

# Prolonged recombinant pregnancy hormone use in BRCA1 and BRCA2 mutation carriers

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**Background** An early first full-time pregnancy substantially reduces the risk of developing breast cancer later in life. Extensive studies indicate that this protective effect is mediated by the pregnancy hormone human chorionic gonadotrophin (hCG).

**Methods** In this proof-of-concept study 33 women with a BRCA mutation received recombinant-hCG (r-hCG). A 4-mm breast biopsy was obtained before (T1) and after 12 weeks of r-hCG injections (T2), as well as 6 months later (T3). The tissue was examined using RNA-sequencing methodology to determine if the 'high-risk' transcriptomic signature was converted to a 'low-risk' signature as in an early first full-time pregnancy. A stringent clinical safety monitoring was performed.

**Results** The r-hCG administration was well tolerated in all participants. No clinically relevant changes were observed. In 25 women, the RNA quality was good for RNA sequencing in all three breast tissue biopsies. In response to the r-hCG, we observed 1907 differentially expressed genes (DEGs) (1032 up, 875 down) at T2 vs. T1 and 1065 DEGs (897 up, 168 down) at T3 vs. T1 in the group of women ( $n = 11$ ) not using any hormonal contraceptives during the study. There was no response at T2 vs. T1 and a

small number of DEGs, 260 (214 up, 46 down) at T3 vs. T1 in the group of 14 women using contraceptives.

**Conclusions** In summary, r-hCG has a remarkable effect on the gene expression profile of breast tissues from BRCA1/2 carriers who did not use any contraception. This opens an opportunity for a novel preventive strategy to reduce the incidence of breast cancer. *European Journal of Cancer Prevention* XXX: 000–000 Copyright © 2021 The Author(s). Published by Wolters Kluwer Health, Inc.

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## Introduction

Large studies indicate that an early first full-time pregnancy (FFTP) substantially reduces breast cancer risk (Lambe *et al.*, 1996; Kobayashi *et al.*, 2012). It effectively restores genomic and epigenetic alterations (Zendehbad *et al.*, 2019; Miranda Furtado *et al.*, 2019). Indeed, when compared to the transcriptomic profile of the nulliparous postmenopausal breast, the parous postmenopausal breast shows significant differences in which cell differentiation, development, apoptosis and chromatin remodeling are the main biological processes (Russo *et al.* 2012; Peri *et al.*, 2012).

The principal hormone responsible for this risk reduction is human chorionic gonadotropin (hCG). Ample in vitro, animal, and clinical studies have shown that hCG induces changes in gene expression and epigenetic markers, induces apoptosis, and protects breast epithelial cells from being transformed, and thereby inhibits breast tumorigenesis (Russo *et al.*, 1990; Bernstein *et al.*, 1995; Srivastava *et al.*, 1997; Jiang *et al.*, 2002; Kocdor *et al.*, 2009; Santucci-Pereira *et al.*, 2013). A recent study has demonstrated that young age at first pregnancy does protect against early-onset breast cancer in BRCA1/2 mutation carriers (Evans *et al.*, 2018). Consequently, treatment with hCG could potentially have similar preventive capabilities compared to a FFTP and could serve as a preventive treatment for high-risk women, such as carriers of BRCA1/2 mutations.

In this study, we explored for the first time the possibility of using r-hCG in a preventive setting in BRCA 1/2 carriers. The primary objectives of this study were to assess clinical safety and potential beneficial influence of prolonged r-hCG treatment in young, nulliparous women.

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**Table 1** This table lists the subject number, age at inclusion and description of mutations

Study number	BRCA	Mutation c1	Mutation p1	Age	Contraception use
101	BRCA1	c.5406+5G>A		25	A
102	BRCA1	c.5266dupC		23	C
103	BRCA1	c.2359dupG (p.Glu787Glyfs*3)		21	A
104	BRCA2	8904del (former name: 9132delC)	Val2969fs	24	A
105	BRCA2	c.4171delG (p.Glu1391Lysfs*19)		19	A
106	BRCA1	3661G>T	Glu1221*	25	B (52 mg levonorgestrel over 5 years)
107	BRCA2	4935delA	Glu1646fs	26	A
108	BRCA2	6275_6276delTT	Leu22092Profs*7	18	A
109	BRCA1	212+3A>G		24	A
110	BRCA2	4935delA	Glu1646fs	22	A
111	BRCA1	397delC	Arg133Valfs*30	25	C
112	BRCA1	3661G>T	Glu1221*	21	A
113	BRCA1	3661G>T	Glu1221*	24	B (52 mg levonorgestrel over 5 years)
114	BRCA1	c.5194-2A>G		24	A
115	BRCA1	134+3A>C		22	B (13.5 mg levonorgestrel over 3 years)
116	BRCA2	6275_6276delTT	Leu209Profs*7	20	C
117	BRCA2	1389_1390delAG	Val464Glyfs*3	22	C
118	BRCA1	2359dupG	Glu787Glyfs*3	19	B (13.5 mg levonorgestrel over 3 years)
119	BRCA1	2359dup (former name: 2478-2479insG)	Glu787fs	24	C
120	BRCA1	3607C>T	Arg1203*	20	C
121	BRCA1	2019del (former name: 2138delA)	Glu673fs	26	A
122	BRCA1	2359dup	Glu787fs	26	C
123	BRCA1	2359dup	Glu787fs	24	C
124	BRCA2	3847_3848del (former name: 4075delGT)	Val1283fs	24	A
125	BRCA2	c.5213_5216del4 (p.Thr1738Ilefs*2)		19	B (EE 0.04 mg + DSG 0.15 mg)
126	BRCA1	c.2359dupG		20	B (E <sub>2</sub> 1.5 mg + Nomac 2.5 mg)
127	BRCA2	4171del (former name: 4399delG)	Glu1391fs	23	C
128	BRCA1	4575_4585delAGAGGAGCTCA	Gln1525Hisfs*2	22	B (19.5 mg levonorgestrel over 5 years)
129	BRCA1	212+3A>G		25	A
130	BRCA1	c.3661G>T		22	B (19.5 mg levonorgestrel over 5 years)
131	BRCA1	c.3661G>T		18	B (19.5 mg levonorgestrel over 5 years)
132	BRCA2	c.662_663del		26	C
133	BRCA2	c.4576dupA (p.Thr1526Asnfs*3)		21	B (etonogestrel 68 mg over 3 years)

To be included in the study, the participants had to be asymptomatic, nulligravid women between 18 and 30 years of age, carriers of the BRCA1 or BRCA2 mutation (diagnosed by the genetics laboratory of the Ghent University Hospital, a CLIA-certified clinical genetics laboratory). The ECOG performance status needed to be 0 (Kornofsky 100%). The contraceptive profile consisted of three categories: (A) participants did not take any hormonal medication during the study and had stopped contraception more than 30 days prior to the start of study medication; (B) in instances where contraception containing any hormone was used, the contraceptive method is listed in this table. Three types of levonorgestrel intrauterine systems were used: Mirena (releasing 52 mg levonorgestrel over 5 years  $N = 2$ ); Jaydess (releasing 13.5 mg of levonorgestrel over 3 years;  $N = 2$ ); and Kyleena (releasing 19.5 mg of levonorgestrel over 5 years;  $N = 3$ ). Etonogestrel (68 mg over 3 years) is an implant inserted 2 years prior to the study participation in one subject. One participant used a natural estradiol-containing oral contraceptive (17 $\beta$ -estradiol (E<sub>2</sub>) 1.5 mg + norgestrel acetate (Nomac) 2.5 mg). Another participant used an oral formulation containing Ethinyl estradiol 0.04 mg combined with desogestrel (DSG) 0.15 mg; (C) participants did not take any hormonal medication during the study but stopped contraception less than 30 days prior to the start of study medication.

## Methods

### Participants

Thirty-three, nulliparous, young women were recruited in this prospective, longitudinal interventional study [ethical board of the University Hospital approval (EudraCT number: 2015-001720-36; EC number: 2015/0588)]. Baseline characteristics, inclusion and exclusion criteria are given in Table 1.

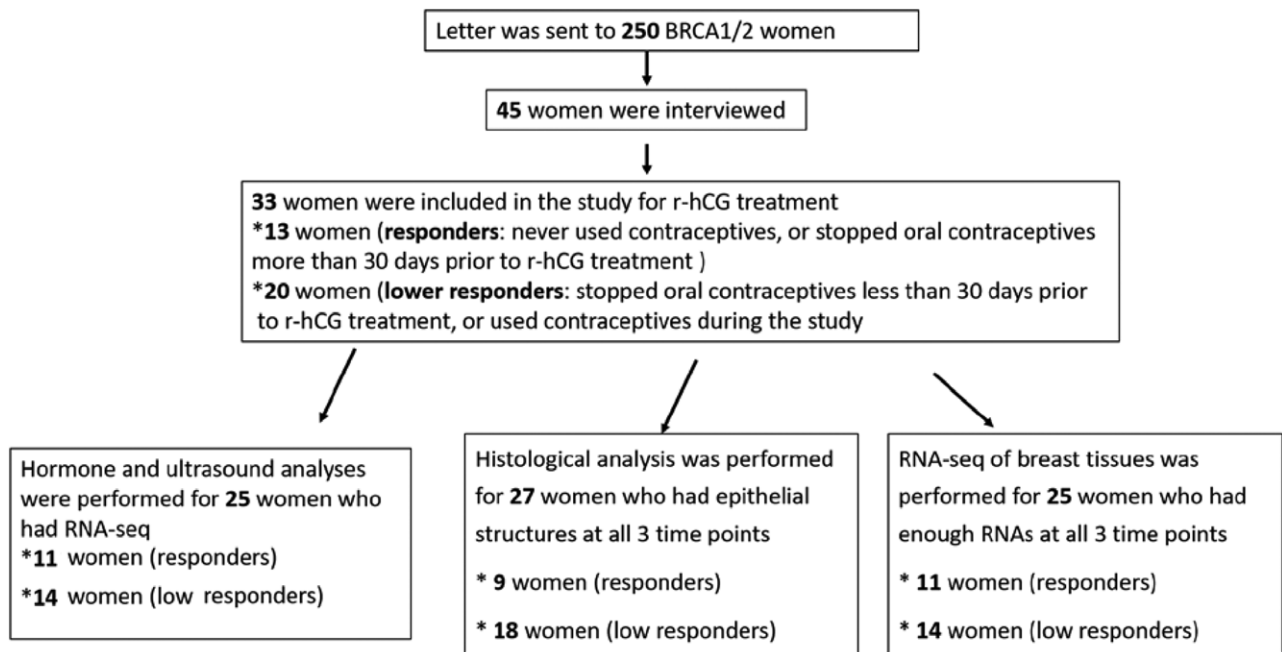
Participants were asked to stop oral contraception prior to the study. We expected that an levonorgestrel-intrauterine system (IUS) would not interfere with the study protocol since it was previously published that the amount of levonorgestrel in the breast epithelium was extremely low (Depypere *et al.*, 2019). As such, seven women with an levonorgestrel-IUS could be included in the study without needing to remove the IUS. One woman had a long-acting reversible contraceptive implant. Two women did not want to stop oral contraception.

### Clinical protocol

Blood was drawn, and an ultrasound of both ovaries and the uterus was performed. If all examinations were normal, a first Spirotome (Bioncise, Belgium) 4-mm breast biopsy was performed. Following the biopsy, the r-hCG treatment was initiated. The r-hCG (Ovidrel Prefilled Syringe, Serono) was purchased from Serono, Inc., Rockland, Massachusetts, USA or Switzerland. Participants received a subcutaneous injection of 250  $\mu$ g r-hCG three times a week for 12 weeks. A rigorous follow-up protocol during and after the study was implemented to monitor acceptance rate, procedural inconsistencies, interferences with clinical parameters, side effects, and safety of prolonged r-hCG administration in these young women (Supplementary S1, Supplemental digital content 1, <http://links.lww.com/EJCP/A320>).

The second and third Spirotome biopsies were taken immediately and 6 months after r-hCG treatment. The third biopsy was done to assess whether transcriptomic

Fig. 1



Flow chart of the participants used for the study.

and histological changes occurring in the breast due to r-hCG treatment persisted after 6 months' follow up. Details of Spirotome biopsies procedure and processing are included in the Supplementary S2, Supplemental digital content 1, <http://links.lww.com/EJCP/A320>.

The hematoxylin and eosin (H&E) staining was performed following a standard protocol (Supplementary S2, Supplemental digital content 1, <http://links.lww.com/EJCP/A320>). Immunohistochemistry (IHC) was performed by staining with primary antibodies using an i6000 BioGenex Autostainer following a standard protocol (Supplementary S2, Supplemental digital content 1, <http://links.lww.com/EJCP/A320>).

Total RNA was extracted within a month after all samples were received using the RNeasy Lipid Tissue Mini kit (Qiagen, Germantown, Maryland, USA) at Fox Chase Cancer Center according to the manufacturer's protocol; the library construction and sequencing were carried out by the BGI Company in Hong Kong (Supplementary S2, Supplemental digital content 1, <http://links.lww.com/EJCP/A320>). In total, there were 166 files sequenced, with each containing from 128–199 million reads (Supplementary S3–S6, Supplemental digital content 1, <http://links.lww.com/EJCP/A320>: library construction/qualified participants/sequence reads/statistics of reads generated from sequencing).

### Ultrasound monitoring

Ultrasound examinations of the endometrium, uterus and ovaries were performed with a vaginal probe (7.5 Hz, Medison, Germany) prior to the start of the r-hCG treatment and every month thereafter.

### Hormone level monitoring

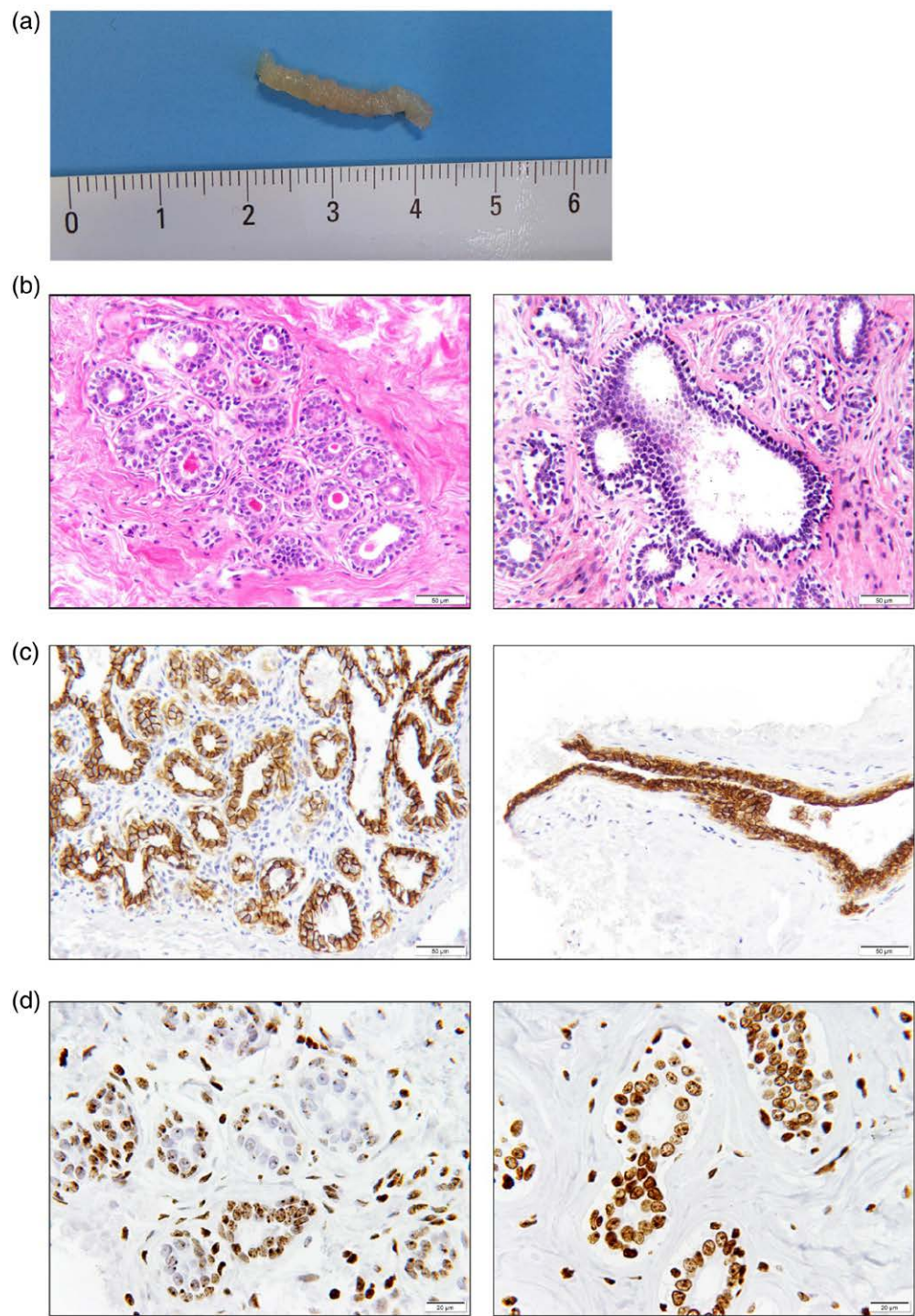
Estradiol and progesterone serum levels were used to monitor the cycle. Since none of the participants had any complaints during the r-hCG administration, and no signs of ovarian dysfunction were observed on ultrasound monitoring, blood was analyzed in one batch at the end of the study. Estradiol, progesterone, follicle stimulating hormone (FSH), luteinizing hormone (LH) and hCG were measured by electrochemiluminescence immunoassay on the Elecsys (Erler, 1998) and Cobas immunoassay analyzers.

### Statistical power sampling for RNA sequencing

Analysis of differential expression between two time points involved adjustment for multiple testing in terms of controlling the false discovery rate (FDR). At a significance level of the FDR of 0.05, and an effect size of 1 with the RNA sequencing to an average of 11× depth, the required sample size of approximately 11 allows the differential gene expression analysis of 90% power [R version 3.4.4 package *RNASeqPower* (Hart *et al.*, 2013)]. In terms of log 2 ratios, an effect size of 1 corresponds to a



Fig.2



The quality of breast biopsy samples for H&E and IHC staining. (a) Picture of one breast biopsy specimen. (b) H&E stained images of breast tissue. Magnification: 200×. (c) IHC images stained with E-cadherin, showing positive membrane staining in the epithelial cells. Magnification: 200×. (d) IHC images stained with H3K27me3, showing positive nuclear staining. Magnification: 400×. H&E, hematoxylin and eosin; IHC, immunohistochemistry.

two-fold change difference between any two time points being compared. A significance level of 0.05 results in 100 false discoveries per 2000 nondifferentially expressed

genes. Due to the paired nature of the comparisons, 11 participants are required for each time point. Taking into account the fact that breast samples would not always

yield enough material for RNA analysis in each biopsy and that we needed study participants where all three biopsies could be compared, we projected that we needed to include 30 women. Some participants were related to each other, and we did not want to choose amongst them for inclusion in the study. Hence, we included three more women to end up with a total of 33 women participating in the trial.

### Statistical analysis for hormones and ultrasound

Linear mixed models for the natural log-transformed hormones were fitted with a random intercept for patient (to account for the correlation between repeated measurements on the same patient) and with the visit as a categorical fixed effect (the visit at week 1 before r-hCG administration was taken as a reference). Confidence intervals were computed using the profile method based on the likelihood ratio test. *P* values were computed via Satterthwaite's degrees of freedom method.

In addition, linear mixed models were fitted with the visit (categorical, with the visit at week 1 before r-hCG administration was taken as the reference group), responsiveness (low to moderate responders versus responders) and the two-way interaction between visit and responsiveness in the fixed effect part of the model.

### Results

In total, 33 women were recruited (Fig. 1). All women were young nulliparous women with a mean age of 22.5 years. Clinical signs were monitored before, during and after the r-hCG administration. The most frequently reported side effects were nausea (12.5%), fatigue (12.5%), headache (29.2%), vasomotor symptoms (<5%) and insomnia (<5%). The side effects of prolonged r-hCG administration were mild and did not cause interference with daily functioning. The complete list of medications used by the participants during the study is provided as Supplementary S7, Supplemental digital content 1, <http://links.lww.com/EJCP/A320>. The compliance with the study medication was 100%. All participants included in the study finished the study and had three biopsies of the breast during the trial.

The breast tissues of BRCA1/2 carriers (Fig. 2) contain very dense stroma and fewer well-defined lobules compared to the breast tissues of BRCA1/2 wild-type women, consistent with previous findings (Russo *et al.*, 2001).

Since the response to r-hCG treatment evaluated by RNA-seq varied between the study participants, we re-analyzed our data according to hormonal contraceptive use during the study. Among these 25 patients, there were 11 women who did not use contraceptives during the r-hCG trial or who stopped oral contraceptives more than 30 days prior to the trial (except one case using a copper IUD before, during, and after the trial) and 14 women using oral contraceptives or a hormonal IUD during the trial or stopping the pills less than 30 days prior to the trial. We observed a strong difference between women

with and without contraceptive use in response to the r-hCG at both T2 and T3 versus baseline, T1. That was clearly reflected in Fig. 3c showing the differentially expressed genes (DEGs) at the cutoff fold change of 1.5 and 2, respectively, with 1907 DEGs (1032 up, 875 down) at T2 vs. T1 and 1065 DEGs (897 up, 168 down) at T3 vs. T1 for the women not using contraceptives (named as responders) while there was almost no response at T2 vs. T1 and a small number of DEGs, 260 (214 up, 46 down) at T3 vs. T1 for the group of 14 women using contraception during or close to start of the trial (named as low responders). Notably, the number of DEGs with the fold change of two accounts for about half of the total number of genes with significant expression changes.

### Ultrasound changes

There was a significant, gradual increase in the size of the ovaries, from 582 (488–694) mm<sup>2</sup> at the beginning of the study to a significantly higher surface of 831 (697–991) mm<sup>2</sup> (mean ratio 1.43 (1.19–1.71), *P* = 0.002) at the end (week 13) of the r-hCG administration. After the study was completed, the size of the ovaries remained within the values before the administration of the medication (Fig. 4). No clinically relevant changes were observed either by ultrasound or reported subjectively by the participants during the study. No cyst formation was observed. There was a marginally significant decrease in endometrial thickness from 3.9 cm (2.98–5.11) to 2.79 cm (2.13–3.66) (mean ratio 0.72 (0.54–0.95), *P* = 0.059). The subsequent values for endometrial thickness were not different.

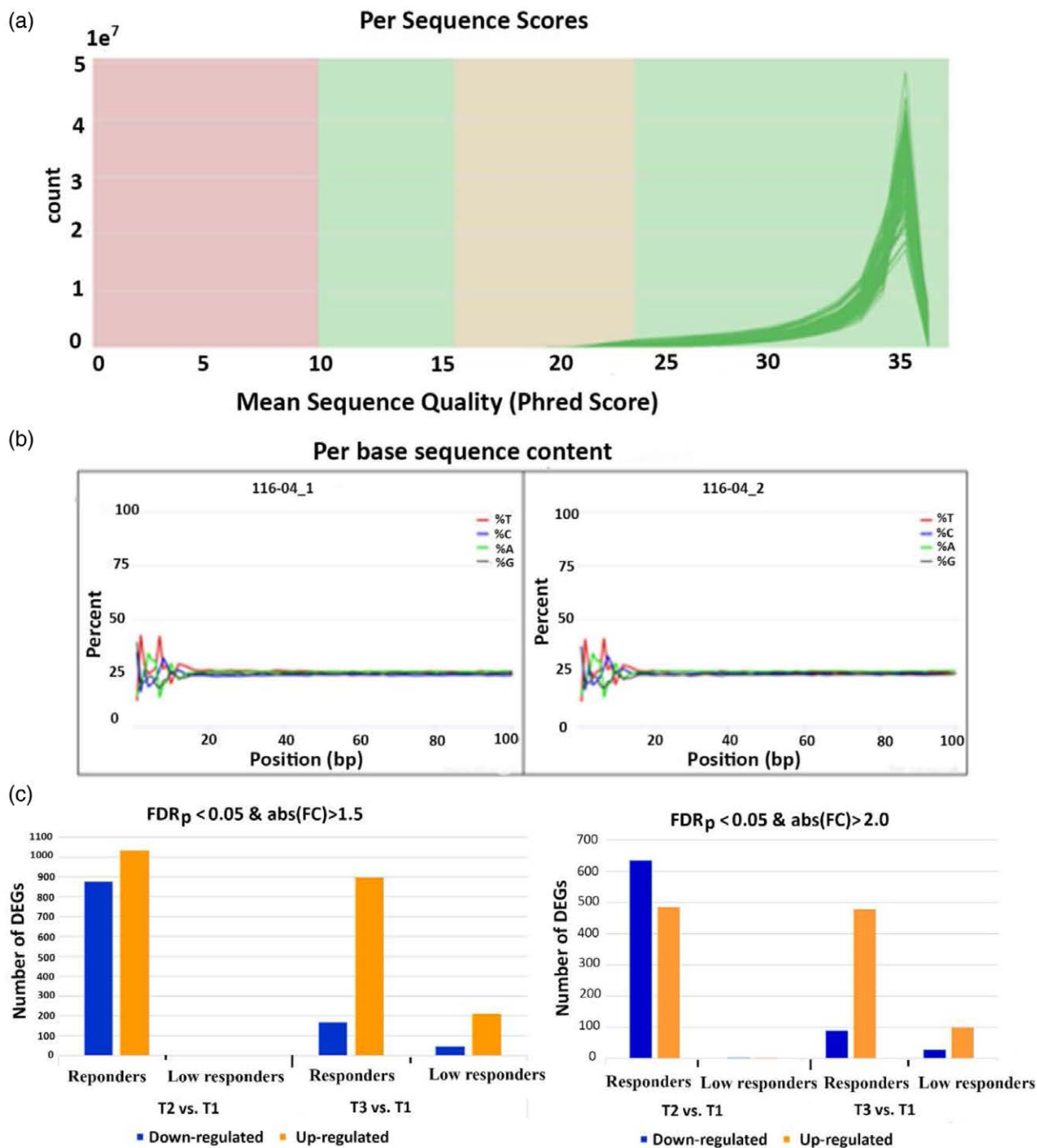
### Hormonal changes

During the r-hCG administration, there was a drop in FSH and LH levels. FSH decreased from 3.6 (2.4–5.2) mIU/mL (reference time: T1 = week 1) at the start of the study to a significantly lower value of 1.9 (1.3–2.8) mIU/mL (mean ratio 0.54 (0.35–0.83), *P* = 0.021) at week 5. The subsequent FSH levels were not significantly different. The LH levels significantly decreased from 5.7 (4.3–7.7) mIU/mL at the start of the study to 1.6 (1.2–2.2) mIU/mL (mean ratio 0.28 (0.2–0.38), *P* < 0.001) at week 5, and 3.9 (2.8–5.7) mIU/mL (mean ratio 0.69 (0.48–0.99), *P* = 0.098) at week 9. During the last month of r-hCG administration, the LH normalized. After the administration of the study medication, LH was not different from values at the beginning of the study.

We observed that the response to r-hCG treatment evaluated by the number of DEGs varied between study participants. The response evaluated in DEGs varied in relation to the history of contraceptive use; we assessed whether this variation could be explained by differences in hormone levels during the study. The following differences were observed:

Firstly, both LH and FSH levels were significantly higher (LH at week 5 (*P* = 0.003, mean ratio = 2.91) and

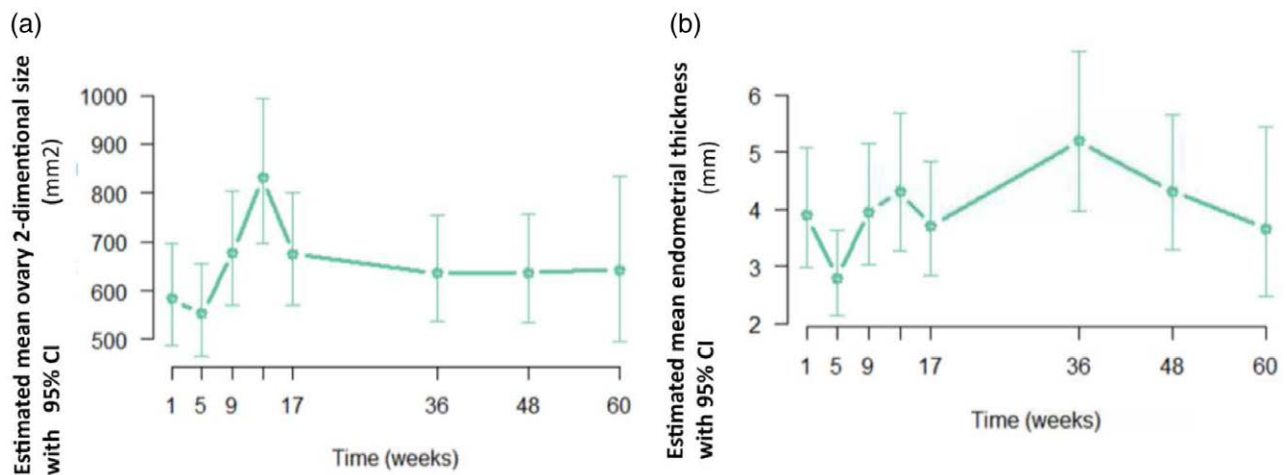
Fig. 3



r-hCG treatment induces a significant amount of gene expression changes in BRCA1/2 mutation carriers without contraceptives exposure. (a) Per sequence quality scores. Overall Phred scores. Each panel on the left-side shows the universal quality values of all reads in main trials and additional cases (paired-end, 166 read files in total). The y-axis shows the total counts corresponding to the Phred scores (x-axis). Green area represents good quality scores. (b) Per base sequence content. Right and left panels show the paired raw reads with very few acceptable biases for the difference between A and T, or G and C greater than 20% at the first of reads. The remaining base pairs of reads showed no bias, and no adapter contamination was observed. (c) Graphs representing the number of DEGs found in the breast tissue of women at different time points after r-hCG treatment compared to the control samples taken from the same patient before treatment [cutoff of false discovery rate-adjusted  $P$  value (FDR<sub>p</sub>) < 0.05 and fold change of 1.5 or 2]. DEGs, differentially expressed genes; r-hCG, recombinant human chorionic gonadotrophin.



Fig. 4



Mean ovary two-dimensional size (a) and endometrial thickness (b) with 95% confidence interval. The changes induced by r-hCG on the uterus were assessed by measuring the endometrial thickness and its appearance (triple lining, luteal appearance), the fundal diameter and isthmus-fundal distance. There was a marginal significance of decrease in endometrial thickness from 3.9 cm (2.98–5.11) to 2.79 cm (2.13–3.66) (mean ratio 0.72 (0.54–0.95),  $P = 0.059$ ). The subsequent values for endometrial thickness were not different. r-hCG, recombinant human chorionic gonadotrophin.

FSH [at weeks 5, 9 and 13 (pooled analysis)] ( $P = 0.028$ , mean ratio low responders to responders = 1.98) in the noncontraceptive-low-responder group (Supplementary S8, Supplemental digital content 1, <http://links.lww.com/EJCP/A320>).

Secondly, responders had a higher level of estradiol ( $P = 0.078$ , mean ratio = 0.55) and progesterone ( $P = 0.01$ , mean ratio = 0.2) compared to low responders at week 1 (Supplementary S8, Supplemental digital content 1, <http://links.lww.com/EJCP/A320>). There was a remarkable reduction for both estradiol and progesterone in responders at week 5 and a peak at week 9, and after that time, the levels of estradiol and progesterone decreased. Generally, the mean levels of estradiol and progesterone in the responders were always higher than those in the low responders at each time point during the first 36 weeks of the trial.

Thirdly, the mean hCG level was 206 (180–237) IU/L at weeks 5, 9 and 13 in the low responders; it was significantly ( $P < 0.005$ ) higher than the mean value 154 (134–178) IU/L in the responders (mean ratio low responders to responders = 1.34). The levels of prolactin were not significantly different between groups, not even when they were pooled (Supplementary S8, Supplemental digital content 1, <http://links.lww.com/EJCP/A320>).

## Discussion

We examined whether the use of r-hCG, a recombinant hormone, mimics pregnancy. By inducing changes in the mammary gland, it could be a novel preventive strategy against breast cancer for high-risk women and, by extension, for all women. This is the first study in which r-hCG

was administered during a prolonged period of 12 weeks in young women as a tentative chemoprophylactic agent in an attempt to mimic the effects of an FFTP.

Overall prolonged r-hCG was well supported, with all participants completing the trial. The side effects were minimal. Initially, we started the administration during the luteal phase. Since the recruitment and maturation of one follicle had taken place, it was considered that this would be a safe option to avoid multiple follicle recruitment. This was done to avoid potential ovarian hyperstimulation (OHSS). As evidenced by laboratory tests and ultrasound monitoring, prolonged administration was safe, and no significant increase in estradiol levels was observed. Since in the initial participants no signs of OHSS were observed, we allowed women to start r-hCG soon after stopping hormonal contraception, not requiring them to start during the luteal phase. Because most participants had an ongoing relationship, we avoided that they had to wait too long prior to the onset of the study, especially since some women took contraceptives because they had irregular or very irregular cycles. Stopping contraceptives would entail these latter participants having to wait for months until ovulation occurred, so they could start the study. We did not require the 7 participants having a hormonal levonorgestrel-IUS to have it removed prior to the study. To our surprise, these women (contraception group) had different responses with a delay and a significant reduction in DEGs.

Generally, it is believed that the action of hCG is luteinizing hormone-chorionic gonadotropin receptor (LHCGR) mediated. The expression of LHCGR has been identified not only in the ovary and testis but also

in extragonadal tissues, such as in the uterus (Sacchi *et al.*, 2018) and breast (Meduri *et al.*, 1997). It was shown that hCG has a role in promoting the weight gain of ovaries and uterus (Lecompte *et al.*, 2010, Rafert *et al.*, 2016), as well as inducing mammary gland development (Russo *et al.*, 1990). Interestingly, estrogen receptor antagonists exert a dose-dependent inhibition of the hCG stimulation of uterine weight gain when injected simultaneously with hCG (Rafert *et al.*, 2016), suggesting that the estrogen level could affect the impact of the hCG on its target organs.

Organs such as the ovary, breast, liver and kidney can take up hCG after administration. The hCG receptor expression differential including (1) cell numbers with receptor, (2) receptors per cell and (3) receptor affinity can affect the distribution of hCG in the breast and ovaries. The liver is responsible for the removal of hCG from the circulation, and the kidney is involved in excretion of hCG (Mizejewski, 1975). All these factors can have an influence on the serum hCG level. Studies have shown that exogenous hCG concentrates preferentially in the ovarian tissues of nonhypophysectomized mice, the maximum concentration occurring at 2–4 h after injection (Kazeto and Hreshchysyn, 1970), although serum hCG levels were reduced hours after hCG administration, the tissue/blood hCG ratio in the ovaries increased (Rafert *et al.*, 2016), suggesting that the serum hCG level does not reflect hCG's bioactivity. In our study, the lower serum hCG level in responders might suggest a higher binding of r-hCG in target organs. Consistently, the serum estrogen and progesterone levels were relatively higher in responders during 36 weeks of the study, indicating a higher hCG response.

Interference from medication, hormonal status and hCG can act in two ways. The influence of hCG on clinical and endocrine parameters seems minimal and even absent. The effect of clinical parameters on hCG efficiency is unexpected. Hormonal use seems to have a paramount effect on molecular biology parameters. This observation is the first of its kind, and subsequent prevention studies should take into account stratification according to contraception techniques and wash-out periods.

In conclusion, this study demonstrates for the first time that prolonged use of r-hCG in young BRCA1/2 mutation carrier women for breast cancer prevention is feasible and safe. The RNA-sequencing analysis showed that r-hCG treatment has a remarkable effect on the gene expression profile of breast tissues from BRCA1/2 carrier women who did not use any hormonal contraceptives, whereas the use of contraceptives during the study delayed the response and significantly reduced the number of DEGs.

## Acknowledgements

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R.J. conceived and supervised the whole project; D.H. carried out the clinical trial, collected samples, analyzed hormone and ultrasound analysis and drafted the manuscript; S.Y. received all biopsy samples, performed histological analysis, extracted total RNAs and drafted the manuscript; D.N. carried out RNA-seq analysis and drafted the manuscript. P.B. is the clinical director of the department of genetics. He was involved in the counseling of high-risk women. He recruited women from his database of BRCA carriers and designed the invitation letters approved by the ethical board of the University Hospital. S.F. helped us during the design of the study and helped interpret the data. J.J. helped us in the design of the study. He shared with us his extensive experience with the Spirotome system, coordinated the handling of the biopsies and cosupervised the project.

## Conflicts of interest

There are no conflicts of interest.

## References

- Bernstein L, Hanisch R, Sullivan-Halley J, Ross RK (1995). Treatment with human chorionic gonadotropin and risk of breast cancer. *Cancer Epidemiol Biomarkers Prev* 4:437–440.
- Depypere HT, Stanczyk FZ, Croubels S, Blondeel PN, Roche NA, Depypere BP, Vanhaecke L (2019). Breast levonorgestrel concentrations in women using a levonorgestrel-releasing intrauterine system. *Contraception* 100:299–301.
- Erler K (1998). Elecsys immunoassay systems using electrochemiluminescence detection. *Wien Klin Wochenschr* 110 (Suppl 3):5–10.
- Evans DG, Harkness EF, Howel S, Woodward ER, Howell A, Laloo F (2018). Young age at first pregnancy does protect against early onset breast cancer in BRCA1 and BRCA2 mutation carriers. *Breast Cancer Res Treat* 167:779–785.
- Hart SN, Therneau TM, Zhang Y, Poland GA, Kocher JP (2013). Calculating sample size estimates for RNA sequencing data. *J Comput Biol* 20:970–978.
- Jiang X, Russo IH, Russo J (2002). Human chorionic gonadotropin and inhibin induce histone acetylation in human breast epithelial cells. *Int J Oncol* 20:77–79.
- Kazeto S, Hreshchysyn MM (1970). Tissue distribution of human chorionic gonadotropin. *Am J Obstet Gynecol* 106:1229–1234.
- Kobayashi S, Sugiura H, Ando Y, Shiraki N, Yanagi T, Yamashita H, Toyama T (2012). Reproductive history and breast cancer risk. *Breast Cancer* 19:302–308.
- Kocdor H, Kocdor MA, Russo J, Snider KE, Vanegas JE, Russo IH, Fernandez SV (2009). Human chorionic gonadotropin (hCG) prevents the transformed phenotypes induced by 17 beta-estradiol in human breast epithelial cells. *Cell Biol Int* 33:1135–1143.
- Lambe M, Hsieh CC, Chan HW, Ekblom A, Trichopoulos D, Adami HO (1996). Parity, age at first and last birth, and risk of breast cancer: a population-based study in Sweden. *Breast Cancer Res Treat* 38:305–311.
- Lecompte F, Harbey E, Cahoreau C, Klett D, Combarnous Y (2010). Use of the immature rat uterotropic assay for specific measurements of chorionic gonadotropins and follicle-stimulating hormones *in vivo* bioactivities. *Theriogenology* 74:756–764.
- Meduri G, Charnaux N, Loosfelt H, Jolivet A, Spyrtatos F, Brailly S, Milgrom E (1997). Luteinizing hormone/human chorionic gonadotropin receptors in breast cancer. *Cancer Res* 57:857–864.
- Miranda Furtado CL, Salomão KB, Verruma CG, Paulino Leite SB, Lopes Rios AF, Bialecka M, *et al.* (2019). Variation in DNA methylation in the KvDMR1 (ICR2) region in first-trimester human pregnancies. *Fertil Steril* 111:1186–1193.
- Mizejewski GJ (1975). Human chorionic gonadotropin: comparative studies of ovarian uptake in mammals. *Comp Biochem Physiol A Comp Physiol* 52:29–34.
- Peri S, de Cicco RL, Santucci-Pereira J, Slifker M, Ross EA, Russo IH, *et al.* (2012). Defining the genomic signature of the parous breast. *BMC Med Genomics* 5:46.



- Rafert S, Mariot J, Klett D, Combarnous Y (2016). Involvement of ovarian estradiol biosynthesis and pituitary FSH expression in the mechanism of human chorionic gonadotropin stimulation of uterine growth in immature female rats. *J Hormones* **2**:1–7.
- Russo IH, Koszalka M, Russo J (1990). Effect of human chorionic gonadotropin on mammary gland differentiation and carcinogenesis. *Carcinogenesis* **11**:1849–1855.
- Russo J, Lynch H, Russo IH (2001). Mammary gland architecture as a determining factor in the susceptibility of the human breast to cancer. *Breast J* **7**:278–291.
- Russo J, Santucci-Pereira J, de Cicco RL, Sherif F, Russo PA, Peri S, *et al.* (2012). Pregnancy-induced chromatin remodeling in the breast of postmenopausal women. *Int J Cancer* **131**:1059–1070.
- Sacchi S, Sena P, Degli Esposti C, Lui J, La Marca A (2018). Evidence for expression and functionality of FSH and LH/hCG receptors in human endometrium. *J Assist Reprod Genet* **35**:1703–1712.
- Santucci-Pereira J, George C, Armiss D, Russo IH, Vanegas JE, Sherif F, *et al.* (2013). Mimicking pregnancy as a strategy for breast cancer prevention. *Breast Cancer Manag* **2**:283–294.
- Srivastava P, Russo J, Russo IH (1997). Chorionic gonadotropin inhibits rat mammary carcinogenesis through activation of programmed cell death. *Carcinogenesis* **18**:1799–1808.
- Zendehbad Z, Izadi P, Daraei A, Yekaninejad MS, Nafissi N, Younossi N, *et al.* (2019). Early parity epigenetic footprint of FOXA1 gene body in normal breast tissue of Iranian women. *Iranian Biomed J* **23**:99–106.