Methodology article

Multilocus analysis of SNP and metabolic data within a given pathway

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Abstract

Background: Complex traits, which are under the influence of multiple and possibly interacting genes, have become a subject of new statistical methodological research. One of the greatest challenges facing human geneticists is the identification and characterization of susceptibility genes for common multifactorial diseases and their association to different quantitative phenotypic traits.

Results: Two types of data from the same metabolic pathway were used in the analysis: categorical measurements of 18 SNPs; and quantitative measurements of plasma levels of several steroids and their precursors. Using the combinatorial partitioning method we tested various thresholds for each metabolic trait and each individual SNP locus. One SNP in CYP19, 3UTR, two SNPs in CYP1B1 (R48G and A119S) and one in CYP1A1 (T461N) were significantly differently distributed between the high and low level metabolic groups. The leave one out cross validation method showed that 6 SNPs in concert make 65% correct prediction of phenotype. Further we used pattern recognition, computing the p-value by Monte Carlo simulation to identify sets of SNPs and physiological characteristics such as age and weight that contribute to a given metabolic level. Since the SNPs detected by both methods reside either in the same gene (CYP1B1) or in 3 different genes in immediate vicinity on chromosome 15 (CYP19, CYP11 and CYP1A1) we investigated the possibility that they form intragenic and intergenic haplotypes, which may jointly account for a higher activity in the pathway. We identified such haplotypes associated with metabolic levels.

Conclusion: The methods reported here may enable to study multiple low-penetrance genetic factors that together determine various quantitative phenotypic traits. Our preliminary data suggest that several genes coding for proteins involved in a common pathway, that happen to be located on common chromosomal areas and may form intragenic haplotypes, together account for a higher activity of the whole pathway.
Background
The challenge of identification and characterization of susceptibility genes for complex multifactorial diseases is partly due to the limitations of parametric statistical methods for detection of gene effects that are dependent solely or partially on interactions with other genes and with environmental exposures [1,2]. These limitations are reduced by non-parametric methods such as the combinatorial partitioning method (CPM) [3], which has been used to study the effect of many marker loci on quantitative phenotypes. The focus of the method is to form subsets of loci or genotypic partitions within which the trait variability is much lower than between the partitions [3]. The loci in such a set of genotypic partitions are then selected as candidates to influence the given trait and are then cross-validated.

A modification of this method is the multifactor dimensionality reduction (MDR) method, which has been used to study the impact of multiple loci on categorical endpoints such as presence or absence of disease or response to treatment. This is accomplished by reducing the dimensionality of the multilocus data where genotypes from multiple loci are pooled into high-risk and low-risk groups, depending on whether they are more common in affected or in unaffected individuals [4,5]. This approach is so far limited to categorical parameters and cannot be applied to quantitative traits. The only possible approach to association mapping would then be to search for patterns of genotypes at different loci. Pattern recognition by machine learning techniques may then be applied to define pattern frequencies or relationships in a data set [6].

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**Figure 1**
The estradiol metabolic pathway. Estradiol is synthesized from cholesterol in a series of consecutive hydroxylation relations.
In the present study we have used a variation of the combinatorial partitioning method and compared that to a pattern recognition method by the machine learning approach to identify subsets of SNPs that may predict the levels of metabolites in the estradiol metabolic pathway in healthy post-menopausal women. We have chosen this pathway since a positive correlation between estradiol exposure and risk of breast cancer among postmenopausal women has been rather well documented [7,8], and a significant correlation between plasma estrogen levels and subsequent risk of breast cancer development has been repeatedly described [9-12]. Estrone is synthesized from cholesterol in a cascade of subsequent hydroxylations [13] (Figure 1). After ovary seizure at menopause, the peripheral aromatization of androgens, mainly androstenedione into estrone, becomes the main source of circulating estrogen contributing to tumor stimulation [14]. A complex system of enzymes is responsible for

<table>
<thead>
<tr>
<th>Gene/SNP</th>
<th>Primer set</th>
<th>Method of analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A1, repeat</td>
<td>(5'-6-FAM-GTC-AGC-TGT-CTT-GAT-GAA-5')</td>
<td>Fragment analysis ABI310, (Applied Biosystems)</td>
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<td>CYP17, rs 743572</td>
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<td>RFLP MspA1 (Promega).</td>
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<tr>
<td>CYP19 3'UTR SNP3, rs255192</td>
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<td>BDT (Big Dye Terminator) sequencing ABI310 (Applied Biosystems)</td>
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<tr>
<td>HSDA3T, rs3138620</td>
<td>(5'-FAM-CAG-TAC-5')</td>
<td>Fragment analysis ABI310, (Applied Biosystems)</td>
</tr>
<tr>
<td>HSD DEL, rs1911194</td>
<td>(5'-FAM-CAG-TAC-5')</td>
<td>Fragment analysis on a ABI310, (Applied Biosystems)</td>
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<tr>
<td>CYP1B1 R48G</td>
<td>(5'-GAC-GGT-GAC-5')</td>
<td>RFLP, Rsr II (Fermentas)</td>
</tr>
<tr>
<td>CYP1B1 A119S</td>
<td>(5'-GAC-GGT-GAC-5')</td>
<td>RFLP, NgoM IV (New England BioLabs)</td>
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<tr>
<td>CYP1B1 V432L, rs1056836</td>
<td>(5'-CCAGGAACCTGTCGAGTGA-5')</td>
<td>RFLP, EcoS77 (Fermentas)</td>
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<td>COMT, rs4680</td>
<td>Cascorbi et al.</td>
<td>See reference</td>
</tr>
<tr>
<td>GSTM1</td>
<td>Cascorbi et al.</td>
<td>See reference</td>
</tr>
<tr>
<td>GSTP1</td>
<td>Cascorbi et al.</td>
<td>See reference</td>
</tr>
<tr>
<td>GSTT1</td>
<td>Cascorbi et al.</td>
<td>See reference</td>
</tr>
</tbody>
</table>

Table 1: Summary of the selected SNPs and the respective method of genotyping. Gene/SNP, rs number given when available, aminoacid change or UTR. Primer set: for the assays developed for this study or otherwise as referred to original publication. Method of analysis: platform and assay selection.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>DHEA</th>
<th>DHEA-SO4</th>
<th>Androstenedione</th>
<th>Testosterone</th>
<th>E1</th>
<th>E2</th>
<th>E1S</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHEA</td>
<td></td>
<td>0.578 p &lt; 0.001</td>
<td>0.494 p &lt; 0.001</td>
<td>0.606 p &lt; 0.001</td>
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<tr>
<td>Androstenedione</td>
<td></td>
<td>0.321 p &lt; 0.001</td>
<td>0.285 p &lt; 0.002</td>
<td>0.217 p &lt; 0.021</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone</td>
<td></td>
<td>0.218 p &lt; 0.034</td>
<td>0.285 p &lt; 0.002</td>
<td>0.217 p &lt; 0.021</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.873 p &lt; 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.873 p &lt; 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1S</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.776 p &lt; 0.001</td>
<td>0.795 p &lt; 0.001</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Correlation between metabolic levels of estradiol and its precursors in the plasma of healthy post-menopausal women. DHEA, dihydroepiandrostenedione, DHEA-SO4, dihydroepiandrostenedione sulfate, androstenedione, testosterone, E1, estrone, E2, estradiol, E1S, estrone- sulphate.
estradiol synthesis and its further metabolism: CYP17, CYP11a, CYP19, 17β-hydroxysteroid dehydrogenase, steroid sulfatase (STS), sulfotransferase (EST), CYP1A1, CYP1B1, Catechol-O-methyltransferase ([15-17] (Figure 1). Polymorphisms in these enzymes have previously been associated with both breast cancer risk and estradiol levels [2]. In the present report we have studied genetic polymorphism in all these enzymes and addressed the methodological challenge of the analysis of multiple loci 1) by free combinatorial approaches 2) in relation to intergenic haplotype structures within a common biochemical pathway.

Results
The levels of 9 metabolites of the estradiol pathway were studied (Figure 1). High correlation was observed between the levels of the different metabolites in the plasma of healthy individuals, metabolites upstream (DHEA, DHEA-S, androstenedione and estrone) as well as downstream (estrone, estradiol, estrone-sulphate) in the pathway (Table 2). Weight and body mass index significantly correlated with the levels of estrone and estradiol, while levels of DHEA and DHEA-S inversely correlated with age. Testosterone levels correlated with height (Table 3).

Chi square analysis
A total of 18 SNPs in 10 genes were genotyped in 109 individuals resulting in a total of 1962 genotypes. The genotype distribution of the studied polymorphisms was significantly different between the groups of individuals with metabolic activities below and above median when Chi square test was applied. The levels of E1 and E2 were significantly associated with two polymorphisms in the 3’UTR of CYP19 as well as two non-synonymous substitutions in the CYP1B1-R48G and A119S (Table 4). The E1 level was also associated to the T461N SNP in CYP1A1. Several other non-significant trends were observed.

Combinatorial partitioning analysis (Mutual Information Score (MIS))
The metabolic groups were further re-defined by using other thresholds than the median, using either one optimal threshold, (partitioning A) or two optimal thresholds (Partitioning B). Several moderately significant SNPs using the optimal thresholds approach were found (all SNPs with p-value < 0.05, Table 5). Leave one out cross validation analysis was performed to find sets of genotypes that jointly predict the value of the trait (high or low levels). Estrone levels partitioned into samples with values < 68.2 pmol/l and >68.2 pmol/l revealing a maximal difference in genotype distribution. A graphical representation (infogram) of the genotypes for each locus and this partition is shown in Figure 2A, where each row corresponds to a SNP and each column – to a sample. Figure 2B shows the stack diagram. The leave one out cross validation method showed that while one SNP can make only ~50% correct predictions of the estrone levels at this partition, combining 6 SNPs, including CYP1A1m4, CYP1B1A119S, CYP1A1m2, CYP19utr3’ SNPS, GSTP1, COMT allows 65% correct prediction (Figure 2C). Two of the selected polymorphisms were known to be functional at the metabolic level from previous studies in vitro. In the case of random labels, the probability of finding a set of SNPs that can make better prediction was found to be 0.16 based on 100 simulations.

Locus CYP11A1 was a microsatellite repeat with 10 variant repeat length alleles. All variant alleles were categorized together: A1/A1 (wt/wt), A1/mut, mut/mut. The QT scores and p-values for this locus and each metabolite were calculated and significant differences were found for several of the metabolites. The variant allele was more frequent in women with DHEA-S level>92.6 µg/dl than in women with DHEA-S levels<92.6 µg/dl (p < 0.042). In the two threshold analysis the significance was even higher (p = 0.004) when comparing individuals with DHEA-S levels<92.6 µg/dl and DHEA-S levels>92.6 µg/dl (Figure 3A); stack diagram is shown in Figure 3B. For estrone the p-value was 0.008 when comparing groups of women with estrone >133 pmol/l to those with estrone< 133 pmol/l. Four women with estrone level > 133 pmol/l have genotypes A2/A2 and A4/A4. Similar to estrone, levels of estradiol were associated with the CYP11A1 variants when comparing individuals with estradiol > 33 pmol/l to those with estradiol < 33 pmol/l (p < 0.06).
Pattern recognition of SNPs in relation to hormone metabolizing enzymes

The optimal threshold of the metabolic levels was found by multiple testing close to the median. Interactions between set of SNPs and physical characteristics like age and weight was identified (Fig 4A, B). Carriers of the wt CYP1A1m1 and wt GSTT1 with age above 64 years and with a body weight above 75 kg were more often in the lower level group of the metabolite DHEA-SO4 (CorrMAX 0.54(49/56), P-value < 0.0001) (Figure 4A). An interaction between the levels of this metabolite and age and weight (r = 0.44, p < 0.002), also seen by the conventional Chi square analysis (Table 2), was detected by this method. Individuals with weight higher than 75 kg carrying the wt GSTM1 had significantly higher plasma levels of E2, (CorrMAX 0.43(43/62), p < 0.003) (Figure 4B).

Another pattern of SNPs was found correlated to the estrone level; the variant allele in the 5' flanking area of CYP11 in combination with the wt GSTT1 was present among 12 individuals with a plasma level of estrone above 68 pmol/l, while none of the individuals with E1 plasma level below 68 pmol/l carried this combination (CorrMAX 0.36(53/52) P-value 0.05) (Figure 4C). Individuals homozygous for the variant alleles in the HSD17/B (A3T), CYP1B1 A119S, and COMT1 had significantly higher levels of sex hormone binding globulin, (CorrMAX 0.38(59/49) P-value 0.05) (Figure 4D). A colored infogram illustrating the significant differences in SNP patterns above and below the different thresholds is given below each frequency diagram.

Haplotype analysis

Since some of the SNPs detected by the above methods reside in 3 different genes in vicinity on chromosome 15 we hypothesized that they could form common intragenic haplotypes, which in concert might account for a higher activity of the whole pathway. Our findings suggest that the SNPs in CYP19, CY11 and CYP1A1 are not inherited at random but form common haplotypes (Figure 5A). Individuals with variant number of repeats in the microsatellite repeat of CYP11 were also carriers of the variant alleles in both loci CYP1A1m1 and m2 (D' 0.350 and 0.194, p < 0.001 (Bonferroni corrected) and p < 0.012, respectively) as well as in CYP19utr3' SNP2 (D' 0.293, p < 0.001 (Bonferroni corrected) (Figure 5B). A schematic presentation of the D' values is given in Figure 5C. High D' values and significant LD was observed in addition between the 3 SNPs in CYP19 and the 2 of the 4 SNPs in CYP1A1. Carriers of the haplotype CTATATC and CTTA(T)C(T)ATC(A) had more often E2 levels below median, while carriers of the TGTTT(C)ATC more often had E2 levels above the median (p < 0.025) (Figure 5D). The SNPs in CYP1B1 were also in strong linkage disequilibrium forming steady haplotype blocks (Figure 6A, B).

While the haplotype CGG, containing the C allele in CYP1B1R48G and the G alleles in CYP1B1A119S and CYP1B1 V432L was associated with high levels of E1 and E2, the haplotype GTC containing the alternative alleles in locus was associated with lower than median levels (p < 0.05).

Discussion

Finding effects of groups of SNPs on metabolite levels is complex since the effects of individual SNPs are small and the number of possible SNP combinations is large. We applied two different methods to help identify sets of SNPs correlated to metabolite levels: one using direct two-way classification based on combination of genotypes at selected loci, and another based on leave-one-out-cross-validation analysis. Direct classification method requires sufficiently big sample set for meaningful evaluation of genotype combination frequencies in groups with different metabolite levels. In studies like this with a small number of samples, the LOOCV method allows the evaluation of larger sets of SNPs, since the classifiers are constructed for each locus individually. In the first "pre-screening" phase of the genotype-phenotype analysis the metabolic levels were divided by median followed by sets of percentiles of the trait values. Finally, instead of pre-defining cut offs, we let the distribution of the genotypes lead us to those cut offs with a maximal difference in allele distribution. Interestingly, often these best thresholds converged to the median, i.e. for estrone in both the Mutual Information Score method as well as the pattern recognition. Whether or not these resulting cut offs have some physiological significance, remains to be investigated.

Long term exposure to estradiol increases the risk of breast cancer. The mechanisms responsible for this effect have not been firmly established. The prevailing theory proposes that estrogens increase the rate of cell proliferation by stimulating estrogen receptor-mediated transcription and thereby the number of errors occurring during DNA replication [19,20]. An alternative hypothesis proposes that estradiol can be metabolized to quinone derivatives, which can react with DNA and then remove bases from DNA through a process called depurination. Error prone DNA repair then results in point mutations [21]. These two processes, increased cell proliferation and genotoxic metabolite formation, may act in an additive or synergistic fashion to induce cancer. It has been suggested that measuring total E2 concentration and SHBG concentration may be sufficient in large epidemiological studies [12]. Our study shows that even in a small size it is sufficient to monitor only few metabolites as we observed tight correlations between them. Several genetic polymorphisms that may influence estradiol metabolism have been associated with different hormone levels. A poly-
Table 4: Statistical significance of distributions of genotypes in the listed estradiol metabolizing enzymes in below and above median levels of metabolites DHEA, dihydroepiandrosterone, DHEA-SO4, dihydroepiandrosterone sulfate, androstenedione, testosterone, E1, estrone, E2, estradiol, E1S, estrone-sulphate, SHBG, sex hormone binding globulin. NS – not significant.

<table>
<thead>
<tr>
<th>Gene/SNP</th>
<th>Below and above median values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DHEA</td>
</tr>
<tr>
<td>HSD DEL</td>
<td>5'flanking 12 bp deletion</td>
</tr>
<tr>
<td>HSD A3T</td>
<td>5'flanking (AAAT)n</td>
</tr>
<tr>
<td>CYP17</td>
<td>5'flanking</td>
</tr>
<tr>
<td>CYP19UTRSNP1</td>
<td>3'untranslated</td>
</tr>
<tr>
<td>CYP19UTRSNP2</td>
<td>3'untranslated</td>
</tr>
<tr>
<td>CYP19UTRSNP3</td>
<td>3'untranslated</td>
</tr>
<tr>
<td>CYP11</td>
<td>5'flanking</td>
</tr>
<tr>
<td>CYP1B1 R48G</td>
<td>coding nonsynon R48G</td>
</tr>
<tr>
<td>CYP1B1 A119S</td>
<td>coding nonsynon A119S</td>
</tr>
<tr>
<td>CYP1B1 V432L</td>
<td>coding nonsynon V432L</td>
</tr>
<tr>
<td>CYP1A1 m1</td>
<td>3'untranslated</td>
</tr>
<tr>
<td>CYP1A1 m2</td>
<td>coding nonsynon I462V</td>
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<tr>
<td>CYP1A1 m3</td>
<td>3'untranslated</td>
</tr>
<tr>
<td>CYP1A1 m4</td>
<td>coding nonsynon T461N</td>
</tr>
<tr>
<td>COMT</td>
<td>coding nonsynon V158M</td>
</tr>
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</tr>
<tr>
<td>GSTTI</td>
<td>gene deletion</td>
</tr>
<tr>
<td>GSTp1</td>
<td>coding nonsynon</td>
</tr>
</tbody>
</table>

*ANOVA analysis
morphism in CYP19, a 3-bp deletion in intron 4 (TTT)$_n$, and a base substitution in exon 3 (G $\rightarrow$ A) have been reported to be associated with levels of estradiol [22,23]. Genetic polymorphism in the enzymes further hydroxylating estradiol and conjugating its metabolites has also been studied. Women carrying the COMT Met/Met genotype had 28% higher 2-hydroxyestrone ($P = 0.02$), compared to women with the Val/Val genotype [22].

The previous studies discussed above analyzed single loci. In a recent breast cancer case-control study, including categorical values only (genotypes), a four-locus susceptibility model including the polymorphisms of COMT, CYP1A1m1, CYP1B1 codon 48, and CYP1B1 codon 432 was found associated with breast cancer [2]. The four-locus model was significant at the $p = 0.001$ level by permutation testing bringing evidence of epistasis, or gene-gene interaction in the case-control setting. Each genotype at a particular locus had an influence on breast cancer disease risk dependent on the genotypes at each of the other three loci. With that in mind, we searched the hormone metabolic pathway for interactions between these loci both at random, combinatorial, without regards to the chromosomal localisation and with regards to the LD in genes that may form common haplotype structures. Two such domains were identified – the polymorphisms in CYP1B1 form stable haplotype structures in Norwegian population (Zimarina et al, submitted), and a block on the long arm of chromosome 15 consisting of CYP11A1 gene close to the CYP1A1 (less than 1 cM) gene and approximately 27.4 cM telomeric to the CYP19 gene. Indeed, we found 2 haplotypes in the CYP1B1, which were significantly overrepresented in individuals with E1 and E2 levels above median. Two of the three genotypes (R48G and V432L) were among the best predictors according to the combinatorial partitioning method as well. Furthermore, the same CYP1B1 haplotypes (CGG and GTC) were associated with breast cancer risk (Zimarina et al, submitted). The CGG haplotype includes both the V432 form of CYP1B1 and the R48 with higher 4-OH/2-OH E2 metabolic ratio and affinity (Km) towards 17b-estradiol respectively [24,25]. More unexpectedly, we found high D’ values among SNPs residing in different genes but coding for proteins in the same metabolic pathway. The haplotype comprising of the T allele of CYP19utr3’ SNP1, the variant number of repeats of CYP11 and the variant alleles of CYP1A1m1 and m2 we associated with high E2 levels (above median). Upregulation of CYP1A1 by dioxin derivatives through the Ah receptor leads to down-regulation of CYP19 and ER. The close location of these genes gives an attractive opportunity to study whether they are regulated by a common regulatory unit. The haplotype structures may vary from population to population – hence explain the variability of the published data on various susceptibility alleles in a number of genes.

The fact that we manage to predict correctly 65% of the individuals according to their metabolic levels based on this limited selection of SNPs in healthy individuals, make us believe that we have identified markers of estradiol levels in the present study. Furthermore, we found similar combinations of SNPs as those involved in the susceptibility combinations from the case control study [2]. We are presently conducting a larger study of both cases and controls with a higher SNP density to improve our 65% prediction value. In the last stage of the preparation of this manuscript another large study of 1975 individuals was published [26] confirming our previous initial report of an association of the polymorphism in CYP19utr and aromatase activity [27] and in concordance with our present observation of association with plasma levels. Here we demonstrate that the association can be discovered in much smaller number of individuals (109) using the multilocus data analysis.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>SNP</th>
<th>p-value Partitioning A</th>
<th>p-value Partitioning B</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHEA</td>
<td>CYP1B1 R48G</td>
<td>NS</td>
<td>0.022</td>
</tr>
<tr>
<td>E1</td>
<td>CYP1B1 A119S</td>
<td>0.039</td>
<td>0.0045</td>
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<td>E1S</td>
<td>CYP1B1 A119S</td>
<td>NS</td>
<td>0.043</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>CYP19 UTRSNP1</td>
<td>0.042</td>
<td>NS</td>
</tr>
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<td>DHEA-SO4</td>
<td>CYP1A1 m1</td>
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<tr>
<td>E1</td>
<td>CYP1A1 m4</td>
<td>0.042</td>
<td>0.028</td>
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</tbody>
</table>

Table 5: Searching for optimal thresholds approach to identify genotypes associated with quantitative trait (all SNPs with p-value < 0.05). Partitioning A shows significant results for one optimal threshold (two partitions), and Partitioning B shows the results for two optimal thresholds (three partitions). The calculated p-values take into account the multiple search over all possible thresholds as described in [3].
Figure 2
(A). Identification of minimal combination of SNPs for maximal prediction value of metabolic expression level of E1 (estrone) using leave one out cross validation analysis. Estrone levels <68.2 pmol/l and >69.2 pmol/l are best predicted by the genotypes in the given order (p < 0.05). Frequency distribution of the genotypes at each locus (row) and each person (column), white- missing value (A). Columns under heat map labeled by '|' correspond to samples with estrone level < 68.2 pmol/l, columns labeled by '-' correspond to samples with estrone level > 69.2 pmol/l. Columns are ordered with respect to increasing estrone level (B). Stacked diagrams of frequency distribution of the genotypes at each locus (C).

Conclusion
These studies provide further evidence that genetic variation may appreciably alter plasma level of sex hormone and thus have an effect on disease risk. We describe an approach for multilocus approach to study multiple low-penetrance genetic factors that together determine quantitative phenotypic traits.

Methods
Blood samples were collected from 109 healthy female volunteers between 55 and 75 years of age on a regular mammographic screening. The women enrolled had 2 consecutive negative mammograms in a period of 2 years and were not on hormone replacement therapy (HRT). The plasma levels of the metabolites E1, E2, E1S, DHEA, androstenedione and testosterone were analysed as described previously in [28]. DNA was isolated from EDTA blood using chloroform/phenol extraction followed by ethanol precipitation according to standard procedures using the Applied Biosystems 340A Nucleic Acid Extractor.

Genotyping
Primer sets and methods for analysis are summarized in Table 1 and as described in [29,30].

Statistical Analysis
Parametric method
Metabolic levels for each metabolite were divided into below and above median and allele and genotype fre-
Let \( (x_i, q) \), \( i = 1, 2, ..., n \) denote the measurements for a particular pair consisting of a SNP and a quantitative variable (metabolite level) across all patients. For any such pair we seek a threshold \( t \), such that the genotype frequencies in the samples with \( q < t \) will be the most different from the genotype frequencies in the samples with \( q \geq t \). This difference is measured by the mutual information score. The search process itself is exhaustive, considering all possible thresholds. More precisely, for a SNP locus \( L \) and a quantitative trait \( q \), let \( G \) be a partition of the sample-set induced by the genotypes at locus \( L \). For a threshold \( t \), let \( C_t \) be a binary partition of the sample-set defined by \( q < t \) and \( q \geq t \). The Mutual Information Score (MIS) is defined as the difference between the entropy of the partition \( C_t \) and the entropy of \( C_t \) conditioned on \( G \):

\[
MIS(C_t, G) = H(C_t) - H(C_t|G),
\]

where \( H \) is the entropy or conditional entropy function, as appropriate. The best threshold \( \hat{t} \) for a pair \((L, q)\) is such that

\[
MIS(C_{\hat{t}}, G) = \max_{\min(q) \leq t \leq \max(q)} MIS(C_t, G).
\]

The corresponding \( p \)-values were also calculated, effectively counting all possible ways of partitioning the samples that would give a score better than \( MIS(C_t, G) \).
olds \((\hat{a}, \hat{b})\) that gave maximum score. The calculated p-values take into account the multiple search over all possible thresholds as described in [3].

Further, for each SNP-trait pair, and a partition of the sample-set \(C\) defined by a single best threshold or by a pair of best thresholds, we ran leave one out cross validation analysis in order to find a set of SNPs such that genotypes at these SNPs can jointly predict the classification of samples with respect to \(C\), i.e. whether the value of the quantitative trait level is above or below threshold [3]. Leave one out cross validation analysis, working with a given subset of SNPs, \(S\), consists of the following steps:

1. Hide a sample

2. For each SNP in the subset \(S\), construct a classifier based on the likelihood of each class of the partition \(C\) given the genotypes of remaining (non hidden) samples at this locus.
Figure 5
Linkage disequilibrium between SNPs in 3 genes from the estradiol metabolising pathway situated within 1 cM on chromosome 15: CYP19, CYP1A1 and CYP1A. Samples in each row, variants in columns, high frequency allele – blue, low frequency allele- yellow (A). LD was observed between the 3 SNPs in the 3'UTR of CYP19 and 2 SNPs in CYP1A1 and the variant allele of CYP1 with one SNP in CYP19 and another in CYP1A1, bold in panel (B). D', R and Fisher exact test values for all 8 SNPs in this chromosomal area (Site 1–8) are given in panel (B) and Fisher exact test results in colour diagram – blue approximating 0.00 (C). Individuals carrying these haplotypes had more often estradiol levels above median (D).
3. Classify the hidden sample using the sum of single-SNP classifiers

4. Repeat steps 1–3 for each sample, and thus determine the number of correct predictions for the subset S

Eventually, we also seek a subset S with the best performance. Several search techniques were used for finding best subset of SNPs in order to avoid evaluating each possible subset for each locus/trait pair. These techniques included ordering SNPs by mutual information score and evaluating sets of top scoring SNPs, as well as performing forward and backward sequential searches [3]. Further, we estimated the probability of finding such predictive subsets of SNPs for random labels by simulations.

**Pattern recognition by the two way classification method**

The problem of finding an association between groups of individuals with metabolic levels above or below a certain threshold, referred to as the positive and negative groups, and combinations of genotypes at specific polymorphic loci may be formulated as a two-way classification problem. When evaluating a specific combination of genotypes against a particular patient record, the outcome can be a true-positive (i.e., the individual has all the genotypes in the given combination, and the patient is in the positive group), a true-negative (i.e., the patient does not have the specific combination of genotypes, and the patient is in the negative group), a false-positive (i.e., the individual has all the genotypes the given combination, and the patient is in the negative group) and a false-negative (i.e.,
the patient does not have the specific combination of genotypes and the patient is in the positive group). For a two-way classification problem, the correlation of a specific combination of genotypes for each combination of SNPs may be computed as

\[
C = \frac{N_{tp}N_{in} - N_{fn} + N_{fp}}{\sqrt{(N_{in} + N_{fn})(N_{in} + N_{fp})(N_{ip} + N_{fp})}}
\]

Where \( N_{ip}, N_{tn}, N_{fn}, \) and \( N_{fp} \) denotes the number of patient records which are respectively true-positive, true-negative, false-negative, and false-positive. The significance of a specific combination of polymorphisms with correlation \( CorrMAX \) is defined as the probability of finding a better, or just as good, combination of polymorphisms by random. This probability is referred to as the p-value, and is computed by Monte Carlo simulation. The steps for the Monte Carlo procedure are as described in [31]. Briefly, for each individual record, permute the "above" or "below" labels randomly from the same distribution as in the original data. Calculate \( CorrMAX \) for the permuted data. If \( CorrMAX \) for the permuted data is larger than, or equal to \( CorrMAX \) from the original data, count 1; otherwise count 0. Repeat steps 1, 2 and 3 \( k \) times. Estimate the p-value, the total count for \( CorrMAX \) divided by the total number of shuffles \( k \). When computing \( CorrMAX \) for the permuted data, we include all polymorphisms, not only the polymorphisms in the combination to test the significance of. The standard value for \( k \) (number of repeated steps) is 2000.

Haplotypes were estimated using PHASE2.0 software. Linkage disequilibrium and D and D' values were calculated using DNAsp software. Significance of the linkage disequilibrium (LD) was estimated using Fisher exact test with Bonferoni correction for the final p-value.

Author's contribution

VNK, final design of the study, supervised AF, carried out the haplotype analysis and drafted the manuscript

AT, carried out the Mutual Information Score and LOOCV analyses

JG, carried out the metabolic profiling

AF, performed the genotyping as a part of her master's project

GIGA, provided technical assistance in the lab and isolated the DNA

OCL, consulted the statistical analysis and comparison of the methods

SF, performed the pattern recognition analysis

ZY, supervised the statistical analysis and edited the manuscript

PEL collected the blood samples and participated in designing the study

ALBD designed the study and DNA preparation

All authors have read and edited the manuscript and approve the final manuscript.

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References

15. Huang CS, Chen HD, Chang KJ, Chng CW, Hsu SM, Shen CY: Breast cancer risk associated with genotype polymorphism of the estrogen-metabolizing genes CYP17, CYP1A1, and...


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