Paper III

Sex hormones and gene expression in peripheral blood from postmenopausal women – the NOWAC postgenome study

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Abstract

The objective was to explore potential associations between sex hormones and gene expression in whole blood from a population based, random sample of postmenopausal women. Gene expression measured by the Applied Biosystems microarray platform was compared between hormone therapy (HT) users and non-users and between high and low hormone plasma concentrations. The gene expression matrix analysed 285 samples and 16185 probes. Gene-wise analysis revealed genes significantly associated with different types of HT use. Gene set analysis revealed 22 gene sets enriched between high and low estradiol concentration. Among them were seven estrogen related gene sets, including our gene list associated with systemic estradiol use, which thereby represents a novel estrogen signature. Seven gene sets were related to immune response. Among 15 gene sets enriched for progesterone, 11 overlapped with estradiol.
Gene expression associated with sex hormones is detectable in a random group of postmenopausal women, demonstrated by the finding of a novel estrogen signature.

Keywords

gene expression, microarray, postmenopausal, sex hormone, population based

Introduction

Previous reports have shown that there is an association between plasma/serum concentrations of endogenous sex hormones and risk of breast cancer among postmenopausal women.[1-3] The Women's Health Initiative[4] and large observational studies[5, 6] have also shown that use of postmenopausal hormone therapy (HT) increases the risk of breast cancer. Exogenous hormones have an influence on endogenous hormone concentrations. Systemically administered estradiol (E_2) containing HT suppresses plasma concentrations of follicle stimulating hormone (FSH), and increases E_2 and sex hormone binding globulin (SHBG) concentrations.[7-9] Tibolone use suppresses both FSH and SHBG concentrations in blood and increases free T due to lower SHBG levels.[10, 11]

Blood is a fluid connective tissue interacting with all other human tissues, and peripheral blood cells have been found to reflect system wide biology.[12, 13] Being easily accessible, peripheral blood could be an excellent surrogate tissue for exploring effects of environmental exposure on gene expression in large epidemiological studies. Microarray analysis of the blood transcriptome may shed light on etiologic pathways between environmental exposure and disease[13] and gene expression signatures are hypothesized to become important tools as diagnostic or prognostic biomarkers.[14]

Except for previous research in the Norwegian Women and Cancer study (NOWAC),[15, 16] population based studies on whole blood gene expression in postmenopausal women are scarce. However, some research on blood cells or tissue biopsies have reported gene expression patterns associated with HT and other menopause related variables.[17-22]

The population based NOWAC postgenome cohort study provides opportunities to conduct nested case-control studies implementing gene expression analyses of whole blood.[23] A first step would be to assess pre-disease impact of known risk factors for female cancer, e.g. circulating sex hormone levels or HT, on gene expression.

The objective of this study was to explore potential associations between different levels of endogenous and exogenous sex hormones and gene expression in whole blood from a random sample of postmenopausal women.

Material and methods

Subjects

An extensive description of NOWAC has been published elsewhere.[24] Briefly; NOWAC is a national, population based cohort study among women aged 30-70 years, with questionnaire data on lifestyle and health collected at 4-6 year intervals. Participants are randomly drawn from the Central Population Register. By June 2007, about 172 000 women were enrolled in NOWAC overall. The study is approved by The Regional Committee for Medical and Health Research Ethics and the Norwegian Data Inspectorate. The NOWAC postgenome cohort comprises questionnaire data and blood samples collected in 2003-2006 from approximately 50 000 women born 1943-57.[23] For the present analyses we used a randomly drawn group of 445 third time participants from the NOWAC postgenome cohort who donated a blood sample in 2005 (overall response rate 74%). The exclusion of subjects with incompletely filled blood collection tubes, >3 days from blood collection to frozen sample, or pre-/perimenopausal status, left 328 PAXgene (PreAnalytiX GmbH, Hembrechtikon, Switzerland) whole blood samples for RNA extraction.

Endogenous hormones measurements

Plasma concentrations of estradiol (E_2), progesterone (P_4), testosterone (T), Follicle Stimulating Hormone (FSH) and Sex Hormone Binding Globulin (SHBG) were measured by immunometry at the Department of Medical Biochemistry, University Hospital of North Norway, Tromsø, Norway[7]. For convenience, SHBG is referred to as a hormone throughout this text.

RNA isolation

Total RNA was isolated using the PAXgene Blood RNA Isolation Kit, according to the manufacturer's manual (PreAnalytiX GmbH, Hembrechtikon, Switzerland). RNA quantity and purity were assessed by the NanoDrop ND-1000 spectrophotometer (ThermoFisher Scientific, Wilmington, Delaware, USA). The absorbance ratio of 260 nm and 280 nm (A260/A280) was between 1.93 and 2.1 for all samples included for further analysis. The Experion automated electrophoresis system (BioRad, Hercules, CA, USA) and the RNA StdSens Analysis Kit was used to evaluate RNA integrity of a randomized 32% of the samples, according to the instruction manual. Thirty nine samples were excluded due to insufficient purity or yield.

Microarray-based profiling and image analysis

The samples were analysed using the Applied Biosystems expression array system (Foster City, LA, USA). From each sample 500 ng of total RNA was amplified using the NanoAmp RT-IVT labelling kit for one round of amplification (Applied Biosystems), in accordance with the manufacturer's manual. The quantity and purity of the cRNA was measured using the NanoDrop ND-1000. Digoxigenin (DIG)-labelled cRNA, 10 µg per sample, was fragmented and hybridized to Applied Biosystems Human Genome Survey Microarray V2.0, in accordance with the Chemiluminescence Detection Kit Protocol. Each microarray chip contains 277 control probes and 32 878 probes representing 29 098 genes. Applied Biosystems Expression System software was used to extract signal intensities, signal to noise ratios (S/N) and flagging values from the microarray images.

Data analysis

The data was analysed using R version 2.8.1 (http://cran.r-project.org), and tools from the Bioconductor project (http://www.bioconductor.org). For genes with a flagging value >8191 the expression intensity was set to missing. Samples where less than 40% of the probes showed S/N ≥3 were excluded. Probes with S/N ≥3 in less than 50% of the samples were filtered out. Subsequently we performed log transformation, quantile normalization and imputation of missing values using the k-nearest neighbourhood method (k=10). The gene expression values were adjusted for significant technical variables (i.e. array lot number, RNA extraction date and time between blood collection and storage) using gene-wise mixed linear modelling.[25] One sample was excluded as an outlier due to very high plasma E_2 and P_4 concentrations, a probable misclassification of menopausal status. The final expression matrix comprised 285 samples and 16 185 probes.

The effect of different HT regimens and hormone concentrations on the expression of individual genes were tested using linear model, limma,[26] adjusted for multiple testing using the false discovery rate (FDR).[27]

Gene sets defined from the limma analyses, curated from literature or found in publicly available web applications like KEGG (Kyoto Encyclopedia of Genes and Genomes)[28] or AmiGO[29] were tested for differential expression between groups with high and low hormone concentration using globaltest.[30] Supplementary Table 1 lists all the 56 gene sets included in the analysis. Gene sets fulfilling the criteria $p<0.05$, FDR<0.25 and comparative $p<0.20$, were defined as differentially expressed. The comparative p-value denotes the percentage of random gene sets of the same size that would have a larger test statistic than the gene set in question. The same method was used to test gene set

enrichment between different categories of HT use versus non-use. Core genes for each significant gene set were defined as the genes with the highest influence on the differences seen (cut-off: z.score>1.5).

The analyses were adjusted for variables which were significantly different between the groups under study (i.e. age and/or body mass index (BMI)).

Information on specific genes was found at GeneCards® (www.genecards.org). Some genes were not assigned an approved gene symbol and are referred to as unassigned or by the Celera Gene ID if provided by the Applied Biosystems annotation.

Searches for gene networks and pathways were performed using DAVID (the Database for Annotation, Visualization and Integrated Discovery)[31] and HEFalMp (Human Experimental/Functional Mapper).[32]

Endogenous hormones were analysed as dichotomized variables: high (fourth quartile) versus low (first quartile) of hormone concentration, with cut-offs defined among non-users of medication. Users of HT and thyroxine (T_4) were excluded from these analyses.

Exogenous hormones were categorized as use of systemic E_2 or E_2/progestogen(P) (tablets or patches), systemic E_2 alone (patches), tibolone or total HT. Although not defined as postmenopausal HT, T_4 use was also defined as a category. The different types of exogenous hormones were compared with non-use. Users of other medication (e.g. blood pressure lowering agents, antibiotics, antihistamins etc.) were excluded from these analyses.

Results

Among 285 women, 182 were medication users (52 used HT, 159 used other medication). Table 1 shows the participants characteristics of the study sample.

Table 2 shows the results from limma analysis, comparing different categories of HT with non-users (users of other medication excluded). Five genes were significantly associated with HT use, and 9 genes with FDR<0.28. The list of differentially expressed genes associated with use of "E_2 or E_2/P systemic" treatment was the longest (n=33).

Overlap between the gene sets from the three HT categories that contain estradiol is shown in figure 2. Tibolone and T_4 use resulted in 400 and 8 differentially expressed genes, respectively. Due to the limited number of tibolone only users (n=2), a complementary analysis comprising all tibolone users was conducted. With an FDR cut-off at 0.50 we found 58 genes, no genes had FDR<0.41. Among these 58 genes, 21 were significantly associated with the tibolone only users.

Using functional annotation clustering in DAVID, the "E2 or E2/P systemic" gene set was enriched in one cluster (enrichment score 1.67) which included six general cellular component GO-terms. Among them was "intracellular membrane bound organelle" with the lowest FDR (0.17). The "E2 alone" gene set and the T_4 gene set revealed no enriched clusters. HEFalMp predicted a few gene networks for the tibolone gene set (Figure 4). The tibolone gene set revealed no significantly enriched annotation clusters in DAVID, but one individual GO-term, "alcohol metabolic process", based on four genes (ALDH2, PRDX1, PDIA and PNPO) was significant (FDR=0.06).

Comparing the high and low hormone concentrations using a gene-wise approach (limma, HT and T_4 users excluded), two genes of unknown biological function (*DGCR9*

and *hCG2018460*) were significantly differentially expressed between high and low levels of FSH. No genes were significant for any of the remaining hormones. However, we observed significant gene sets enriched between high and low concentrations of E2 and P4 (Table 3, HT and T_4 users excluded).

Out of 22 gene sets significant for E_2, seven were estrogen or HT related, including the "E_2 or E_2/P systemic" gene set. Seven gene sets extracted from four publications were related to white blood cells and immune response while the remaining eight (five publications) were related to proto-oncogenes, exercise, age, carbohydrate/protein breakfast, transcription factors and drug metabolizing enzymes. Among the 15 gene sets differentially expressed for P_4, 11 were also significant for E_2, although with slight differences in core genes. Among the 186 different core genes, 151 (81%) were up-regulated in low E_2/P_4. Among these, 71 (47%) were present for E_2, 34 (23%) were present for P_4 while 45 (30%) appeared on both lists. Among the 35 core genes up-regulated in high E_2/P_4, 19 (54%) genes were present for E_2, 14 (40%) were present for P_4 while 2 (6%) were on both lists. Investigating FSH, SHBG and T, no gene set filled all three criteria for differential expression in the gene set analysis. Of note, the same analysis conducted without excluding HT and thyroxine users only marginally altered the ranking of the gene sets by p-value, but the p-values and FDRs increased slightly (data not shown).

Our "E_2 or E_2/P systemic" gene set was only significant for E_2 which thereby represents a novel estrogen gene expression signature. Among the six core genes for the "E_2 or E_2/P systemic" gene set (Figure 3), five have unknown function while *RAI1* may function as a transcription regulator. Comparing the direction of gene expression (up- or down-regulation) for the "E_2 or E_2/P systemic" gene set between endogenous and

exogenous E_2 (Figures 2 and 3), we found 61% concordance for all 33 probes and 80% concordance among the top 10 influencing probes. The second most influential gene (*C3orf14*) was up-regulated in high E_2 but down-regulated among users of systemic E_2 relative to non-users. Gene number three (*LOC344178*) was down-regulated in high E_2 but up-regulated among users of systemic E_2.

Discussion

This study confirms that a population based cohort study like NOWAC provides the opportunity to use high throughput technology, e.g. microarray analysis, to explore biologic variation in gene expression related to both endogenous and exogenous sex hormones.

Hormone concentrations did not show a profound influence on gene expression according to the gene-wise analysis. This is not surprising given the low variability that is present in a study group representing the general postmenopausal population. Conversely, all categories of HT use produced differentially expressed genes when compared with non-users. This is attributable to the wider range of hormone concentrations between the groups in this analysis. Intake of exogenous E_2, particularly by systemic administration, increases endogenous E_2 and suppresses FSH toward premenopausal plasma levels.[7] Apart from the direct hormonal effects, a probable cause is the supply of synthetic medical substances, e.g. tibolone, progestogens and their metabolites, to the blood.

The gene set enrichment analysis showed a fair amount of overlap between P_4 and E_2, a plausible result considering the positive correlation between the two hormones ($r=0.43$, $p<0.01$). Among the 58 subjects present in both the E_2 and the P_4 analyses, 49 were concordantly in low or high group for both hormones. Hence, it might be difficult to disentangle gene expression associated with E_2 and P_4. Still, there are differences. For instance, the "estrogen up-regulated" gene set (Frasor/KEGG) were only significant for E_2, and though the total "estrogen regulated" gene set (Frasor/KEGG) was significant for both E_2 and P_4, there was no overlap between the core genes up-regulated in high

group. In general, we found a much larger overlap of core genes up-regulated in low than high group for the gene sets significant for both hormones.

The "E_2 or E_2/P systemic" gene set turned out to be a more reliable estrogen signature than the "E_2 alone" gene set, probably due to the inclusion of oral high-dose E_2 users (n=7) and/or the generally larger group of users in the "E_2 or E_2/P systemic" category. Interestingly, as opposed to most of the other significant gene sets, the majority of the genes in this gene set were up-regulated in the high E_2 group. There was a high although not complete concordance in the direction of gene expression between endogenous and exogenous E_2 for this gene set. Opposing directions for some genes might be due to progestogen content in several of the products in this HT category, or possibly differential feed back mechanisms between endogenous and exogenous hormones. Further research might reveal the functions and regulation of these core genes. The tibolone and thyroxine gene sets did not meet our significance criteria for any of the hormones. One might have expected some association with FSH, but the number of users in these categories was probably too small to generate reliably specific expression sets.

None of the gene sets were differentially expressed between high and low levels of FSH, SHBG or T. Compared with the wide variety of target tissues and acknowledged effects of steroid hormones, FSH and SHBG would be expected to have a more limited association with gene expression. The biological effect of FSH is essentially the stimulation of gonadal E_2 and P_4 synthesis, and in postmenopausal women FSH has lost its gonadotropic potency. Although SHBG is suggested to possess some signalling properties,[33] it is mainly a transport protein. Adding the moderate variation in FSH and SHBG levels across the study population, a difference in gene expression might be

difficult to detect. Testosterone is not a main steroid in women, and though a potent steroid, differences in gene expression relative to low levels of T are probably non-detectable in a high background variability setting.

Seven gene sets related to immune responses or cells active in the immune system were differentially expressed between high and low E_2 concentration. Additionally, two gene sets associated with exercise (stress response and inflammatory response) and the proto-oncogene gene set could be viewed as immune system related. Sex hormones have been found to influence the immune system through steroid receptors in white blood cells[34]. In general, female sex hormones are viewed as suppressors of the immune response. It has been shown that plasma levels of both interleukin 6 (IL6) and interleukin 2 (IL2) increases after menopause, i.e. with decreasing levels of E_2, and that HT opposes this effect.[34] Though neither IL6 nor IL2 are among the 16 185 probes in our data set, the higher expression of the respective receptors, *IL6R* and *IL2R*, in low E_2 concentration indicates suppressive effects of E_2. Other interesting core genes include the heat shock proteins (*HSP*s) in the "Stress response from exercise" gene set. *HSP*s function as intracellular chaperones for other proteins (integrity and folding) and some have been found to play a role in rapid non-genomic effects of steroid hormones,[35] which is interesting considering the rapid responses seen for these genes following exercise.[36] *FOS* is a high influence core gene for both E_2 and P_4. In fact all *FOS* containing gene sets were differentially expressed. However, contradictory to Frasor et.al.,[37] *FOS* is up-regulated in the low E_2 category together with *EPB41L3* and *AP1G1*. On the other hand, *CXCL12*, the steroid 21-hydroxylase *CYP21A2* and *PDZK1* are congruously up-regulated in the high E2 category. According to Kendall et.al.,[20] *FOS* is up-regulated by estrogen deprivation which supports our results, while *SGK3* and *TAGLN* are down-

regulated, opposing our results. These contradictions in gene expression direction might arise from methodological differences or from regulations and feed-back mechanisms similarly to the above mentioned disconcordance for the "E_2 or E_2/P systemic" gene set. Though the differential expression of the tibolone gene set lacks statistical significance, the network mapping suggests that further research is warranted. A larger data set would contain a larger group of tibolone users and provide a more solid basis for finding tibolone associated genes.

Strengths and limitations

The NOWAC study subjects are randomly drawn from the Central Population Register and are representative for the population in which future microarray based diagnostic and/or prognostic tests for breast cancer will be applied. The fact that we were able to detect subtle effects in a dataset with high degree of random variation is reassuring. Among the limitations of this study is the lack of information regarding the relative proportions of peripheral blood cell types. If differences in hormone concentrations or HT use is associated with the number of particular type(s) of peripheral blood cells this may influence our results. Research into the influence of sex hormones on leukocyte cell count reveals conflicting results.[34] Although the women were healthy enough to visit a physician's office, we have limited information regarding disease and immune system status beyond what can be extracted from self reported drug use. However, a systematic difference in disease prevalence between hormone concentration levels is unlikely.

Our FDR cut-off of<0.25 might exceed conventional limits where FDR≤0.10 is considered acceptable.[38] However, at least when analysing gene sets curated from previous publications and thereby supported by research, a higher FDR can be accepted.

Also, in the gene-wise analysis we are not looking for singe genes, but for groups of genes that might explain known effects. For instance, among the 33 genes in the "E_2 or E_2/P systemic" gene set, 9 had FDR≤0.10, but only two of these genes were among the core genes differentially expressed between high and low E_2. Hence, using the ≤0.10 FDR cut-off we might have overlooked this estrogen signature.

Our results are based on a snapshot measurement; we have only one blood sample from each woman and can infer nothing about intra-individual variation or variation over time. However, previous reports have shown low intra-individual variation in gene expression compared with inter-individual variation.[39, 40]

The study design prevents an extensively standardized blood sampling protocol with regards to fasting, blood sample handling and transport etc. However, the main source of technical variation in this data set is associated with the performance of the assay and not with pre-analytical processing.[25]

The gene set enrichment analyses were adjusted for age and/or BMI. We found no significant differences between the compared categories regarding fasting and smoking. However, residual confounding may have influenced the differences found between high and low concentrations of E_2 and P_4.

Differentially expressed genes have not been validated using an independent data set. Our results must be interpreted accordingly.

In summary, we have identified a novel estrogen gene expression signature and further research may reveal the function of these genes in relation to E_2. A potential tibolone signature was also defined that warrants further research. Several gene sets, particularly immune system related, but also gene sets related to drug metabolism, exercise and

carbohydrate/protein ingestion, were differentially expressed between high and low levels of E_2 and P_4.

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Conflict of interest

The authors declare no conflict of interest.

Supplementary information is available at *The Pharmacogenomics Journal*'s website.

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Tables

Table 1 Participant characteristics given as mean (sd) or frequency (%)

Age, years		55.7 (3.6)		
BMI, kg/m^2		25.6 (4.3)		
Sex hormone concentration			Among women not using medication	
			1.quartile (low)	4.quartile (high)
			Cut-off (mean)	Cut-off (mean)
Estradiol mmol/L		0.10 (0.09)	<0.05] (0.05)	>0.08 (0.14)
Progesterone mmol/L		0.99 (0.67)	<0.55] (0.44)	>1.21 (1.79)
Testosterone mmol/L		1.16 (0.74)	<0.66] (0.50)	>1.54 (2.14)
FSH IU/L		70.2 (28.0)	<56.4] (44.5)	>91.2 (106.4)
SHBG mmol/L		47.6 (21.8)	<32.0] (24.2)	>61.0 (75.1)
Medication use		182 (64%)		
HT		52 (18%)		
E2 and E2/P systemic	32 (62%)			
E2 systemic alone	9 (17%)			
Tibolone	10 (19%)			
Vaginal treatment	9 (17%)			
Thyroxine		20 (7%)		
Other medication		159 (56%)		
No medication		98 (34%)		
Number of medications used (n=182)				
1 medication		97 (53%)		
2 medications		62 (34%)		
>2 medications		23 (13%)		
Current smoker				
Yes		75 (26%)		
No		209 (73%)		

Table 2 Number of genes differentially expressed between HT users and non-users (Limma analysis)

	Total sample	Single users[1]	Number of genes
HT type	**N**	**N**	**FDR<0.25**
HT all types	52	23	5(9)[2]
E_2 or E_2/P systemic	32	15	33
E_2 systemic	9	7	10
Tibolone	10	2	400
Tibolone2[3]	10	10	0(58)[3]
Thyroxine	20	5	8
Non-users	98	98	

[1] Users of other medication excluded.

[2] FDR<0.30, 5 gene with FDR<0.25

[3] All tibolone users included in the analysis, 58 genes with FDR<0.50

Table 3 Gene sets differentially expressed between low and high hormone concentration, age adjusted, HT and thyroxin users excluded

Gene sets	N total probes	N tested probes	p-value	FDR	Comp. p-value	Core genes[§] (probes) up-regulated in 1.quart. N	Gene symbols	Core genes[§] (probes) up-regulated in 4.quart. N	Gene symbols
Estradiol (N=99)									
Stress response from exercise[36]	15	12	0,004	0,08	0,01	5(4)	*DUSP5, HSPA1A/HSPA1B, HSPH1, HSPCA*	0	
T-cell receptor signaling, KEGG[28]	114	72	0,007	0,08	0,00	27(26)	*FOS, RELA, NFKB1, RHOA, CD3Z, LCP2, CD3E, CHP, NFATC1, NFATC3, PIK3CG, MAP3K8, PIK3R5, PAK1, CDK4, FYN, ZAP70, MAP3K14, PPP3CA, CARD11, CD40LG, GRB2, CBL, LCK, LAT/SPIN1, PIK3CD*	2	*HRAS, CD8B1*
* Estrogen responsive genes (GO:0043627)[29]	27	16	0,008	0,08	0,02	3(5)	*STAT3, CRIPAK, TGFB1*	1	*RNF14*
Neutrophil signature[41]	38	31	0,011	0,08	0,01	11	*IL6R, SIGLEC5, SLA, ZFP276, FPRL1, FPR1, CSF3R, GBP1, LYN, PSCDBP, PIK3CD*	1	*FANCA*
* Predictors of HT use[15]	112	52	0,011	0,08	0,02	9(10)	*PILRA, ACTC, TLE4, HLA-DQA1, GNLY, RNF24, IRF2, AVIL, GNAZ*	3	*C8B, GPR116, MALAT1*
* E2 or E2/P systemic	33	33	0,014	0,08	0,02	1	*LOC344178*	5	*RAI1, C3orf14, 3 unassigned (hCG1993395, hCG2002980.1, one obsolete)*
High carbohydr. and protein breakfast[40]	33	29	0,017	0,08	0,04	6	*SIGLEC5, DAPK1, PDCD4, C1QR1, KLRF1, DHRS9*	0	
Monocytes in PBMC signature[42]	61	50	0,017	0,08	0,03	10	*APLP2, ATP6V1B2, CDA, ADRBK2, BRI3, CCND2, SERPING1, NRGN, LCK, FES*	3	*RNASE3, RIRPB1, PTPNS1*
Transcription factors and drug metabolizing enzymes[43]	39	23	0,018	0,08	0,03	3	*NR1H2, CYP4F2, TCF7*	1	*GSTT1*
* Estrogen related, Frasor/KEGG, up-regulated[28, 37]	68	23	0,019	0,08	0,05	3(4)	*FOS, EPB41L3, AP1G1*	3	*CXCL12, CYP21A2, PDZK1*
Age[42]	15	9	0,019	0,08	0,05	3	*NEDD9, CHIC2, UTF1*	1	*HLA-DQB1*
Natural killer cells in PBMC sign.[42]	35	25	0,020	0,08	0,05	7	*CNOT2, KIR2DL4, CTBP2, MLC1, CX3CR1, KLRF1, CTSW*	1	*CD8B1*
Proto-oncogenes[44]	8	6	0,022	0,08	0,07	2	*FOS, NFKB1*	0	
Drug metabolizing enzymes[43]	23	12	0,022	0,08	0,04	1	*CYP4F2*	1	*GSTT1*
PBMC signature[41]	105	89	0,023	0,08	0,05	20(15)	*KIAA1219, GZMB, CSF1R, HLA-DRB1/HLA-DRB3, IL2RB, FAIM3, C1QR1, HLA-DQA1, GNLY, TRAJ17/TRDV2/TRAC/TRAV20/TRA@, PGD,*	1	*HLA-DQB1*

							TNFRSF7, CTSW, TRBV19/TRBC1		
	Trauma; down-regulated genes[39]	138	119	0,023	0,08	0,03	26(22)	*LEF1, LBH, FAM102A, CD3E, RABGAP1L, IL2RB, FAIM3, P2RY10, SPOCK2, TP53, HLA-DQA1, HNRPA1, GNLY, TRAJ17/TRDV2/TRAC/TRAV20/TRA@, RPS4X, KLRF1, NOV, PRF1, LCK, TRBV19/TRBC1, RARRES3*	1	*TNFRSF25*
*	HT use, core genes, incl. PNA[16]	19	12	0,024	0,08	0,06	3	*LEF1, FOS, TLE4*	1	*GPR116*
*	Estrogen related, Frasor/KEGG[28, 37]	175	79	0,026	0,08	0,05	11(14)	*FOS, KYNU, EPB41L3, KIAA0922, ABCG1, AP1G1, LITAF, DBN1, GNE, KLF6, KRT7*	3	*CXCL12, CYP21A2, PDZK1*
	Lymphocyte signature[41]	73	61	0,029	0,09	0,06	16(12)	*KIAA1219, GZMB, CSF1R, IL2RB, FAIM3, HLA-DQA1, GNLY, TRAJ17/TRDV2/TRAC/TRAV20/TRA@. TNFRSF7, CTSW, TRBV19/TRBC1*	0	
*	Response to estrogen deprivation, breast tissue[20]	57	18	0,032	0,09	0,12	3	*FOS, SGK3, TAGLN*	1	*MALAT1*
	Inflammatory response to exercise[36]	27	25	0,038	0,10	0,12	8	*IL6R, GZMB, IL2RB, NCR3, GNLY, CSF3R, PRF1, CTSW*	0	
	High interindividual variability genes[40]	28	24	0,044	0,11	0,14	4(4)	*HLA-DRB1/HLA-DRB3, HLA-DQA1, IFIT2,*	1	*HLA-DQB1*
Progesterone (N=104)										
*	HT use, core genes, incl. PNA[16]	19	12	0,005	0,14	0,010	4	*LEF1, FOS, CREB5, TLE4*	1	*GPR116*
	Interleukins[44]	17	5	0,007	0,14	0,018	2	*IL1B, IL15*	1	*IL7*
	Monocytes in PBMC signature[42]	61	50	0,008	0,14	0,005	16(18)	*SERPING1, BRI3, LMO2, CDA, ATP6V1B2, TIMP2, IGSF6, AP1LP2, CREB5, SERPINA1, FLJ20273, RAB31, PLSCR1, SLC31A2, BCL6, ADRBK2*	1	*RIN2*
	Stress response from exercise[36]	15	12	0,010	0,14	0,022	3(2)	*DUSP5, HSPA1A/HSPA1B*	1	*SPON2*
	High carbohydr. and protein breakfast[40]	33	29	0,016	0,14	0,030	8(8)	*SIGLEC5, DHRS9, PDCD4, PSAP, DAPK1, TNFSF13/TNFSF13-TNFSF12, HAL*	0	
*	Estrogen related, Frasor/KEGG[28, 37]	175	79	0,019	0,14	0,041	14(14)	*LITAF, FOS, KYNU, IFI30, ABCG1, AP1G1, KIAA0922, DBN1, RAB31, ENC1, IER3, HIST2H2AA/HIST2H2AC, CBX6*	5(6)	*RAP1GA1, TFF1, SELENBP1, ADCY9, AP1M2*
	Neutrophil signature[41]	38	31	0,022	0,14	0,048	9	*GBP2, GBP1, IL6R, SIGLEC5, LYN, LILRA2, CSF3R, BCL6, SLA*	0	
	Growth factor, transcription factor, excercise[36]	27	16	0,022	0,14	0,097	2	*FOS, ECGF1*	1	*PDGFRB*
	Proto-oncogenes[44]	8	6	0,023	0,14	0,077	2	*FOS, NFKB1*	0	
*	Estrogen responsive genes (GO:0043627) [29]	27	16	0,025	0,14	0,104	2(4)	*STAT3, TGFB1*	2	*TFF1, GH1*
	T-cell reseptor signalling, KEGG[28]	114	72	0,028	0,14	0,114	20(23)	*PAK1, CHP, PPP3CA, FOS, NFKBIE, LCP2, NFATC1, CDC42, RELA, MAP3K14, LAT/SPIN1,*	0	

							CRB2, MAP3K8, CD40LG, PTPN6, AKT1, NFATC3, GRAP2, NFKB1		
* Predictors of HT use[15]	112	52	0,030	0,14	0,108	11(10)	*PILRA, RNF24, GNAZ, AVIL, SLC12A6, CREB5, TLE4, IRF2, HIST2H2AA/HIST2H2AC, QPCT*	5	*GPR116, GPHA2, C8B, GPR75, SLC36A1*
* HT use, no globin reduction[16]	14	11	0,039	0,17	0,130	3	*FOS, CREB5, TLE4*	0	
* Response to estrogen deprivation, breast tissue[20]	57	18	0,048	0,17	0,179	2	*FOS, TAGLN*	1	*IFT122*
General cytokines[44]	11	5	0,050	0,17	0,156	3	*LTB, TGFB1, FAS*	0	

* Gene sets related to steroid hormones, § The core genes are listed according to z.score from highest to lowest (above 1.5).

Figures

Figure 1 Study population

Figure 2 Overlap between gene sets from three different HT categories, all including users of systemic estradiol.

Among the 9 HT genes, *LOC344178, SET, MGC4618* and *MORC2* have FDR <0.30, the remaining 5 genes have FDR<0.25. Gene symbols in red denote genes up-regulated in users and gene symbols in green denote genes up-regulated in non-users.

Figure 3 Gene plot of the "E_2 or E_2/P systemic" gene set (33 genes) in relation to E_2 concentration ("0"=low, "1"=high)

The reference line for each bar represents the expected height under the null hypothesis (i.e. that the gene is not associated with hormone concentration), marks indicate the number of standard deviations above the reference line.

Figure 4 The tibolone gene set (21 genes) in HEFalMp

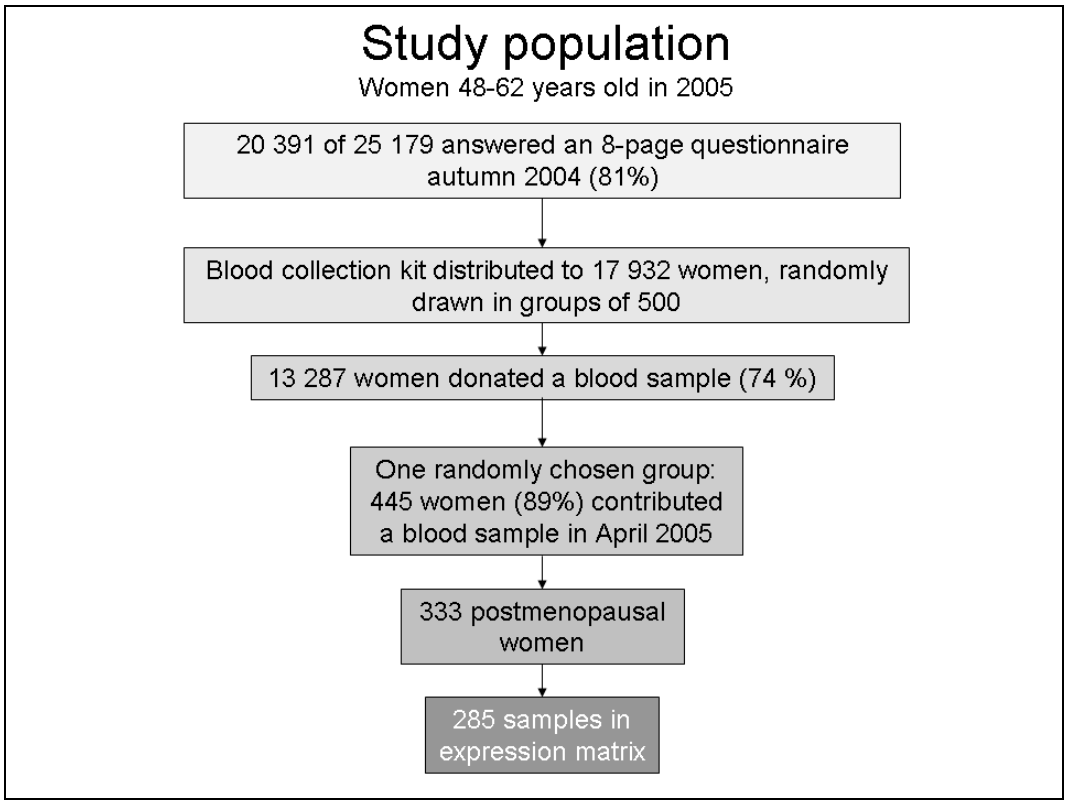

Figure 1

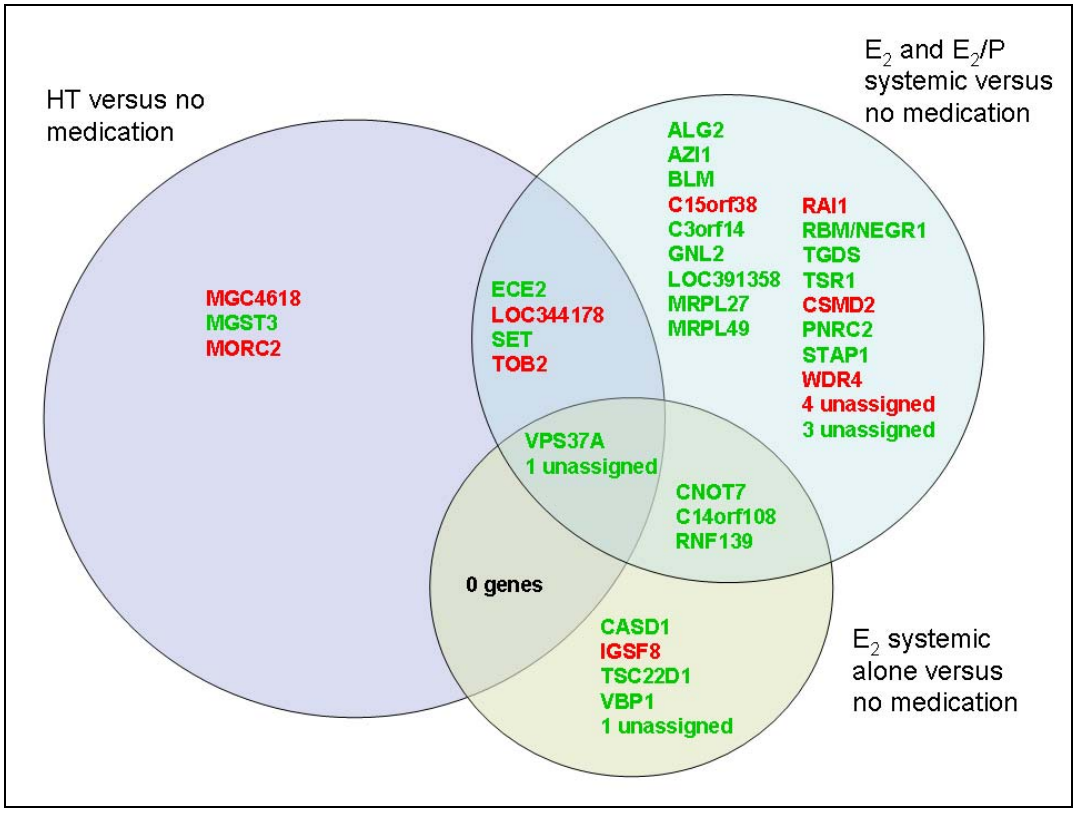

Figure 2

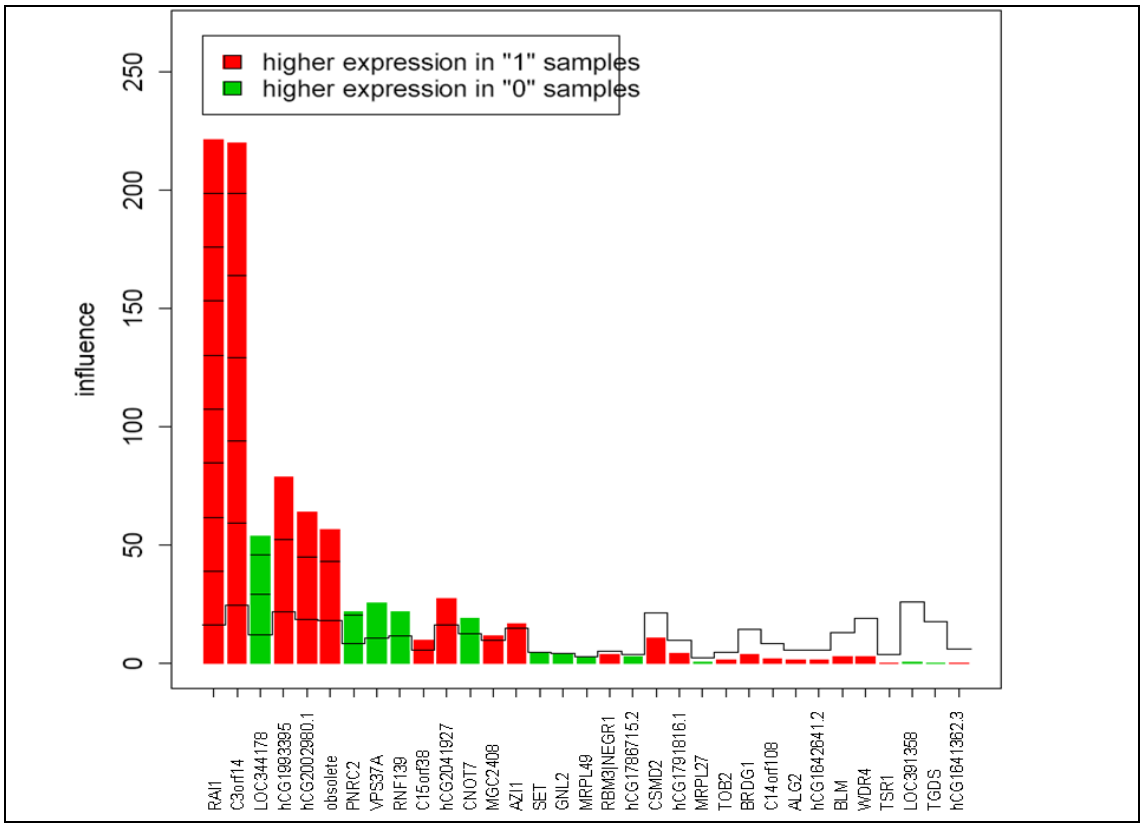

Figure 3

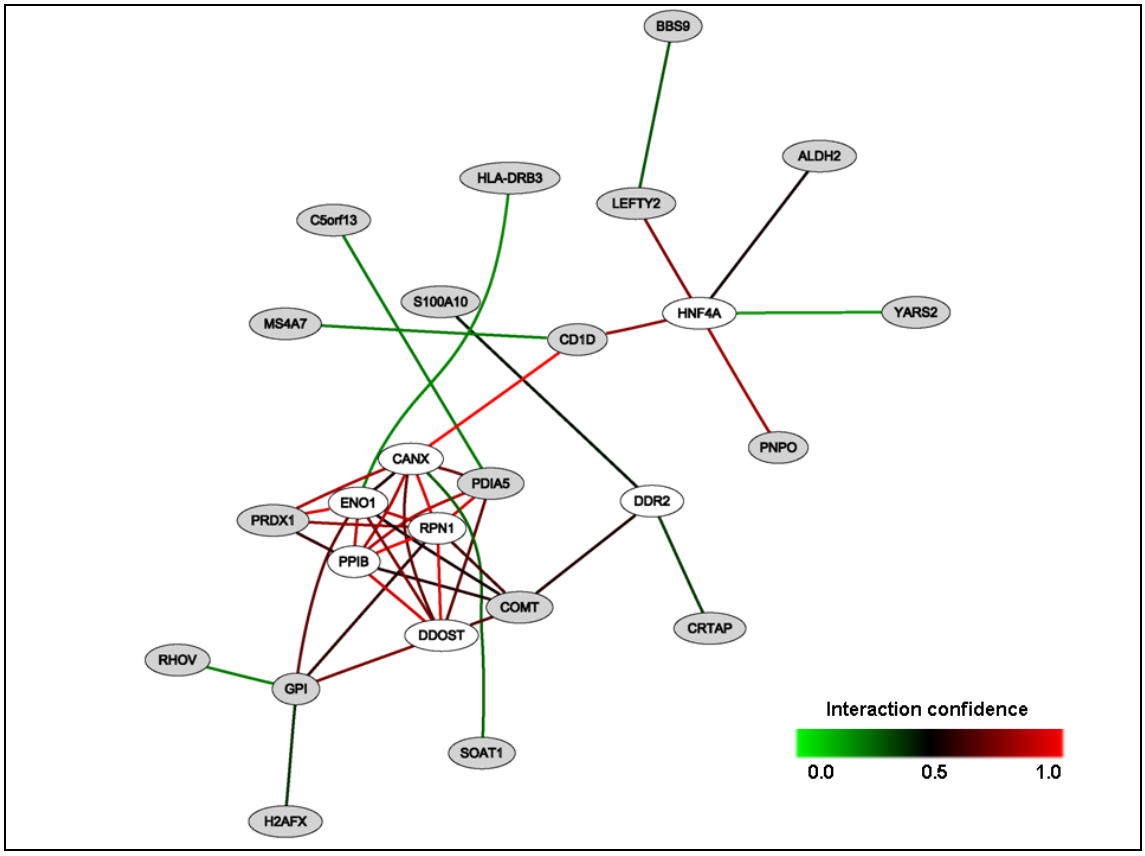

Figure 4

Supplementary Table 1 Gene sets included in the gene set enrichment analysis

Reference	RNA source	Expression profiling method	Gene sets	genes N	probes N	Subjects N/sex*
Gene sets related to female sex hormones or menopause						
Present study	Whole blood	Applied Biosystems	Genes associated with hormone therapy use	9	9	121w
		Applied Biosystems	Genes associated with systemic E2 or E2/P use	33	33	113w
		Applied Biosystems	Genes associated with systemic E2 use	10	10	105w
		Applied Biosystems	Genes associated with tibolone use alone	347	400	100w
		Applied Biosystems	Genes associated with tibolone use, all users	54	58	108w
		Applied Biosystems	Genes associated with tibolone use combined	21	21	-
		Applied Biosystems	Genes associated with thyroxine use	8	8	103w
AmiGO [1]			Response to estrogen stimulus, GO:0043627. GO database release 2009-09-17	23	27	-
Frasor [2]	MCF-7 (breast cancer cell line)	Affymetrix Hu95A GeneChips	Estrogen up-regulated genes (incl. 28 hormone related genes from KEGG (ref))	56	68	-
			Estrogen regulated genes (incl. 28 hormone related genes from KEGG (ref))	134	174	-
Dumeaux [3]	Whole blood	Agilent 22k oligoarray	Genes predicting hormone therapy use	83	112	100w
Ji[4]	MCV152 (ovarian surface epithelium)	Affymetrix Human Genome HGU95Av2 GeneChips, 10,000 full-length genes.	FSH treatment at 200 mIU/mL for 72 hr, or no treatment. (Differential exprs. defined by fold change)	113	142	26w
Kendall[5]	Breast biopsies	in-house Breakthrough cDNA microarrays, two color, dye swap	Normal and cancer biopsies before and after Letrozol treatment (estrogen deprivation)	45	57	13w
Dumeaux[6]	Whole blood	Applied Biosystems	Genes associated with hormone therapy use, core genes, 3 methods for globin RNA reduction	7	10	12w
			Genes associated with hormone therapy use after globin reduction using peptide nucleic acids (PNA)	14	19	12w
			Genes associated with hormone therapy use, no globin reduction	11	14	12w
Pöllänen[7]	Muscle biopsies	Sentrix HumanRef-8 Expression BeadChips (BD-25-201, Illumina)	Hormone therapy use vs. non-use	35	50	15w
Dvornyk[8]	Monocytes	Affymetrix GeneChip Human U133A	Pre- vs. postmenopausal women	40	57	19w
Xiao[9]	B-cells	Affymetrix HG-U133A GeneChip	Low vs. high Bone Mineral Density (BMD)	29	34	20w
KEGG[10]			Estrogen-androgen metabolism	18	20	-
			Steroid hormone metabolism	10	10	-
Gene sets related to blood cells and the immune system						
KEGG[10]			T cell receptor signalling	94	110	-
Tanner[11]	Whole blood	5' Nuclease PCR assay	Interleukins	15	17	1m
			General cytokines	11	11	1m
			CD markers	20	23	1m
Whitney[12]	PBMC and whole blood	Standford cDNA microarray	Lymphocyte signature	55	76	35w/40m
			Monocyte signature	25	29	35w/40m
			PBMC signature	78	105	35w/40m
			Neutrophil signature	31	38	35w/40m
			Red blood cell signature	37	44	35w/40m
			Reticulocyte signature	9	7	35w/40m
			Red blood cell and reticulocyte signature	48	51	35w/40m
Martinelli[13]	Neutrophils	Affymetrix HG-U95Av2 GeneChip	Immature vs. mature neutrophils	25	29	-
Cobb[14]	Whole blood	Affymetrix U133A or U133 Plus GeneChip	T cell enriched vs. leukocytes	104	121	5
			Monocyte enriched vs.leukocytes	83	102	5
			Up-regulated genes associated with trauma	77	89	31
			Down-regulated genes associated with trauma	103	139	31
Other			Immunoglobulin gene set	51	51	-
Eady[15]	PBMC	In house oligo-microarray	Natural killer cells in PBMC signature	26	35	10w/6m
			Monocytes in PBMC signature	47	61	10w/6m
Gene sets related to other factors						
Eady[15]	PBMC	In house oligomicroarray	Genes related to age	15	15	10w/6m
			BMI-specific genes	3	3	10w/6m

Tanner[11]	Whole blood	5'Nuclease PCR assay	Proto-oncogenes	7	8	1m
Connolly[16]	PBMC	Affymetrix HU133A GeneChip.	Stress response associated with exercise	12	15	15m
			Growth factor and transcription factor associated to exercise	23	27	15m
			Inflammatory response to exercise	24	27	15m
Radich[17]	Leukocytes	Agilent Hu25K microarray	Individual specific genes	50	66	8w/7m
Lampe[18]	Whole blood	Agilent Hu25k microarray	Genes correlated with cotinine levels	28	43	37w/48m
			Positive correlation with cotinine levels	20	30	37w/48m
			Negative correlation with cotinine levels	8	13	37w/48m
van Erk[19]	Leukocytes	Affymetrix U133A GeneChip	Genes associated with high protein high carbohydrate breakfasts	27	33	8m
			Protein synthesis genes associated to high protein breakfast	32	73	8m
			High inter-individual variability genes	20	28	8m
Siest[20]	Lymphocytes	In-house microarray (Visvikis-Siest et al., 2007)	Drug metabolizing enzymes	16	23	9w/11m
			Transcription factors	12	16	9w/11m
			Transcription factors and drug metabolizing enzymes	28	39	9w/11m

* w=women, m=men

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