BRCA1 mRNA levels following a 4–6-week intervention with oral 3,3′-diindolylmethane

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Background: Haploinsufficiency may contribute to the development of breast cancer among women with a *BRCA1* mutation. Thus, interventions that enhance *BRCA1* expression may represent avenues for prevention. Studies have shown that 3,3′-diindolylmethane (DIM) can upregulate *BRCA1* expression in breast cancer cells. This has yet to be demonstrated *in vivo*.

Methods: We conducted a study to evaluate the ability of oral DIM to upregulate *BRCA1* mRNA expression in white blood cells. A total of 18 women were enroled in the study, including 13 *BRCA1* mutation carriers who received 300 mg per day of Rx Balance BioResponse DIM for 4–6 weeks (intervention group) and 5 *BRCA1* mutation carriers who did not take DIM (control group). *BRCA1* mRNA expression was assessed at baseline and at 4–6 weeks by real-time, quantitative PCR and the relative change in *BRCA1* mRNA expression (that is, $2^{-\Delta\Delta C_T}$) was calculated.

Results: The relative change in *BRCA1* mRNA expression among women in the intervention group achieved borderline significance (*P* paired *t*-test = 0.05). In the intervention group, *BRCA1* mRNA expression increased in 10 of the participants, decreased in 2 and remained unchanged in 1 of the participants following DIM intervention (*P* sign test = 0.02). On average, women in the intervention group experienced a 34% increase in *BRCA1* mRNA expression (range −24 to 194%). There was no significant difference in the relative change in *BRCA1* mRNA expression among women in the control group (*P* paired *t*-test = 0.45).

Conclusions: Under the tested conditions, oral DIM was associated with an increase in *BRCA1* mRNA expression in women with a *BRCA1* mutation. The possibility of mitigating the effect of an inherited deleterious *BRCA1* mutation by increasing the physiologic expression of the gene and normalising protein levels represents a clinically important paradigm shift in the prevention strategies available to these high-risk women. Future studies with a larger sample size and higher doses of DIM are warranted.

The inheritance of a deleterious mutation in the breast cancer susceptibility gene, *BRCA1*, confers high lifetime risks of developing breast and ovarian cancer, estimated at 80 and 40%, respectively (Ford *et al*, 1994; The Breast Cancer Linkage Consortium, 1999; Robson, 2002; Thompson and Easton, 2002; Antoniou *et al*, 2003; Chen and Parmigiani, 2007). Women with *BRCA*-associated breast cancers are often diagnosed at a young age (Narod, 2010), and have elevated risks of developing contralateral (Metcalfe *et al*, 2011a) and ipsilateral breast cancer (Metcalfe *et al*, 2011b). *BRCA1*-associated breast cancers exhibit features of an aggressive phenotype (for example, triple negative;

Da Silva and Lakhani, 2010). Genetic testing permits the identification of high-risk women before cancer; however, prevention is limited to prophylactic surgery and chemoprevention with tamoxifen (Narod, 2010; Finch *et al*, 2014).

The incomplete penetrance associated with a *BRCA* mutation suggests that non-genetic modifiers may have an important role (Narod *et al*, 1993; Foulkes *et al*, 2002; Antoniou *et al*, 2003; King *et al*, 2003). Various reproductive factors have been shown to influence the risk (Jernstrom *et al*, 2004; Kotsopoulos *et al*, 2005a); however, the role for dietary and lifestyle factors is less clear (Kotsopoulos and Narod, 2005). There is some evidence that

caffeine (Nkondjock *et al*, 2006), selenium (Kowalska *et al*, 2005; Kotsopoulos *et al*, 2010) and weight loss in early adult life (Kotsopoulos *et al*, 2005b) protect against *BRCA1*-related breast cancer. In addition, we have recently reported possible relationships between plasma iron and antimony levels and *BRCA1*-associated breast cancer (Kotsopoulos *et al*, 2012). Cancer risk reduction options that are nonsurgical and modifiable are needed.

When identifying possible lifestyle interventions that may help in the prevention of breast cancer, it is important to consider that the mechanism underlying the predisposition is likely to be different in *BRCA1* mutation carriers from that of women in the general population (Salmena and Narod, 2012). BRCA1 helps maintain genomic integrity through participating in the cellular response to DNA damage, specifically in the repair of double-stranded DNA breaks (Scott, 2004). Haploinsufficiency refers to a state in which an individual has only one functional copy of a gene due to mutation or gene loss, and thus may produce an insufficient amount of protein (Berger and Pandolfi, 2011; Berger *et al*, 2011; Konishi *et al*, 2011). Although limited, there is evidence to support that the predisposition to breast cancer among *BRCA1* mutation carriers is due to haploinsufficiency associated with heterozygosity, which increases genomic instability and accelerates the mutation rate of other critical genes, including the second copy of *BRCA1* (Konishi *et al*, 2011; Salmena and Narod, 2012). Thus, factors that might increase the cellular expression of the normal copy of the gene and thereby help normalise protein levels may mitigate against the effect of the mutation.

One dietary supplement that is thought to have potential for preventing cancer in *BRCA1* mutation carriers is 3,3′-diindolyl-methane (DIM), a phytochemical derived from cruciferous vegetables (Higdon *et al*, 2007). DIM has been found to have various cancer-protective effects, particularly for hormone-dependent cancers such as that of the breast (Higdon *et al*, 2007). *In vitro* administration of a relatively low dose of the phytochemical DIM (and its precursor indole-3-carbinol (I3C)) can significantly upregulate both the *BRCA1* and *BRCA2* mRNA and the protein expression in breast and prostate cancer cells (Meng *et al*, 2000a, b, c; Fan *et al*, 2006, 2009).

If haploinsufficiency predisposes mutation carriers to cancer development, ultimately, the ability to upregulate BRCA1 expression in female *BRCA1* heterozygotes may translate into a reduced cancer risk. To date, no studies have evaluated whether or not oral administration of I3C or DIM enhances the expression of normal *BRCA1* mRNA or protein *in vivo*. We conducted a 4–6-week dietary intervention trial to evaluate the ability of DIM, a stable metabolite of I3C, to upregulate *BRCA1* mRNA expression in women with a *BRCA1* mutation.

MATERIALS AND METHODS

Study population. Potential study participants were identified from the Familial Breast Cancer Research Unit, Women's College Hospital (Toronto, Ontario, Canada) and included women who were enroled in previous and ongoing clinical research protocols. Eligible subjects were healthy women with no personal history of cancer, who were not pregnant or breastfeeding and were between the ages of 25 and 65 years. Women who had taken DIM were eligible to participate if they discontinued DIM use for at least 1 month before study enrolment. We included 21 healthy *BRCA1* mutation carriers ($BRCA1^{+/-}$). All study subjects provided their written informed consent. This study was approved by the Women's College Hospital research ethics board and an independent Data Safety Monitoring Board was in place during the study.

Data collection. Women were invited to participate in the study by letter. After a woman expressed interest in the study, a package

consisting of a consent form and a short questionnaire about diet and lifestyle was sent to her. The research coordinator contacted the participants to arrange a preliminary clinic visit.

DIM intervention. The intervention group included 15 *BRCA1* mutation carriers who were assigned to receive 300 mg per day (150 mg twice daily) of Rx Balance BioResponse DIM for 4–6 weeks provided kindly by Michael Zeligs, BioResponse, LLC, Boulder, CO, Canada. The control group consisted of six *BRCA1* mutation carriers who did not take DIM. At the first clinic visit, the research coordinator assigned the *BRCA1* mutation carriers to either the intervention group or control group. DIM was not randomly assigned, given that women who did not want to take the intervention were assigned to the control group. A study diary was provided to the participants in the intervention group to record information on compliance and any side effects. The research coordinator also completed a reporting form for all participants to log clinic visits, compliance with the medication and any side effects. Adverse events were reported to the Data Safety Monitoring Board at the Women's College Hospital.

Two women in the intervention group reported adverse side effects with DIM use and withdrew from the study, thus only providing baseline blood and urine samples and one woman in the intervention group did not return for a second clinic visit. One of these subjects experienced nausea and painful menstrual cramps, whereas the other woman had an adverse reaction to the gadolinium received during MRI appointment and not due to the study intervention. One of the subjects in the control group did not complete the study. Eight of the 16 women in the intervention group reported side effects on initiation of DIM supplementation, which were of mild severity and self-limited, resolving with continued use. Side effects included loose stools, change in urine colour, headaches, decreased appetite, feeling thirsty, joint pain and abdominal pain. All adverse events were reviewed by the Data Safety Monitoring Board at the Women's College Research Institute and deemed to be of minimal severity.

Biological sample collection. Participants were invited to come to the Familial Breast Cancer Research Unit for a 15-min visit for biological sample collection and dietary intervention assignment. All participants had a blood sample collected at baseline during the first clinic visit and an additional sample collected 4–6 weeks after the first appointment. Subjects arrived to the clinic between 9 am and 10 am after an overnight fast. Approximately 20 ml of blood was collected from the participants by venipuncture into two 10 ml EDTA-containing vials. Women were also asked to bring their first morning urine sample on ice that was collected at home. Samples were labelled, placed on ice and delivered immediately (within half an hour) to the laboratory of Dr Steven Narod (Women's College Hospital). The blood was immediately processed for RNA extraction, and 5 ml of urine was aliquotted into two 15 ml tubes containing 10 mg of ascorbic acid and stored for future use at – 80 °C in freezers, which are alarmed and continuously monitored.

BRCA1 mRNA expression. *BRCA1* mRNA expression was quantified in the white blood cells of the study subjects using real-time, quantitative PCR in the laboratory of Dr Steven Narod (Women's College Hospital) at baseline and following DIM supplementation. The LeukoLOCK Total RNA Isolation System (Ambion, Austin, TX, USA) was used for total RNA extraction from the leukocyte population according to manufacturer's instructions. The Taqman RNA-to-C_T 1 Step Kit (Applied Biosystems, Foster City, CA, USA) was used for combined reverse-transcription real-time PCR and the ABI 7500 Fast Real-Time PCR System (Applied Biosystems) was used to quantify mRNA levels. The glyceraldehyde phosphate dehydrogenase (GAPDH) gene was used as a control measuring changes in *BRCA1* mRNA by the comparative C_T method ($\Delta\Delta C_t$ method). The Taqman

assay IDs for BRCA1 and GADPH were Hs01556194_m1 and Hs99999905_ml, respectively (Applied Biosystems). All the samples were run in triplicate to evaluate accuracy and reproducibility. The largest coefficient of variation was < 2% and the mean s.e. for the C_T was 0.09 and 0.10 for *BRCA1* and *GADPH*, respectively. Samples from the same study subject (both baseline and second visit samples) were assayed in the same batch. Data were collected by the Applied Biosystems 7500 software v2.0.6 in the form of threshold cycle number (C_T) for the target gene and the endogenous control gene. The mean C_T values of the three replicate runs for each sample were used for calculations. The C_T values were normalised to the endogenous control gene and the corresponding ΔC_T values were obtained by subtracting the mean GAPDH value from the mean *BRCA1* C_T value. The corresponding $\Delta\Delta C_T$ values were obtained by subtracting the ΔC_T of the mRNA sample at baseline from the ΔC_T of the mRNA at the second clinic visit. The relative change in *BRCA1* mRNA expression is given by $2^{-\Delta\Delta C_T}$, which represents the change in *BRCA1* mRNA expression at the second visit compared with the baseline levels (Livak and Schmittgen, 2001).

Statistical analyses. For descriptive statistics, the *t*-test and χ^2 test were used to test for differences in continuous (including $2^{-\Delta\Delta C_T}$) and categorical variables, respectively. A paired *t*-test was used to compare the mean ratio of the change in *BRCA1* mRNA levels at baseline versus the second clinic visit (that is, specifically the $2^{-\Delta\Delta C_T}$) compared with one (which represents the ratio for no change in *BRCA1* mRNA expression) in the intervention group and control group separately. A sign test was used for differences in the distribution in the number of women in the intervention group that experienced an increase versus decrease in the $2^{-\Delta\Delta C_T}$. A *P*-value of ⩽ 0.05 was considered statistically significant and all analyses were carried out using SAS Version 9.1 (SAS Institute, Cary, NC, USA).

RESULTS

Table 1 shows the baseline characteristics of the 18 participants in the intervention ($n = 13$) and control ($n = 5$) groups who provided two blood samples. Participants in the intervention group were on average significantly younger than those in the control group ($P = 0.02$), and thus were more likely to be premenopausal ($P = 0.0004$). There was no significant difference with respect to the other baseline characteristics between the two groups, including current oral contraceptive use, alcohol consumption and multivitamin use.

Figure 1 shows the distribution of the $2^{-\Delta\Delta C_T}$ values among all the study participants. The relative change in *BRCA1* mRNA expression among women in the intervention group achieved borderline significance (*P* paired *t*-test = 0.05). In the intervention group ($n = 13$), *BRCA1* mRNA expression increased in 10 of the participants, decreased in 2 and remained unchanged in 1 of the participants following DIM intervention (*P* sign test = 0.02). On average, these women experienced a 34% increase in *BRCA1* mRNA expression (range − 24 to 194%). There was no significant difference in the relative change in *BRCA1* mRNA expression among women in the control group (*P* paired *t*-test = 0.45). Among women in control group ($n = 5$), *BRCA1* mRNA expression decreased in two of the participants, increased in two of the participants and remained unchanged in one of the participants when comparing baseline and second visit levels with a mean increase of 15% (range − 18 to 81%). There was no significant difference in the relative change in *BRCA1* mRNA expression between women in the intervention group and those in the control group ($P = 0.43$).

Table 1. Principal characteristics of the study participants in the intervention group and control group			
Variable	**Intervention group BRCA1$^{+/-}$ ($n = 13$)**	**Control group BRCA1$^{+/-}$ ($n = 5$)**	***P*-value**
Year of birth, mean (s.d.)	1971.9 (10.6)	1959.8 (7.1)	0.03
Age at menarche, mean (s.d.)	12.7 (2.7)	13.3 (1.9)	0.71
Parity[a], mean (s.d.)	1.7 (0.8)	1.5 (0.7)	0.73
BMI (kg m^{-2}), mean (s.d.)	23.7 (3.7)	25.7 (4.1)	0.32
Menopausal status, *n* (%)			
Premenopausal	10 (77)	0	0.003
Post menopausal	3 (23)	5 (100)	
Hormone replacement therapy use[b], *n* (%)			
Non-user	13 (100)	4 (80)	0.10
Current user	0	1 (20)	
Oral contraceptive use[b], *n* (%)			
Non-user	11 (85)	5 (100)	0.35
Current user	2 (15)	0	
Smoking status[b], *n* (%)			
Non-user	13 (100)	5 (100)	1.00
Current user	0	0	
Multivitamin use, *n* (%)			
Non-user	4 (31)	0	0.16
Current user	9 (69)	5 (100)	
Coffee consumption, *n* (%)			
Non-drinker	2 (15)	2 (40)	0.26
Current drinker	11 (85)	5 (60)	
Alcohol consumption, *n* (%)			
Non-drinker	0	1 (20)	0.10
Current drinker	13 (100)	4 (80)	
Total number of hours of physical activity per week, mean (s.d.)	9.6 (4.1)	9.0 (7.4)	0.84
Duration on DIM (days), mean (s.d.)	33.9 (8.4)	NA[c]	

Abbreviations: BMI = body mass index; DIM = 3,3′-diindolylmethane. All *P*-values are univariate and were derived using the *t*-test for continuous variables and the χ^2 text for categorical variables.
[a]Among parous women.
[b]Current use.
[c]Not applicable.

There were three common mutations among the women in this study: (1) 2190delA (exon 11), (2) 185delAG (exon 2) and (3) 5382insC (exon 2). We found no significant difference in *BRCA1* mRNA levels among the three (*P* from ANOVA = 0.32; data not shown).

DISCUSSION

The objective of this study was to evaluate the effect of a 4–6-week intervention with oral DIM on *BRCA1* mRNA levels in women with an inherited *BRCA1* mutation. We found that supplementation with 300 mg per day of Rx Balance BioResponse DIM for 4–6 weeks resulted in a borderline significant 34%
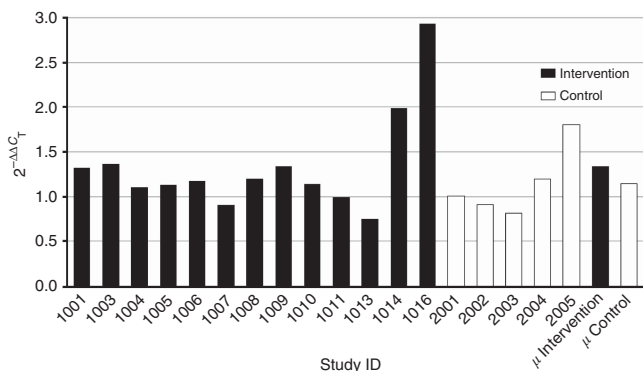
Figure 1. Relative change in BRCA1 expression normalized to GAPDH in all participants. The paired *t*-test used to test for the relative change in *BRCA1* mRNA expression for women in the intervention group and in control group ($P = 0.05$ and 0.45, respectively). The student's *t*-test was used to test for differences in the relative change in *BRCA1* mRNA expression between women in the intervention group and those in the control group ($P = 0.43$).

increase in *BRCA1* mRNA expression in lymphocytes from women with a *BRCA1* mutation. To our knowledge, this represents the first study to demonstrate modulation, and more importantly an increase, in *BRCA1* mRNA expression with a dietary intervention. This study was designed as a 'proof of principal' experiment to evaluate whether mRNA expression can be modified by non-genetic exposures and thus, these findings do not translate into a reduction in breast cancer risk with this level of DIM supplementation.

Cruciferous vegetables such as cauliflower, cabbage and broccoli are rich sources of glucosinolates (Higdon *et al*, 2007). Crushing or cooking these vegetables results in the formation of sulphur-containing isothiocyanates and indole-based I3C and DIM (Higdon *et al*, 2007; Ciska *et al*, 2009). Under the acidic environment of the stomach, I3C further dimerises to the biologically active and stable DIM and its associated oligomers, collectively referred to as acid condensation products, which are believed to be responsible for most of the biological effects associated with I3C (Shertzer and Senft, 2000). Numerous cancer-preventive properties have been associated with I3C and its derivatives, including cell cycle arrest, induction of apoptosis and modulation of oestrogen metabolism (Aggarwal and Ichikawa, 2005). Of particular relevance for *BRCA1* mutation carriers is the ability of DIM to enhance *BRCA1* expression (Fan *et al*, 2006).

In cell culture media, I3C is known to spontaneously auto-react, yielding DIM (Bradlow and Zeligs, 2010). In a series of cell culture experiments, Fan *et al* (2006) demonstrated that incubation of human breast cancer cells (that is, MCF-7 and T47D) with I3C significantly upregulated *BRCA1* and *BRCA2* mRNA and protein expression in human breast and prostate cancer cells in a dose- and time-dependent manner. The authors proposed that the induction of BRCA expression is oestrogen independent and may be via an endoplasmic reticulum stress-like pathway. This group has also shown that *in vitro* exposure to low doses of DIM (< 0.5–$1 \mu M$) for 72 h (rather than I3C) significantly increased BRCA1 and BRCA2 protein expressions and protects against oxidative stress (Fan *et al*, 2009). This dose of DIM was ~ 40 times lower than that of I3C required to produce similar effects. Collectively, the findings of Fan *et al* provide preliminary evidence that expression of the normal BRCA1 protein may also be enhanced in *BRCA1*-deficient cells by exposing them to low doses of DIM. Zhang and colleagues have shown that intraperitoneal administration of DIM (10–$50 \, \mathrm{mg \, kg}^{-1}$ body weight) is able to significantly increase expression of *BRCA1* in colon and cardiac tissue from mice with experimental colitis and

adriamycin-induced cardiac fibrosis, respectively (Huang *et al*, 2011; Yao *et al*, 2013). This group also demonstrated a concomitant reduction in levels of oxidative stress.

Various groups (including ours) have evaluated mutagen sensitivity (as a marker of impaired cellular response to DNA damage) in fibroblasts, lymphoblastoid cell lines and peripheral blood lymphocytes of heterozygous *BRCA1* (and *BRCA2*) mutation carriers; however, the results have been conflicting (Speit and Trenz, 2004; Kotsopoulos *et al*, 2007). Although limited, there is evidence to support the idea that the predisposition to breast cancer among *BRCA1* mutation carriers is the haploinsufficiency associated with a heterozygosity, which increases genomic instability and accelerates the mutation rate of other critical genes, including the second copy of *BRCA1* (Foray *et al*, 1999; Rothfuss *et al*, 2000; Baldeyron *et al*, 2002; Coupier *et al*, 2004; Kowalska *et al*, 2005; Konishi *et al*, 2011; Salmena and Narod, 2012). Baldeyron *et al* (2002) have shown that BRCA1 protein levels in lymphoblastoid cell lines established from peripheral blood lymphocytes of heterozygous *BRCA1* mutation carriers were 50% lower in comparison with the normal control cell lines. Further, these heterozygous cells displayed impaired DNA end joining, a major double-strand break repair pathway in mammals. Similarly, Konishi *et al* (2011) have reported reduced capacity for homologous recombination-mediated DNA damage repair in human cell lines with a single mutant copy of *BRCA1* derived from noncancerous human breast epithelial cells. In contrast, we and others have failed to demonstrate differences in DNA repair BRCA mutagen sensitivity using fibroblasts or lymphocytes with heterozygous mutations (Baria *et al*, 2001; Nieuwenhuis *et al*, 2002; Kotsopoulos *et al*, 2007; Lovelock *et al*, 2007).

Although some of the initial *in vitro* studies of I3C and BRCA expressions were conducted using I3C, the current study utilised DIM supplementation for several reasons. I3C is highly reactive and poorly absorbed as it does not leave the stomach or circulates in the bloodstream after oral administration to humans (Reed *et al*, 2006). *In vitro* studies have shown that at least 50% of I3C spontaneously dimerises to form DIM within 24 h of incubation (Bradlow and Zeligs, 2010), whereas *in vivo* studies have shown that following administration of I3C to humans, only DIM and not I3C is detectable in the bloodstream (Reed *et al*, 2006). Further, following oral administration of I3C in humans, only DIM and other non-DIM reaction products are found in the stomach (Reed *et al*, 2008). Thus, it is believed that DIM is the active agent and that I3C is a pro-drug that requires bioactivation in the acidic environment of the stomach (De Kruif *et al*, 1991).

A major limitation of our study was the quantification of *BRCA1* mRNA in the genetic material derived from peripheral blood lymphocytes, which might not be representative of the breast tissue. Studies that directly quantify *BRCA1* mRNA (or protein) levels in the breast before and following DIM administration are warranted. Despite this, the goal of this study was to elucidate the role of DIM in *BRCA1* expression using a convenient, minimally invasive approach. Our small sample size may have precluded us from detecting a greater effect of DIM on *BRCA1* expression; however, this was due to the need for immediate processing of the blood samples for RNA extraction. Nonetheless, we anticipated a substantial increase in *BRCA1* mRNA with the intervention. Perhaps, the dose of DIM was not high enough or the duration of intervention was not long enough to induce a substantial change in gene expression, although this is unlikely given that this duration and dose were based on prior studies reporting plasma and tissue accumulation of DIM as well as shifts in oestrogen metabolism (Dalessandri *et al*, 2004; Reed *et al*, 2008; Heath *et al*, 2010; Rajoria *et al*, 2011), changes in gene expression (Kong *et al*, 2012) and activation of DNA repair (Fan *et al*, 2013). Pertinent to our study is that Fan *et al* (2009) showed increases in both BRCA1 mRNA and protein expression in cell lines exposed to $\leqslant 0.5 \mu M$ of DIM.

This concentration of DIM corresponds to $\leqslant 125\,ng\,ml^{-1}$ of DIM in the human plasma, which has been achieved at lower doses of DIM (that is, $\sim 200\,mg$ per day; Reed *et al*, 2008). Thus, the literature suggests that the dose and duration of intervention we used were adequate to induce changes in gene expression. Further, we did not have information on intake of cruciferous vegetables; however, we asked women to discontinue DIM use for at least 1 month before study enrolment. Finally, we did not evaluate the effect of DIM on a non-carrier population. Despite these limitations, strengths of the current study include the inclusion of unaffected participants with a known *BRCA1* mutation status and the use of a single technician (who was blinded to the intervention group) who analysed all of the samples from the same participant in a single batch under the same conditions.

In summary, the findings from this study suggest that an intervention of 300 mg per day of Rx Balance BioResponse DIM significantly modulates *BRCA1* mRNA expression in *BRCA1* mutation carriers. The resultant effect on BRCA1 protein expression, and more importantly cancer risk, requires further exploration. Future studies that evaluate dietary and/or lifestyle determinants of *BRCA1* expression at the gene or protein level in a large sample of *BRCA1* mutation carriers may help delineate a role of non-genetic factors in the aetiology of this disease. The possibility of mitigating the effect of an inherited deleterious *BRCA1* mutation by increasing the physiologic expression of the gene and normalising protein levels represents a clinically important paradigm shift in the prevention strategies available to these high-risk women.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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