Associations of obesity and circulating insulin and glucose with breast cancer risk: A Mendelian randomization analysis

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ABSTRACT

Background: In addition to the established association between general obesity and breast cancer risk, central obesity and circulating fasting insulin and glucose have been linked to the development of this common malignancy. Findings from previous studies, however, have been inconsistent, and the nature of the associations is unclear.

Methods: We conducted Mendelian randomization analyses to evaluate the association of breast cancer risk, using genetic instruments, with fasting insulin, fasting glucose, 2-hour glucose, body mass index (BMI), and BMI-adjusted waist-hip-ratio ($WHR_{adj\ BMI}$). We first confirmed the association of these instruments with type 2 diabetes risk in a large diabetes genome-wide association study consortium. We then investigated their associations with breast cancer risk using individual-level data obtained from 98 842 cases and 83 464 controls of European descent in the Breast Cancer Association Consortium.

Results: All sets of instruments were associated with risk of type 2 diabetes. Associations with breast cancer risk were found for genetically predicted fasting insulin [odds ratio (OR)=1.71 per standard deviation (SD) increase, 95% CI=1.26-2.31, $p=5.09\times10^{-4}$], 2-hour glucose (OR=1.80 per SD increase, 95% CI=1.30-2.49, $p=4.02\times10^{-4}$), BMI (OR=0.70 per 5-unit increase, 95% CI=0.65-0.76, $p=5.05\times10^{-19}$), and $WHR_{adj\ BMI}$ (OR=0.85, 95% CI=0.79-0.91, $p=9.22\times10^{-6}$). Stratified analyses showed that genetically predicted fasting insulin was more closely related to risk of ER-positive cancer, while the associations with instruments of 2-hour glucose, BMI, and $WHR_{adj\ BMI}$ were consistent regardless of age, menopausal status, estrogen receptor status, and family history of breast cancer.

Conclusions: We confirmed the previously reported inverse association of genetically predicted BMI with breast cancer risk and showed a positive association of genetically predicted fasting insulin and 2-hour glucose, and an inverse association of $WHR_{adj\ BMI}$ with breast cancer risk. Our

study suggests that genetically determined obesity and glucose/insulin-related traits have an important role in the etiology of breast cancer.

Key words: breast cancer; insulin; glucose; obesity; genetics; Mendelian randomization analysis

Key messages

- Mendelian randomization studies eliminate potential influence of reverse causation on study results and are less susceptible to bias and confounding than conventional observational studies. We utilized this approach to evaluate the association of obesity and glucose/insulin-related traits with breast cancer risk using the data of a large consortium.

- We found genetically predicted fasting insulin and 2-hour glucose levels were positively associated with breast cancer risk, while genetically predicted body mass index and waist-hip-ratio with adjustment of BMI were inversely associated with the risk.

- Our study has uncovered complex inter-relations of genetics, obesity and breast cancer risk and provided novel findings regarding roles of circulating glucose and insulin in the risk of this common cancer.

Introduction

General and central obesity have been linked to breast cancer risk in previous studies. [1,2] Body mass index (BMI) and waist-hip-ratio (WHR) are commonly used to measure general and central obesity, respectively. Obesity, particularly central obesity, is a major risk factor for insulin resistance and type 2 diabetes, which are often characterized by elevated fasting insulin and glucose as well as impaired glucose tolerance (usually measured by blood glucose level 2 hours after oral glucose challenge). [3] Previous studies have linked fasting insulin and glucose levels to increased risks of multiple cancers. [4–6] Proposed mechanisms for these associations include cancer-promoting effects mediated by insulin and insulin-like growth factor (IGF) signaling pathways. [7] However, the relationship between these biomarkers and breast cancer remains controversial and findings from epidemiological studies are inconsistent. [8,9] Concerns regarding the validity of these observational study findings include potential selection biases, reverse causation, confounding effects, small sample size, and differences in assays used to measure the biomarkers of interest.

Mendelian randomization analysis has been used to evaluate potential causal relationships between exposures and the disease. [10,11] Genetic variants are used as instrumental variables in the analysis. Random assortment of alleles at the time of gamete formation results in a random assignment of exposures that are related to an allele (or a set of alleles). Thus, Mendelian randomization analyses may reduce potential biases that would afflict conventional observational studies. In the current study, we performed Mendelian randomization analyses to assess associations of obesity (i.e. BMI and WHR) and glucose/insulin-related traits (i.e. fasting glucose, 2-hour glucose, and fasting insulin) with breast cancer risk using data from the Breast Cancer Association Consortium (BCAC).

Methods

Study Population

Included in this analysis are 182 306 participants of European ancestry whose samples were genotyped using custom Illumina iSelect genotyping arrays, OncoArray (56 762 cases and 43 207 controls) or iCOGS array (42 080 cases and 40 257 controls). Institutional review boards of all involved institutes approved the studies. Selected characteristics of the two datasets are presented in Supplementary Table 1. Details of the genotyping protocols in the BCAC are described elsewhere (iCOGS: http://ccge.medschl.cam.ac.uk/research/consortia/icogs/; OncoArray: https://epi.grants.cancer.gov/oncoarray/). [12,13] Genotyping data were imputed using the program IMPUTE2 [14] with the 1000 Genomes Project Phase III integrated variant set as the reference panel. SNPs with low imputation quality (imputation $r^2 < 0.5$) were excluded. Top principal components (PCs) were included as covariates in regression analysis to address potential population substructure (iCOGS: top eight PCs; OncoArray: top 15 PCs).

Selection of SNPs associated with glucose/insulin-related traits

In December 2016, we searched the National Human Genome Research Institute-European Bioinformatics Institute Catalog of Published Genome-Wide Association Studies and the literature for SNPs associated with the following traits: levels of 2-hour glucose (2hrGlu), fasting glucose (FG), fasting insulin (FI), BMI, and waist-hip-ratio with adjustment of BMI ($WHR_{adj\ BMI}$). [15–19] SNPs associated with any of these traits at the genome-wide significance level ($p<5\times10^{-8}$) in populations of European ancestry were included. For each GWAS-identified locus, a representative SNP with the lowest *p* value in the original GWAS publication was selected (linkage disequilibrium $r^2<0.1$, based on 1000 Genome Phase III CEU data).

Construction of instrumental variables

Weighted polygenic scores for each trait (ie. wPRS-2hrGlu, wPRS-FG, wPRS-FI, wPRS-BMI, and wPRS-WHR$_{adj\ BMI}$) were constructed followed the formula: $\mathrm{wPRS} = \sum_i \ \beta_{i,GX} * SNP_i$, where $\beta_{i,\ GX}$ is the beta coefficient of the i th SNP for the trait of interest from the published GWAS (Supplementary Table 2). SNP_i is the imputed dosage of the effect allele in BCAC data (range: 0 to 2). To reduce potential pleiotropic effects, we excluded BMI and WHR$_{adj\ BMI}$ -associated SNPs from instruments of 2hrGlu, fasting glucose and insulin (r^2<0.8), and *vice versa*. The pleiotropic SNPs associated with more than one trait were presented in Supplementary Table 2. The F-statistic was taken to indicate whether an instrumental variable is well-powered for Mendelian randomization analysis with 10 being a commonly used threshold. [20] Variance explained (%) and F statistics were calculated following the formulae: $\sum_i \ 2 * \beta_{i,GX}^2 * f_{effect\ allele} * \frac{(1-f_{effect\ allele})}{var(X)} * 100$ and $R^2*(n-1-k)/(1-R^2)/k$, respectively, where R^2 is percentage of variance explained by used SNPs; *f* is the frequency of the effect allele reported by GWAS for the trait; var(X) is the variance of trait, see below; *n* is the sample size of BCAC data; and *k* is the number of SNPs used in the instrument. [21]

For 2-hour glucose, fasting glucose and insulin, $\beta_{i,\ GX}$ were further transformed to represent 1 standard deviation (SD) increase with the unit in the original GWAS (2-hour glucose: 1 SD= 2 mmol/L, variance= 4; fasting glucose: 1 SD= 0.65 mmol/L, variance= 0.42; fasting insulin: 1 SD= 0.60 ln[pmol/L], variance= 0.36) [17,22] by the formula: $\beta_{i\ ,SD} = \beta_{i,\ GX}$ [2*f* (SNP_i)(1-*f* (SNP_i)]^0.5/SD. wPRS-BMI and wPRS-WHR$_{adj\ BMI}$ represented the adjusted 1-SD increase of transformed BMI and WHR$_{adj\ BMI}$ as the original GWAS performed the inverse normal transformation for both phenotypes. [18,19,23] We further scaled wPRS-BMI to be equivalent to 5 units of BMI by performing a linear regression among controls in our dataset: observed BMI ~

wPRS-BMI+error. Then we calculated the transformed BMI as $BMI_{wPRS}=\beta_0 + \beta_1 *$ (wPRS-BMI), where β_0 and β_1 are slope and coefficient from the linear regression model mentioned above.

Statistical analysis

Given an established association between impaired glucose/insulin traits and type 2 diabetes, an association between constructed instruments and risk of type 2 diabetes are expected. We utilized summary statistics from the DIAbetes Genetics Replication And Meta-analysis (DIAGRAM) Consortium and conducted an Mendelian randomization analysis of our traits using the inverse-variance-weighted two-sample method [10,24]. The Mendelian randomization estimate and standard error were calculated as $\sum_i \ \beta_{i,GX} * \beta_{i,GY} * \sigma_{i,GY}^{-2} / (\sum_i \ \beta_{i,GX}^2 * \sigma_{i,GY}^{-2})$ and $1/(\sum_i \ \beta_{i,GX}^2 * \sigma_{i,GY}^{-2})^{0.5}$, respectively. *GY* represents the association between a SNP and type 2 diabetes risk, thus $\beta_{i,\,GY}$ and $\sigma_{i,\,GY}$ are beta coefficient and standard error, respectively. The *p* value was based on Student's *t* distribution, where the degrees of freedom were determined by the number of SNPs included in the instrument for the trait of interest. We calculated Pearson's correlations between each pair of wPRSs in the control data before and after removal of pleiotropic SNPs. Egger's regression, as described in Bowden et al, [25] was performed to detect potential pleiotropy of our instruments. We also included all instruments in the same model to evaluate possible independent associations of each instrument with breast cancer risk.

Associations of wPRSs with breast cancer risk were evaluated separately in the iCOGs and OncoArray datasets by treating these scores as continuous variables. A logistic regression was performed with age at interview/diagnosis, study site/country, and PCs as covariates. The results were then combined using meta-analyses in METAL with a fixed-effects model. [26] We performed additional analyses adjusting for certain known breast cancer risk factors listed in

Supplementary Table 1. Finally, we conducted sub-analyses by estrogen receptor (ER) status, age at interview/diagnosis (<50 versus ≥50), menopausal status at interview/breast cancer diagnosis, and family history of breast cancer. All statistical analyses were conducted using R statistical software (version 3.1.2).

Results

Approximately 90% of cases included in this study were diagnosed at age 40 or above. A total of 278 SNPs were selected to construct the instruments, for which the number of SNPs for each trait ranged from 4 to 166 (Table 1). The variance of each trait explained by its associated variants ranged from 0.23% for 2-hour glucose to 2.89% for BMI (Table 1). (Table 1 here)

Using data from DIAGRAM, we demonstrated that all genetic instruments were associated with risk of type 2 diabetes in the direction that would be expected (Table 2). The strongest association was observed for the genetic instrument for fasting glucose (OR=6.37, p=5.77×10^{-16} and OR=4.32, p=1.12×10^{-11} before and after the exclusion of pleiotropic SNPs, respectively). (Table 2 here)

We observed associations of breast cancer risk with genetically predicted 2-hour glucose, BMI, and $WHR_{adj\ BMI}$ prior to the removal of pleiotropic SNPs (Table 3). Removing pleiotropic SNPs did not appreciably change the associations. A one-SD increase in genetically predicted 2-hour glucose levels was associated with an 80% increased risk of breast cancer (OR=1.80, 95% CI=1.30-2.49, p=4.02×10^{-4}). An inverse association was observed for both genetically predicted BMI and $WHR_{adj\ BMI}$ (per 5 units of BMI increase: OR=0.70, 95% CI=0.66-0.77, p=5.05×10^{-19}; per unit increase of genetic risk score for $WHR_{adj\ BMI}$: OR=0.85, 95% CI= 0.79-0.91, p=9.22×10^{-6}). The association of breast cancer risk with genetically predicted fasting

insulin became significant after excluding pleiotropic SNPs (OR=1.71, 95% CI= 1.26-2.31, p=5.09×10^{-4}). No association was observed for genetically predicted fasting glucose. Results of iCOGS and OncoArray were shown separately in Supplementary Table 3. (Table 3 here)

Genetically predicted fasting insulin was correlated with both genetically predicted 2-hour glucose and $WHR_{adj\ BMI}$ (Supplementary Table 4). Exclusion of pleiotropic SNPs attenuated these correlations. Mutual adjustment of all instruments did not materially change the observed associations with breast cancer risk described above (Supplementary Table 5). We evaluated the associations of genetically predicted obesity and glucose/insulin-related traits with traditional risk factors for breast cancer and found that genetically predicted fasting insulin and $WHR_{adj\ BMI}$ were associated with BMI in controls. Further, genetically predicted BMI were correlated with age at menarche, age at first live birth, and breast feeding history (Supplementary Table 6). Adjusting for these covariates did not materially change the observed associations of genetically predicted fasting insulin, BMI, and WHR_{adjBMI} with breast cancer risk (Supplementary Table 7). Finally, using Egger's regression, we found that the intercept in the model was noticeable for genetically predicted 2-hour glucose, BMI, and $WHR_{adj\ BMI,}$ indicating a strong pleiotropic effect for these instruments (p<0.005 for β_0, Supplementary Table 8). [25] No apparent pleiotropy was found for genetically predicted fasting insulin. The Mendelian randomization estimates from Egger's regression remained significant after accounting for detected pleiotropy for genetically predicted BMI and $WHR_{adj\ BMI}$ (Supplementary Table 8).

Stratified analysis was performed by age, menopausal status, ER status, and family history of breast cancer. The association with genetically predicted fasting insulin was restricted to ER(+) cancer (Figure 1-B, P_{het}=0.007, exclusion of pleiotropic SNPs). Genetically predicted 2-hour glucose, BMI, and $WHR_{adj\ BMI}$ were consistently associated with breast cancer across all strata (Figure 1-A, C, D, P_{het}>0.05, exclusion of pleiotropic SNPs). The results of stratified

analysis are shown for other sets of instrumental variables in Supplementary Figures 1 (inclusion of pleiotropic SNPs) and 2 (fasting glucose, exclusion of pleiotropic SNPs).

Discussion

In this large study we found that genetically predicted obesity, 2-hour glucose and fasting insulin were associated with breast cancer risk. Measured BMI has been well established to be positively associated with breast cancer risk in postmenopausal women but inversely related to the risk in premenopausal women. Results from epidemiologic studies investigating the association of breast cancer risk with WHR, fasting insulin and glucose have been inconsistent. Traditional epidemiological studies are prone to biases, including confounding, selection biases, recall biases and reverse causality. Mendelian randomization analyses take advantage of the random assignment of genetic alleles during gamete formation to minimize the biases commonly encountered in traditional epidemiological studies. When an instrumental variables are not associated with any potential confounders and are not linked to the outcome via any alternative pathway, Mendelian randomization analysis using such instrumental variables resemble randomized clinical trials and thus could provide strong results for causal inference for the exposure of interest .[10]

We found that the risk of breast cancer increased approximately 70% for each SD increase of genetically predicted fasting insulin levels. Previous epidemiological studies were unable to reach a conclusion regarding the association between fasting insulin and breast cancer risk. A meta-analysis reported a null association for fasting insulin. [8] However, the I^2, an indicator of heterogeneity across studies, was considerable. Our results provide strong evidence to support a positive association. Insulin is an important growth factor with cancer-promoting features such as stimulating cell mitosis and migration and inhibiting apoptosis. Its mitogenic

effects involve the activation of Ras and the mitogen-activated protein kinase pathway, [27] of which the role in cancer development have been recognized. [28] Further, insulin may inhibit the production of sex hormone binding globulin and lead to elevated bioavailable estrogen levels. [29] It also has been shown that knockdown of insulin and IGF-1 receptors inhibits hormone dependent growth of ER(+) breast cancer cells. [30] It may explain the association of fasting insulin with ER(+) breast cancer observed in this study.

Previous epidemiological studies have suggested that fasting glucose may be a risk factor for breast cancer, but few have assessed 2-hour glucose levels, as the latter is difficult to investigate in large prospective cohort studies. Overall, a meta-analysis of prospective studies showed no strong evidence to support an association of fasting glucose levels and risk of breast cancer in nondiabetic women. [9] In the current study, we found a positive association with breast cancer for genetically predicted 2-hour glucose levels but not for fasting glucose. Although fasting glucose and 2-hour glucose are closely correlated, [31] they represent different biological processes. The genetically determined fasting glucose levels primarily reflect the glycogenolysis activity in liver and hepatic insulin sensitivity. [32] On the other hand, the levels of post-challenge glucose are mainly determined by the amount and pace of insulin released into blood stream in response to the challenge as well as by the glucose uptake in skeletal muscle cells (in other words, it primarily reflects beta cell function and skeletal muscle insulin sensitivity [33]). The reasons why genetically predicted 2-hour glucose is associated with increased risk of breast cancer but not fasting glucose are not clear. One animal study has provided evidence that transgenic mice with inactivated insulin and IGF-1 receptors in skeletal muscles (impaired skeletal muscle insulin sensitivity) can lead to hyperinsulinemia and an accelerated development of breast cancer. [34] Since genetically predicted 2-hour glucose is correlated with instruments for other traits, we cannot completely rule out the possibility that the association of

2-hour glucose may be mediated by other insulin-related traits; even these traits were carefully adjusted and having pleiotropic SNPs excluded in our analyses.

We reported previously that genetically predicted BMI was inversely associated with breast cancer risk in both pre- and post-menopausal women. [35] We have now confirmed this finding with a much larger sample size and more BMI-associated SNPs. While our finding for pre-menopausal breast cancer is consistent with previous observational studies, the inverse association observed in our study between genetically predicted BMI and post-menopausal breast cancer risk contradict prior findings based on measured BMI. Multiple lines of evidence suggest that early life body size may be inversely associated with both premenopausal and postmenopausal breast cancer risk. [36,37] It has been speculated that reduced serum estradiol and progesterone levels, due to an increased frequency of anovulation, play a role. In addition, the association is further supported by the observation that early life fatness was inversely correlated with IGF-1 levels measured in later adulthood. [38] We hypothesize that genetically predicted BMI may be more closely correlated to early life body weight, while obesity determined using measured BMI later in life may be more closely related to environmental and lifestyle factors that are associated with breast cancer risk. Indeed, one previous study found that a BMI-genetic score was positively associated with weight gain before reaching middle age but inversely associated with weight gain after reaching middle age. [39] If the hypothesis is correct, our study may provide additional support for preventing weight gain later in life to reduce the risk of breast cancer.

Results from previous studies regarding the association of WHR with breast cancer risk have been inconsistent. While several previous studies reported that measured WHR was associated with breast cancer risk, [40] we recently found that this association was substantially attenuated after adjusting for BMI using data from a large prospective cohort study conducted among Chinese women. [41] In the current study, we observed an inverse association between

genetically predicted $WHR_{adj\ BMI}$ and breast cancer risk in both pre- and post-menopausal women. This finding was unexpected given the close association of measured WHR with type 2 diabetes. [42] As discussed previously for the BMI findings, we hypothesize that genetically predicted $WHR_{adj\ BMI}$ may reflect visceral adipose tissue level in early life, while measured WHR in late adulthood reflect accumulation of visceral fats later in life. Additional research is needed to understand the inter-relationship of genetically predicted WHR, measured WHR and breast cancer risk.

We showed that genetically predicted obesity and circulating insulin and glucose levels were positively correlated with risk of type 2 diabetes. Epidemiologic studies have shown that a prior diagnosis of type 2 diabetes is related to an elevated risk of breast cancer risk, although the association was weak to moderate. [43] However, in a previous study, we found a null association between a polygenetic risk score for type 2 diabetes and breast cancer risk. [44] It is possible that lifestyle changes after diabetes diagnosis and/or diabetes treatment may have confounded this association. Given the significant association we found in this study for breast cancer risk with genetically predicted fasting insulin and 2-hour glucose, two factors that are strongly associated with type 2 diabetes risk, we suggest that type 2 diabetes may be associated with breast cancer risk.

The sample size of our study is very large, providing us sufficient statistical power for Mendelian randomization analyses of multiple obesity and glucose/insulin-related traits with breast cancer risk. Our ability to perform Mendelian randomization analysis is limited by the genetic variants identified to date in GWAS, and the variance explained by these genetic variants for some traits is small. We used 10 instruments in our main analysis, which could lead to false-positive findings due to multiple comparisons. However, the associations reported in this study for 2-hour glucose, fasting insulin, BMI, and $WHR_{adj\ BMI}$ were robust, reaching the stringent Bonferroni corrected significance level (p<0.05/10=0.005). Pleiotropy was found for the

associations of obesity but it is not likely that the observed associations can be primarily explained by pleiotropic effects.

In summary, this study provided new evidence that genetically predicted fasting insulin, 2-hour glucose, BMI, and $WHR_{adj\ BMI}$ are associated with breast cancer risk in women. Further research into the complex association of genetics, obesity, glucose/insulin-related traits, and breast cancer risk will help to improve the understanding of underlying biological mechanisms for the associations observed in this study and provide tools to reduce breast cancer risk.

Figure Legend

Figure 1. Associations of genetically predicted obesity and levels of circulating glucose and insulin with overall breast cancer risk: stratified analysis

The $P_{heterogeneity}$ was obtained from heterogeneity test across strata.

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