Multicenter study of genotype-guided tamoxifen dosing increases active metabolite exposure in women with reduced CYP2D6 metabolism

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Data Supplement

Appendix A

CYP2D6 genotyping

CYP2D6 genotyping was performed in a CLIA (Clinical Laboratory Improved Amendments)-certified clinical laboratory using the AmpliChip® CYP450 test (Roche Diagnostics, Indianapolis, IN) for the major CYP2D6 alleles, including the active alleles (*1, *2, and *35), inactive alleles (*3, *4, *5, *6, *7, *8, *11,*15, *19, *20, and *40), reduced activity alleles (*9, *10, *17, *29, *36, and *41), and duplication alleles (*1XN, *2XN, *4XN, *10XN, *17 XN, *35XN, and *41XN). Genomic DNA purified from whole blood was subjected to PCR amplification, labeling, and hybridization of the amplified products to glass microarrays bound with probes complementary to wild-type and mutant sequences. After staining of the bound products and scanning of the microarray, CYP450 genotype was determined by the AmpliChip® software based on the amount of hybridization to wild-type or mutant probes. The turnaround time for the CYP2D6 genotyping was 1-2 weeks from the time of the blood draw to the time of the patient notification. Treating physicians and patients (unless they opted “no”) were notified of their genotype group.

Metabolite determination

The HPLC-MS/MS was performed on an Applied Biosystems (Foster City, CA) model API 2000 triple-quadropole mass spectrometer, coupled with a Shimadzu USA (Addison, IL) HPLC system consisting of a model LC-20AB binary solvent delivery pump and model SIL-20A HT autosampler. The separation system was comprised of a Luna 3-um C18-2 column (100 x 2.00 mm i.d.; Phenomenex, Torrance, CA) and a nitrile guard column (4 x 3.0mm; Phenomenex). A gradient elution profile was used to separate tamoxifen and its metabolites. The initial mobile phase consisted of 55% methanol and 45% (v/v) formic Acid (0.05%) in water (adjusted to pH = 3.5 with 10mM potassium hydroxide). The secondary mobile phase consisted of 90% methanol and 10% (v/v) Formic Acid (0.05%) in water (adjusted to pH = 3.5 with 10mM potassium hydroxide). The secondary mobile phase percentage was increased from 0 to 100% linearly from 0 to 15 minutes; the initial mobile phase conditions were resumed after 15 minutes and remained constant for an additional 10 minutes, allowing the column to equilibrate. The eluate was introduced, without splitting, at 0.200mL/min to the electrospray ionization source. Parent and fragment ions were detected in multiple-reaction monitoring (MRM) mode. The electrospray voltage was set at +5500mV and the dwell time 100ms per detection channel with unit mass resolution on the Q1 and Q3 mass analyzers. All data were collected in the positive ion mode with the temperature of the interface set at 500°C. Nitrogen was used as the nebulizer (10.00 psi), turbo/heater (50.00 psi), curtain (20.00 psi) and collision activated dissociation (10.00 psi) gasses. The multiple reaction monitoring (MRM) used for quantification were 372.3/72.1 amu for

tamoxifen, 358.28/58.06 amu for N-desmethyltamoxifen, 374.29/58.09 amu for endoxifen, and 388.25/72.1 amu for 4-hydroxytamoxifen.

Extraction procedures. To patient plasma sample (250µL), the internal standard diphenyhydramide (25µL of 10µg/mL in H2O) was added and extracted with ethylacetate under alkaline pH (1.0mL of 1.0 M Glycine/1.0 M NaOH buffer, adjusted to pH=11.3 with 85% phosphoric acid). After sample was centrifuged at 3600RPM for 15 minutes at 0°C, the organic phase was removed and evaporated to dryness using a speed vacuum. The residual sample was then reconstituted with 100µL of mobile phase and 25µL was injected onto the HPLC-MS/MS system. Tamoxifen and its metabolites were quantified by using the ratio of peak area of the metabolite to peak area of the internal standard and by using the slope of the standard curves constructed using known concentrations of tamoxifen and its known metabolites.

Statistical methods

The Generalized Estimating Equation (GEE) method with unstructured covariance matrix and identity link was used to compare the three genotype groups in terms of their patterns of change from baseline in the median endoxifen concentrations. The models were fit with covariates of genotype group (EM, IM and PM), time (baseline and 4 months) and their interaction terms. Separate models for the other metabolites and pro-drug (tamoxifen, N-desmethyltamoxifen, 4-hydroxytamoxifen and for the endoxifen/N-desmethlytamoxifen ratio) were established and fit with the above covariates.[*]

[*] Zeger SL, Liang KY: Logitudinal data analysis for discrete and continuous outcomes. Biometrics 42:121-130, 1986

Appendix B:

Table 4: Distribution of non-adherence by genotype group (% = % missing doses/group)

Genotype Group	N	Missing 5 or fewer doses Count (%)	Missing 6-10 Count (%)	Missing 11 or more Count (%)	Total (%)
EM	29	7 (24)	2 (7)	0 (-)	9 (31)
IM	51	21 (41)	7 (14)	5 (10)	33 (65)
PM	9	1 (11)	0 (-)	1 (11)	2 (22)
Total	89	29 (33)	9 (10)	6 (7)	44 (49)

Appendix C:

Table 5: Median endoxifen concentrations for the IM CYP2D6 genotype subgroups and change over time (all increased tamoxifen dose); the baseline endoxifen concentrations for EM/EM and EM/PM were significantly different to each other (p=0.0048), while the baseline endoxifen concentrations for EM/EM and EM/IM were not (p=0.27).

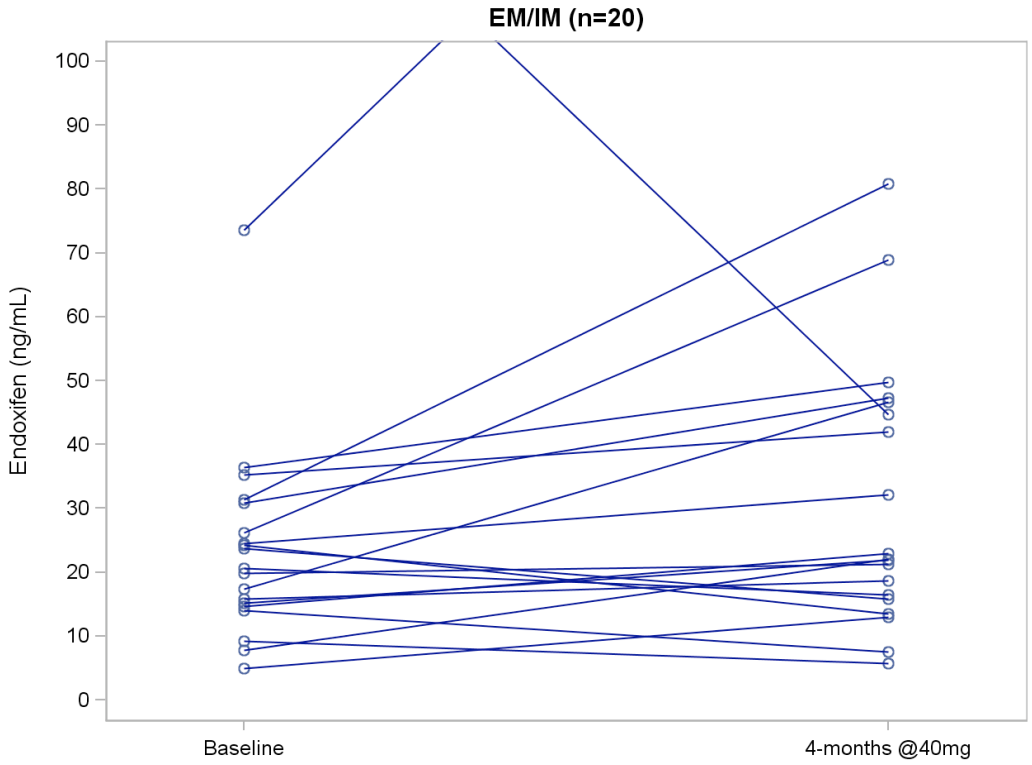
Genotype subgroup	N	Median endoxifen baseline (ng/mL)	Median endoxifen 4 months later* (ng/mL)	Median intrapatient change from baseline (Interquartile range) ng/mL	P-value**
EM/IM	20	20.2	21.9	+7.2 (-96.2 to 96.7)	0.021
EM/PM	19	18.5	22.0	+12.2 (-38.8 to 61.1)	0.0080
IM/IM	4	20.2	32.8	+19.7 (-7.8 to 37.3)	0.38
IM/PM	8	12.4	14.1	+2.5 (-10.8 to 7.7)	0.74

*Represents aggregate data within genotype groups, not intrapatient data

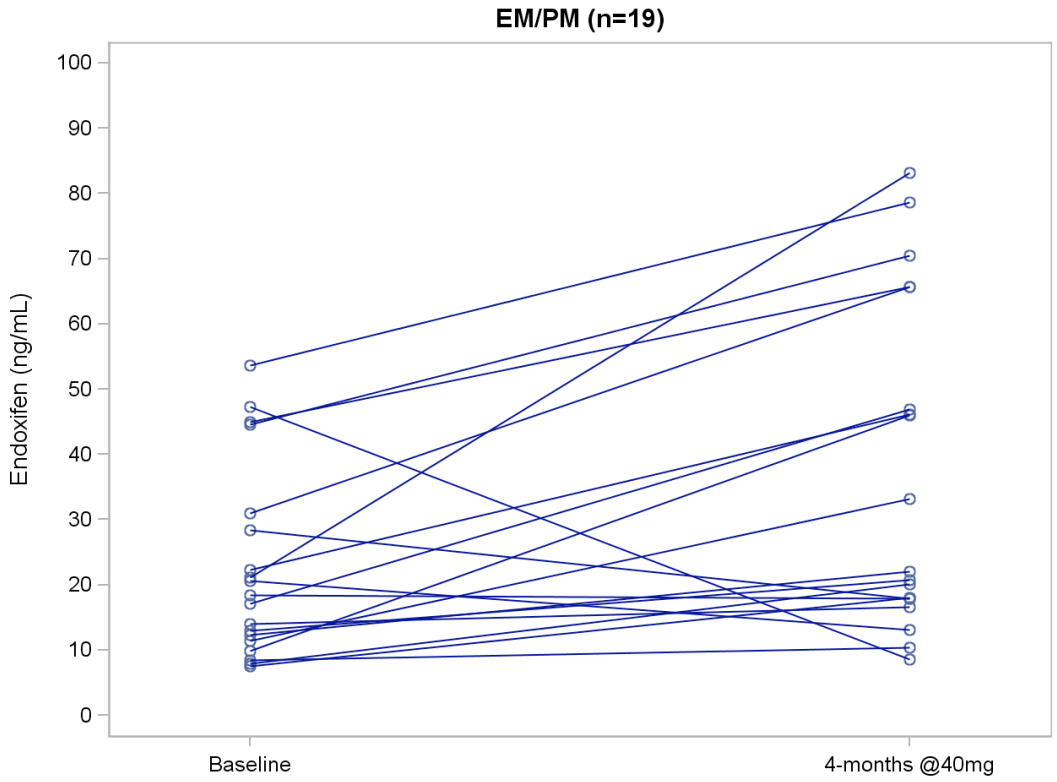
**Relates to the median intrapatient change from baseline

Figure 5: Endoxifen concentration change over time for the A. EM/IM, B. EM/PM, C. IM/IM and D. PM/IM (the IM genotype group) patients. The EM/IM group (p=0.021) and the EM/PM (0.0080) group show a significant increase in endoxifen concentration after 4 months of the increased dose of tamoxifen.

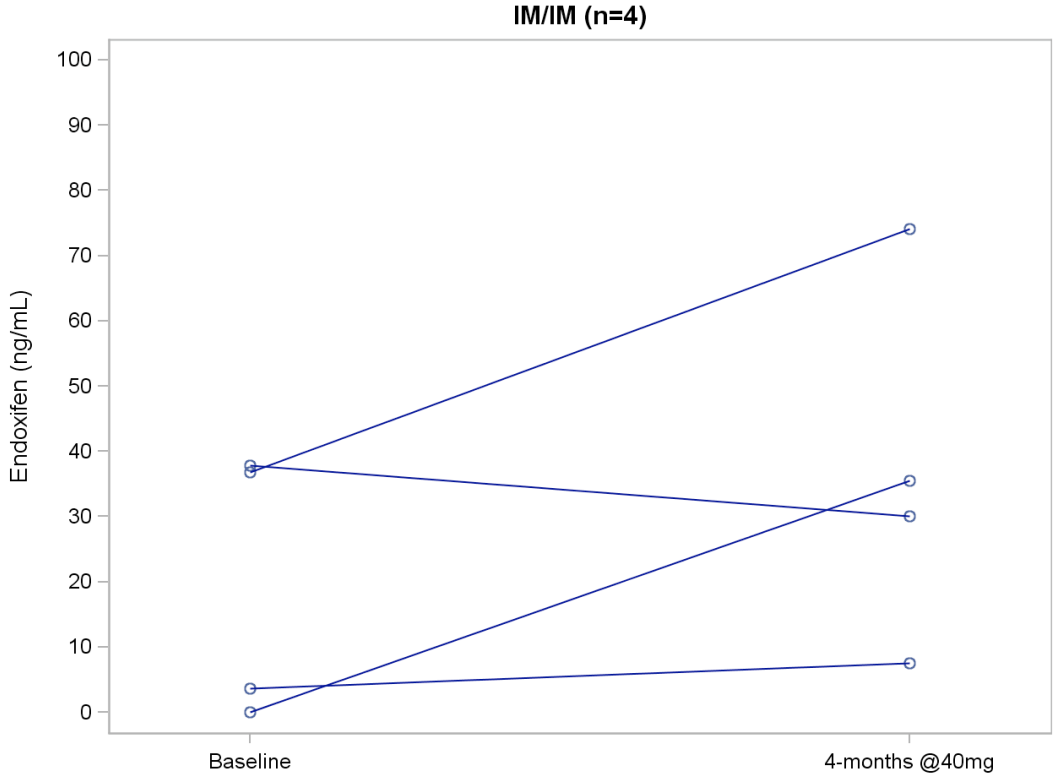
A. Value outside range of figure for 4-months = 170 ng/mL
and value outside figure for baseline = 141 ng/mL



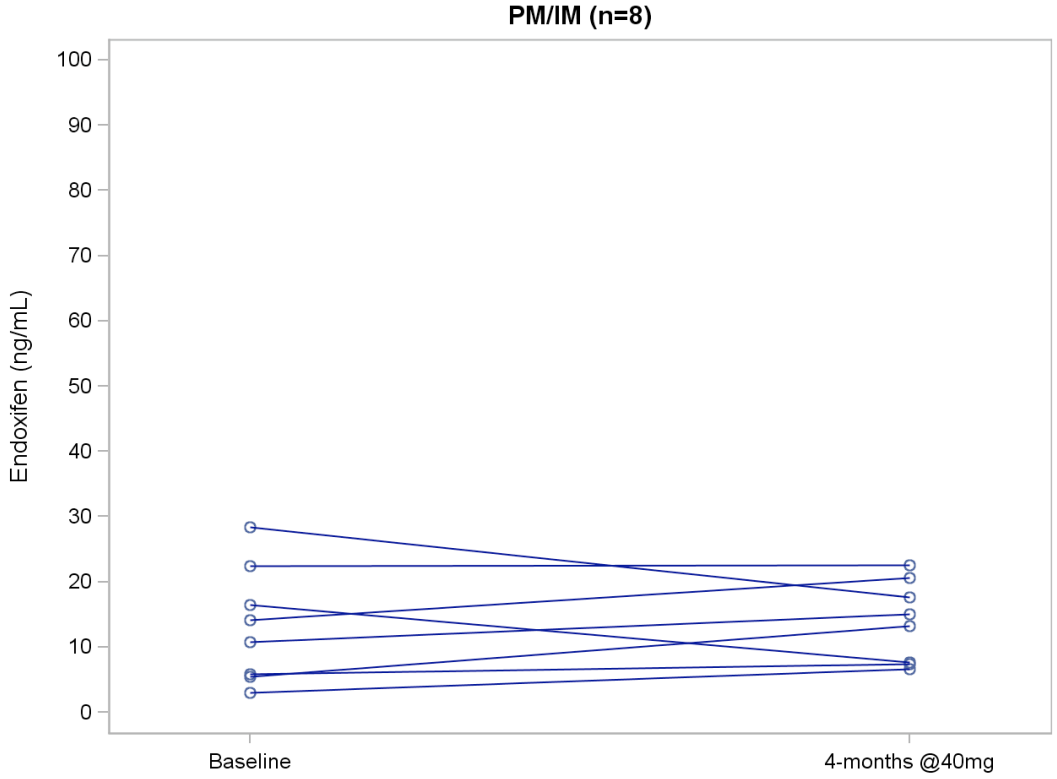
B.


EM/PM (n=19)
Endoxifen (ng/mL)
100
90
80
70
60
50
40
30
20
10
0
Baseline
4-months @40mg

C.


IM/IM (n=4)
Endoxifen (ng/mL)
100
90
80
70
60
50
40
30
20
10
0
Baseline
4-months @40mg

D.


PM/IM (n=8)
Endoxifen (ng/mL)
100
90
80
70
60
50
40
30
20
10
0
Baseline
4-months @40mg

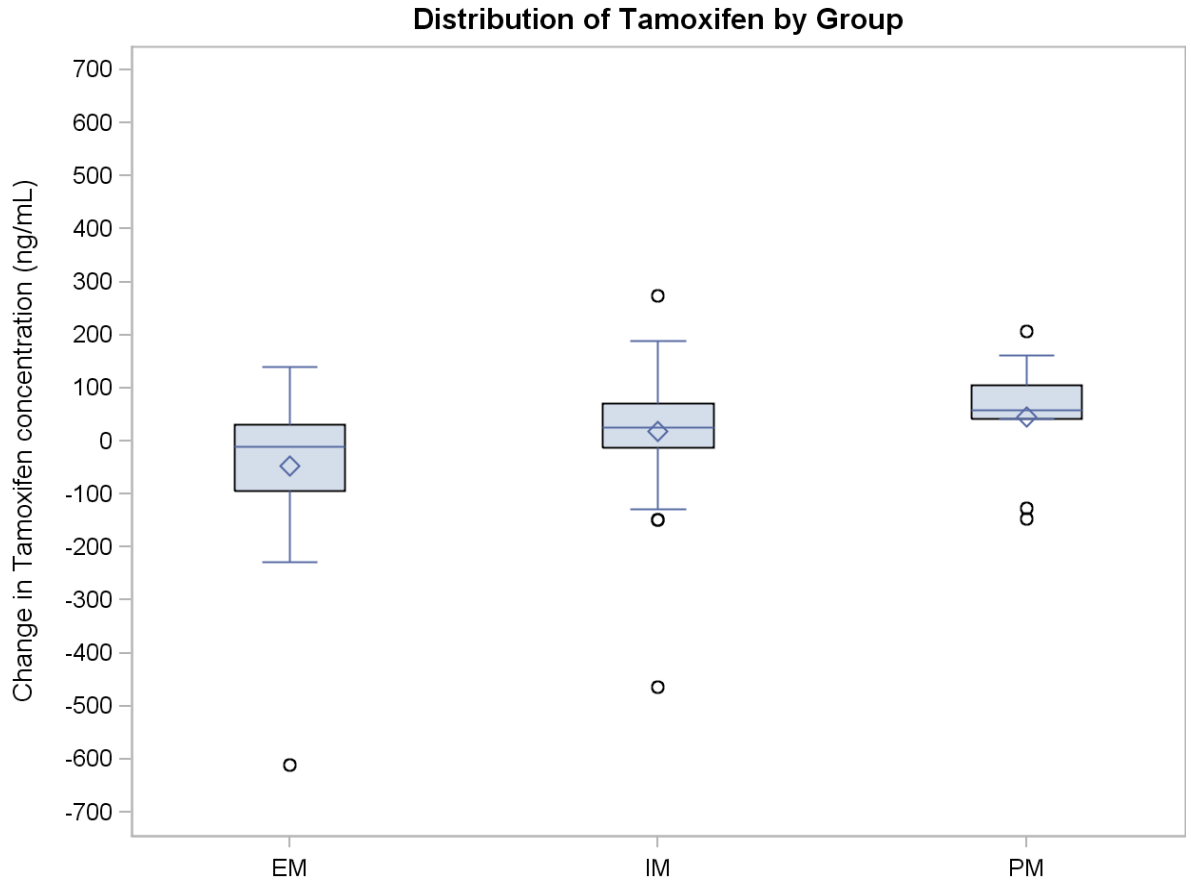
Appendix D:

For the EM group, there was no statistical difference in the concentrations of tamoxifen (p=0.17), N-desmethyltamoxifen (p=0.31), 4-hydroxytamoxifen (p=0.30), and the endoxifen/N-desmethyltamoxifen (E/NDT) ratio (p=0.29) between the baseline and 4 month timepoints. For the IM group, after increasing tamoxifen dose, there was a significant difference in the measured concentrations of tamoxifen (median change after the increase in dose, +25.0 ng/mL, p=0.033), N-desmethyltamoxifen (+58.4 ng/mL, p=0.023), 4-hydroxytamoxifen (+0.3 ng/mL, p=0.0094), and the E/NDT ratio (+0.00834, p=0.043). In the PM group, no statistical difference was detected in the concentrations of tamoxifen (p=0.30), N-desmethyltamoxifen (p=0.30), and 4-hydroxytamoxifen (p=0.055) between the different doses; however the E/NDT ratio (+0.00196, p=0.004) was significantly higher.

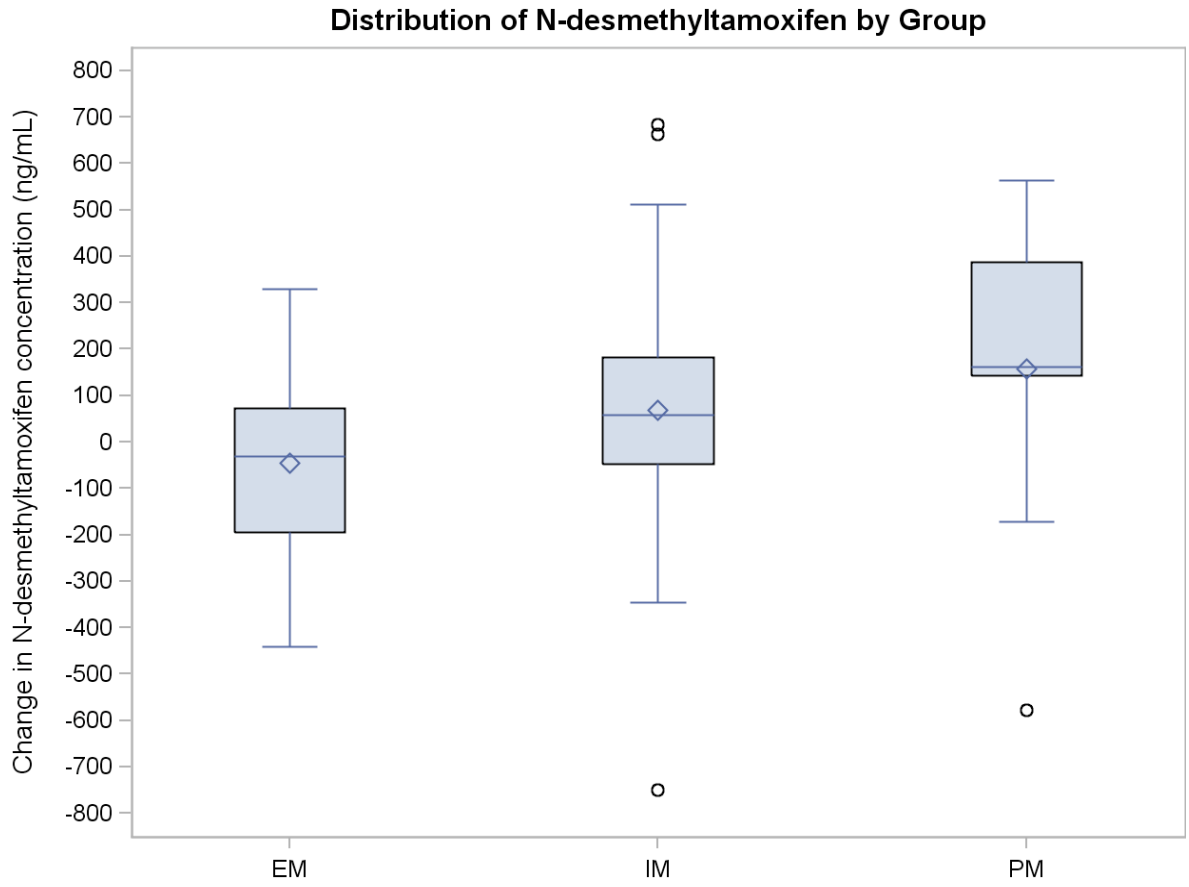
In GEE, the pattern of change between PM and EM was significant in tamoxifen (p=0.031, EM decrease), N-desmethyltamoxifen (p=0.037, PM increase) and in 4-hydroxytamoxifen (p=0.0041, PM increased compared to EM). Such change was also significant between IM and EM in tamoxifen (p=0.025, IM increased compared to EM) and in N-desmethyltamoxifen (p=0.33, EM had a higher increase). The pattern of change in the E/NDT ratio was not significant.

Figure 6: Change in tamoxifen and the other non-endoxifen metabolites over time by group. A. Change in tamoxifen by group. B. Change in N-desmethyltamoxifen by group C. Change in 4-hydroxytamoxifen by group D. Change in E/NDT ratio by group

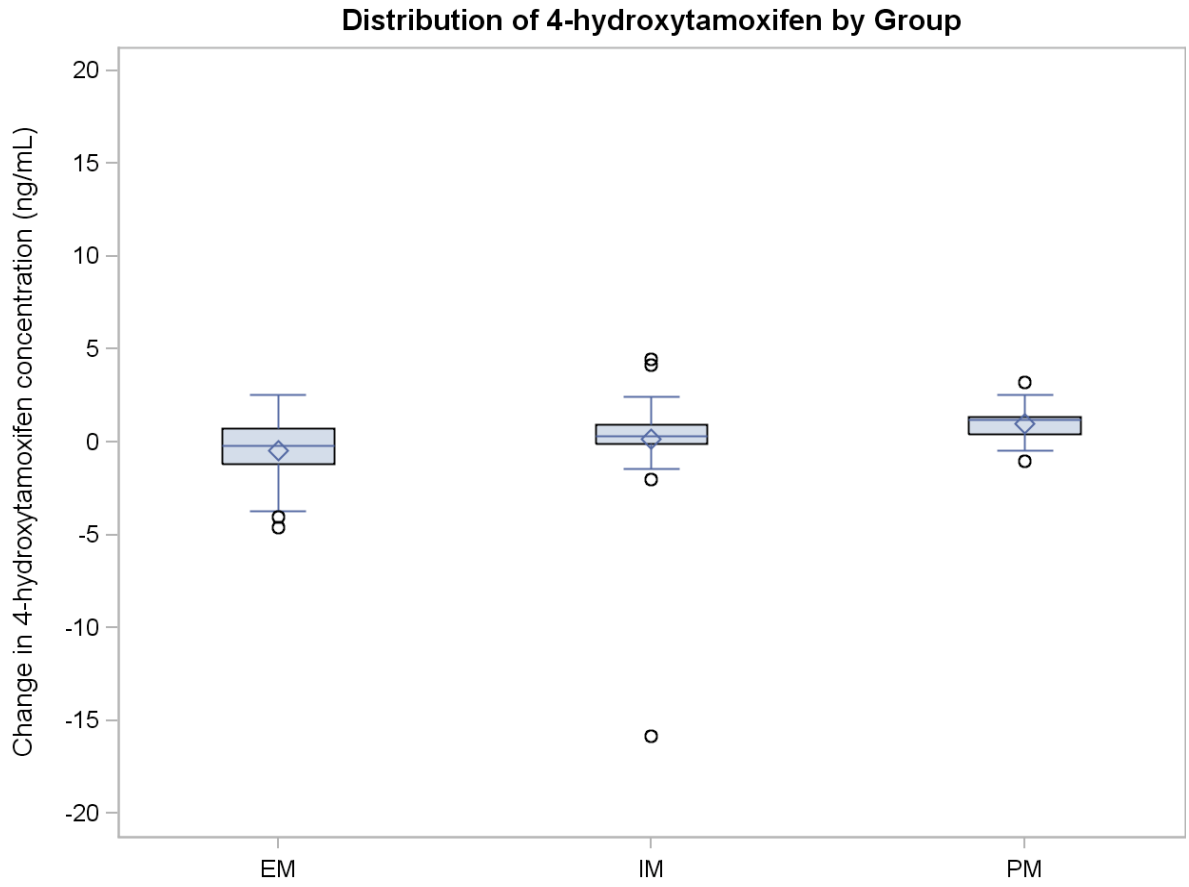
A. The change in the EM/UM patient (EM group) = -95 ng/mL, just below the lower quartile (-94.1 ng/mL) (baseline = 131 ng/mL; 4-month = 36 ng/mL)



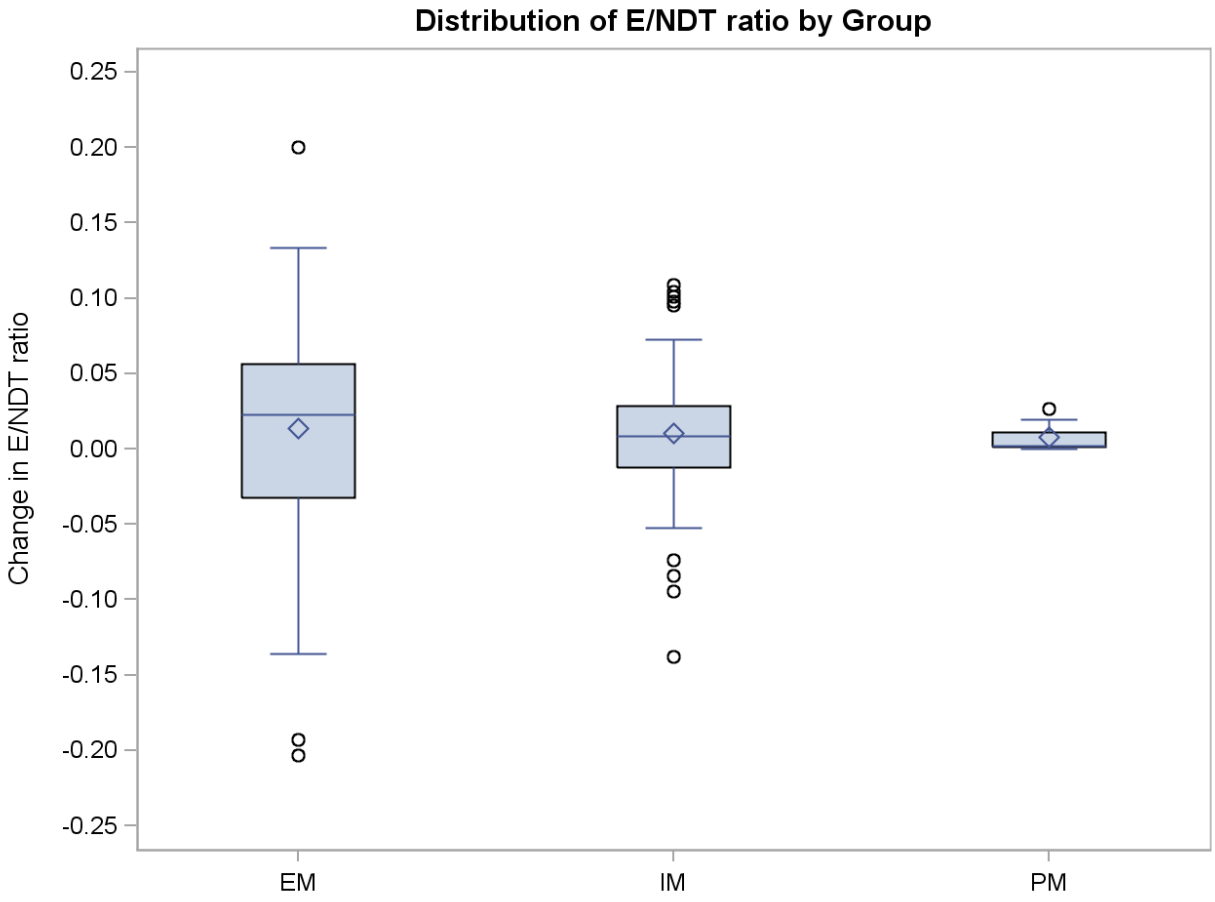
B. The change in the EM/UM patient (EM group) = -150 ng/mL, between the upper and lower quartiles (baseline = 269ng/mL; 4-month = 119 ng/mL)



C. The change in the EM/UM patient = -1.354 ng/mL, just below the lower quartile (-1.2 ng/mL) (baseline = 1.78 ng/mL; 4-month = 0.426 ng/mL)



D. The change in the EM/UM patient = 0.031, within the upper and lower quartiles (baseline = 0.137; 4-month = 0.168)



Appendix E:

The BCPT Menopausal Symptom Scale asks during the past 4 weeks, how much were you bothered by on a scale of 0-4 (not at all, slightly, moderately, quite a bit, extremely): hot flashes, nausea, vomiting, difficulty with bladder control when laughing or crying, difficulty with bladder control at other times, vaginal dryness, pain with intercourse, general aches and pains, joint pains, muscle stiffness, weight gain, unhappy with the appearance of my body, forgetfulness, night sweats, difficulty concentrating, easily distracted, arm swelling (lymphedema), and decreased range of motion (ROM) in arm on surgery side.

The FACT B Endocrine Subscale asks how true each statement has been for you during the past 7 days on a scale of 0-4 (not at all, a little bit, somewhat, quite a bit, very much): I have hot flashes, I have cold sweats, I have night sweats, I have vaginal discharge, I have vaginal itching/irritation, I have vaginal bleeding or spotting, I have vaginal dryness, I have pain or discomfort with intercourse, I have lost interest in sex, I have gained weight, I feel lightheaded/dizzy, I have been vomiting, I have diarrhea, I get headaches, I feel bloated, I have mood swings, I have breast sensitivity/tenderness, and I am irritable.

Table 6. Fact-B (es) analysis – Change in response score over time (by genotype group)

Fact-B (es)	p-values		
	EM	**IM**	**PM**
Q1 I have hot flashes	0.0598	0.8330	0.4398
Q2 I have cold sweats	0.0965	0.7328	0.2059
Q3 I have night sweats	0.9242	0.6516	0.5536
Q4 I have vaginal discharge	0.3227	0.1690	0.1624
Q5 I have vaginal itching/irritation	0.4093	0.7739	0.2446
Q6 I have vaginal bleeding or spotting	0.1582	0.6013	0.3257
Q7 I have vaginal dryness	0.8538	0.5495	0.5006
Q8 I have pain or discomfort with intercourse	0.8404	0.5772	0.7288
Q9 I have lost interest in sex	0.7190	0.4609	0.0686
Q10 I have gained weight	0.2596	0.6861	0.5017
Q11 I feel lightheaded/dizzy	0.1187	0.3638	0.3096
Q12 I have been vomiting	0.3178	0.3166	-*
Q13 I have diarrhea	0.3366	0.4794	0.2909
Q14 I get headaches	0.8650	0.1277	0.8598
Q15 I feel bloated	0.6746	0.7172	0.1239
Q16 I have mood swings	0.3531	0.2872	0.6708
Q17 I have breast sensitivity/tenderness	0.2787	0.2366	0.0762
Q18 I am irritable	0.7259	0.0028	0.1481

*=Not enough responses to calculate

1. Mean response score for Q18 in IM are:
Mean: Baseline (0.9) vs. 4-months (0.6)

	IM
Q18 I am irritable	
- Baseline	0.9
- 4-months	0.6

2. Mean score for Q1 (Hot flashes) across the groups are:

	EM	IM	PM
Q1 I have hot flashes			
- Baseline	2.3	1.9	2.5
- 4-months	1.9	1.9	2.3

Table 7. Fact-B (es) analysis – Change in response score among genotype group (by time)

Fact-B (es)	p-values	
	Baseline	**4-months**
Q1 I have hot flashes	0.1873	0.7147
Q2 I have cold sweats	0.8268	0.0900
Q3 I have night sweats	0.8608	0.7067
Q4 I have vaginal discharge	0.5819	0.1858
Q5 I have vaginal itching/irritation	0.6496	0.6252
Q6 I have vaginal bleeding or spotting	0.9420	0.2169
Q7 I have vaginal dryness	0.5296	0.2721
Q8 I have pain or discomfort with intercourse	0.9648	0.9976
Q9 I have lost interest in sex	0.3266	0.9635
Q10 I have gained weight	0.8352	0.7128
Q11 I feel lightheaded/dizzy	0.3498	0.6413
Q12 I have been vomiting	0.6019	0.6046
Q13 I have diarrhea	0.4787	0.0573
Q14 I get headaches	0.9558	0.5687
Q15 I feel bloated	0.2013	0.8419
Q16 I have mood swings	0.9410	0.6165
Q17 I have breast sensitivity/tenderness	0.0046	0.9476
Q18 I am irritable	0.2765	0.2845

1. For Q17 at baseline:
EM vs IM: p=0.5273

EM vs PM: p=0.0011

Mean response score: EM (0.9) vs. PM (0.0)

PM vs IM: p=0.0015

Mean: IM (0.8) vs. PM (0.0)

2. Mean response scores for Q1 (Hot flashes) across the time are:

		Baseline	**4-months**
Q1	I have hot flashes		
	- EM	2.3	1.9
	- IM	1.9	1.9
	- PM	2.5	2.3

Table 8. BCPT-Menopausal Symptom Scale – Change in response score over time (by genotype group)

Menopausal symptom scale	P-values		
	EM	**IM**	**PM**
Q1 Arm swelling	0.9584	0.9194	0.3209
Q2 Decreased ROM in arm on surgery side	0.6723	0.0032	0.6595
Q3 Difficulty concentrating	0.8662	0.4006	0.1988
Q4 Difficulty w/bladder control at other times	0.1392	0.2875	0.2533
Q5 Difficulty w/bladder control when laughing or crying	0.5924	0.4534	0.0395
Q6 Easily distracted	0.0111	0.8409	0.0181
Q7 Forgetfulness	0.3341	0.9216	0.4875
Q8 General aches and pains	0.4065	0.7227	0.8199
Q9 Hot flashes	0.1944	0.7046	0.3071
Q10Joint pains	0.1021	0.3123	0.3849
Q11Muscle stiffness	0.1353	0.3315	0.5553
Q12Nausea	0.7558	0.9196	0.2173
Q13Night sweats	0.5539	0.8333	0.3848
Q14Pain with intercourse	0.9016	0.6269	0.3995
Q15Unhappy with the appearance of my body	0.4520	0.5568	0.9499
Q16Vaginal dryness	0.9699	0.3343	0.8091
Q17Vomiting	0.3176	0.0832	-*
Q18Weight gain	0.8840	0.8179	0.6539

*=Not enough responses to calculate

1. Mean response scores for Q2 in IM are:

		IM
Q2	Decreased ROM in arm on surgery side	
	- Baseline	0.6
	- 4-months	0.2

2. Mean response scores for Q5 in PM are:

		PM
Q5	Difficulty w/bladder control when laughing or crying	
	- Baseline	0.1
	- 4-months	0.9

3. Mean response scores for Q6 in EM and PM are:

		EM	PM
Q6	Easily distracted		
	- Baseline	0.9	0.6
	- 4-months	1.3	1.2

4. Mean response scores for Q9 (Hot flashes) across the group are:

		EM	IM	PM
Q9	Hot flashes			
	- Baseline	2.1	1.7	2.2
	- 4-months	1.8	1.8	1.9

Table 9. BCPT-Menopausal Symptom Scale – Change in response score among genotype group (by time)

Menopausal symptom scale		**p-values**	
		Baseline	**4-months**
Q1	Arm swelling	0.8194	0.0395
Q2	Decreased ROM in arm on surgery side	0.5628	0.0249
Q3	Difficulty concentrating	0.6144	0.7619
Q4	Difficulty w/bladder control at other times	0.9543	0.1791
Q5	Difficulty w/bladder control when laughing or crying	0.0534	0.5096
Q6	Easily distracted	0.3739	0.5679
Q7	Forgetfulness	0.8540	0.9282
Q8	General aches and pains	0.9976	0.5449
Q9	Hot flashes	0.3650	0.9837
Q10	Joint pains	0.4163	0.4849
Q11	Muscle stiffness	0.7492	0.2657
Q12	Nausea	0.5229	0.9858
Q13	Night sweats	0.5575	0.7884
Q14	Pain with intercourse	0.9014	0.9439
Q15	Unhappy with the appearance of my body	0.7688	0.6331
Q16	Vaginal dryness	0.2061	0.1997
Q17	Vomiting	0.2124	-*
Q18	Weight gain	0.2949	0.4440

*=Not enough responses to calculate

1. Group comparison in Q1 & Q2

		EM vs. IM	EM vs, PM	IM vs. PM
Q1	Arm swelling		0.0383	0.0211
	- At 4-months, mean response score of: EM (0.3), IM (0.2), PM (0.0)			
Q2	Decreased ROM in arm on surgery side	0.0073		
	- At 4-months, mean response score of: EM (0.7), IM (0.2), PM (0.4)			

2. Mean response score for Q1 (Hot flashes) across the time are:

		Baseline	4-months
Q1	I have hot flashes		
	- EM	2.1	1.8
	- IM	1.7	1.8
	- PM	2.2	1.9

Appendix F:

Table 10: Relationship between hot flashes and endoxifen for the FACT-B (es) and the BCPT-MSS

FACT-B (es)

Response	Hot flashes	p-value
Endoxifen (baseline)	Baseline (continuous variable)	0.67
Endoxifen (4-month)	4-months (continuous variable)	0.0502
Endoxifen (baseline)	Baseline (in quartiles)	0.1241
Endoxifen (4-month)	4-months (in quartiles)	0.769

BCPT-MSS

Response	Hot flashes	p-value
Endoxifen (baseline)	Baseline (continuous variable)	0.97
Endoxifen (4-month)	4-months (continuous variable)	0.14
Endoxifen (baseline)	Baseline (in quartiles)	0.82
Endoxifen (4-month)	4-months (in quartiles)	0.96