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Adiposity and estrogen receptor-positive, postmenopausal breast cancer risk: quantification of the mediating effects of fasting insulin and free estradiol

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ER: estrogen receptor; SHBG: sex hormone binding globulin; BMI: body mass index; RR: relative risk CI: confidence interval; IGF-1: insulin-like growth factor-1; WHIOS: Women's Health Initiative Observational Study; HR: hazard ratio; PLCO: Prostate, Lung, Colorectal, and Ovarian Cancer; MCCS:

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Melbourne Collaborative Cohort Study; ICC: intraclass correlation; TE: total effect; IDE: interventional direct effect; IIE: interventional indirect effect; IQR: interquartile range

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Novelty and Impact

Adiposity increases the risk of estrogen receptor-positive postmenopausal breast cancer. Better understanding of mechanisms underlying this association could help with identifying new targets for cancer prevention. In this study of 1,178 women (149 cases), we applied novel statistical methods to quantify mediating effects of insulin and free estradiol on this association. Our results suggest that free estradiol explains adiposity-breast cancer association only in part. Identifying other pathways contributing to this relation should be a priority.

Abstract

Adiposity increases estrogen receptor (ER)-positive postmenopausal breast cancer risk. While mechanisms underlying this relation are uncertain, dysregulated sex-steroid hormone production and insulin signaling are likely pathways. Our aim was to quantify mediating effects of fasting insulin and free estradiol in the adiposity and ER-positive postmenopausal breast cancer association. We used data from a case-cohort study of sex hormones and insulin signaling nested within the Melbourne Collaborative Cohort Study. Eligible women, at baseline, were not diagnosed with cancer, were postmenopausal, did not use hormone therapy, and had no history of diabetes or diabetes medication use. Women with ER-negative disease or breast cancer diagnosis within the first follow-up year were excluded. We analysed the study as a cumulative sampling case-control study with 149 cases and 1,029 controls. Missing values for insulin and free estradiol were multiply imputed with chained equations. Interventional direct (IDE) and indirect (IIE) effects were estimated using regression-based multiple-mediator approach. For women with body mass index (BMI) >30kg/m^2 compared with women with BMI 18.5kg/m^2 to 25kg/m^2, the risk ratio (RR) of breast cancer was 1.75 (95% confidence interval (CI) 1.05-2.91). The estimated IDE (RR) not through the mediators was 1.03 (95% CI 0.43-2.48). Percentage mediated effect through free estradiol was 72% (IIE-RR 1.56; 95% CI 1.11-2.19). There was no evidence for an indirect effect through insulin (IIE-RR 1.12; 95% CI 0.68-1.84; 28% percentage mediated). Our results suggest that circulating free estradiol plays an important mediating role in the adiposity-breast cancer relationship but does not explain all of the association.

Introduction

There is convincing evidence that adiposity increases the risk of estrogen receptor (ER)-positive breast cancer in postmenopausal women [1, 2]. The mechanisms underlying this association are uncertain, but disturbed production of sex-steroid hormones related to increased mass and activity of adipose tissue is recognized as one of the main candidate pathways [3]. Circulating estrogen concentrations are elevated in overweight and obese postmenopausal women while sex hormone binding globulin (SHBG) concentrations are decreased, thereby raising bioavailable estrogens (estradiol and estrone) [4, 5]. Estrogens might increase breast cancer risk through their oxidative metabolites, and by affecting cell proliferation and apoptosis through their interaction with the ER in breast tissue [6]. A positive association between circulating estrogens and the risk of postmenopausal breast cancer has been documented consistently by prospective epidemiological studies [7-9]. In addition, suggestive of a mediating effect of estrogens on the adiposity-postmenopausal breast cancer association, the association between body mass index (BMI) and postmenopausal breast cancer is attenuated after adjusting for circulating estrogens, in particular, free estradiol [10, 11]. A pooled analysis of eight prospective studies showed that adjusting for free estradiol changed the relative risk (RR) of postmenopausal breast cancer for a 5 kg/m^2 increment in BMI from 1.19 (95% confidence interval (CI) 1.05 to 1.34) to 1.02 (95% CI 0.89 to 1.17) in women not using hormone therapy at the time of blood draw [10].

Insulin resistance, a condition with a well-established association with adiposity, is another pathway hypothesized to link adiposity with postmenopausal breast cancer [3]. Insulin could affect cancer risk directly by its mitogenic properties and by increasing secretion of insulin-like growth factor-1 (IGF-1) and down-regulating IGF-1 binding proteins [12]. Some evidence suggests that insulin might increase breast cancer risk by downregulating SHBG production and increasing bioavailable estrogen levels [3, 13]. Epidemiological studies investigating the association between insulin or C-peptide (a marker for insulin production) and postmenopausal breast cancer risk have been inconclusive [3, 14]. However, in a case-cohort study within the Women's Health Initiative Observational Study (WHIOS), a positive association was observed between fasting insulin and postmenopausal breast cancer in women not using hormone therapy and adjusting for fasting insulin substantially attenuated the association between BMI and postmenopausal breast cancer risk (hazard ratio (HR) for BMI≥30 kg/m^2 compared with 18.5 to 24.9 kg/m^2 prior to and after adjustment were 2.12 95% CI 1.26 to 3.58) and 1.50 (95% CI 0.80 to 2.83) respectively) [15].

Quantification of the effects of biomarkers as intermediaries of the adiposity-postmenopausal breast cancer association using formal mediation analysis might contribute to a better understanding of the potential mechanistic pathways [16]. This knowledge might be instrumental in identifying targets for pharmacological and lifestyle interventions for the prevention of adiposity-related breast cancer [4]. Two studies within the WHIOS [17] and the Prostate, Lung, Colorectal, and Ovarian Cancer (PLCO) study [18] have used modern methods for mediation analysis based on the potential outcomes approach [16] to quantify the mediating effects of insulin [17] and estradiol [17, 18] in the BMI-breast cancer association. Neither of these studies had a measure of free estradiol, which, compared with total estradiol, might play a more important role in mediating the effect of adiposity on breast cancer risk [10, 11].

For this study, we used data from a case-cohort study of sex hormones and insulin signaling and cancer nested within the Melbourne Collaborative Cohort Study (MCCS) [7, 19]. We used a regression-based multiple-mediator approach to estimate interventional indirect effects [20] quantifying the extent to which the associations between two proxy measures for adiposity - BMI and waist circumference – and ER-positive postmenopausal breast cancer were mediated through fasting insulin and free estradiol.

Materials and Methods

The Melbourne Collaborative Cohort Study

The MCCS includes 24,469 women recruited in Melbourne, Australia, in 1990-1994 [21]. Almost all participants (99%) were aged between 40 and 69 years at baseline. Clinical data collection, including blood sample (74% fasting) and height, weight, and waist circumference measurements were administered at baseline based on standard procedures by nurses at dedicated recruitment clinics [21]. Plasma was stored in liquid nitrogen. Women were interviewed about lifestyle factors, use of hormone therapy and oral contraceptives, and reproductive history. The study protocol was approved by the Cancer Council Victoria Human Research Ethics Committee and participants provided written informed consent to participate. The data that support the findings of this study are held by the Cancer Epidemiology Division, Cancer Council Victoria. Restrictions apply to the availability of these data, which were used under license for this study. Information on how data from the MCCS (Health 2020) data can be accessed is available at https://www.pedigree.org.au/.

Participants

Details of the case-cohort study have been published [21]. For this analysis, women were ineligible if, at baseline, they did not have body size measurements recorded or did not provide blood, had BMI<18.5kg/m^2, had been diagnosed with any cancer, were current users of hormone therapy, had a history of diabetes or diabetes medication use, or were not postmenopausal. Women were classified as postmenopausal if their periods had stopped naturally for more than 12 months, or their menopausal status was unknown but they were at least 56 years old [22], or they had a hormone measurement recorded, with estradiol concentration <109 pmol/L [22]. The sample included all eligible women diagnosed with invasive adenocarcinoma of the breast between baseline and 30 June 2002 (although for this analysis women with ER-negative or unknown ER status cancers were excluded), and a random sample of eligible women from the cohort (sub-cohort) (**Figure 1**).

Diagnoses of breast cancer were ascertained through record linkage to the population-based Victorian Cancer Registry. ER status was determined from histopathology reports or, if not reported, from immunohistochemical analysis of archival tumor tissue [7].

For the mediation analysis, because methods for handling multiple mediators with survival outcomes or complex designs have only recently been proposed and are still being developed [23], we analyzed the study as a case-control study by using as the control group women in the sub-cohort who had not been diagnosed with breast cancer (**Figure 1**). Sub-cohort members who left Australia or died before 30 June 2002 (identified by record linkage to Victorian death records and the National Death Index) were also excluded. Hence, the remaining sub-cohort participants were known to be genuine "controls" and constituted the control group of the case-control analysis.

Plasma Analysis

Free estradiol concentration was calculated from measured circulating total estradiol and SHBG. Insulin, total estradiol, and SHBG were not measured for women who had insufficient or contaminated plasma (**Figure 1**). Insulin was only measured for women who were fasting at the time of blood draw (**Figure 1**). A detailed description of the laboratory measurements has been published [7]. Briefly, plasma samples were retrieved from liquid nitrogen, aliquoted into 450-μL amounts, randomly allocated to batches with approximately equal number of cases and sub-cohort participants across batches, and shipped to the laboratory of H.M. One scientist, blinded to the case-control status of the samples, performed all the measurements. Samples were thawed in a warm water bath, vortexed rapidly for a few seconds, and

centrifuged at 2,000 (210 × g) for 10 minutes. The assays used for measurement of total estradiol and SHBG were electrochemiluminescence immunoassay (Elecsys 2010 analyzer, Roche Diagnostics GmbH, Mannheim, Germany) and immunometric assay (IMMULITE analyzer, DPC, Los Angeles, CA, USA) respectively. The overall coefficients of variation from pooled plasma were 10% at 157 pmol/L for total estradiol and 7% at 45.0 nmol/L for SHBG. From a reliability study done in 45 women with blood samples drawn at baseline and approximately 1 year later, the intraclass correlation (ICC) was 0.90 (0.85 – 0.95) for SHBG; the ICC was not estimated for total estradiol because of insufficient samples [7]. Free (protein-unbound) estradiol concentration was calculated from measured total estradiol and SHBG, assuming a fixed albumin concentration of 40 g/L and using the law of mass action [7].

For the fasting participants, plasma insulin was measured using AxSYM Microparticle Enzyme Immunoassay (Abbott, North Ryde, NSW, Australia).

Statistical Analysis

Waist circumference and BMI were used as proxy measures for adiposity in separate analyses. The World Health Organization cut-off values were used to generate binary variables comparing obese with normal weight (BMI, ≥30 vs <25 kg/m^2; waist circumference, >88 vs ≤80 cm) for each measure. The choice to primarily treat these exposure variables as binary was made to assist with the interpretation of the estimated mediated effects (see Mediation Analysis) and utility for policy-making.

To remove batch effects, we first fitted linear mixed effects models, including a random effect for batch, to the log-transformed biomarker values for women in the sub-cohort. Next, for all women, the predicted batch-specific deviations from the overall mean were subtracted from the observed values.

Exposure-mediator association

Linear regression models were fitted to describe the relationship between fasting insulin and calculated free estradiol concentrations and adiposity quantified as BMI or waist circumference; for each biomarker, ratios of geometric means and corresponding 95% CIs were calculated for women classified as overweight/obese compared with those classified as normal BMI and waist circumference. Models were fitted to the sub-cohort participants with available biomarker measurements (complete-case) and adjusted for potential confounders identified using prior knowledge in the literature and depicted in a causal diagram (**Figure 2**).

Exposure-outcome and mediator-outcome associations

Cox regression with age as the time axis was fitted to estimate the HR and 95% CI for the association between postmenopausal breast cancer and each adiposity measure or biomarker. The Prentice approach with robust standard errors was used to take the case-cohort design into account [24]. For the sub-cohort, observation time started at age at recruitment and ended at breast cancer diagnosis, death, last age known to be in Australia, or the end of follow-up, whichever occurred first. All models included potential confounders (**Figure 2**). Graphical inspection of the Schoenfeld residuals suggested no departure from proportionality of the hazards [25].

Mediation Analysis with Multiple Mediators

For mediation analysis, the binary case-control status was the outcome. The exposure was the binary measure of adiposity; mediators were the continuous normalized variables for fasting insulin and calculated free estradiol. All mediation analyses were guided by the causal diagram in **Figure 2**, which was developed with reference to the existing literature [2, 26, 27]as well as expert opinion and took into account the potential confounders of the exposure-outcome, mediator-outcome, and exposure-mediator relationships [16]. The total effect (TE) of adiposity on the risk of ER-positive breast cancer was estimated using logistic regression, with the result in this case-control analysis providing similar results to those from the original case-cohort study using Cox regression. The TE was decomposed into the interventional direct effect (IDE) not through either of the mediators, the interventional indirect effect (IIE) through fasting insulin, IIE through calculated free estradiol, and the difference between the TE and the sum of the estimated IDE and IIE [20]. This latter effect essentially captures the IIE through the dependence between fasting insulin and calculated free estradiol [20]. **Table 1** summarizes the interpretations and **Supplementary Table 1** the expressions used for estimating the IDE and IIEs, which are based on [20, 28]. The effects were estimated on the risk difference and risk ratio scales, based on the following models: 1) linear regression of fasting insulin conditional on exposure and baseline confounders; 2) linear regression model of calculated free estradiol conditional on the exposure and baseline confounders; 3) linear regression model of calculated free estradiol conditional on the exposure, fasting insulin, an interaction term between exposure and fasting insulin, and baseline confounders, 4) logistic regression of the outcome conditional on the exposure, both mediators, interaction terms between exposure and each mediator, interaction term between the two mediators, and baseline confounders. Models 1, 3 and 4 were combined to estimate the IDE, and models 1, 2 and 4 to estimate the

IIEs through fasting insulin or calculated free estradiol. The case-control design was taken into account by fitting the models for the mediators to the sub-cohort [29] and by subtracting the log of (1/sampling fraction of controls) from the constant term of the outcome model prior to calculating risks [30].

Using the estimated absolute risk differences, we further calculated the proportion mediated, which could be interpreted as the importance of the biomarker in explaining the adiposity-cancer risk, by dividing the IIE risk difference by the sum of IDE and IIE risk differences [31].

Missing data

We had missing values for fasting insulin and calculated free estradiol (**Figure 1**). For mediation analysis, we used multiple imputation based on chained equations [32] with 40 iterations to impute missing values for each mediator, as well as for the interaction terms between each mediator and the outcome, and for the interaction term between the two mediators [33]. The imputation models were stratified on BMI and waist circumference (in separate models) [33], and included all the variables in the final mediation models and additional auxiliary variables (height, hip circumference, weight, and sub-cohort membership). The imputed mediator variables were then used to generate the interaction terms between each mediator and exposure [33].

Simulation-based regression-standardization was used to estimate mediation effects separately for each of the imputed datasets. This approach entails averaging estimates based on a large number of Monte-Carlo draws (in our case, 600,000) from the mediator distributions, with the distribution of the confounders set to be the same as their observed distribution in the sample, as suggested in [20]. Standard errors for the TE, IDE, and IIE (and 95% CI) within each imputed dataset were estimated using 1000 bootstrap samples. The estimates and standard errors derived for each imputed dataset were then pooled using Rubin rules [34] to calculate the multiple imputation estimate and its variance.

All analyses were performed in Stata version 14 [35].

Sensitivity Analysis

Mediation analyses were repeated to compare postmenopausal breast cancer risk for women with BMI≥25 vs <25 kg/m^2 and waist circumference >80 vs ≤80 cm. We also present results for continuous exposure measures (per 5 kg/m^2 increase in BMI or 10 cm increase in waist circumference).

Results

Of all women in the MCCS who met the eligibility criteria, 1,196 were randomly selected for the case-cohort study (**Figure 1**). Eighty-three women were excluded because of missing menopausal status and 13 women due to missing values for potential confounders. For the case-control analysis 45 women who had died or left Australia before the end of follow-up were additionally excluded. During follow-up, 264 women who met the eligibility criteria were diagnosed with breast cancer; 26 of these were also members of the sub-cohort; in the mediation analysis, these 26 women were included as cases only. Of the breast cancer cases, we excluded 13 with missing menopause status, 88 with ER-negative or unknown ER status tumors, 2 with missing values for potential confounders, and 12 who were diagnosed within the first year after recruitment. The final sample for mediation analysis comprised 149 ER-positive breast cancer cases and 1,029 controls. Of these, 330 women (45 cases) were not fasting at the time of blood draw and 108 (13 cases) had insufficient or contaminated plasma and were therefore not included in the complete-case analyses involving biomarkers, but they were in multiple imputation analyses.

The median age at cancer diagnosis was 68 ((interquartile range (IQR) 64 to 71) years and the median time to cancer diagnosis from baseline was 5.5 (IQR 3.7 to 7.0) years. For controls, the median follow-up time was 9.3 (IQR 8.4 to 10.3) years. **Table 2** summarizes the baseline characteristics of participants included in the case-control analysis.

Exposure-mediator associations

The geometric mean fasting insulin was 1.73 (95% CI 1.56 to 1.93) times higher for women with BMI ≥30 kg/m^2 compared with women with BMI <25 kg/m^2, and the geometric mean for calculated free estradiol was 1.49 (95% CI 1.36 to 1.62) times greater. Similar associations were observed for waist circumference (**Table 3**).

Exposure-outcome and mediator-outcome associations

The relationship between ER-positive postmenopausal breast cancer risk and adiposity measures and biomarkers are reported in **Table 4**. For both BMI and waist circumference, women categorized as obese had an increased HR for breast cancer compared with the reference group (BMI ≥30 vs <25kg/m^2 HR 1.75; 95% CI 1.10 to 2.77; waist circumference >88 vs ≤80cm hazard ratio 1.85; 95% CI 1.22 to 2.81). There was

also an increased risk of postmenopausal breast cancer associated with calculated free estradiol (HR 1.56, 95% CI 1.12 to 2.16), but not with fasting insulin (HR 1.00, 95% CI 0.65to 1.53).

Mediation Analysis with Multiple Mediators

From mediation analysis with multiple imputation to handle missing insulin and estradiol data, when using BMI as proxy for obesity, the TE risk ratio for obese women compared with normal women was 1.75 (95% CI 1.05 to 2.91). The estimated IDE risk ratio not through either of the mediators was 1.03 (95% CI 0.43 to 2.48). The risk ratio was 1.12 (95% CI 0.68 to 1.84) through fasting insulin and 1.56 (95% CI 1.11 to 2.19) through calculated free estradiol (point estimate on the risk difference scale corresponding to 28% and 72% mediated effect respectively). The estimated IIE risk ratio through the interdependence between fasting insulin and calculated free estradiol (effect #4 **Table 1**) was 0.90 (95% CI 0.63 to 1.29) (**Table 5**).

When using waist circumference as proxy for obesity, the TE risk ratio for overweight or obese versus normal women was 1.96 (95% CI 1.23 to 3.13); the IDE was 1.02 (95% CI 0.47 to 2.22); and the IIEs were 1.23 (95% CI 0.81 to 1.88) through fasting insulin (32% mediated effect), 1.42 (95% CI 1.09 to 1.87) through calculated free estradiol (54% mediated effect), and 1.00 (95% CI 0.81 to 1.25) through the interdependence between the two biomarkers (**Table 5**).

Sensitivity analyses

Results comparing ER-positive breast cancer risk for women with BMI≥25 vs <25 kg/m^2 or waist circumference >80 vs ≤80 cm, as well as results for continuous exposures are presented in Supplementary Table 3. For all exposure variables, there was evidence for a mediated effect through free estradiol (with the proportion mediated ranging from 30% for waist circumference per 10 cm and 46% for waist circumference >80 vs ≤80 cm to 51% for BMI per 5 kg/m^2 and 71% for BMI ≥25 vs <25 kg/m^2) but not for fasting insulin.

Potential influence of missing data

Biomarker measurements were not available for 37% of the participants. When compared with women who had missing data for the mediation analysis, women with complete data had similar proportions of breast cancer cases (12% vs. 13%), as well as distributions of BMI (median (IQR) 26.7kg/m^2 (24.2 to 30.1) vs 26.9kg/m^2 (24.1 to 30.5)) and waist circumference (median (IQR) 81 cm (74 to 90) in both groups). Also, no

notable differences were observed for the distribution of other variables between the two groups (**Supplementary Table 2**).

Results from complete case analysis are provided for all mediation analyses (**Table 5** and **Supplementary Table 3**). Compared with multiple imputation analyses, for both BMI and waist circumference, the TEs estimated from complete case analyses were weaker, while the IIEs through calculated free estradiol were stronger. There was no evidence for mediating effect through fasting insulin using the complete case analysis.

Discussion

We estimated that increased calculated free estradiol concentrations explain approximately half of the association between measures of adiposity (BMI or waist circumference) and ER-positive breast cancer in postmenopausal women. There was no evidence of a mediating effect through insulin.

To our knowledge, of the existing studies that have quantified the mediating effect of estradiol on adiposity-postmenopausal breast cancer association [17, 18], our study is the only study with a measure for free estradiol. A strength of mediation analysis based on interventional effects is that it allowed us to quantify the mediating effects of fasting insulin and calculated free estradiol without assuming that the two mediators do not influence each other directly [20]. The method also allowed for inclusion of possible exposure-mediator and mediator-mediator interactions in the models [20, 36]. These are important advantages over the traditional approaches used to assess mediation, which are invalid in the presence of exposure-mediator interactions or multiple interrelated mediators [16]. While we controlled for the known measured confounders of exposure-outcome, mediator-outcome, and exposure-mediator relationships, we did not measure biomarkers in other biological mechanisms that could also serve as mediators of the obesity-breast cancer relationship such as the inflammatory pathway. Inflammatory factors might influence fasting insulin and free estradiol levels, as well as postmenopausal breast cancer risk [3]. Ignoring these potential mediator-outcome confounders might have led to overestimation of the indirect effect through calculated free estradiol and fasting insulin. Our results might have also been biased due to residual confounding from unmeasured (e.g. family history of breast cancer) or imperfectly measured confounders. Another limitation of our study was the lack of longitudinal measurements for adiposity and biomarkers (we used measures collected at baseline), which precluded us from ruling out reverse causation with respect to the exposure and mediator. Preclinical cancer

might have influenced adiposity or levels of the biomarkers; to address this, we excluded all cases diagnosed within the first year after recruitment.

BMI and waist circumference were measured following standard protocols, and breast cancer cases were ascertained through linkage to a population-based cancer registry, minimizing measurement error. The limitations of using BMI to define adiposity are well known [4]. Using the percentage of body fat measured by dual-energy x-ray absorptiometry scans for validation, it has been observed that, depending on age and race, BMI has a sensitivity ranging from 42.1% (95% CI 36.2% to 48.1%) to 73.7% (95% CI 65.5% to 82.8%) to identify postmenopausal obese women [37]. The non-differential misclassification of obesity based on BMI (i.e. misclassification of exposure independent of outcome) might have led us to underestimate the BMI-breast cancer association in our analyses. We also report results for waist circumference as another proxy for adiposity, which is considered a better indicator of active visceral adipose tissue and the preferred measure of adiposity [4]. Circulating total estradiol and SHBG, which were used in calculation of free estradiol, were well-measured and stable over time, as demonstrated by high ICCs in the reliability study and/or low coefficient of variation for the assays [7]. Sex-steroid hormones levels in serum and breast adipose tissue are highly correlated [8, 38] and it is likely that circulating hormones levels are reflective of hormone concentration in breast tissue [8]. We did not have measures of reliability for the assay used to measure fasting insulin. We had measures for insulin-like growth factor-1 and binding protein-3 in our cohort but did not include these as potential mediators. Although there is strong evidence supporting a positive association between these biomarkers and postmenopausal breast cancer risk[39], their relation with obesity is uncertain [27]. Our study excluded women who, at baseline, had a history of diabetes, because insulin production might decrease in response to long-term hyperglycemia [40]. This exclusion might have led to an underestimation of the indirect effect through insulin. Perhaps the most important limitation of our study was that biomarker measurements were not available for 37% of the women because they were either not fasting at the time of blood collection (28%) or had insufficient or contaminated plasma. This missingness was handled using multiple imputation and assuming that the data were missing dependent only on measured variables.

Results of a causal mediation analysis from the WHIOS suggested that of the overall BMI- postmenopausal breast cancer association (additional cases per 100,000 women for a 5 kg/m^2 increment in BMI 50.0 (95% CI 23.2 to 76.6)), 24% could be attributed to total estradiol (indirect additional cases per 100,000 11.9 (95% CI 1.7 to 22.6)) [17]. The proportion mediated through total estradiol was higher for ER-positive cancers (49%). A sub-study from the PLCO, reported the estimated total effect for a 5 kg/m^2 increment in BMI on

risk of ER-positive postmenopausal breast cancer as 7.0 (95% CI 2.1 to 11.9) additional cases per 100,000, of which 20% was through total estradiol (indirect additional cases per 100,000 1.4 (95% CI -1.0,3.9)) [18]. In our study, 51% of the TE of BMI per 5 kg/m^2 and 30% of the TE of waist circumference per 10 cm on ER positive breast cancer could be attributed to calculated free estradiol. The stronger indirect effect through free estradiol estimated in our study compared with the reported indirect effects through total estradiol in the other two studies is in line with the evidence suggesting that bioavailable estradiol is a more important player in explaining the adiposity-breast cancer association than total estradiol [10, 11]. Together, these observations imply that estradiol, particularly free estradiol, plays a substantial role in mediating the effects of adiposity on breast cancer risk. But despite its importance, free estradiol does not explain all the association between adiposity and breast cancer.

In the WHIOS, about 66% of the BMI-postmenopausal breast cancer association in non-diabetic women was mediated through insulin (additional cases per 100,000 for the overall effect for 5 kg/m^2 increment in BMI 52.0 (95% CI 12.1 – 91.3) and indirect effect through insulin 34.2 (95% CI 9.4 – 59.0)) [17]. In our study, there was no evidence for an indirect effect through insulin. In line with this difference, the observed association between fasting insulin and postmenopausal breast cancer was weaker in our study compared with that reported for the WHIOS (HR for postmenopausal breast cancer in non-hormone therapy users for highest vs. lowest quartiles of fasting insulin 2.40 (95% CI 1.30 to 4.41)) [15]. Different assays used to measure insulin might explain some of the difference in the insulin-postmenopausal breast cancer association observed in our study and WHIOS [14]. In a meta-analysis investigating the association between serum insulin and breast cancer, the summary relative risk for the association between insulin and breast cancer was 1.02 (95% CI 0.53 to 1.97) for six prospective studies. The association did not change when analysis was limited to studies with postmenopausal breast cancer as the outcome [14]. A high heterogeneity was reported across studies (I^2 73%), which was suggested to be in part explained by the variety of assays used to measure insulin levels [14]. Further research is required to examine the presence and magnitude of a mediating role of the insulin signaling pathway in the adiposity-breast cancer association.

In summary, we applied novel mediation methods to quantify the mediating effect of two potentially interrelated pathways on the association between adiposity and ER-positive postmenopausal breast cancer. Our results provide additional evidence that free estradiol plays a key role in mediating the adiposity-postmenopausal breast cancer relationship. However other pathways must also be important for explaining

this association; identifying other contributing pathways and quantifying their contribution to breast cancer development should be a priority.

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Data availability

The data that support the findings of this study are held by the Cancer Epidemiology Division, Cancer Council Victoria. Restrictions apply to the availability of these data, which were used under license for this study. Information on how data from the Melbourne Collaborative Cohort Study (Health 2020) data can be accessed is available at https://www.pedigree.org.au/.

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Tables

Table 1- The interpretations of interventional direct and indirect effects estimated in this study

#	Effect	Interpretation [20,26]
1	Interventional direct effect not through fasting insulin or calculated free estradiol	Average relative change in probability of ER-positive postmenopausal breast cancer risk for overweight or obese versus normal weight women, when fixing the joint distribution of fasting insulin and calculated free estradiol to a random draw from their observed joint distribution adjusted for baseline confounders in normal weight women.
2	Interventional indirect effect through fasting insulin	Average relative change in probability of ER-positive postmenopausal breast cancer risk for overweight or obese women, while intervening to shift the distribution of fasting insulin from its observed distribution in overweight or obese women adjusted for baseline confounders, to its observed distribution in normal weight women adjusted for the baseline confounders and fixing the distribution of calculated free estradiol to a random draw from its observed distribution in normal weight women adjusted for the baseline confounders.
3	Interventional indirect effect through calculated free estradiol	Average relative change in probability of ER-positive postmenopausal breast cancer risk for overweight or obese women, while fixing the distribution of fasting insulin to a random draw from its observed distribution in overweight or obese women, adjusted for the baseline confounders and intervening to shift the distribution of calculated free estradiol from its observed distribution in overweight or obese women adjusted for the baseline confounders to its observed distribution in normal weight women adjusted for the baseline confounders
4	Interventional indirect effect through dependence of fasting insulin and calculated free estradiol	Difference between the total effect and the sum of direct and indirect effects (effects 1 to 3). This effect captures the average relative change in probability of ER-positive postmenopausal breast cancer for overweight or obese women through the dependence between fasting insulin and calculated free estradiol. This effect is generally expected to diverge from null in the presence of mediator-mediator and exposure-mediator interactions [20].

The corresponding expressions used for the effects are provided in Supplementary Table 1.

Table 2 – Baseline characteristics of cases and controls

	Breast cancer cases (N=149)	Controls (N=1,029)
Age at baseline, *years*; median (interquartile range)	63 (59 – 66)	61 (57 – 65)
Was fasting at baseline and had plasma available for biomarker measurement; n (%)	91 (61)	649 (63)
Country of birth, *grouped*; n (%)		
Australia/New Zealand/ Northern Europe	119 (80)	758 (74)
Southern Europe	30 (20)	271 (26)
Socioeconomic position, *quintile*; n (%)		
1 (most disadvantaged)	30 (20)	228 (22)
2	31 (21)	244 (24)
3	23 (15)	133 (13)
4	31 (21)	183 (18)
5 (least disadvantaged)	34 (23)	241 (23)
Physical activity score; n (%)		
None	24 (16)	217 (21)
Low	36 (24)	217 (21)
Medium	60 (40)	407 (40)
High	29 (20)	188 (18)
Smoking status; n (%)		
Never smoked	110 (74)	749 (73)
Former smoker	34 (23)	200 (19)
Current smoker	5 (3)	80 (9)
Alcohol consumed per day in the last decade, *grams per day*; n (%)		
0	69 (46)	562 (55)
1-19	66 (44)	382 (37)
≥20	14 (9)	85 (8)
Personal history of cardiovascular disease; n (%)	13 (9)	57 (6)
Personal history of arthritis; n (%)	67 (45)	493 (48)
Age at menarche, *years*; n (%)		
<12	25 (17%)	141 (13.7%)
12	24 (16%)	210 (20.4%)
13	36 (24%)	263 (25.6%)
>13	64 (43%)	415 (40.3%)
Personal history of contraceptive pill use; n (%)	62 (42)	455 (44.2%)
Personal history of hormone therapy use; n (%)	21 (14)	143 (13.9%)
Parity and age at first live birth; n (%)		
Nulliparous	16 (11)	120 (12)
1	11 (7)	70 (7)
>1 & <25 years	60 (40)	421 (41)
>1 & ≥25 years	62 (42)	418 (41)
Lactation duration; *months*; n (%)		
Never	28 (19)	177 (17)
Up to 6	46 (31)	293 (29)
7-12	27 (18)	174 (17)
>12	48 (32)	385 (37)
Body mass index, kg/m^2; median (interquartile range)	27.5 (24.4-32.3)	26.7 (24.2-30.0)
≥18.5 - <25	46 (31)	346 (34)
≥25 - <30	52 (35)	425 (41)
≥30	51 (34)	258 (25)

Waist circumference, *cm*; median (interquartile range)	82.5 (76-94.5)	80.9 (74.0-89.0)
≤80	61 (41)	492 (48)
>80 - ≤88	32 (22)	265 (26)
>88	56 (38)	272 (26)
Fasting insulin, *pmol/L*, median (interquartile range)	5.3 (3.8-8.8)	5.5 (3.8-7.7)
Calculated free estradiol, *pmol/L*, median (interquartile range)	0.9 (0.6-1.2)	0.8 (0.6-1.0)

Table 3 – Estimated relative change in geometric mean of fasting insulin and calculated free estradiol levels associated with measures of adiposity in a random sample of the cohort eligible for this study and available for complete-case analysis (sub-cohort members; n=649; 9 with breast cancer)

	Median value	Relative change in geometric mean (95% CI)	
Exposure		Fasting insulin	Calculated free estradiol
Body mass index, kg/m^2			
<25	23.2 kg/m^2	1 (Reference)	1 (Reference)
≥25 - <30	27.1 kg/m^2	1.25 (1.15-1.38)	1.21 (1.12-1.30)
≥30	33.2	1.73 (1.56-1.93)	1.49 (1.36-1.62)
Waist circumference, cm			
≤80	73.5 cm	1 (Reference)	1 (Reference)
>80 - ≤88	84 cm	1.33 (1.21-1.47)	1.19 (1.10-1.29)
>88	94	1.65 (1.50-1.81)	1.37 (1.26-1.48)

Estimates are from linear regression models of log transformed normalized biomarker values on measure of adiposity (obese/overweight versus normal classifications), adjusted for age at baseline, country of birth, socioeconomic index for area, physical activity, smoking status, alcohol intake, history of cardiovascular disease, history of arthritis, age at menarche, history of hormonal contraceptive use, history of hormone therapy use, parity and age at first live birth, and lactation duration

Table 4 – Association between measures of adiposity, fasting insulin, and calculated free estradiol and ER-positive postmenopausal breast cancer risk

	Case-cohort study (n=1,222) *		Case-control sub-study (n=1,178) †
	No cases/ person-years	Hazard Ratio (95% CI)	Odds Ratio (95% CI)
Body mass index			
<25	46/3,274	1 (Reference)	1 (Reference)
≥25 - <30	52/4,201	0.98 (0.63-1.52)	1.03 (0.67 to 1.60)
≥30	51/2,567	1.75 (1.10-2.77)	1.78 (1.12 to 2.83)
Waist circumference;			
≤80	61/4,676	1 (Reference)	1 (Reference)
>80 - ≤88	32/2,585	1.07 (0.67-1.69)	1.08 (0.68-1.73)
>88	56/2,781	1.85 (1.22-2.81)	1.93 (1.26-2.95)
Biomarkers, *per doubling concentration* ‡			
Fasting insulin	91/6,321	1.00 (0.65 to 1.53)	1.06 (0.72 to 1.57)
Calculated free estradiol		1.56 (1.12 to 2.16)	1.54 (1.07 – 2.22)

* For these analyses, age was the timescale, observation time started at age at recruitment and ended at age at breast cancer diagnosis, death, last known age known to be in Australia, or age at the end of follow-up, whichever occurred first. Prentice method and robust standard errors were used to account for the case-cohort design. † For these analyses, controls who left Australia or died before 30 June 2002 were excluded (n=45). ‡ Estimates are from complete case analysis (i.e. excluding women who did not have biomarker measurements) and models that included log-2 transformed biomarkers values after normalization, and were additionally adjusted for body mass index.

All models were adjusted for country of birth, socioeconomic index for area, physical activity, smoking status, alcohol

intake, history of cardiovascular disease, history of arthritis, age at menarche, history of hormonal contraceptive use, history of hormone therapy use, and parity and age at first live birth and lactation duration

Table 5 – Results of multiple-mediator analysis with fasting insulin and free estradiol as the mediators of interest, body mass index (≥30 vs <25 kg/m^2) or waist circumference (>88 vs ≤80 cm) as exposure, and non-estrogen receptor negative postmenopausal breast cancer as outcome.

		Risk ratio (95% confidence interval)		Risk difference, per 10,000 (95% confidence interval)	
	Effect	Complete case analysis*	Multiple imputation†	Complete case analysis*	Multiple imputation†
BMI ≥30 vs <25 kg/m^2	Total effect	1.17 (0.55 to 2.47)	1.75 (1.05 to 2.91)	18.7 (-56.9 to 94.2)	80.6 (11.5 to 149.8)
	Interventional direct effect not through insulin and free estradiol	0.39 (0.08 to 1.92)	1.03 (0.43 to 2.48)	-51.8 (-114.9 to 11.3)	18.1 (-67.4 to 103.7)
	Interventional indirect effect through insulin	1.21 (0.56 to 2.60)	1.12 (0.68 to 1.84)	25.0 (-66.1 to 116.1)	28.0 (-70.6 to 126.6)
	Interventional indirect effect through free estradiol	2.08 (1.14 to 3.82)	1.56 (1.11 to 2.19)	71.5 (0.9 to 142.1)	72.2 (16.7 to 127.7)
	Interventional indirect effect through the interdependence of insulin and free estradiol	0.99 (0.60 to 1.63)	0.90 (0.63 to 1.29)	-10.8 (-64.3 to 42.8)	-18.7 (-81.3 to 43.9)
Waist Circumference >88 vs ≤80 cm	Total effect	1.15 (0.62 to 2.13)	1.96 (1.23 to 3.13)	18.3 (-51.0 to 87.5)	97.4 (29.6 to 165.1)
	Interventional direct effect not through insulin and free estradiol	0.29 (0.08 to 1.02)	1.02 (0.47 to 2.22)	-53.3 (-107.2 to 0.6)	23.1 (-41.7 to 88.0)
	Interventional indirect effect through insulin	1.42 (0.83 to 2.42)	1.23 (0.81 to 1.88)	27.0 (-48.9 to 102.8)	36.0 (-42.7 to 114.8)
	Interventional indirect effect through free estradiol	1.89 (1.23 to 2.91)	1.42 (1.09 to 1.87)	68.8 (7.3 to 130.3)	61.2 (13.8 to 108.7)
	Interventional indirect effect through the interdependence of insulin and free estradiol	1.14 (0.86 to 1.51)	1.01 (0.81 to 1.25)	-4.0 (-40.6 to 32.6)	-6.8 (-40.0 to 26.4)

*Models for mediation analyses were based on 600,000 Monte Carlo draws and 95% confidence intervals were constructed from 1,000 bootstrap draws. † Mediation analyses were performed within each imputed dataset (including 97 cases and 604 controls for BMI ≥30 vs <25 kg/m^2 and 117 cases and 764 controls for waist circumference >88 vs ≤80 cm) and resulting estimates and standard errors (derived from bootstrap draws) were pooled to obtain the multiple imputation estimates and 95% confidence intervals. All models were adjusted for country of birth, socioeconomic index for area, physical activity, smoking status, alcohol intake, history of cardiovascular disease, history of arthritis, age at menarche, history of hormonal contraceptive use, history of hormone therapy use, parity and age at first live birth, and lactation duration

Figure Legends

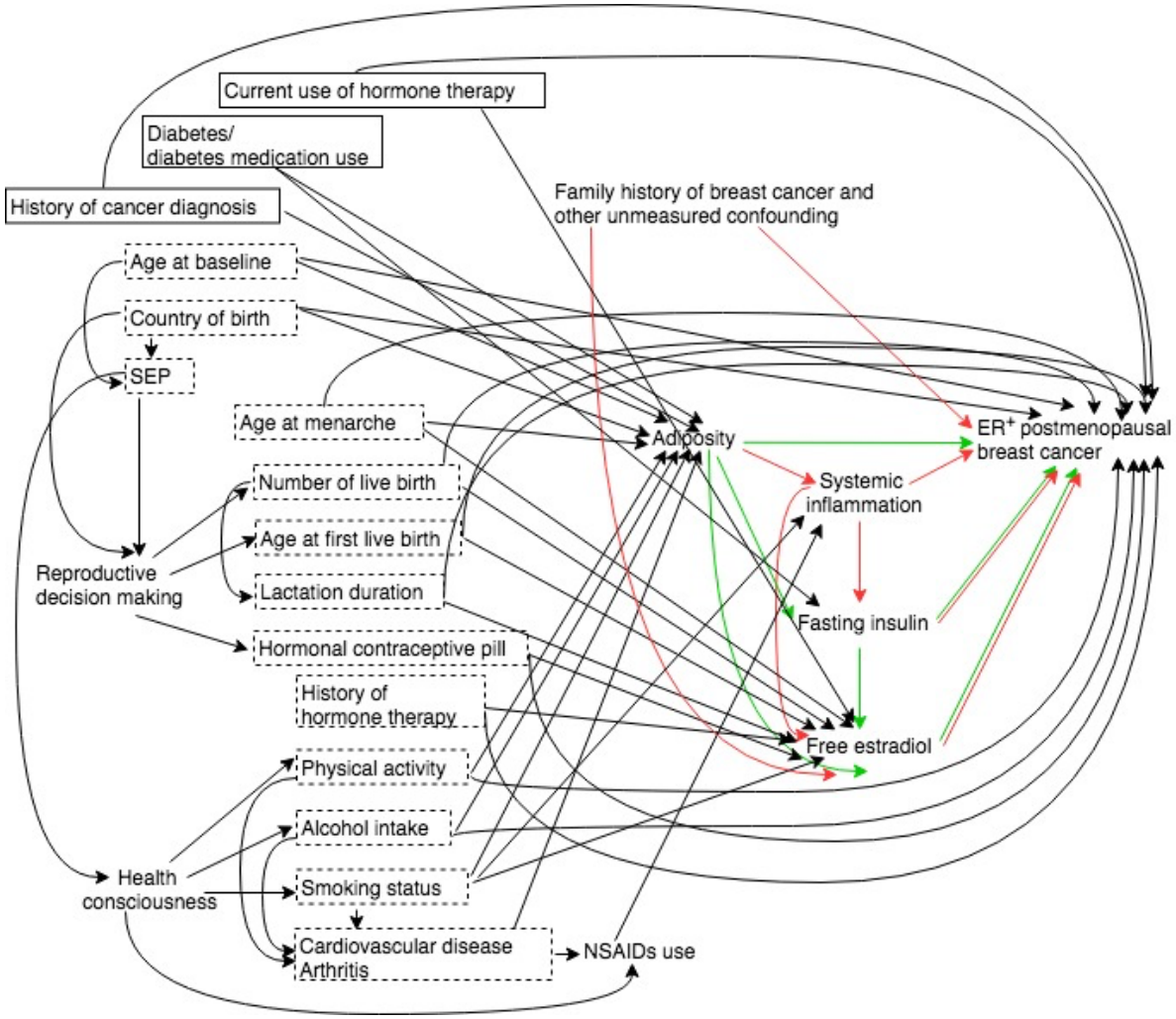
Figure 1 – Flowchart of female participants in the Melbourne Collaborative Cohort Study; abbreviation: ER estrogen receptor

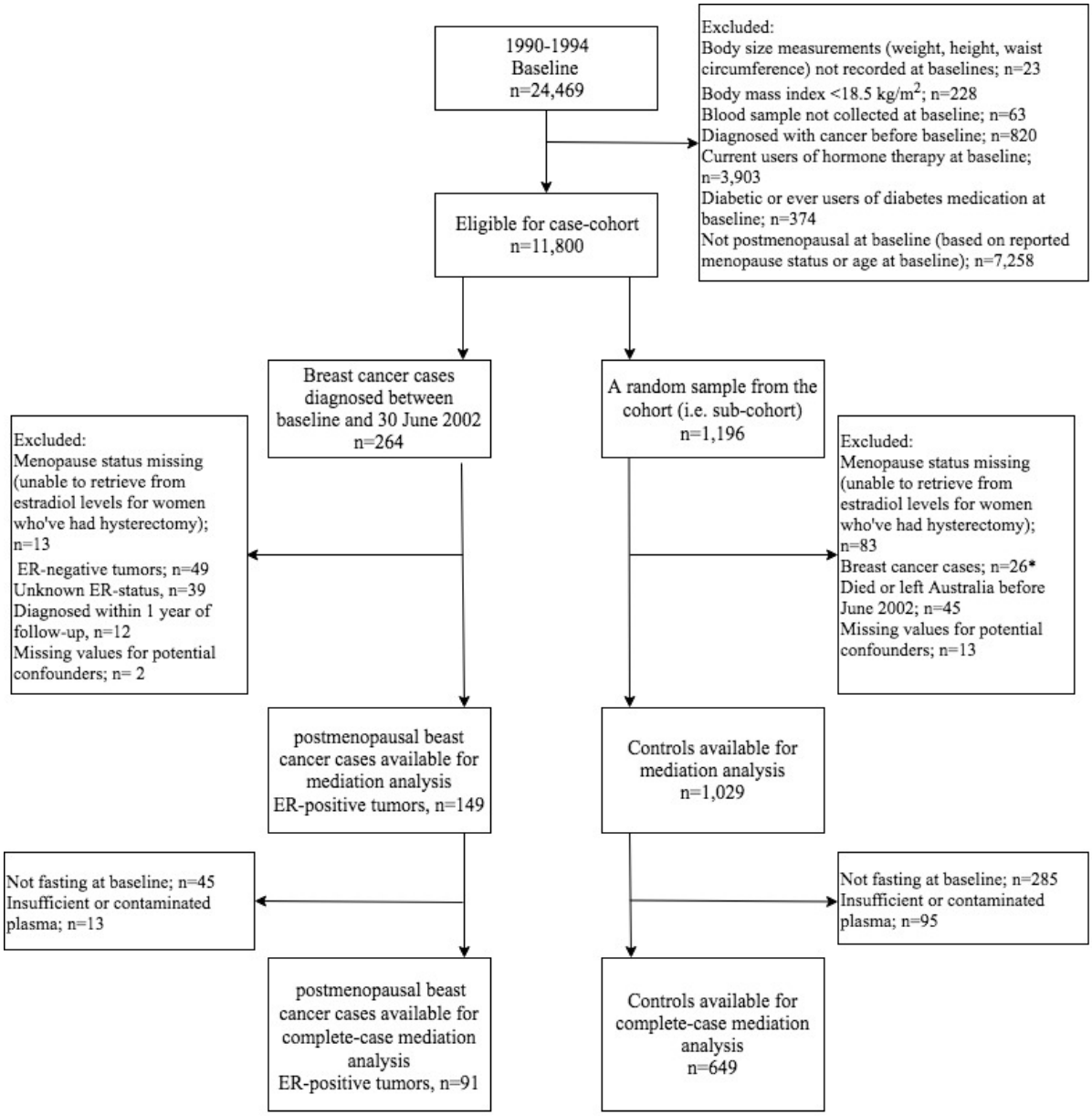
Footnote: for the purpose of mediation analysis women in the sub-cohort who were diagnosed with breast cancer were not included as controls but they were kept as cases if they met the eligibility criteria.

Figure 2– Assumed causal diagram for quantifying the mediating effects of fasting insulin, and free estradiol on adiposity- estrogen receptor-positive postmenopausal breast cancer association; abbreviations: ER estrogen receptor

Footnote: To simplify the diagram of the assumed causal relations between adiposity (exposure), estrogen receptor-positive postmenopausal breast cancer (outcome), fasting insulin and free estradiol (mediators) and potential confounders, we only included the arrows that were sufficient to flag a variable as a common cause (i.e. confounder) of exposure-outcome, or exposure-mediator, or mediator-outcome. Therefore, this is not a causal diagram *per se*. It is assumed that all potential confounders preceded the exposure, mediators, and the outcome. The diagram was developed with reference to the literature [2, 26, 27] and expert opinion. The green arrows depict the direct and indirect pathways we were interested in, the red arrows the pathways that might have introduced biased due to unmeasured confounding, and the black arrows the backdoor pathways that were blocked by conditioning on the boxed variables. Dashed boxes indicate variables that were included in multivariable analyses, and solid boxes variables that were used as eligibility criteria. Based on the assumed causal

ordering of variables in our diagram, no collider bias would have been introduced by conditioning on any of the identified confounders in the figure.





Abbreviations: ER estrogen receptor

*Note: for the purpose of mediation analysis women in the sub-cohort who were diagnosed with breast cancer were not included as controls but they were kept as cases if they met the eligibility criteria.