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Table 2

Heritability estimates for severity of neuropathy captured by SNPs in subsets of the GO Axonogenesis set

	Heritability Estimates					Pathway Characteristics		
GO Axonogenesis Children	V(G)/V(p)[1]	SE	P[2]	Padj[3]	Empirical P[4]	# Genes	Size (Mb)	#SNPs
Axonal Fasciculation	0.000	0.025	0.5	1	0.999	15	2.89	922
Peripheral Neuron Axonogenesis	0.005	0.010	0.3	1	0.203	2	0.13	15
Axon Guidance	0.000	0.019	0.5	1	0.999	362	57.51	699
Axonogenesis in Innervation	0.011	0.015	0.2	1	0.146	3	0.15	19
Axon Regeneration	0.000	0.013	0.5	1	0.999	29	3.31	314
CNS Neuron Axonogenesis	0.051	0.031	0.020	0.2	0.028	26	6.32	935
Axon Extension	0.097	0.050	0.020	0.2	0.003	70	8.88	1,862
Regulation of Axonogenesis	0.130	0.059	0.009	0.09	0.001	104	20.85	3,239
Collateral Sprouting	0.012	0.019	0.3	1	0.26	13	3.10	396
Axon Target Recognition	0.000	0.010	0.5	1	0.999	4	0.27	34

[1] Heritability was estimated for sets of SNPs within ±10 kb of genes in children (subsets) of the GO Axonogenesis set.

[2] P-value from GCTA. Software upper limit for p-value is 0.5; maximal values are noted as 1.

[3] P-value corrected for ten observations.

[4] P-value from permutation analysis.

CYP2D6 Genotype and Adjuvant Tamoxifen: Meta-Analysis of Heterogeneous Study Populations

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The International Tamoxifen Pharmacogenomics Consortium was established to address the controversy regarding cytochrome P450 2D6 (*CYP2D6*) status and clinical outcomes in tamoxifen therapy. We performed a meta-analysis on data from 4,973 tamoxifen-treated patients (12 globally distributed sites). Using strict eligibility requirements (postmenopausal women with estrogen receptor–positive breast cancer, receiving 20 mg/day tamoxifen for 5 years, *criterion 1*), CYP2D6 *poor metabolizer status was associated with poorer invasive disease–free survival* (IDFS: hazard ratio = 1.25; 95% confidence interval = 1.06, 1.47; $P = 0.009$). However, *CYP2D6* status was not statistically significant when tamoxifen duration, menopausal status, and annual follow-up were not specified (criterion 2, $n = 2{,}443$; $P = 0.25$) or when no exclusions were applied (criterion 3, $n = 4{,}935$; $P = 0.38$). Although *CYP2D6* is a strong predictor of IDFS using strict inclusion criteria, because the results are not robust to inclusion criteria (these were not defined *a priori*), prospective studies are necessary to fully establish the value of *CYP2D6* genotyping in tamoxifen therapy.

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Received 3 June 2013; accepted 9 September 2013; advance online publication 18 December 2013. doi:10.1038/clpt.2013.186

Tamoxifen, the pioneering antiestrogenic medicine targeted to the tumor estrogen receptor (ER), is used successfully for long-term adjuvant therapy in breast cancer.[1,2] Extensive analyses of clinical trials demonstrate a major increase in patient survivorship in ER-positive patients. In this age of personalized medicine, any opportunity to improve response rates with tamoxifen should be rigorously investigated. Tamoxifen is considered a prodrug, given that hepatic cytochrome P450 2D6 (CYP2D6) metabolizes tamoxifen to metabolites (4-hydroxy tamoxifen and 4-hydroxy-*N*-desmethyl tamoxifen (endoxifen)) that exhibit significantly greater potency in terms of ER-binding affinity[3] and suppression of estradiol-stimulated cell proliferation.[4] CYP2D6-mediated metabolism is the rate-limiting enzymatic step for the formation of endoxifen, the most abundant active metabolite.

There has been great inconsistency among studies that have reported the association of known genetic and drug factors influencing CYP2D6 enzyme activity with tamoxifen efficacy. Therefore, the International Tamoxifen Pharmacogenomics Consortium (ITPC) was conceived, and researchers were invited to submit their data—both published and unpublished data sets regarding *CYP2D6* genetic variants and clinical outcomes in women treated with tamoxifen in the adjuvant breast cancer setting—to allow a meta-analysis of the potential associations between *CYP2D6* and clinical outcomes.

RESULTS

The ITPC comprises 12 research projects from nine countries and three continents that contributed clinical and genetic data for a total of 4,973 breast cancer patients treated with tamoxifen. In **Table 1**, we show the sample size by site and criteria. Further details for each site are shown in **S3c and S5 online**. We reported preliminary analyses of these collected cohorts before complete curation by pooling the data from each site.[5] For our meta-analyses, three detailed criteria, which ranged from the most restrictive (criterion 1) to the most inclusive (criterion 3), were defined before final curation (see **S4** online). In brief, criterion 1, derived from the NCCTG 89-30-52 clinical trial, consisted of postmenopausal women with surgically resected nonmetastatic invasive ER-positive breast cancers who received adjuvant tamoxifen monotherapy at a dose of 20 mg/day for an intended duration of 5 years, and were followed at least annually for recurrence. In addition, analysis of at least *CYP2D6*4* was required (detailed in **S4a** online). Criterion 2 included criterion 1 but allowed both pre- and postmenopausal patients who had received any duration of tamoxifen; moreover, annual follow-up was not required. Criterion 3 included all samples not excluded by any exclusion test for missing data or data inconsistencies (least restrictive). Patient characteristics according to each criterion are provided in **Table 2**.

The meta-analysis results combining the hazard ratio (HR) estimates (and the corresponding standard errors (SEs)) from each site are shown for all three criteria groups and both clinical outcomes in **Table 3**. For each of the six clinical outcome/criteria groups, we give the combined meta-analysis estimate across all 12 sites, its SE, and the results of two statistical tests: a test of the significance that the meta-HR differs from 1 and a test of "homogeneity of the estimates" across sites (a significant value for the latter test indicates that there is more variability than the derSimonian and Laird random-effects model can reasonably accommodate, suggesting that the meta-estimate and its associated *P* value are suspect). As can be seen for invasive disease–free survival (IDFS), the meta-analyses for criteria 2 and 3 are nearly significantly heterogeneous, whereas there was no indication of heterogeneity for criterion 1 ($P = 0.899$). For patients meeting criterion 1, the meta-HR for IDFS was 1.25 (95% confidence interval = 1.06, 1.47), and for breast cancer–free interval, it was 1.27 (95% confidence interval = 1.01, 1.61). These are both statistically significant, at $P = 0.009$ and $P = 0.04$, respectively. However, for the criterion 2 ($P = 0.25$) and criterion 3 ($P = 0.38$) subsets, the *CYP2D6* HR was not significant for either outcome.

In **Figure 1**, we show the individual HRs for each site for subjects meeting criterion 1, assuming an additive genetic model for *CYP2D6* (coded 0 = extensive metabolizer (EM), 1 = intermediate metabolizer (IM), and 2 = poor metabolizer (PM)) as estimated from a Cox proportional-hazards model using additional risk covariates to predict clinical outcome. Corresponding figures for criteria 2 and 3 are provided in **S6** online. (Note that the list of covariates used in the Cox models included age at primary diagnosis, menopause status at diagnosis, metastatic disease at primary diagnosis, maximum tumor dimension, number of positive nodes, grade, smoking status, ER and progesterone receptor status, intended tamoxifen dose and duration, systemic therapy before surgery, chemotherapy, radiation treatment, adjuvant aromatase inhibitor therapy, and additional hormone therapy. The specific set of covariates used for each site was chosen from this list so as to retain at least 70% of the patients from that site; hence, the exact set of covariates used differs in each site's Cox model. Moreover, several of these covariates were used as inclusion/exclusion items in the basic definitions of the three basic criteria subset groups and thus became irrelevant for those analyses.)

Table 1 Sample size by site and criteria

	Site (*N*)												
Criterion	1	2	3	4	5	6	7	8	9	10	11	12	Total
1	0	70	124	60	212	243	0	847	5	222	179	34	1,996
2	0	127	208	98	212	304	0	898	10	289	228	69	2,443
3	174	320	282	265	214	391	801	1,140	165	516	397	270	4,935
Total	174	320	282	267	214	423	801	1,140	165	519	398	279	4,973

Table 2 Baseline patient and tumor characteristics

Characteristic	Criterion 1 (1,996 patients)		Criterion 2 (2,443 patients)		Criterion 3 (4,935 patients)	
Age at diagnosis, years: data reported in binned ages						
Median	[65–69]		[60–64]		[60–64]	
Range	55 (ages 41–95)		75 (ages 21–95)		76 (ages 21–96)	
Menopausal status—no. (%)	Menopausal status	By age	Menopausal status	By age	Menopausal status	By age
Premenopausal (age ≤ 50)	0 (0.0%)	54 (2.7%)	241 (9.9%)	414 (16.9%)	607 (12.3%)	1,207 (24.5%)
Postmenopausal (>50)	1,688 (84.6%)	1,922 (96.3%)	1,714 (70.2%)	1,997 (81.7%)	3,267 (66.2%)	3,642 (73.8%)
Not available	308 (15.4%)	20 (1.0%)	488 (20.0%)	32 (1.3%)	1,061 (21.5%)	86 (1.7%)
Tumor size—no. (%): maximum dimension of tumor reported (if multiple tumors, largest one is ≤2 cm)						
≤2 cm	1,071 (53.7%)		1,327 (54.3%)		2,303 (46.7%)	
>2 cm	752 (37.7%)		882 (36.1%)		2,182 (44.2%)	
Unknown	173 (8.7%)		234 (9.6%)		450 (9.1%)	
Nodal status—no. (%): number of positive nodes						
Zero nodes	1,243 (62.3%)		1,531 (62.7%)		2,423 (49.1%)	
1–3 nodes	407 (20.4%)		461 (18.9%)		1,281 (26.0%)	
4–9 nodes	103 (5.2%)		111 (4.5%)		438 (8.9%)	
> 9 nodes	43 (2.2%)		45 (1.8%)		185 (3.7%)	
Not available	200 (10.0%)		295 (12.1%)		608 (12.3%)	
Grading—no. (%): 0.5 to 1.49 considered G1, 1.5 to 2.49 G2, etc.						
G1	249 (12.5%)		317 (13%)		456 (9.2%)	
G2	1,148 (57.5%)		1,324 (54.2%)		1,965 (39.8%)	
G3	330 (16.5%)		398 (16.3%)		838 (17.0%)	
Unknown	269 (13.5%)		295 (12.1%)		1,676 (34.0%)	
ER status—no. (%)						
ER-positive	1,996 (100.0%)		2,443 (100.0%)		4,675 (94.7%)	
ER-negative	0 (0.0%)		0 (0.0%)		158 (3.2%)	
Unknown	0 (0.0%)		0 (0.0%)		102 (2.1%)	
PgR status—no. (%)						
PgR-positive	1,479 (74.1%)		1,847 (75.6%)		3,634 (73.6%)	
PgR-negative	273 (13.7%)		302 (12.4%)		665 (13.5%)	
Unknown	244 (12.2%)		294 (12.0%)		102 (2.1%)	
Radiotherapy—no. (%): radiation therapy						
Yes	1,138 (57.0%)		1,412 (57.8%)		2,868 (58.1%)	
No	720 (36.1%)		842 (34.5%)		1,507 (30.5%)	
Unknown	244 (12.2%)		189 (7.7%)		560 (11.3%)	
CYP2D6 metabolizer status						
Extensive	893 (44.7%)		1,077 (44.1%)		2,286 (46.3%)	
Intermediate	985 (49.3%)		1,230 (50.3%)		2,311 (46.8%)	
Poor	118 (5.9%)		136 (5.6%)		244 (4.9%)	
Unknown	0 (0.0%)		0 (0.0%)		94 (1.9%)	
CYP2D6 metabolizer types						
EM/UM	17 (0.9%)		23 (0.9%)		49 (1.0%)	
IM/UM	2 (0.1%)		2 (0.1%)		4 (0.1%)	
EM/EM	874 (43.8%)		1,052 (43.1%)		2,233 (45.2%)	
PM/UM	7 (0.4%)		7 (0.3%)		12 (0.2%)	

Table 2 Continued on next page

Table 2 Continued

Characteristic	Criterion 1 (1,996 patients)	Criterion 2 (2,443 patients)	Criterion 3 (4,935 patients)
EM/IM	327 (16.4%)	407 (16.7%)	693 (14.0%)
EM/PM	496 (24.8%)	616 (25.2%)	1,230 (25.1%)
IM/IM	64 (3.2%)	94 (3.8%)	174 (3.5%)
IM/PM	91 (4.6%)	106 (4.3%)	192 (3.9%)
PM/PM	118 (5.9%)	136 (5.6%)	244 (4.9%)
Unknown	0 (0.0%)	0 (0.0%)	94 (1.9%)
DNA source			
Blood	996 (49.9%)	1,344 (55.0%)	2,513 (50.9%)
Tumor—Frozen	431 (21.6%)	500 (20.5%)	1,575 (31.9%)
Tumor—FFPE	569 (28.5%)	598 (24.5%)	659 (13.4%)
Normal—FFPE	0 (0.0%)	0 (0.0%)	174 (3.5%)
Unknown	0 (0.0%)	1 (0.0%)	14 (0.3%)

CYP2D6, cytochrome P450 2D6; EM, extensive metabolizer; ER, estrogen receptor; FFPE, formalin-fixed–paraffin-embedded; IM, intermediate metabolizer; PgR, progesterone receptor; PM, poor metabolizer; UM, unknown metabolizer.

Table 3 Meta-analyses of CYP2D6 HRs on clinical outcome in inclusion/exclusion criteria subsets

	IDFS				BCFI			
	Meta-estimates		*P* value		Meta-estimates		*P* value	
	HR	95% CI	Homog[a]	Association[b]	HR	95% CI	Homog[a]	Association[a]
Criterion 1	1.25	(1.06,1.47)	0.899	0.009	1.27	(1.01,1.61)	0.858	0.041
Criterion 2	1.17	(0.90,1.52)	0.055	0.249	1.21	(0.889,1.65)	0.130	0.224
Criterion 3	1.07	(0.92,1.26)	0.099	0.382	1.10	(0.868,1.35)	0.114	0.352

BCFI, breast cancer–free interval; Homog, homogeneity; HR, hazard ratio; IDFS, invasive disease–free survival; ITPC, International Tamoxifen Pharmacogenomics Consortium.

[a]The homogeneity *P* value tests the hypothesis that the individual ITPC site estimates meet the statistical random-effects modeling assumptions of the meta-analysis. A significant value indicates that there is significant heterogeneity among the sites, which casts doubt on the "combinability" of the studies for that parameter and on the validity of the corresponding association test. [b]The association *P* value tests the hypothesis that the combined meta-analysis estimate of the HR is significantly different from the null hypothesis value of HR = 1.

Site-specific product-limit estimates of the three CYP2D6 metabolizer status genotype groups (EM, IM, and PM) are shown in **Figures 2** and **3** for criterion 1 patients. Sites 1 and 7 had no subjects who met inclusion/exclusion for criterion 1. The corresponding figures for patients meeting criteria 2 and 3 are shown in **S6** online. As seen in **Figure 2**, for IDFS sites, 3, 5, and 8 show a strong significant effect in the direction expected by the known pharmacokinetic effects of *CYP2D6* on endoxifen exposure, namely, a poorer clinical response for the IM and/or PM genotype groups. Other sites show a trend in the expected direction between the IM and EM groups, but the much smaller PM group is often inconsistent with the expectation, and the separation in the three survival curves is not strong enough to reach statistical significance (e.g., sites 6 and 12). For some sites, there is no hint of any significant difference (e.g., sites 2, 4, 10, and 11), and for one of these, site 2, the direction of effect is exactly opposite than expected. There is a danger in overinterpreting such "trends" (either in favor or against expectation) when there is no statistically significant difference, because some level of site-to-site variation is to be expected. The key question is not whether such variation exists but whether it centers over the null hypothesis or over the alternative; this is the question that the meta-analysis is designed to answer. However, these simple product-limit survival curves show great study-to-study heterogeneity, which complicates both the analyses and the interpretation. We have similar heterogeneous results for the breast cancer–free interval outcome, shown in **Figure 3**. The corresponding figures in **S6** online show a similar pattern for the subsets of patients meeting criteria 2 and 3, although the heterogeneity seems to be even more pronounced as the exclusion criteria are loosened. This is not a surprising result, considering that the criteria themselves impose a certain level of homogeneity.

DISCUSSION

Prospective pharmacology studies consistently demonstrate that *CYP2D6* genetic variants are associated with variable plasma concentrations of endoxifen.[4,6] Endoxifen exposure is related to duration of tamoxifen use and dose, wherein an increase in the tamoxifen dose (from 20 to 40 mg daily) significantly increases endoxifen exposure in patients with reduced or null CYP2D6 metabolism but not in CYP2D6 EMs.[7] However, coadministration of CYP2D6-inhibiting drugs[4] reduces CYP2D6 enzyme activity, and nonadherence to tamoxifen is more commonly observed in patients with normal or increased CYP2D6 metabolism.[8]

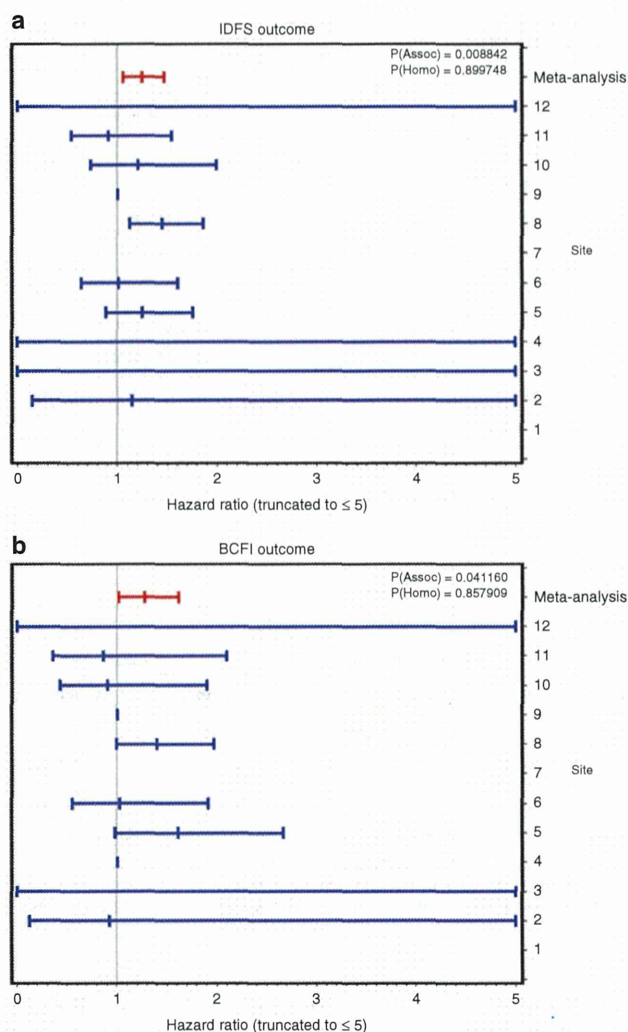

Figure 1 Individual site estimates of hazard ratios of *CYP2D6* genotype on clinical outcome, along with the meta-analyses for the criterion 1 subset. (**a**) Invasive disease–free survival (IDFS) outcome. (**b**) Breast cancer–free interval (BCFI) outcome.

Despite the consistent pharmacogenetic effects of *CYP2D6* on endoxifen exposure, there is considerable controversy regarding the validity of *CYP2D6* as a predictor of tamoxifen outcome.[9,10] Although recent secondary analyses from the Arimidex, Tamoxifen, Alone or in Combination (ATAC) trial and the Breast International Group (BIG) 1-98 study[11,12] did not demonstrate an association between *CYP2D6* and tamoxifen outcome, these studies provoked criticism due to concerns regarding genotyping error and the analysis of small subsets of the main trials.[13–16]

By contrast, a secondary analysis from another large prospective adjuvant tamoxifen trial, the Austrian Breast and Colorectal Cancer Study Group 8 (ABCSG 8), demonstrated that for women treated with 5 years of adjuvant tamoxifen at a dose of 20 mg/day, CYP2D6 PMs had a statistically significant higher odds of recurrence or death as compared with CYP2D6 EMs, and CYP2D6 PMs/IMs and PMs/EMs tended to exhibit a higher odds of recurrence as compared with patients without the PM alleles. However, this effect was not observed for patients who had switched to anastrozole, a drug not metabolized by CYP2D6. These data suggest that the effects of *CYP2D6* genotype may be masked if patients receive a shorter duration of tamoxifen or other active drugs besides tamoxifen, which alter the hazard for recurrence.[17]

We approached the tamoxifen controversy by performing a global meta-analysis of available clinical and *CYP2D6* genetic data of tamoxifen-treated breast cancer patients. All groups from across the world with both published and unpublished *CYP2D6* data were invited to participate. We initially presented a pooled analysis of these data,[5] in which we found no association between *CYP2D6* and IDFS. Following this presentation, we developed a new analysis plan (not defined before the initial negative presentation), which included the following: (i) articulation of three criteria to analyze the data according to the quality of the genetic and clinical data, (ii) additional curation to obtain missing clinical and genetic data, and (iii) a new statistical analysis plan, which applied a random-effects meta-analysis strategy instead of a pooled analysis strategy. Notably, Criterion 1 is most stringent, requiring strict control for as many pharmacologic factors as possible known to affect endoxifen exposure, which include use of tamoxifen monotherapy, genotyping of multiple *CYP2D6* alleles for accurate CYP2D6 phenotype assignment, use of one tamoxifen dose (20 mg), and intended duration of tamoxifen use for 5 years. In addition, eligibility for this cohort was restricted to women with invasive ER-positive status, postmenopausal breast cancer, and the requirement for annual follow-up, parameters required in any prospective clinical trial and that were requirements of criterion 1 (patients who were knowingly not followed were excluded from criterion 1), but not from criteria 2 and 3. These factors may have contributed to the substantial increase in heterogeneity comparing criterion 1 with criteria 2 and 3. However, it should be noted that these criteria impose a certain bias because the majority of negative studies submitted to the ITPC were observed in criteria 2 and 3.

In general, a substantial number of subjects comprising criterion 3 had misclassification of the predicted drug metabolism phenotype due to the lack of a comprehensive coverage of loss-of-function alleles.[18,19] More than 20 loss-of-function alleles out of 100 known *CYP2D6* genetic variants contributed to a frequency of ~8% of PMs in a population of European descent. Limiting the analysis to the most common such allele, *CYP2D6*4*, as was frequently done in the older published literature, will result in misclassification of 35% of PMs, thereby falsely assigning the undetected PMs to the EM or IM groups. Notably, 871/1,996 patients comprising criterion 1 had optimal CYP2D6 phenotype assessment obtained by AmpliChip genotyping, and this may have contributed to the robustness of criterion 1 results, which demonstrated an association between *CYP2D6* and tamoxifen treatment outcome (breast cancer–free interval: HR = 1.27, 95% confidence interval = 1.01–1.61).

The ITPC intended to perform a global study including several thousand patient samples; however, the majority of the subjects were not comprehensively genotyped because DNA was not of sufficient quality. We performed a subgroup analysis using patient samples for which full coverage of alleles by the

AmpliChip genotyping platform was available using criterion 1 (871/1,635 AmpliChip-genotyped subjects met criterion 1). When confined to the Amplichip subjects, the estimates of the pharmacodynamic HRs for *CYP2D6* were similar to what they were for the entire set of subjects meeting criterion 1.

A major source of potential genotyping errors may be related to DNA source. *CYP2D6* is one of the most difficult genes to genotype because of the numerous polymorphisms and adjacent pseudogenes. Some platforms cannot detect the presence of the *5 deletion, particularly in DNA derived from

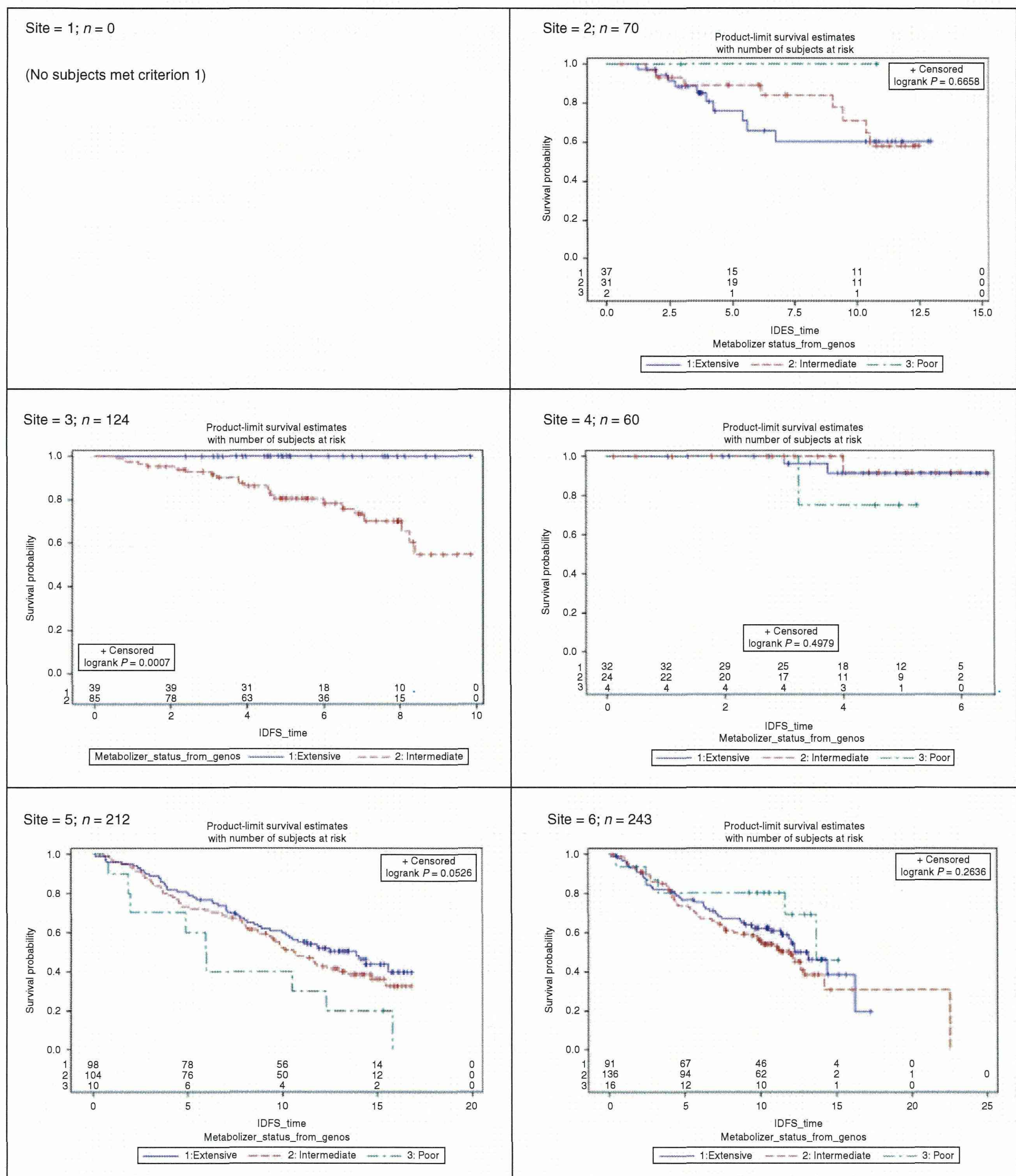

Figure 2 Site-specific effects of CYP2D6 metabolizer status on clinical outcomes for subjects meeting inclusion criterion 1 (outcome = invasive disease–free survival (IDFS)).

formalin-fixed–paraffin-embedded (FFPE) tissue. However, several sites used multiple platforms to validate their genotyping data, reducing potential genotyping errors across the entire data set. Importantly, *CYP2D6* genotypes obtained from blood-derived DNA reflect the patients' germ-line genotypes, known to influence endoxifen plasma concentrations. By contrast, *CYP2D6* genotypes from tumor-derived DNA may be subject to error due to somatic mutation by loss of heterozygosity, known to affect the *CYP2D6* locus at 22q13 in up to 30% of breast tumors.[20–22] Thus, when *CYP2D6* genotype is derived from tumor samples, an excess number of homozygotes may result as a consequence of loss of heterozygosity. This form of genotyping error is revealed by Hardy–Weinberg Equilibrium (HWE) testing, as was observed in the Breast

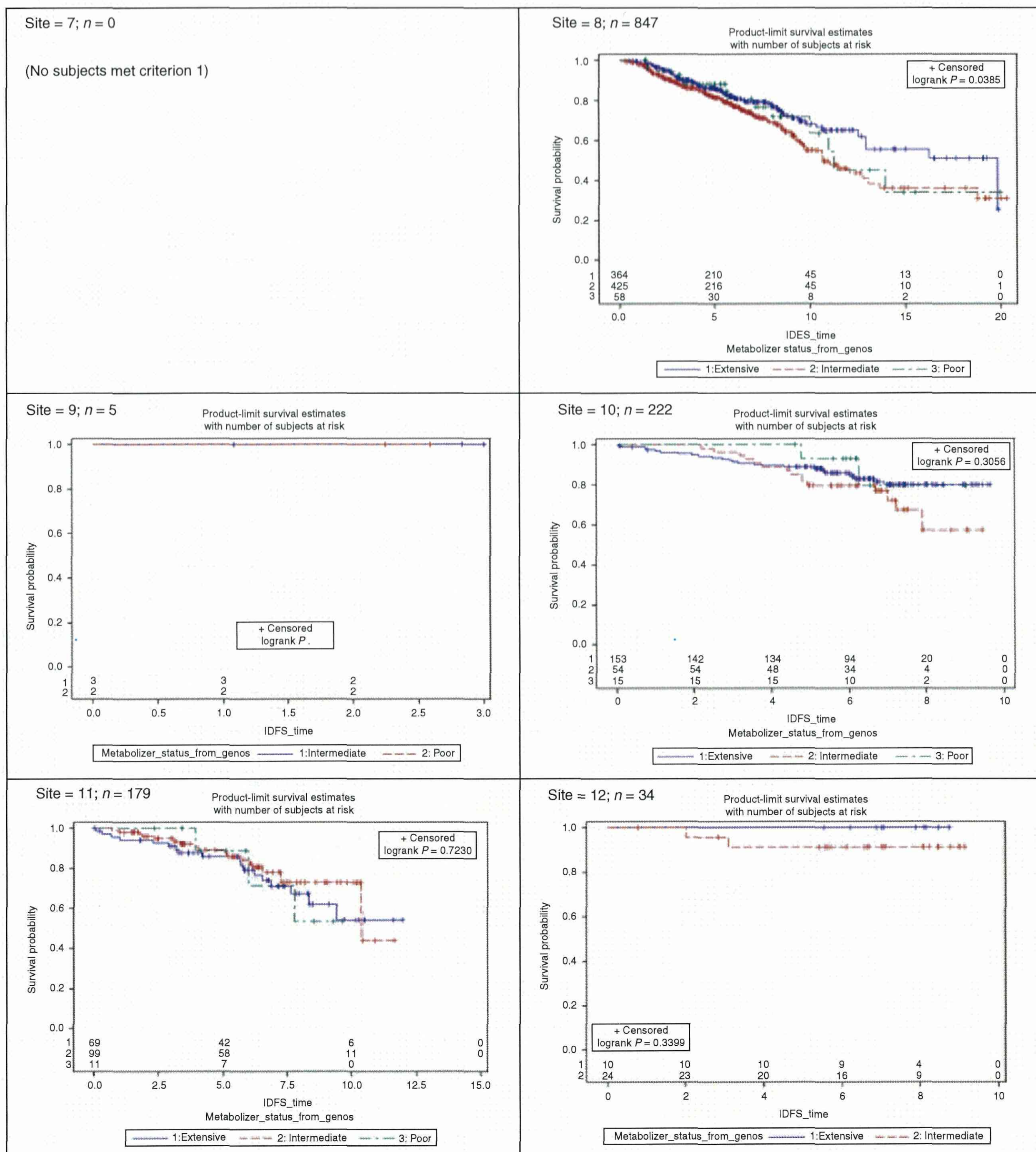

Figure 2 Continued

International Group 1-98 study, in which strong departures from HWE (to a magnitude of 10^{-92}) were observed, leading to a call for retraction of this article.[3,12,16]

For criterion 1, 49.9% of our patient DNA samples originated from blood, 21.6% from fresh-frozen tissues, and 28.5% from FFPE tissues. For criterion 2, 55.0% samples originated from blood, 20.5% were fresh-frozen tissues, and 24.5% from FFPE tissues. For criterion 3, 50.9% of DNA samples originated from blood, 31.9% from fresh-frozen tumor, 13.4% from FFPE tumor tissues, and 3.5% from FFPE normal tissue. Although we cannot exclude the presence of somatic events leading to misclassification of *CYP2D6* genotype, as evident from HWE deviation identified in data from

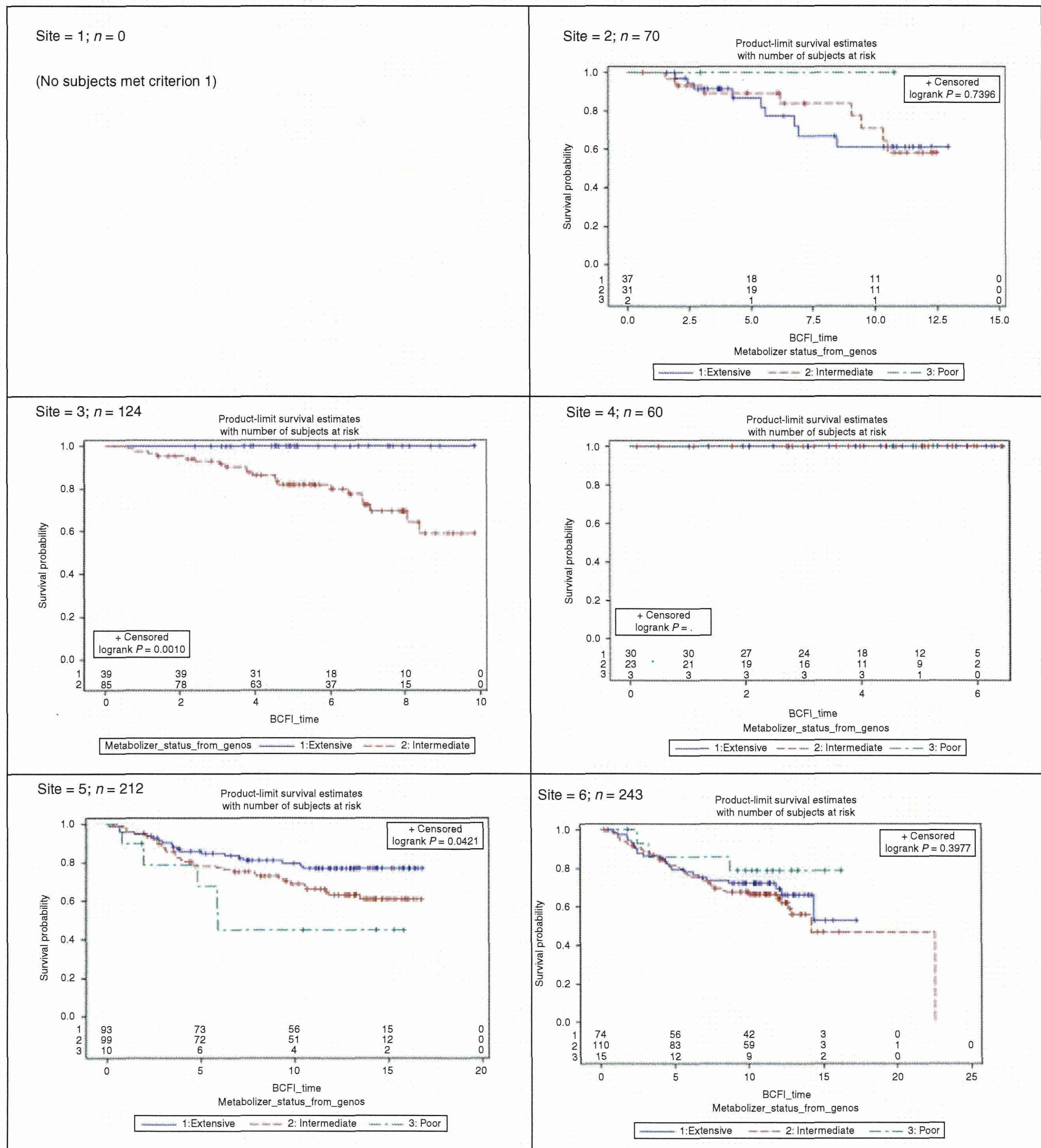

Figure 3 Site-specific effects of CYP2D6 metabolizer status on clinical outcomes for subjects meeting inclusion criterion 1 (outcome = breast cancer–free interval (BCFI)).

some sites, comprehensive testing for HWE did not reveal significant violations across most sites. Moreover, the extent of deviation from HWE in the *4 allele was not associated with sites that evinced less clinical benefit from tamoxifen in patients who were assessed to be PMs in terms of their *CYP2D6* status. This suggests that genotyping errors are unlikely to be a major issue in our analyses.

Our findings are subject to the shortcomings commonly encountered when performing retrospective "biomarker" studies. In our study, most sites were unable to collect or control for the factors known to alter endoxifen exposure, including dose and duration of tamoxifen administration and patients' adherence to the regimen. Although tamoxifen adherence is increasingly recognized as a critical factor for drug efficacy,[23] most studies evaluating tamoxifen biomarkers have not controlled for adherence. Other confounders include limited *CYP2D6* allele coverage and lack of information

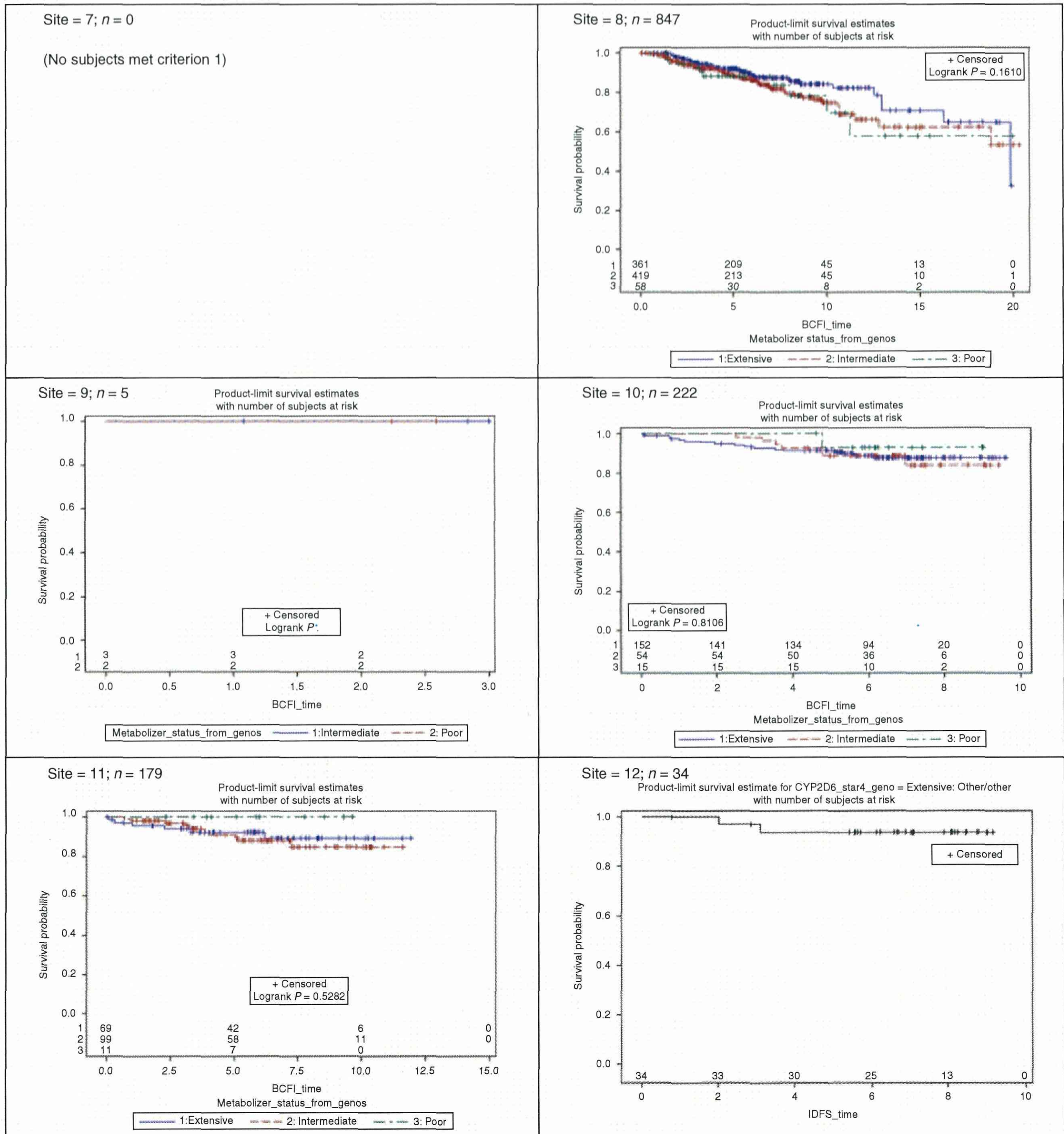

Figure 3 Continued