

Portable NMR for the investigation of models of mammographic density *ex vivo*: Androgens antagonise the promotional effect of oestrogen

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Abstract.

BACKGROUND: Increased mammographic density (MD) is a strong and independent risk factor for breast cancer. Lifetime oestrogenic exposure is associated with increased MD, however androgenic effects on MD have not been widely investigated.

METHODS: We studied the effect of 17β -oestradiol (E2) alone or in combination with an androgen receptor (AR) agonist (5α -dihydrotestosterone [DHT]) or a selective AR modulator (Enobosarm), in modulating MD as measured via single-sided Portable NMR in a patient-derived explant (PDE) model of normal human mammary tissue.

RESULTS: We observed an upward trend in explants treated with E2 alone in 3/6 cases, an effect which appeared to be somewhat influenced by menopausal status. Co-treatment of E2 with the AR agonists DHT or Enobosarm however effected a downward trend in regards to MD. E2 significantly upregulated the ER regulated genes (ERGs) *CELSR2* and *AR*, and the AR regulated genes *SEC14L2* and *GRPC5A*, whereas E2 in combination with AR agonist Enobosarm downregulated ERGs *SERPINA3*, *ATP6V1B1*, *TFE1* and PR regulated gene *RANK*. Only *GREB1* and *CLIC6* were upregulated by E2 and downreg-

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**Denotes shared last author.

ulated by the combination of E2 with Enobosarm. DHT + E2 exhibited no significant difference in expression of ER, AR or PR genes examined, compared with the effect of E2 alone. A negative trend was observed between ER protein levels and MD increase in PDEs. Since ER protein gets degraded upon hormone activation, this observation supports active ER signalling in the promotion of MD.

CONCLUSIONS: Collectively, these findings supports the utility of single-sided Portable NMR for the measurement of MD in explanted human mammary tissue to detect MD change in response to exogenous hormone treatments. A mechanism through which activation of AR may reduce MD as a potential mechanism for reducing breast cancer risk associated to high MD is discussed.

Keywords: Mammographic density, oestrogen, androgen, single-sided portable NMR, patient-derived explants, *ex vivo* culture

Abbreviations

AR	androgen receptor
ARGs	androgen-regulated genes
DHEA(S)	dehydroepiandrosterone (sulphate)
DHT	5 α -dihydrotestosterone
GEE	generalised estimated equation
E2	17 β -oestradiol
ETH	0.00001% ethanol control
ER	oestrogen receptor
ERGs	E2-regulated genes
HMD	(regions of) high mammographic density
HR	regulatory hormone receptor
IHC	immunohistochemistry
LMD	(regions of) low mammographic density
MD	mammographic density
(c)MHT	(combined) menopause hormonal therapy
NMR	Nuclear Magnetic Resonance
PDE	patient-derived explant
PR	progesterone receptor
PRGs	progesterone-regulated genes
SARM	selective androgen receptor modulator
T	testosterone
WT	wild type

1. Introduction

Breast fibroglandular tissue (epithelium and stroma) can attenuate x-rays and appear light on a mammogram; this light, radio-opaque area is known as mammographic density (MD) [6]. When adjusted for age and body mass index, MD is a strong and independent risk factor for breast cancer – women with breasts in the upper MD quartile (75% or more dense tissue area) are at 4- to 6-fold greater risk of breast cancer compared to women in the lowest MD quartile [6,18,39]. Assessment of MD complements other well-established breast cancer risk factors including multi-generational family history, polygenic

risk scores, high risk mutations, family history, parity / breastfeeding, age at menarche, and age at first childbirth [29].

Female sex hormone (oestrogen, progesterone) exposure is strongly correlated with MD [20,23,36]. MD fluctuates during the menstrual cycle due to the periodic changes of endogenous sex hormone production from the ovaries [8,30,57]. Menopause decreases MD because of reduced sex hormone levels [9]. Menopause hormonal therapy (MHT), especially formulations containing an oestrogen and a synthetic progestogen (progestin) (e.g. Medroxyprogesterone [MPA] and Norethisterone acetate [NETA]), can increase MD and sustain density at a high level, consequently increasing breast cancer risk [4,10,21,26,46]. Breast cancer risk associated with progestin-containing MHT disappears after adjustment for treatment-induced changes in MD, indicating that an increase in MD with combined MHT (cMHT) is an intermediate surrogate marker of breast cancer risk [10].

Androgens are another type of sex hormone found in women and the androgen receptor (AR) is expressed in luminal epithelia of normal mammary tissues [24]. AR activity is required to inhibit breast development in males and its anti-estrogenic, anti-proliferative activity in normal and malignant female breast tissues indicates a tumour suppressor role [17,25]. However, the effect of AR signalling on MD is unknown. Only one study has evaluated AR expression in the context of mammographic density; Sartor et al. reported that high MD (HMD) was associated with AR-negative breast tumours, although evidence for this association was considered weak [48].

Testosterone (T) and 5α -dihydrotestosterone (DHT) are endogenous AR ligands, whereby circulating T can be metabolised to the more potent AR ligand DHT, via the 5α -reductase enzyme within breast tissue [24]. Enobosarm is a nonsteroidal selective AR modulator (SARM) that was developed for the treatment of muscle wasting and osteoporosis [14], but has AR agonist activity in breast cancer cells [25,31]. AR agonists, including Enobosarm, have been shown to potently inhibit ER signalling in ER+ breast cancers via reprogramming of the ER cistrome and sequestration of common co-regulatory factors (p300 and SRC-3) from ER to AR target genes [25,43]. Hence, AR agonists may affect MD through regulation of ER signalling in breast epithelial cells.

To date, exploration of androgenic effects on MD have mainly focused on serum androgens; as yet no prospective randomised trial to study MD reduction with androgenic treatment in women has been performed. Relationships between MD and levels of different types of circulating androgens, including testosterone (total and free), androstenedione, dehydroepiandrosterone (DHEA) and DHEA sulphate (DHEAS) have been widely studied, but the results are conflicting and potentially confounded by conversion of circulating androgens to oestrogens [1,5,16,38,41]. Thus, the direct effect of androgen on MD is still unclear.

The suitability of single-sided Portable NMR for compositional and microstructural profiling of biological tissues has been well-demonstrated [7,56]. We have previously demonstrated that single-sided Portable NMR measurements of MD of patient-derived explants (PDEs) *in vitro* closely correlate with the MD measured by MicroCT and X-ray mammography [2,3,32,54], and Portable NMR measurements in healthy human volunteers *in vivo* enabled depth profiling of MD within the breast [40]. We have also used Portable NMR to accurately measure MD in PDEs of human breast tissue cultured *ex vivo* over two weeks [33]. To study the effect of AR signalling on MD, we tested the effects of DHT and Enobosarm and utilised Portable NMR measurements to monitor MD change. The hormonal mechanism regulating MD change was also investigated by analysing hormone receptors and their respective downstream signalling molecules at the mRNA and protein level.

2. Materials and methods

2.1. Patient cohort

Human breast tissue specimens surplus to pathology needs and with no evidence of malignancy were accrued from prophylactic mastectomy surgeries, primarily in women with high BC risk as determined by family history of breast cancer and/or contralateral benign or malignant disease. Six females (2 postmenopausal, 4 premenopausal) were included in this study, equally divided in regards to BRCA 1 or BRCA 2 mutation, or WT status, and ranged in age from 27 to 48, with the average age being 38 years old. Information about the patient cohort and sample treatments is summarised in Table 1.

Outline of the study design can be viewed in Supplementary Fig. 1. Resected breast tissue was placed on ice and taken from surgery to the Pathology Department at Princess Alexandra Hospital, cut into approximately 1 cm thick slices, and checked for lesions by qualified pathologists (Dr Linda Shen and Dr Simone Birch). Up to 3 slices per patient were obtained and used in this project. Some tissues were immediately processed for downstream analyses and some were delegated to culture as PDEs. This study was approved by the Metro South Hospital and Health Services, Queensland (HREC/16/QPAH/107) and Queensland University of Technology (1600000261).

Table 1

Patient demographics and summary of the PDE treatments. Treatment abbreviations: ethanol control (ETH), oestradiol (E2), enobosarm (SARM), dihydrotestosterone (DHT)

Patient ID	Age	Menstrual status	BRCA1/2 pathogenic variant status	BI-RADS	PDE treatments
PAH032M	48	Postmenopausal	WT	3	ETH E2 E2 plus SARM E2 plus DHT
PAH037M	27	Premenopausal	BRCA1+	unknown	ETH E2 E2 plus SARM E2 plus DHT
PAH040M	48	Postmenopausal	WT	4	ETH E2
PAH043M	33	Premenopausal	WT	3	ETH E2 E2 plus SARM E2 plus DHT
PAH044M	36	Premenopausal	BRCA2+	2	ETH E2 E2 plus SARM E2 plus DHT
PAH045M	34	Premenopausal	BRCA1+	2	ETH E2 E2 plus SARM E2 plus DHT

2.2. *Ex vivo culture of patient-derived explants of normal human breast tissue*

Mammography was used to determine high, medium (defined as a mixture of low and high MD) or low MD regions of the mammary slices as interpreted by the clinical radiologist (TL). PDEs were cultured *ex vivo* as previously described [12,33]. Briefly, medium MD regions (used to allow observations of both increased or decreased MD) were cut into smaller pieces of approximately 0.5 cm³ in size and 1-2 pieces randomly placed onto a gelatin sponge scaffold (Spongostan dental sponge, Ark Health, Australia), which was semi-submerged in basal media (phenol red-free RPMI containing 10% charcoal-stripped foetal bovine serum (FBS; ThermoFisher Scientific, Australia), 1 x penicillin and streptomycin (Merck, Australia), 100 µg/mL hydrocortisone (Merck, Australia) and 100 µg/mL insulin (Merck, Australia)) in a well of a 24-well culture plate. For hormonal treatments, basal media was supplemented with ETH control (0.00001% ethanol (Sigma-Aldrich), or 1nM E2 (Merck, Australia), 1 nM E2 plus 100 nM Enobosarm (GTx-024, a gift from TE Hickey) or 1 nM E2 plus 100nM DHT (Sigma). PDEs were first cultured in the basal medium for 36 hrs to allow tissue equilibration to culture, then treated and maintained for up to 2 weeks, a technique has been shown to sustain tissue viability and hormone responsiveness [33]. Media and treatments were replenished twice weekly. Explant-specific PDE treatments are summarised in Table 1.

2.3. *Portable NMR measurements*

Individual PDEs were removed from the underlying sponge and placed in a well within a dry 24 well plate for Portable NMR measurements using a PM5 or PM25 NMR-MOUSE instrument (Magritek, Aachen, Germany) at day 0 (baseline) and day 14 (endpoint). For all treatments, the relative tissue water and fat contents of these explants were measured from the T_2 relaxation spectra obtained from Carr-Purcell-Meiboom-Gill (CPMG) decay curves, as described previously [2]. All Portable NMR measurements were made at room temperature, 22 ± 1 °C.

2.4. *RNA extraction and RT-qPCR*

At endpoint, cultured PDEs were cut in half for RNA isolation or fixation and immunohistochemistry. The tissue for RNA extraction was firstly homogenised in TRIzol (Ambion, Life Technologies) using a Qiagen Tissue Lyser II with pre-autoclaved tungsten carbide beads (Qiagen). Bioline RNA extraction set (Catalog no. BIO-52075) was used for RNA isolation, and purified RNA quantitated and validated using spectrophotometry (NanoDrop 2000/2000c, ThermoFisher Scientific). The SensiFAST cDNA Synthesis Kit was applied for reverse transcription of 100 ng of total RNA. RT-qPCR was performed as previously described [34]. Primer sequences are detailed in Table 2.

2.5. *Immunohistochemistry*

The remaining tissue was fixed overnight in 4% paraformaldehyde (Sigma) or 10% neutral buffered formalin (Sigma-Aldrich) at 4 °C, processed and embedded in paraffin, sectioned and stained with AR, ER or PR hormone receptor antibodies. The staining process was performed either at Mater Pathology, Brisbane or at the Translational Research Institute Histology Core Facility, on a Ventana Benchmark ULTRA IHC machine. The percentages of DAB/nuclear area for ER, PR and AR were calculated by ImmunoRatio image analysis software installed as a plugin in ImageJ (National Institutes of Health) [55]. All epithelial units were analysed at 20× magnification. Each glandular field was considered as

Table 2
Primer sequence details

Gene	Sequence	Gene	Sequence
5' L32	GATCTTGATGCCAACATTGGTTATG	3' L32	GCACTTCCAGCTCCTTGACG
5' ALCAM	GTGCAGTACGACGATGTACCAGAATACAA	3' ALCAM	TGTCCTCAGTTACTAGCATGCACACAAA
5' STMN1	CAGAATTCCTCCCTTTCCCTCCAAAGAA	3' STMN1	TTTGTGGGTCAGTTTCTCTTCTGCCATTTT
5' GREB1	AGGACCTGCGCCTTGCTCCATTT	3' GREB1	CTGCTTCTTGGGTTGAGTGGTCAGTTT
5' PDZK1	CTGCAGGCTGGCTAAAGGTGAAAAT	3' PDZK1	AAGTGGATCAGCCAGGGAGGAAACAATA
5' RASSF5	GGCAAAGTCTCTTCATGGACAAGTGTTT	3' RASSF5	TCAAAGAGGAATCAATGCCAGAGAAAGAA
5' FKBP5	GGACATTGGGGTGGCTACCATGAAGAAA	3' FKBP5	CTCCGGATAATGCCTCCATCTCAAATAAA
5' AR	GGGCCCCACTTTCCCGGCTTAA	3' AR	GCACAGCGGGTGGAACTCCCAAAA
5' ATP6V1B1	ATGCCAGGAAGACCACTGCGAATTTA	3' ATP6V1B1	TCAATAGGAGAAATGCCCGTCTGAATCAT
5' IGSF1	CCCTTACTGAGTCCAATGCAGGTCTTTA	3' IGSF1	CCAGCCATGGCAGAGGATGTTAACATTA
5' CELSR2	GGGCGTTTCAATGAGAAGCATGACTTT	3' CELSR2	GGTTCACGCAAGTGCCCCATTGT
5' SERPINA3	TGGTGGAGCTGAAGTACACAGGCAAT	3' SERPINA3	GCTTCTGTGCCCTCCTCAAATACATCAA
5' CLIC6	AGGAAACCCGCAGACCTGCAGAA	3' CLIC6	CTCGGTGCTGTAGGCATCTATTTTCAT
5' MSMB	TGTTCTCCTGGGCAGCGTTGTGA	3' MSMB	GGGTCCTTCTTCTCCACCAGATATACTT
5' SHR00M3	CCATGCAGATACTGGTGCCTCTAACTT	3' SHR00M3	CATCGGGTTGCTTCTCCCCAGATTTA
5' EAF2	AAGTTAGGGGAGAGTTTCGAGAA	3' EAF2	CCAACCTCAAGTATCCTTCAG
5' EL0VL5	GTGCACATTCCTCTTGGTT	3' EL0VL5	TTCAGGTGGTCTTTCCTTCG
5' GPRC5A	CTCACTCTCCCGATCCTCGT	3' GPRC5A	CAGTCCGATGATGAAGGCGAA
5' SCP2	CCACTGATAAGCATGTTGACCT	3' SCP2	TGAACTGGGAATACGGGTTA
5' SEC14L2	GCCGAATCCAGATGACTATTTTCT	3' SEC14L2	GATGTTGTCAATGTCCTTTTGCTT
5' ZBTB16	GAGATCCTCTTCCACCGCAAT	3' ZBTB16	CCGCATACAGCAGGTCATC
5' TFF1	CGAATGGCCACCATGGAGAACAA	3' TFF1	GAGGGCGTGACACCAGGAAAA
5' MYB	CAGGAAGGTTATCTGCAGGAGTCTTCAAAA	3' MYB	CTATAGGCGGAGCCTGAGCAAAA
5' PGR	CGAGACTCAGCCAAATGTCATTTCTGTAA	3' PGR	CTGCTTGAAGACTCAGGGAAGATT
5' ESR1	GTGCCTTACACAGGGGTGAAGTGT	3' ESR1	GCCCCTGCTCCTTTCAACTACCATT
5' RANK	GCTGCAGGTGGCTTTGCAGAT	3' RANK	CCGGGCCACAGGGCAGACATA
5' RANKL	GCAAGCTGAAGCTCAGCCTTTT	3' RANKL	CATGGTACCAAGAGGACAGACTCACTTTAT

an independent data point. The expression of the hormone receptor was determined by averaging % positivity via the ImmunoRatio approach.

2.6. Statistics

Student's T-test (used to assess the differences in MD changes and gene expression levels between the different treatment groups), were calculated using GraphPad Prism version 8.4.3. Statistical parameters characterizing the correlations between gene expression and MD change were calculated using IBM SPSS v.27.

3. Results

3.1. MD changes with hormonal treatment of breast PDEs

Breast PDEs of medium MD were treated with E2 alone or E2 combined with an AR agonist for 2 weeks. Portable NMR measurements were conducted at baseline and endpoint to determine MD changes. As shown in Fig. 1, MD changed in all explants over the 2 weeks of treatment. In our study, 6 independent cases (from 4 pre- and 2 post-menopausal women) were treated with E2, 5 of which provided

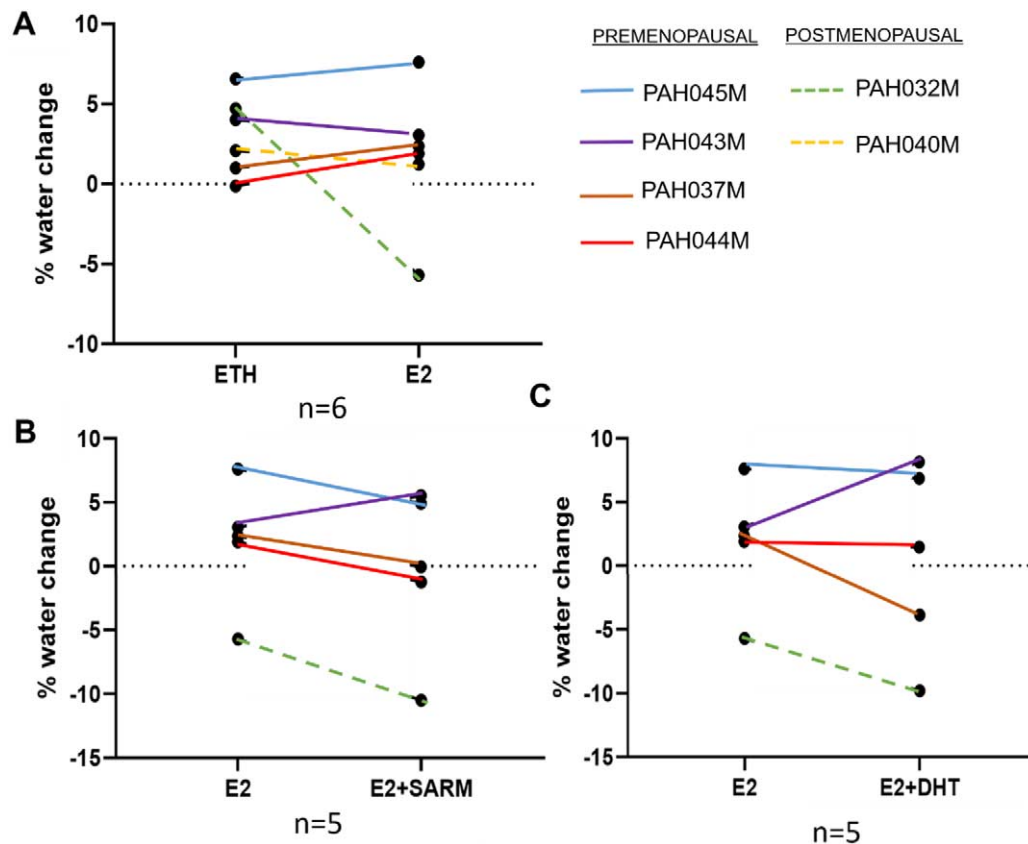


Fig. 1. Tissue water content change obtained from Portable NMR measurements. The differences of tissue water content (“% water”) between endpoint and baseline (day 14 – day 0) are shown, representing the MD modulation due to different hormonal treatments (ETH CONT = ethanol equivalent control, E2 = 1nM oestradiol, SARM = 100nM Enobosarm, DHT = 10 nM 5- α dihydrotestosterone). (A) ETH control vs E2 (n = 6). (B) E2 vs E2 plus SARM (n = 5). (C) E2 vs E2 plus DHT (n = 5 each dot represented % water change in one patient sample with one treatment. Different treatments in the same sample are connected with one colour line. Full lines represent the explants excised from premenopausal women, dotted lines the explants excised from postmenopausal women. Student’s paired T-test was used to determine significance; none of these comparisons were significant.

sufficient material to include co-treatment with an AR agonist. Compared to the patient-matched vehicle control, 3 cases showed E2-induced increases in MD while the other 3 samples exhibited a decrease (Fig. 1A). The oestrogenic effects on MD were relatively small (1.03% to 2.03% increase; -0.96% to -0.85% decrease), except for one case (PAH032M) in which E2 treatment induced a 10.4% difference in MD (Fig. 1A). Notably, the E2-induced promotion of MD occurred in the tissues from premenopausal women. Considering premenopausal cases we previously reported in terms of E2 effects on MD in breast PDEs [33], 6 out of 7 (85.7%) of PDEs from premenopausal women displayed an increase in MD with E2. Conversely, the two postmenopausal PDEs in the current study displayed a decrease in MD over the treatment period. Although the numbers are small, this data suggests that oestrogenic promotion of MD in excised tissues may only occur prior to menopause.

To study the potential anti-estrogenic effect of AR signalling on MD, PDEs were also treated with E2 plus either Enobosarm or DHT. We observed a downward trend in MD in 4 out of 5 cases co-treated with E2 plus either AR agonist compared with E2 alone (Figs 1B and 1C). This downward

trend occurred irrespective of the effect of E2 on MD. The only exception was the premenopausal case PAH043M, in which E2 alone appeared to decrease MD and both AR agonists increased MD compared to E2 treatment (Figs 1B–1C). PDEs treated with E2 plus Enobosarm showed comparatively moderate decreases in MD, in the range of -2.48% to -4.8% , compared with PDEs treated with E2 plus DHT, which varied considerably, from -0.44% to -6.24% . This variation may be due in part to the ability of DHT to be metabolised in breast tissues [31,44].

3.2. Hormone receptor expression levels in untreated PDEs

To explore the possible reason for the outlier PAH043M in regards to hormonal effects on MD, we analysed hormone receptor expression in the original tissue (not explanted) from the 5 patients treated with E2 alone or in combination with an AR agonist. The % DAB positivity per glandular area after immunostaining was calculated. As shown in Figs 2A and 2B, PAH043M had significantly higher expression levels of ER (19.8% vs 1.3% – 7.5% ; $p < 0.05$) and AR (50.6% vs 16.2% – 28.2% ; $p < 0.0001$) compared to the other 4 cases. As an indicator of ER activity, we also examined PR as an E2-response marker. Similar to ER and AR, PAH043M expressed PR at a significantly higher level than PAH044M and PAH045M. Hence, differences in hormone receptor levels in the original tissues may influence hormone responses of the PDEs.

3.3. Oestrogen receptor levels predict MD change

Correlation between percentages of DAB-stained ER/nuclear area per glandular region and MD changes were analysed for the 20 PDEs treated with the four treatments (ETH control, E2, E2 plus DHT, E2 plus SARM) from the 5 patients in Fig. 2 (PDEs and treatments detailed in the methods section). Generalised estimated equation (GEE) analyses were applied to determine association between hormone receptor levels and effects of these combined treatments on MD, according to patient number. As shown in Fig. 3, expression of ER protein appeared to be associated with MD change. Although the GEE statistics indicate significance, more cases are needed to determine if this is indeed a significant finding. Interestingly, a similar association was also observed for PR and MD change, however with a lower Wald Chi-Square value (23.38 vs 7.67). AR protein expression was not associated with MD change. The images of ER positivity are shown in Fig. 2.

3.4. Hormone receptor mRNA expression after treatment with E2 alone or E2 combined with an AR agonist

The mRNA levels of ER, PR and AR in hormone-treated PDEs were analysed by RT-qPCR at harvest. As expected, treatment with E2 alone reduced the mRNA expression of oestrogen receptor (*ESR1*) (Fig. 4A), however, mRNA levels of progesterone receptor (*PGR*) were unchanged at this time point (Fig. 4B). Interestingly, E2 treatments increased androgen receptor (*AR*) expression (Fig. 4C, $p < 0.05$). Given that this data was from whole mammary tissue of varying epithelial content, and hormone receptor expression is largely contributed by epithelial cells, we assumed that these small changes in receptor expression may be attributable to differences in epithelial content.

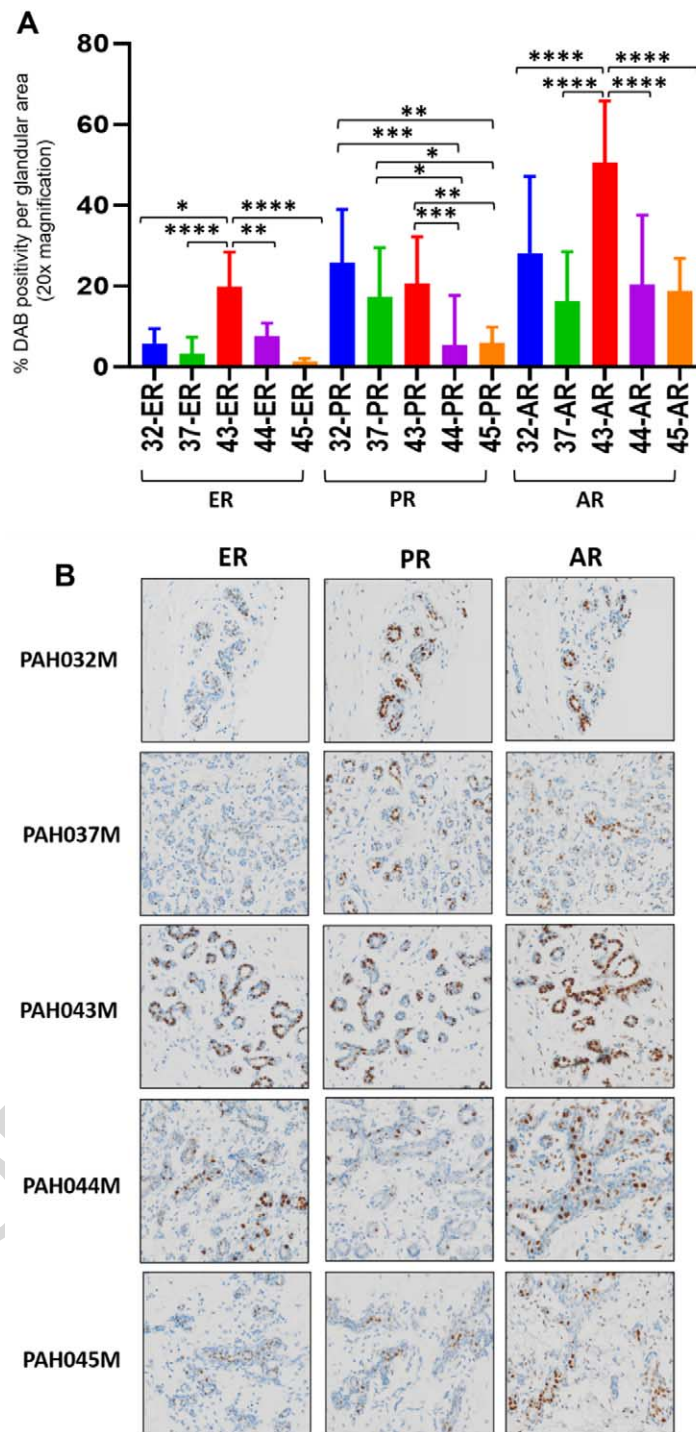
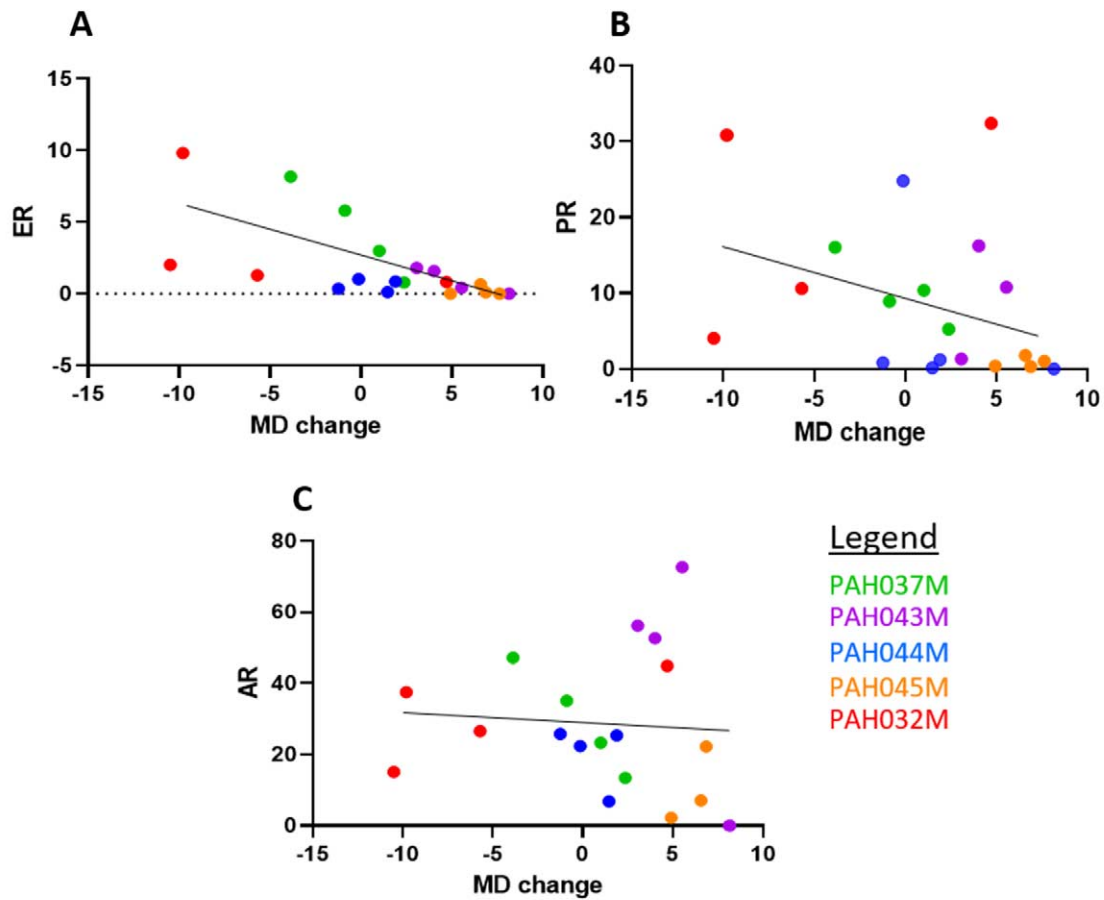


Fig. 2. IHC analysis of hormonal receptors in original tissue from 5 cases treated with AR agonists. A. Comparisons of positivity of ER, PR and AR in original mammary tissue from 5 different. One-way ANOVA was used to determine significance; * denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.001$, **** denotes $p < 0.0001$. B. Representative images of ER, PR and AR IHC staining at 20 \times magnification.



D				95% Wald Confidence Interval		Hypothesis Test		
				Lower	Upper	Wald Chi-Square	df	Sig.
Receptor expression	Parameter	B	Std. Error					
ER	MD change	-0.33	0.07	-0.46	-0.19	23.38	1	<0.001
PR	MD change	-0.69	0.25	-1.18	-0.20	7.67	1	0.006
AR	MD change	-0.55	0.85	-2.21	1.11	0.42	1	0.52

Fig. 3. Generalised estimated equations analyses of MD change and ER (A), PR (B) and AR (C) levels were conducted in 20 explants from 5 patients, representing the controls and all 4 treatments (ETH, E2, E2 plus SARM and E2 plus DHT) for each patient. Hormonal receptor levels were shown as % DAB positivity per glandular area. D. Parameter estimates obtained from the application of generalised estimated equations.

3.5. The effect of hormone treatments on expression levels of oestrogen, progesterone or androgen-regulated genes in PDEs

To further investigate oestrogen and androgen signalling in PDEs, RT-qPCR analysis was performed to assess mRNA expression of 14 known E2-regulated genes (ERGs) and 5 progesterone-regulated genes

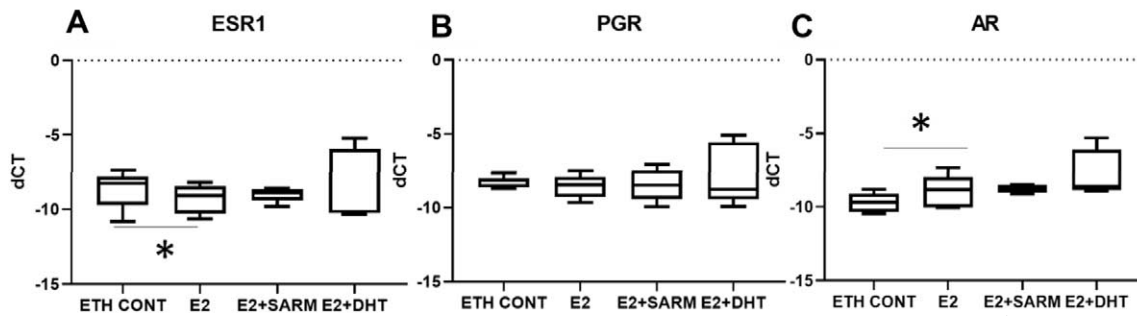


Fig. 4. Hormonal receptor mRNA expression level in treated explants. CT values from RT-qPCR were normalised with L32, with delta-CT shown. A Expression levels of *ESR1*. B Expression levels of *PGR*. C Expression levels of *AR*. Student's paired T-test was used to determine significance, * denotes $p < 0.05$.

(PRGs) as defined from previous studies [22,49], Fig. 5A. Although progesterone was not included as an explant treatment in this study, it is known that activation of the ER can influence PR activity [35]. In addition, 6 AR target genes identified in ER+ breast cancers [25] were assessed. To adjust for the potential confounding effect of varied epithelial content in our PDEs, we normalised target gene expression data to mRNA expression data for the regulatory hormone receptor (HR). Student's paired t test was used to determine significance.

As shown in Fig. 5A, genes were divided into two clusters according to their response to hormonal treatments. Most ERGs were up-regulated in response to treatment with E2 alone (compared to ETH control) and down-regulated in response to E2 plus an AR agonist compared to E2 alone (Cluster A). Conversely, Cluster B was characterised by moderate up-regulation of ARGs in the presence of E2 plus an AR agonist compared to E2 alone, and potent down-regulation of ARGs in the presence of E2 alone compared to ETH, indicative of anti-androgenic effects. The statistical comparison of gene expression averaged across the groups (ERGs, ARGs and PRGs) according to treatment can be seen in Supplementary Tables 1 and 2, where the gene expression trends visible in Fig. 5A were evident. E2 treatment alone did not significantly affect gene expression across the groups, however the combination of E2 and Enobosarm (SARM) significantly reduced ERG ($p = 0.007$) and PRG expression ($p = 0.011$), indicative of anti-estrogenic effects (Supplementary Table 2). ARG expression were not significantly changed by either of the AR agonists, either alone or in combination. To investigate potential drivers of these trends, we assessed each gene in isolation across the treatment groups (Supplementary Tables 3–6). E2 significantly upregulated the ER regulated genes (ERGs) *CELSR2* and *AR*, and the AR regulated genes *SEC14L2* and *GRPC5A*, whereas E2 in combination with AR agonist Enobosarm downregulated ERGs *SERPINA3*, *ATP6V1B1*, *TFF1* and PR regulated gene *RANK*. Only *GREB1* and *CLIC6* were upregulated by E2 and downregulated by the combination of E2 with Enobosarm. DHT + E2 exhibited no significant difference in expression of ER, AR or PR genes examined, compared with the effect of E2 alone.

3.6. Correlations between MD and hormone-regulated gene expression signatures

In addition to the regulation of gene expression by hormones, putative associations between these gene expression patterns and MD were studied to provide insight into the possible mechanism of hormonal effects on MD. Given sample size was small, we report our observations as trends only. Generalised estimated equations analyses were conducted on gene expression in PDEs, regardless of treatment, but taking patient ID into account, to study correlative trends of endpoint gene expression and endpoint

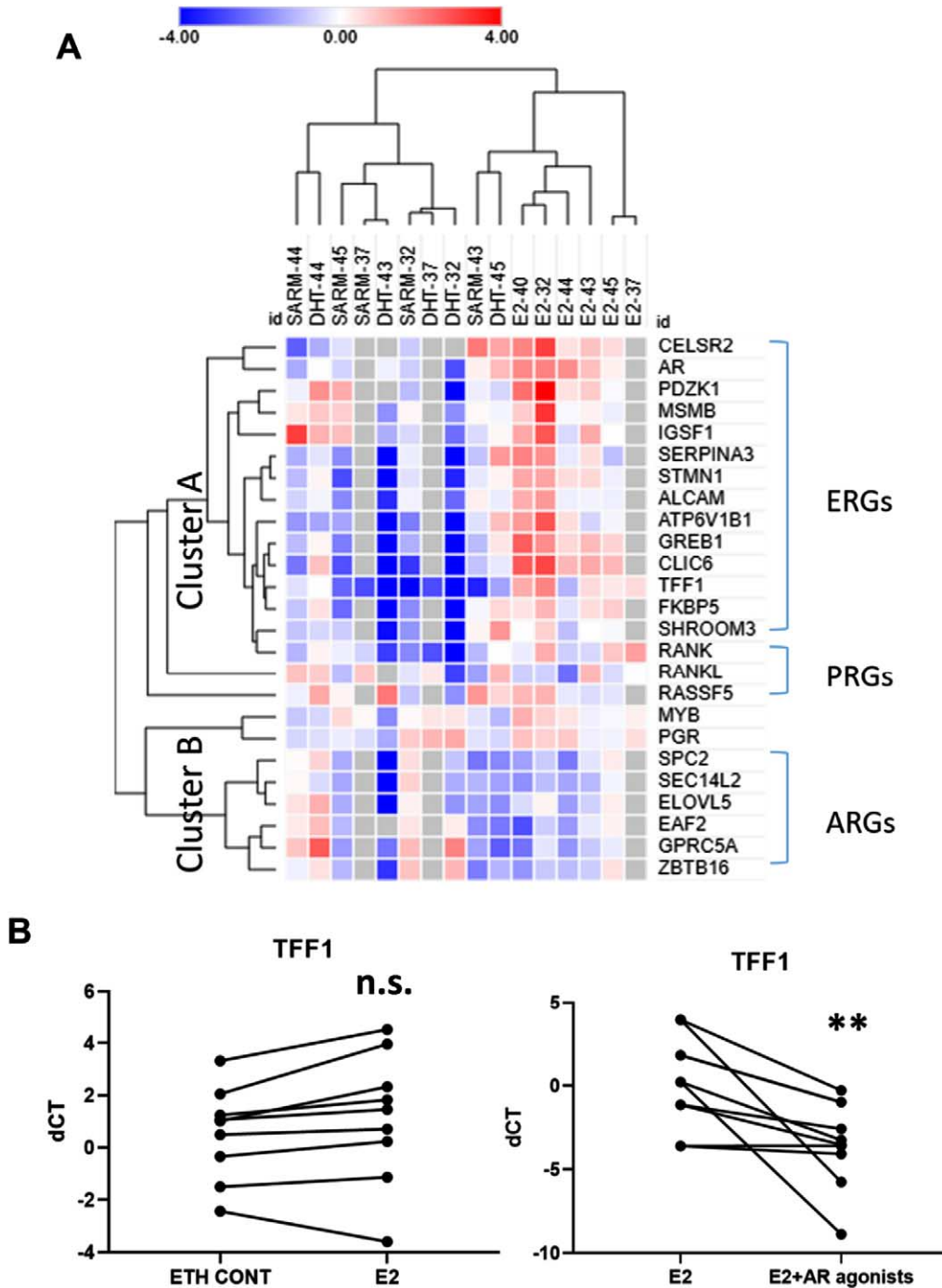


Fig. 5. A. Heatmap of expressions of ERGs, PRGs and ARGs in PDEs treated with E2 compared to ETH control ($n = 6$) and E2 plus SARM or DHT (note that in these cases only SARM or DHT is labelled) treatment compared to E2 alone ($n = 5$). Each cube represents one gene expression level after normalisation with respective HR expression (ddCT value). B. TFF1 expression levels in ETH vs E2 (left) and E2 vs E2 plus AR agonists (right). Student's paired T-test was used to determine the significance of correlations; ** denotes where $p < 0.01$, n.s. denotes the absence of a significant correlation.

Table 3

Generalised estimated equation analysis of gene expression versus MD value at endpoint. The correlation was analysed among all treated explants following normalization with respective hormone receptor expression CT

Gene	Wald chi-square	<i>p</i> value
PDZK1	2.256	0.133
SERPINA3	0.927	0.336
CELSR2	0.002	0.961
AR	2.074	0.150
ATP6V1B1	1.284	0.257
GREB1	3.895	0.048
IGSF1	4.430	0.035
CLIC6	1.873	0.171
MSMB	13.184	<0.0001
TFF1	0.675	0.411
MYB	2.421	0.120
PGR	6.607	0.010
ALCAM	17.034	<0.0001
STMN1	1.343	0.246
RASSF5	3.801	0.051
SHROOM3	17.620	<0.0001
FKBP5	3.525	0.060
RANK	16.501	<0.0001
RANKL	9.158	0.002
SPC2	0.412	0.521
EAF2	1.490	0.222
SEC14L2	0.459	0.498
ZBTB16	1.271	0.260
GPRC5A	6.090	0.014
ELOVL5	3.788	0.052

MD. Wald chi-square values and respective *p* values are listed in Table 3. The full GEE output for genes with *p* values <0.05 have been presented in Supplementary Table 7, including the direction of correlation (positive or negative). Gene expression which trended to negatively correlate with endpoint MD were the ERGs *IGSF1* (Wald chi-square = 4.430), *MSMB* (Wald chi-square = 13.184), *PGR* (Wald chi-square = 6.607), *ALCAM* (Wald chi-square = 17.034) and the androgen receptor associated gene *GRPCA* (Wald chi-square = 6.090). Three of these genes negatively associated with MD (*IGSF1*, *MSMB*, *ALCAM*) share similarities in that they contain an immunoglobulin-like domain and/or immunoglobulin binding [47] and thus may be related functionally. Gene expression which trended to positively associate with MD included the ERGs *GREB1* (Wald chi-square = 3.895), *SHROOM3* (Wald chi-square = 17.620) and the two functionally paired PR response genes *RANK* (Wald chi-square = 16.501) and its ligand, *RANKL* (Wald chi-square = 9.158). The presentation of these potential correlations, in an insufficient number of cases, have been made for discussion purposes only and need to be further investigated in a larger number of cases.

Table 4

Generalised estimated equations of RANK/RANKL expression versus MD separated by BRCA 1 and BRCA 2 mutation status. The correlation was analysed among all treated explants

Gene	BRCA mutated		BRCA WT	
	Wald chi-square	<i>p</i> value	Wald chi-square	<i>p</i> value
RANK	28.821	<0.001	0.85	0.771
RANKL	604.944	<0.001	2.314	0.128

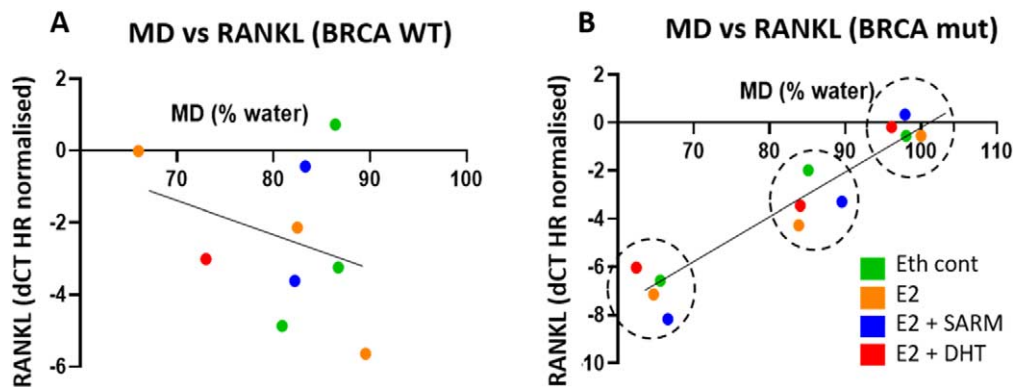


Fig. 6. Plots of MD and RANKL expression levels from 3 patient samples classified as WT (A) or BRCA 1 and BRCA 2 (B) pathogenic variant (mut), with RANKL expression data normalized using hormone receptor of respective treatments. Each colour represents one treatment, and each of the 3 clusters (groups of red, green, orange and blue, graphs B and D) represents one of the 3 different BRCA 1 and BRCA 2 mutated gene subjects.

3.7. Positive correlations between RANK/RANKL and MD exist only in BRCA 1 AND BRCA 2-mutated patients

We investigated these MD-negative or positively associated genes further to see if BRCA mutation status influenced the strength of association. This involved separating samples by BRCA 1 and BRCA 2 pathogenic variant status versus WT. In this study, 3 out of 6 cases were BRCA 1 and/or BRCA 2-mutated. RANK receptor *and* its ligand RANKL both displayed a strengthened correlation when the BRCA-WT patient data was removed, while the relationship of RANK/RANKL and MD disappeared when only BRCA-WT data was considered. This data is shown in Table 4. We report a similar pattern for SHROOM3 (Supplementary Table 8).

To study the influence of hormone treatments on these relationships, generalised estimated equation analysis was applied to PDEs with the same treatment (E2 or E2 plus AR agonists). The Wald Chi-Square values generated from explants with different hormone treatments did not differ ($p = 0.151$), indicating RANK/RANKL were positively correlated with MD in BRCA 1 and BRCA 2 mutated tissues regardless of whether RANK/RANKL signalling was modulated. The correlations are shown in Fig. 6 with colour coding according to treatment.

4. Discussion

We have previously pioneered the use of single-sided Portable NMR for quantitative measurement of MD in human breast tissue and demonstrated a strong linear correlation between tissue water content

measured from Portable NMR and %MD obtained from MicroCT ($R^2 = 0.96$, $p < 0.0001$) [32]. We have further validated the use of Portable NMR-measured tissue water content as a surrogate for MD change effected by hormonal treatment *ex vivo* [33]. We have shown that the PDE model for MD analysis of freshly excised mammary tissue [33] has utility in the investigation of the effects of hormone treatments on MD. In that study, 6 women were recruited and 22 groups of explants were treated with various hormones. Analysis of data from these explants have provided impetus to further investigate a role for AR agonism in reducing MD in normal human mammary tissue. High MD (>50%) increases the likelihood of a woman developing breast cancer [28], and is also associated with increased breast cancer recurrence [13] and treatment resistance [19]. The present study provides a means through which this risk associated with high MD may be further investigated *ex vivo*.

We observed modest relative MD increases, within a range of 1.03%–2.03%, in 6 out of 9 mammary tissue explants from premenopausal women (considering cases from this and the previous study), treated with E2 alone compared to ETH control. In comparison, although the numbers were small, oestrogen reduced density in the explant issues from two postmenopausal women. This is consistent with studies that report that MHT containing oestrogen only does not increase MD or BC risk in *postmenopausal* women [20,21]. The first of its kind, our pilot study provides a tissue-specific direct assessment of the effect of E2 on MD, quantitatively assessed using Portable NMR, in pre-menopausal normal breast tissues. Our data suggest that E2 may promote MD in this demographic, and further follow-up studies will be particularly informative in regards to breast cancer risk in pre-menopausal women, dictated by E2 exposure.

A downward trend in MD was seen in 4 out of 5 explants treated with E2 plus AR agonists in comparison with E2 alone, providing insights into the effect of AR agonism on MD in our physiologically relevant PDE model. With the treatment of E2 plus AR agonists (DHT or Enobosarm), 4 samples showed a consistent decrease in MD in the range of -0.44% to -6.24% , compared to E2 treatment alone. PAH043M was the only outlier that responded differently to combination treatments of E2 and AR agonists from the other four samples. Interestingly, the original ER and AR levels in this sample were prominently higher (Fig. 2), which may account for the observed divergent response. Further study is needed to determine the prevalence and implications of such high hormone receptor levels.

Circulating free testosterone has been negatively associated with MD [5,38], although testosterone therapy was found to have no effect on MD among both MHT users and non-users [15,27]. In addition to metabolism to the most potent androgen, DHT, testosterone can also be converted to oestrogen via aromatase activity in breast adipose tissue, which is a major source of oestrogen in postmenopausal women [51]. This aromatization possibly accounts for the lack of effect of testosterone on MD in the studies referred to previously [15,27]. In our study, the PDEs were treated with DHT or Enobosarm, direct agonists of AR, in the presence of E2, and evoked a downward trend on MD consistent with an anti-estrogenic effect. Indeed, the repressive effect of the estrogen signalling molecule TFF1 in explants treated with AR agonists was indicative of this effect (Fig. 5B).

Associations were observed between ER positivity levels by immunohistochemistry in the normal mammary epithelium and MD, with a comparatively diminished association seen with PR and MD (Fig. 3A). This data has been colour coded to illustrate that the association of ER and PR in regards to MD change may not be driven by one patient but is perhaps a phenomenon observed in explants from all patients. The relationship with ER, though the sample size is small, is consistent with what is normally observed in ER signalling, where the ER becomes degraded following transcriptional activation [45]. Similarly, PR is associated with ER signalling [35] therefore it is interesting that we observed a positive association with MD in our small case set. While AR levels in the donor tissue did not correlate with MD,

treatment with an AR agonist appeared to somewhat reduce MD in PDEs (Figs 1B, 1C). This finding is consistent with previous work showing that AR agonists oppose ER signalling in normal and malignant breast tissues [25], however requires further investigation in mammary tissue from more cases.

ERGs, PRGs and ARGs were differentially regulated in the PDEs treated with the various hormones. ERGs were increased in E2-treated explants but decreased in patient-matched explants co-treated with an AR agonist. Anti-estrogenic effects of androgen / AR signalling in normal and malignant breast tissues involves AR-mediated reprogramming of ER and its co-activators p300 and SRC-3 on DNA to down-regulate ER target genes and up-regulate AR target genes [25]. The AR-mediated down regulation of ERGs, especially TFF1 (Fig. 5B), in the current study suggest that the observed effect of AR agonists in decreasing MD in the small number of cases we examined, may be via ER modulation and inhibition of the ER signalling pathway.

Among the ERGs, expression of *GREB1* and *CLIC6* were significantly altered by both E2 alone and E2 in combination with Enobosarm, showing good sensitivity to ER signalling regulation. During the menstrual cycle, *GREB1* expression has been shown to be upregulated along with increased MD in the late follicular and luteal phases of the menstrual cycle, which corresponds to intermediate to high oestrogen milieu [22,57]. As an ER-regulated gene, *GREB1* was also required for oestrogen-stimulated proliferation of breast cancer cells, and appears to regulate proliferation via PI3K/Akt/mTOP signalling [11]. *CLIC6* is an intracellular chloride channel, of which SNPs within this gene has been linked to the onset of breast cancer [37]. Our data also showed a positive correlation between *GREB1* expression and MD at endpoint, but not for *CLIC6* (Table 3). Taken together, our data suggest that ERGs, particularly *GREB1*, may be better predictors of ER activity and potentially MD change than traditional approaches dependent on ESR1 mRNA or ER IHC levels.

The potential significance of RANK receptor expression and signalling in association with MD has been highlighted in this study. We found that RANK expression was significantly reduced by Enobosarm (Fig. 5 and Supplementary Table 4). Furthermore, RANK and RANKL appeared to positively correlate with MD in the small number of cases that we examined, regardless of what the PDE was treated with, which we found to be specific to BRCA 1 and BRCA 2-mutated explants. Toriola *et al* also reported similar correlations with MD, although that study did not take BRCA 1 and BRCA 2 pathogenic variant carrier status into account [52,53]. They found that circulating RANK was positively associated with volumetric percent density in premenopausal women, while women with higher circulating soluble RANKL and RANKL mRNA levels in breast tissue had higher volumetric percent density, indicating a possible regulatory role of RANKL signalling on MD. Although requiring further investigation, we observed a correlation between RANKL expression and MD in PDEs from women with BRCA 1 and BRCA 2 pathogenic variations (Table 4), which appeared to be consistent even when RANK/RANKL expression varied with different hormonal treatments. Following further study of more cases, this data may indicate that the expression level of RANKL could be a strong marker of MD and a putative a predictor for MD. RANKL/RANK is a mediator of cancer formation in BRCA 1 and BRCA 2 pathogenic variant carriers [50], where RANK positive, tumorigenic luminal progenitor cells abound in BRCA 1 and BRCA 2 mutated breast tissue [42]. Our finding that RANK/RANKL associated with high MD in our small number of BRCA 1 and BRCA 2 pathogenic variant carriers provides impetus for further study to determine whether interplay between MD and luminal progenitor cell abundance exists. Indeed, a clinical trial is underway to test whether the RANK pathway inhibitor Denosumab can induce the incidence of breast cancer in BRCA1 mutation positive women, and changes in mammographic density are being used as indicators of efficacy (Geoff Lindeman, NBCF).

Future work. The statistical sample used in the present study (6 patients, 22 treated PDEs) is small and does not allow us to confidently analyse the effects of patient-specific factors on the tissue response to treatment. Such factors include (but are not limited to) patient age, menstrual status, BRCA1/2 status, and breast density (BI-RADS category). Therefore, the present results call for a more expansive study on a larger patient cohort that would include several patients for each combination of these parameters. Younger women are known to have higher levels of MD than older women. Similarly, postmenopausal status is known to be associated with a relatively low MD compared to an equivalent pre-menopausal cohort. Therefore, it would be valuable to determine whether age, menstrual status, number of child-births, breast-feeding and other similar factors affect only the “baseline” MD values, or whether they potentially can have an effect on the change in MD in response to a treatment. Furthermore, our latest results suggest that the precision and accuracy of Portable NMR MD quantification in tissue explants are not uniform but influenced by the tissue fat and water contents. Therefore, an investigation of the relationship between baseline tissue composition and treatment response would also be important and topical.

Summary. Our investigation of the effects of oestrogen alone or in combination with an AR agonist on MD via *ex vivo* culture of normal breast PDEs support the efficacy of this model in further investigations of the effect of AR agonism on MD. Initial clinical indications have demonstrated that combining testosterone with an aromatase inhibitor can result in significant reduction in mammographic breast density by enhancing intramammary DHT (personal communication by Dr Stephen Birrell, Havah Therapeutics Pty Ltd). Indeed, commercial development of androgenic products to reduce mammographic density is currently underway (Birrell SN 2016, Methods of reducing mammographic breast density and/or breast cancer risk, USTPO US 9,351,977 B2). Given that the AR has been found to be a tumour suppressor in ER-driven breast cancers, our preliminary data suggests that activation of this pathway has the potential to be used for BC prevention via reducing MD and subsequently decrease BC risk. Further investigation is needed to determine if this is indeed the case.

5. Conclusions

The present study further demonstrates the utility of single-sided Portable NMR for quantitative measurement of the dynamics of Mammographic Density in explanted human mammary tissue in response to exogenous hormonal treatments. The study suggests that androgen receptor agonism can reduce MD, and therefore may be a novel means by which to reduce the risk of breast cancer associated with high MD.

Supplementary data

Supplementary material is available at: <http://dx.doi.org/10.3233/BSI-230000>.

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Author contributions

XH performed all experiments contained within this manuscript, GR provided assistance with ex-planting of human tissue, KIM developed the measurement procedure and performed the analysis of raw Portable NMR data, TL viewed the patient in-life and slice mammograms to determine BI-RADS density and regions of HMD versus LMD, respectively, WDT and TEH provided the ER, PR and AR gene signatures and provided expert input in the interpretation of data, CES performed AR, PR and ER IHC of human tissue specimens, EWT and HJH equally provided expert guidance, helped conceive the study and directly influenced its direction, with HJH directly providing assistance for XH in human tissue accruals and experimentation. All authors provided intellectual input into the interpretation of data and reviewed and edited the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Research ethics

Informed consent was obtained from all subjects involved in the study.

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