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Detectible mosaic truncating *PPM1D* mutations, age and breast cancer risk

Mitchell J. Machiela[#1], **Timothy A. Myers**[#1], **Christopher J. Lyons**[1], **Roelof Koster**[1], **William D. Figg Jr.**[1], **Leandro M. Colli**[1], **Lea Jessop**[1], **Thomas U. Ahearn**[1], **Neal D. Freedman**[1], **Montserrat García-Closas**[1], **Stephen J. Chanock**[1]

[1]Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

[#] These authors contributed equally to this work.

Abstract

Mosaic protein truncating variants (PTVs) in the phosphatase, Mg2+/Mn2+dependent 1D (*PPM1D*) gene in blood-derived DNA have been associated with increased risk of breast cancer. We analyzed *PPM1D* PTVs in blood from 3817 breast cancer cases and 3058 controls by deep sequencing of a previously defined region in exon 6 of *PPM1D*. We identified 50 of 6875 (0.73%) participants having a mosaic *PPM1D* PTV. We observed a higher frequency of mosaic *PPM1D* PTVs with increasing age ($P_{trend} = 2.9 \times 10^{-6}$). We did not observe an overall association between *PPM1D* PTVs and increased breast cancer risk (OR = 1.51, 95% CI = 0.84–2.71). Evidence for an association was observed in a subset of cases with DNA collected 1-year or more before breast cancer diagnosis (OR = 3.44, 95% CI = 1.62–7.30, *P*-value = 0.001); however, no significant association was observed for the larger series of cases with DNA collected post diagnosis (OR = 1.01, 95% CI = 0.51–2.01, *P*-value = 0.98). Our study indicates that the *PPM1D* PTVs are present at higher rates than previously reported and the frequency of *PPM1D* PTVs increases with age. We observed limited evidence for an association between mosaic *PPM1D* PTVs and breast cancer risk, suggesting mosaic *PPM1D* PTVs in the blood likely do not influence risk of breast cancer.

Introduction

The protein phosphatase, Mg2+/Mn2+dependent 1D (*PPM1D*, also known as *WIP1*) is a member of the PP2C family of Ser/Thr protein phosphatases, an evolutionarily conserved family known to negatively regulate cellular response to stress [1]. *PPM1D* expression is induced in a p53-dependent manner in response to environmental stressors, such as DNA damage, and negatively regulates MAPK/p38 kinase, which reduces the phosphorylation of p53 and results in growth inhibition and suppression of apoptosis [2, 3]. *PPM1D* is located

*Stephen J. Chanock chanocks@mail.nih.gov.
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Compliance with ethical standards
Conflict of interest The authors declare that they have no conflict of interest.

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on chromosome 17q23 in a region commonly amplified in breast cancer, suggesting a potential oncogenic role of *PPM1D* overexpression in breast cancer [4, 5].

A prior large-scale sequencing study of 6912 breast cancer cases and 5861 cancer free controls identified mosaic PTVs in *PPM1D* of leukocyte-derived DNA as a potential risk factor for breast and ovarian cancer (crude unadjusted odds ratio (OR) = 15; adjusted retrospective likelihood risk ratio = 2.7) [6]. The study identified a mutation cluster region of 370 base pairs in the carboxy-terminal to the phosphatase catalytic domain within the final exon of *PPM1D* (exon number 6). Interestingly, the study indicated that *PPM1D* PTVs enhance suppression of p53 following exposure to ionizing radiation, suggesting these mutations may result in a gain-of-function rather than a loss-of- function [6]. Subsequent studies have investigated the presence of mosaic *PPM1D* PTVs in a variety of tumor types including lung [7], ovarian [8–10], and prostate [11] cancer. Results from these studies are mixed and have not sup- ported an association between mosaic *PPM1D* PTVs in blood and cancer risk, but rather suggest the Ruark et al. [6] association may be an artifact of treatment.

PPM1D mutations detected in exon 6 can generate constitutively activated *PPM1D* proteins that have been shown to suppress both phosphorylation and activation of p53 after DNA damage [12]. DNA-damaging chemotherapies could inadvertently select for clones with *PPM1D* mutations that can circumvent the *TP53* checkpoint control. An investigation of ovarian cancer patients found elevated frequencies of *PPM1D* PTVs in ovarian cancer cases compared with controls; however, all PTVs were blood-drawn post treatment, which included DNA-damaging agents, suggesting that treatment might select for *PPM1D* PTVs [8]. A subsequent study on ovarian cancer also found an association with chemotherapy and *PPM1D* PTVs [10]. Likewise, an investigation in non-small cell lung cancer found all individuals with *PPM1D* PTVs in blood-derived DNA were exposed to chemotherapy [7].

Our aim was to perform ultra-high depth (>2500x target coverage) sequencing of the *PPM1D* mutation cluster region (chr17:58,740,349–58,740,815; GRCh37) in a sample of 3817 breast cancer cases and 3058 controls from two epidemiologic studies to describe the frequency of *PPM1D* PTVs in circulating leukocytes and evaluate whether mosaic *PPM1D* PTVs are associated with increased breast cancer risk.

Materials and methods

Sample population

Samples for our analysis were drawn from two studies: the Polish Breast Cancer Study (PBCS) [13], a population- based breast cancer case-control study conducted from 2000–2003 in two major cities in Poland (Warsaw and Lodz); and the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO) [14], a prospective trial initiated in 1993 and subsequent cohort study conducted in the United States with enrollment age between 55 and 74. All female participants with pathologically confirmed breast cancer diagnosis and available, high-quality DNA were selected for inclusion as breast cancer cases. In PLCO, all but 2 breast cancer cases had breast cancer as their first diagnosed cancer. Female cancer-free controls with avail- able, high-quality DNA were selected and frequency matched on

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age at blood collection (Supplementary Fig. 1). In total, we successfully sequenced blood-derived DNA from 1659 breast cancer cases and 1126 controls from PBCS and 2158 breast cancer cases and 1932 controls from PLCO. All participants provided informed consent prior to study participation and sample collection. The study was reviewed by the Institutional Review Board of the National Cancer Institute and all participating study centers.

PPM1D screening by deep PCR amplicon sequencing

Multiplex amplicon PCR was performed using two sets of target-specific primer pairs (Supplementary Table 1) that were designed to amplify the entire mutation cluster region, as defined by Ruark et al. (chr17:58740349–58740815, GRCh37/hg19) [6], and incorporated Illumina adapter overhang sequences (Supplementary Fig. 2). The full sequences are listed in Supplementary Table 1.

The amplified PCR product was purified using solid- phase reversible immobilization (SPRI) (Agencourt Ampure XP, Beckman Coulter, Inc., Brea, CA; Cat. No. A63882) beads in a SPRI to sample volume ratio to remove fragments less than 200 bp [15]. We employed a dual-index strategy to pool 384 samples per Illumina MiSeq run. Subsequently, we performed low-cycle amplification to add the Illumina sequence adapters and dual indices (24 index sequences were used for index 1, and 16 index sequences for index 2; Supplementary Table 2) [16]. Following low-cycle PCR, SPRI beads were used to cleanup and remove fragments less than 200 bp. The purified libraries were quantified by qPCR (NEBNext Library Quant Kit for Illumina, New England Biolabs, Inc., Ipswich, MA; Cat. No. E7630L) to deter- mine size-adjusted concentration, subsequently normalized and pooled. The pooled library was quantified by qPCR, diluted to a loading concentration of 8 pM, denatured and loaded with a >25% PhiX (Illumina, Inc. San Diego, CA; Cat. No. FC-110–3001) spike-in onto an Illumina MiSeq (Illumina, Inc., San Diego, CA) instrument and sequenced using a 2 × 300 bp read kit (Illumina, Inc., San Diego, CA; Cat. No. MS-102–3003).

Variant detection

Base calls were generated on-instrument. Secondary data analysis was performed by the MiSeq Reporter 2.6.2.3 PCR Amplicon Workflow and aligned to human genome build 37. All samples were required to pass a quality control step of 250x coverage across 80% of the amplicon to be included in subsequent analyses. Variants were detected using the Illumina Somatic Variant Caller, designed to detect low- frequency mutations below 5% (Illumina Technical Note– Somatic Variant Caller, https://www.illumina.com/documents/products/technotes/technote_somatic_variant_ca ller.pdf). We filtered out variants with variant allele frequencies below 1% and above 35% as potential sequencing noise and germline variation, respectively.

Replication/validation by ultra-deep sequencing

Samples successfully identified with mosaic PTVs during the screening phase were selected for validation by ultra-deep sequencing (Supplementary Fig. 1). Input DNA was prepared by amplifying the *PPM1D* mutation cluster region using the same primer pair (Supplementary

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Table 3) designed by Ruark et al. that generates a 467 bp amplicon [6]. Accurate quantification of the PCR product was obtained with the Qubit dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific, Waltham, MA; Cat. No. Q32854). The Illumina Nextera XT DNA Library Preparation and Index Kits (Illumina, Inc., San Diego, CA; Cat. No. FC-131–1024 and FC-131–1001) were used to prepare dual indexed, paired-end libraries per the manufacturer's protocol. Libraries were pooled and loaded with a 5% PhiX (Illumina, Inc. San Diego, CA; Cat. No. FC-110–3001) spike-in onto an Illumina MiSeq instrument and sequenced using a 2 × 300 bp read kit (Illumina, Inc., San Diego, CA; Cat. No. MS-102–3003).

Statistical analysis

All statistical analyses were performed using R version 3.3.0 "Supposedly Educational" in the R console on a macOS Sierra 10.12.6 operating system. Statistical tests were two sided with a *P*-value less than 0.05 considered statistically significant. Associations with breast cancer risk were adjusted for woman's age at blood collection as a continuous covariate in the logistic regression model, since age was found to be a significant predictor of *PPM1D* PTV frequency. Results from PBCS and PLCO were merged by fixed effects meta-analysis using the R metaphor library. Fisher's exact tests were used to evaluate the effect of smoking and treatment on *PPM1D* PTV frequency with results from PBCS and PLCO merged by fixed effects meta-analysis. The R package trackViewer was used to plot detected *PPM1D* PTVs.

Results

Our study population consisted of 1659 breast cancer cases and 1126 controls from PBCS and 2158 breast cancer cases and 1932 controls from PLCO (Table 1). The mean age of breast cancer diagnosis was higher in PLCO (68.59) as compared to PBCS (55.53, *P*-value < 0.001) because enrollment for PLCO began at age 55 whereas PBCS enrolled all ages. Likewise, age at DNA collection was higher in PLCO versus PBCS (65.73 and 55.83, respectively; *P*-value < 0.001).

Our deep targeted sequencing of the *PPM1D* mutation cluster region detected a total of 50 (0.73%) women with validated mosaic PTVs in a total of 6875 women sequenced (Supplementary Table 4, Supplementary Fig. 3). These PTV mutations were present in 32 (0.84%) of 3817 breast cancer cases and 18 (0.59%) of 3058 controls (unadjusted OR = 1.43, 95% CI = 0.78–2.71). Variant allele frequencies for detected mosaic *PPM1D* PTVs ranged between 1.0 and 30.6% (Supplementary Fig. 4). In addition, 42 non-PTV mosaic mutations (e.g., synonymous or missense mutations, not plotted in Supplementary Fig. 3) were detected across 25 (0.36%) women, although we did not attempt to validate these mutations by subsequent sequencing of Nextera generated libraries.

We observed a positive association between increasing age at blood collection and frequency of a mosaic *PPM1D* PTV (Fig. 1, Supplementary Table 5). For a one year increase in age, the odds of a mosaic *PPM1D* PTV increased by 10% (OR = 1.10, 95% CI = 1.06–1.15, *P*-value = 2.9×10^{-6}). This association with age was similar for both breast cancer cases (OR

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= 1.10, 95% CI = 1.05– 1.16, *P*-value = 0.0002), as well as in cancer-free controls (OR = 1.11, 95% CI = 1.03–1.19, *P*-value = 0.004).

In a combined overall analysis adjusted for the effect of age at blood collection, we did not observe an association between *PPM1D* PTVs and increased breast cancer risk (OR = 1.51, 95% CI = 0.84–2.71, *P*-value = 0.17, Fig. 2).

In a subanalysis restricted to a reduced set of PLCO breast cancer cases collected 1-year or more before breast cancer diagnosis, we observed an association between the presence of *PPM1D* PTVs and breast cancer risk (OR = 3.44, 95% CI = 1.62–7.30, *P*-value = 0.001). However, in a larger analysis that included cases with DNA collected at or after breast cancer diagnosis, no overall association was observed between the presence of a mosaic *PPM1D* PTV and breast cancer (OR = 1.01, 95% CI = 0.51–2.01, *P*-value = 0.98).

This was observed in both PBCS (OR = 1.99, 95% CI = 0.54–7.38, *P*-value = 0.42) and the set of PLCO cases with DNA collected at or after breast cancer diagnosis (OR = 0.78, 95% CI = 0.35–1.75, *P*-value = 0.55, Fig. 2), with no statistical evidence for study heterogeneity (Q = 1.40, *P*-value(het) = 0.24). Furthermore, there was no difference in years between DNA collection and breast cancer diagnosis when comparing PLCO cases with pre-diagnostic DNA samples with mosaic *PPM1D* PTVs (N = 13) to those without mosaic *PPM1D* PTVs (N = 451, *P*-value = 0.98).

Although our study had limited power to assess the impact of smoking and treatment on acquiring *PPM1D* PTVs, we performed exploratory analyses for subsets of women with available exposure data. Data on smoking was available for PBCS and PLCO participants. Of the 50 women with detected *PPM1D* PTVs, 22 (44%) report ever smoking cigarettes compared to 3371 (49%) of 6824 women that report ever smoking in women without PTVs. No overall association was observed between smoking and frequency of mosaic *PPM1D* PTVs (OR = 0.87, 95% CI = 0.46–1.63, *P*-value = 0.67). In addition, sensitivity analyses that adjusted for age and smoking found no differences in association between mosaic *PPM1D* PTVs and breast cancer. Treatment data was available for the PBCS breast cancer cases. Of the 9 PBCS breast cancer cases with detected mosaic *PPM1D* PTVs, 6 (67%) had undergone some form of chemotherapy, radiation, or hormonal treatment prior to blood collection compared to 551 (33%) women receiving some form of chemotherapy, radiation, or hormonal treatment of the 1650 women with no detectable mosaic *PPM1D* PTV (OR = 3.99, 95% CI = 0.94–18.46, *P*-value = 0.07).

Fisher exact tests indicate varying levels of evidence for chemotherapy (OR = 3.08, 95% CI = 0.78–11.53, *P*-value = 0.10), radiation (OR = 4.52, 95% CI = 0.97–19.30, *P*-value = 0.05), and hormonal treatment (OR = 5.48, 95% CI = 1.38–20.55, *P*-value = 0.02, Supplementary Table 6) prior to blood collection being associated with increased risk of mosaic *PPM1D* PTV, although further studies are needed to support any such association. No changes in overall conclusion with respect to the PBCS association between *PPM1D* PTVs and breast cancer was observed when removing PBCS cases with treatment from the analysis(OR = 0.92, 95% CI = 0.11–5.92, *P*-value = 1).

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Discussion

Our deep sequencing approach detected mosaic PTVs in the last exon of *PPM1D* in as few as 1% of circulating leuko- cytes. We detected mosaic *PPM1D* PTVs in blood-derived DNA of breast cancer cases (0.82%) and controls (0.59%). An earlier reported study of breast cancer by Ruark et al. [6] likewise detected the presence of mosaic *PPM1D* mutations in blood-derived DNA of breast cancer cases (0.26%), although the sequencing techniques employed in this study had a detection limit of variant allele frequencies greater than 5%. Filtering our detected *PPM1D* PTVs to only include breast cancer cases with 5% or more leukocytes affected with a mosaic mutation, we find 15 of 3817 (0.39%, 95% CI = 0.22–0.65%) breast cancer cases affected.

While the Ruark et al. investigation suggested mosaic *PPM1D* PTVs are associated with breast cancer risk, with an estimated crude unadjusted odds ratio of approximately 15, our overall analysis did not find evidence for a relationship between mosaic *PPM1D* truncating variants and breast cancer risk. Our overall 95% confidence interval ranged from 0.84 to 2.71, excluding crude odds ratio effects as large as 15 observed in the Ruark et al. study. Our OR estimate of 3.44 (95% CI = 1.62–7.30) from the prospectively collected PLCO breast cancer samples is similar to the relative risk estimate from a Rurak et al. [6] sub-analysis using retrospective likelihood modeling of unrelated women free of *BRCA1/2* (RR = 2.7, 95% CI = 1.3–5.3), however; our sample of women collected at or after breast cancer diagnosis did not indicate evidence for an association between *PPM1D* PTVs and breast cancer risk (OR = 1.01, 95% CI = 0.51– 2.01). It is unclear why some evidence for an association would be observed for *PPM1D* PTVs and breast cancer risk in samples with DNA collected prior to breast cancer diagnosis, but no evidence for an association in a larger set of samples collected after diagnosis as was previously observed by Ruark et al. It is unlikely DNA storage time or conditions that could explain this observed difference since detected *PPM1D* PTVs are clonal and have high variant allele frequencies. Future investigations with longitudinal blood sample collections before and after breast cancer diagnosis and from more than one tissue source would offer improved understanding of mosaic *PPM1D* PTV frequency relative to breast cancer diagnosis.

We found a notable association between increasing age and increasing frequency of mosaic *PPM1D* PTVs, consistent with previous reports [10]. Associations between somatic mosaicism and increasing age are commonly observed for large structural autosomal mosaicism [17–19], sex chromosome mosaicism [20–22], and clonal hematopoiesis [23–25]. Exome-wide scans of mosaic point mutations, also referred to as clonal hematopoiesis, have identified several genes that are commonly affected by age-related mosaic point mutations (e.g., *DNMT3A*, *TET2*, *ASXL1*) [23–25]. Interestingly, mosaic *PPM1D* PTVs are also age-related, and occur at higher frequencies than observed for most genes which could have impacts on cellular growth and survival, and may impact risk of non- breast cancer outcomes such as hematologic cancer, cardiovascular disease, and mortality [23–25], although further studies are needed to refine these associations in relation to mosaic *PPM1D* mutations.

Studies of ovarian [8, 10] and non-small cell lung [7] cancer suggest mosaic *PPM1D* PTVs in the blood are potential sequelae of cancer treatments. We performed analyses in women

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from the PBCS exposed to a variety of breast cancer treatments (e.g., chemotherapy, radiation, and hormonal treatments) to explore links between breast cancer treatment and the frequency of *PPM1D* PTVs in blood DNA. Our analyisis found limited evidence that chemotherapy, radiation, and hormonal treatment is associated with increased risk of mosaic *PPM1D* PTVs, although better powered studies are needed. Differences in breast cancer treatment and treatment in ovarian and lung cancer may also account for this observed difference in effect. It is important to note that we were unable to adjust for other historic drug exposures that may have influenced *PPM1D* PTV formation or in some way may alter the observed association of *PPM1D* PTVs with breast cancer risk. Additionally, we assessed the effect of smoking on the development of *PPM1D* PTVs and found no significant evidence indicating smoking is associated with mosaic *PPM1D* PTVs.

Our study indicates *PPM1D* PTVs are present at various cellular fractions in women from two studies of breast cancer and finds limited evidence for an association between *PPM1D* PTVs and breast cancer risk. Although prior studies in other tumor types have suggested cancer treatment could be confounding the relationship between mosaic *PPM1D* PTVs and cancer risk, our study had limited ability to assess effects of treatment on *PPM1D* PTVs. Our observations indicate mosaic *PPM1D* PTVs are commonly observed in studies of clonal hematopoiesis and increase with age suggesting these events are likely not specific to breast cancer risk.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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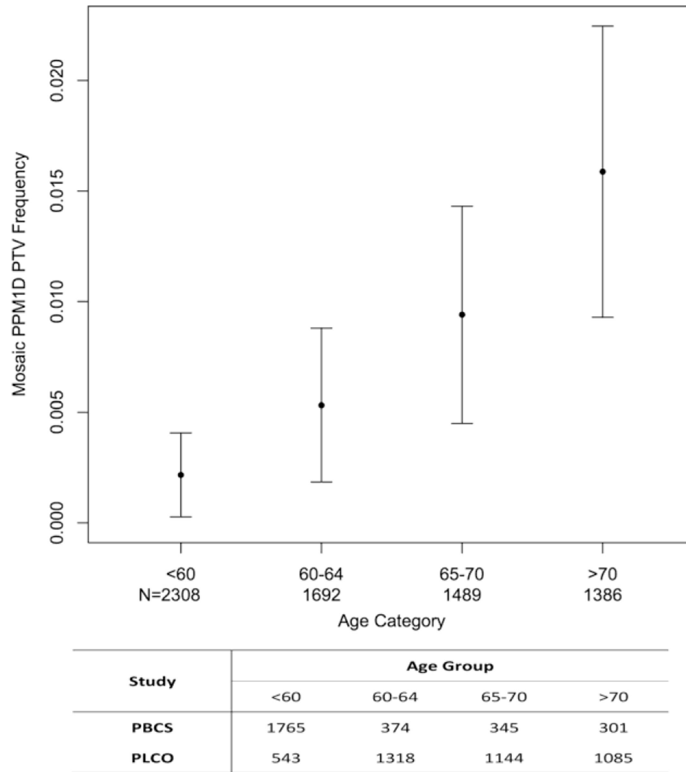

Study	Age Group			
	<60	60-64	65-70	>70
PBCS	1765	374	345	301
PLCO	543	1318	1144	1085

Fig. 1.

Association between 5-year age category and mosaic *PPM1D* PTVs for all breast cancer cases and controls from PBCS and PLCO

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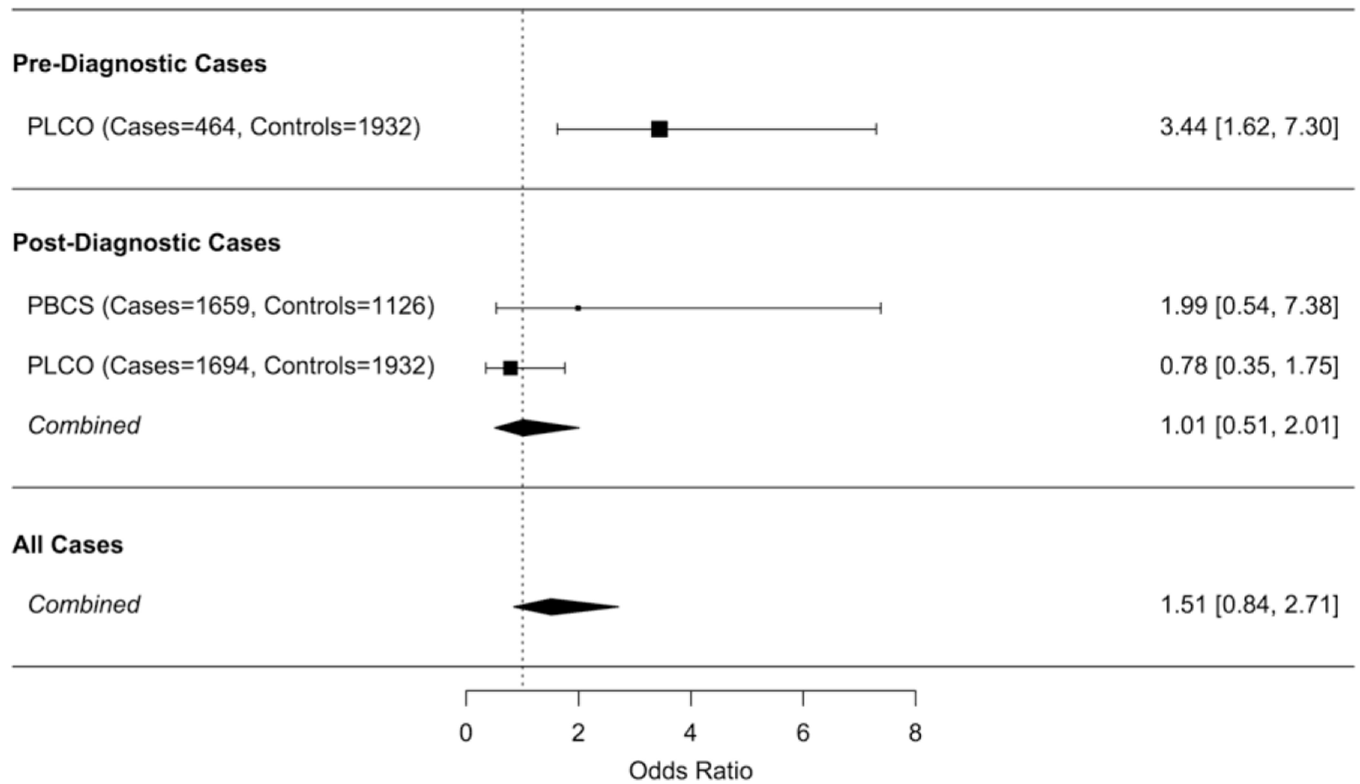

Fig. 2.

Association of detected mosaic *PPM1D* PTVs and breast cancer by strata of cases with blood DNA collected before and at/after breast cancer diagnosis, and for overall breast cancer risk

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Table 1

Descriptive statistics of PBCS and PLCO study populations

	Cases			Controls		
	Mean	**Median**	**Range**	**Mean**	**Median**	**Range**
Age at blood collection						
PBCS	55.9	55	(27.0 –76.0)	55.7	55	(30.0 – 75.0)
PLCO	65.6[a]	65	(55.0 – 81.0)	65.9	65	(55.0 – 81.0)
Age at diagnosis						
PBCS	55.5	55	(27.0 – 74.0)	—	—	—
PLCO	68.6	68	(55.0 – 87.0)	—	—	—
Years between blood collection and cancer diagnosis						
PLCO (PTV positive)	2.5	3	(1.0 – 5.0)	—	—	—
PLCO (PTV negative)	2.5	2	(1.0 – 8.0)	—	—	—
	Cases				**Controls**	
	No PTV	**PTV**			**No PTV**	**PTV**
PPM1D PTVs identified				PBCS (cancer-free)	1123	3
PBCS (Post-diagnosis)	1650	9				
PLCO (Pre-diagnosis)	451	13		PLCO (cancer-free)	1917	15
PLCO (Post-diagnosis)	1684	10				

[a]Higher mean age of PLCO is due to enrollment criteria of 55–74 years old