse and cancer-specific outcomes in a luminal breast cancer population. The Neven et al. [26] paper, studied patients who were treated in multiple settings – neoadjuvant, metastatic firstline and metastatic secondline – thus introducing multiple possible confounding factors.

In our study, we also acknowledge the small size of our study, and that extensive patient characteristics were not analysed-thus our results should be interpreted in this context and with these considerations. We have previously shown that if endoxifen levels are measured and are found to be lower than 15 nM, (24% of patients) incremental dose increase in tamoxifen will lead to higher levels in 75% of patients who had endoxifen level <15 nM at baseline on a daily 20 mg dose of tamoxifen [21].

We have previously argued that the best way to ensure effective endoxifen level is to undertake therapeutic monitoring [21]. However, we acknowledge the popularity of CYP2D6 genotype which may infer endoxifen level. For CYP2D6 genotype to be clinically useful it should accurately predict slow metabolizer status (and low endoxifen level) or 'not-slow' metabolizer status (and effective endoxifen level). We contend that it is not clinically relevant to have a complex categorization that includes more than

Table 2
Current phenotype vs. simplified phenotype classification for SM in test cohort.

Test Cohort	Current genotype classification			Simplified classification		
	UM/EM N = 135	IM N = 8	PM N = 18	wt/wt N = 68	wt/v N = 54	v/v N = 39
Slow metabolizer (NDMT/EDF > 35) N = 44	23/135 (17%)	6/8 (75%)	15/18 (83%)	3/68 (4%)	11/54 (20%)	30/39 (77%)
Sensitivity					44%	
Specificity					96%	
Sensitivity		81%				
Specificity		83%				

these two categories for tamoxifen treatment. Using our simplified system, we found that 93% of slow metabolizer patients had any variant present (wt/V or V/V) and this was 100% in the validation cohort. Conversely, slow metabolizer patients displayed wt/wt genotype in only 7% and 0% of the test and validation cohorts, respectively.

In our study, the standard CYP2D6 phenotype system, which consists of four categories, was poor at identifying patients with slow metabolism. Only 48% of slow metabolizer patients were IM or PM using the standard phenotype system (57% in the validation cohort). Conversely, slow metabolizer patients were inaccurately classified as EM status in 52% of cases (43% of validation cohort). This comparison clearly illustrates that, in our patient group, the simplified phenotype method was superior to the standard method in identifying patients who are not CYP2D6 slow metabolizers. If validated by further groups, our findings will have clinical relevance since it has the potential to simplify the interpretation of CYP2D6 by clinicians.

Using the simplified phenotype system, patients with wild type diplotype (*1 or *2), (represented by approximately 40% of our test cohort patients), are highly likely to have normal metabolism with an endoxifen level above 15 nM, if compliant with treatment and have no issues with absorption. We propose these patients should start tamoxifen with a 20 mg daily and have an endoxifen level measured after at least 2 months of treatment.

Our study indicates that patients with *any* variant present are more likely to have a low endoxifen level than those without a genotype variant. Importantly, this means that determination of diplotype (as opposed to single alleles) may not be required. Patients with any CYP2D6 genetic variant present (alleles other than *1 or *2), represented by approximately 60% of the test cohort, have a moderate to high chance of having slow metabolism, and the diplotype alone is not accurate enough to predict this. In this population, it would be wise to start with a higher dose to ensure adequate endoxifen levels. As side effects are not dose-related [21,27], we recommend starting with a 40 mg tamoxifen dose and then perform therapeutic monitoring of endoxifen concentrations. In those patients who still have a low endoxifen level, a further dose escalation could be considered [21].

Variant diploid patients using the simplified system (V/V) or those who are PM by the standard categorization, have a higher chance of being slow metabolizers and very low endoxifen levels. Such patients may not achieve adequate endoxifen levels even with tamoxifen dose increase [6,7], and we advise the consideration of alternative endocrine therapy, such as an aromatase inhibitor.

We acknowledge that tamoxifen is not used in all patients with breast cancer and that alternatives exist [28] [-33]. In the adjuvant setting, for premenopausal women whose disease characteristics are adverse enough to require adjuvant chemotherapy, adjuvant aromatase inhibitor in combination with ovarian function suppression is the gold standard endocrine manouvre [34–36]. However, for premenopausal women with relatively low-risk disease, adjuvant tamoxifen is still standard of care and remains an option for postmenopausal women who do not tolerate aromatase inhibitors. In the metastatic setting where many therapies are available, tamoxifen is still a valid later line therapy. Irrespective of how tamoxifen treatment is used, we propose that tamoxifen dose should be determined by direct measurement of endoxifen and by checking CYP2D6 phenotype.

5. Conclusions

In conclusion, our results demonstrate that a simplified method for classifying *CYP2D6* phenotype using the presence or absence of *any* genotype variant is superior to the standard phenotype classification in determining slow metabolizer status in tamoxifen-treated patients. We have made recommendations based on this simplified system regarding the starting dose of tamoxifen, when to use therapeutic monitoring, and when to consider an alternative endocrine therapy.

Ethical approval

Informed consent was obtained from participants and the study was conducted with approval from the local Human Research Ethics Committee, Westmead Hospital, Sydney.

Declaration of competing interest

Clara Inkyung Lee, Peter Fox, Bavanthi Balakrishnar, Bo Gao, Sally Coulter, Christopher Liddle, Mark Wong, and Nicholas Wilcken have no conflicts of interest to declare.

Rina Hui has conflicts to declare including: Advisory board member for Merck Sharp and Dohme, Astra Zeneca, Novartis, Roche and Bristol-Myers Squib, speaker honorarium for Merk Sharp and Dohme.

Howard Gurney has conflicts to declare including: Advisory board/consulting role for Bristol-Myers Squib, Ipsen, Merck Sharp and Dohme, Astra Zeneca, Janssen-Cilag. Honorarium for Pfizer.

Ron H.J. Mathijssen has conflicts to declare including: Speakers' Bureau: Novartis.

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Stijn L.W. Koolen has conflicts to declare including: Speakers' Bureau: Novartis, Pfizer, Research Funding: Cristal Therapeutics (Inst), Novartis (Inst), Travel, Accommodations, Expenses: Ipsen.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.breast.2020.10.008.

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