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Clinical Studies

Mammographic density and molecular subtypes of breast cancer

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BACKGROUND: Gene expression profiling has led to a subclassification of breast cancers independent of established clinical parameters, such as the Sorlie–Perou subtypes. Mammographic density (MD) is one of the strongest risk factors for breast cancer, but it is unknown if MD is associated with molecular subtypes of this carcinoma.

METHODS: We investigated whether MD was associated with breast cancer subtypes in 110 women with breast cancer, operated in Stockholm, Sweden, during 1994 to 1996. Subtypes were defined using expression data from HGU133A + B chips. The MD of the unaffected breast was measured using the Cumulus software. We used multinomial logistic models to investigate the relationship between MD and Sorlie–Perou subtypes.

RESULTS: Although the distribution of molecular subtypes differed in women with high *vs* low MD, this was statistically non-significant ($P = 0.249$), and further analyses revealed no association between the MD and Sorlie–Perou subtypes as a whole, nor with individual subtypes.

CONCLUSION: These findings suggest that although MD is one of the strongest risk factors for breast cancer, it does not seem to be differentially associated with breast cancer molecular subtypes. However, larger studies with more comprehensive covariate information are needed to confirm these results.

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Mammographic density (MD) is a well-established and very strong risk factor for breast cancer (McCormack and dos Santos Silva, 2006). Women with a percentage density (PD) of more than 75% have a four to six times higher risk for breast cancer than women with a PD <5% (McCormack and dos Santos Silva, 2006). Mammographic density is defined by the relative amounts of radiodense stromal and epithelial tissue compared with radiolucent fatty tissue. Consequently, higher MD is characterised by larger amounts of stromal and/or epithelial tissue and vice versa.

Mammographic density differs between women, as well as within the same woman throughout her life course, being influenced by many well-established breast cancer risk factors, such as age, menopausal status, body mass index, hormone replacement therapy (HRT), and parity (Boyd *et al*, 2009). However, the biological basis for this association is not well understood. It has been suggested that if MD is a marker of cumulative exposure to oestrogens, it may be more strongly associated with oestrogen receptor (ER)-positive breast cancers. Studies investigating this to date are highly inconsistent (Boyd *et al*, 2011a; Heusinger *et al*, 2012; Phipps *et al*, 2012).

Mammographic density is radio-dense, as are tumours. Consequently, density can hide tumours, a phenomenon referred to as masking (Boyd *et al*, 2007). In accordance, density decreases mammographic sensitivity (Kerlikowske *et al*, 1996) and is associated with an increased risk of interval cancers (Boyd *et al*, 2007). Whether the latter relationship is solely based on masking or whether density gives rise to more highly proliferative tumours is unknown. If the latter is true, density may be associated with a more aggressive subtype, specifically triple-negative tumours (ER-negative, PR-negative, and HER2-negative) and the basal subtype (see below for description), which have been found to be more frequent in interval cancers than screening-detected cancers (Collett *et al*, 2005; Gluz *et al*, 2009).

Advances in microarray technology and pathology have led to improved techniques of subclassifying tumours. Global gene expression profiling enables a subdivision of tumours into five individual subclasses (known as the Sorlie–Perou subtypes) found to convey a distinct prognostic and biological message in breast cancer above and beyond established clinical markers. The five groups are the luminal A, luminal B, basal-like, ERBB2 +, and the normal breast-like subtypes (Perou *et al*, 2000; Sorlie *et al*, 2003). Luminal A tumours are mostly ER-positive, have a low proliferation rate, and are of low grade, whereas luminal B tumours are also mostly ER-positive but may express low levels of hormone receptors, and are usually of high grade and have a higher proliferation rate. The basal-like subtype, on the other hand, is often characterised by triple-negative tumours (ER-, PR-, and HER2-negative) and a certain cytokeratin pattern, and the ERBB2 + subtype shows amplification and high expression of the *ERBB2* gene (also known as *HER2* or *HER2-neu*). Lastly, there is the normal breast-like subtype, which shows expression of many genes expressed by adipose tissue and other non-epithelial cell types, strong expression of basal epithelial genes, and low expression of luminal epithelial genes. It is, however, unclear

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whether the latter subtype is a distinct group or represents poorly sampled tissue (Sorlie, 2007).

We have previously used gene expression analyses to characterise the genetic alterations behind tumour differentiation and p53 mutations (Miller *et al*, 2005; Ivshina *et al*, 2006). In the present study, we explore a possible association between MD at diagnosis and gene expression patterns from breast tumours in 110 Swedish women operated for breast cancer, taking established prognostic and risk factors for breast cancer into consideration.

MATERIALS AND METHODS

This is a case-only study consisting of 110 women. The source population was all women with breast cancer operated at a large university hospital in Stockholm between 1 January 1994 and 31 December 1996 ($n=524$), as previously described (Pawitan *et al*, 2005). The women were identified through the population-based Stockholm-Gotland Breast Cancer Registry established in 1976. Exclusion was because of refusal of participation ($n=6$), emigration ($n=7$), lack of frozen tumour ($n=231$), insufficient amount or quality of RNA ($n=89$), lack of gene expression profiling on U133 A and B chips ($n=14$), neoadjuvant therapy ($n=12$), *in situ* cancer ($n=5$), or stage IV cancer ($n=1$).

The subjects excluded because of lack of frozen tumour had a lower mean tumour diameter (16 mm compared with 23 mm) and fewer individuals had affected lymph nodes (16% compared with 38%) than included women. There was no difference in mean age (57 years for both groups).

We collected information pertaining to status at diagnosis on age, menopausal status, HRT, family history, oral contraceptive use, and tumour characteristics from the medical records for the remaining 159 patients. Family history includes history of breast cancer in both first- and second-degree relatives. Menopausal status was self-assessed by the patient as either pre- or postmenopausal. Two women had unknown menopausal status. Both oral contraceptive use and HRT use were assessed according to status at time of referral to the Karolinska Hospital (former, current, and non-use, collapsing former and current use into one category because of few observations). Non-users of HRT were postmenopausal women actively stating no current or previous use of HRT. Of the HRT users, approximately two out of three used a combined oestrogen and progesterone regimen, and one out of three used oestrogen only. Local oestrogen treatment was not considered as HRT use. Oral contraceptive use included all preparations.

The mammogram closest to diagnosis was retrieved for 141 subjects. Mammograms were digitised with an Array 2905HD Laser Film Digitizer (Array Corporation, Hampton, NH, USA). Density resolution was set at 12 bit, spatial resolution 5.0 μm and optical density 0–4.7. The size of the images was 4770×3580 pixels.

Tumours appear white on a mammogram and can thus distort density measurements. As MD is highly correlated between the two breasts (Byng *et al*, 1996b), we measured the mediolateral oblique view of the breast contralateral to the tumour. Women with bilateral breast cancer ($n=10$) and subjects with breast implants ($n=3$) were excluded. We thus had density measurements for 128 women.

For all subjects, but three, date of mammography was within 1 month of the date of diagnosis. For the remaining three patients, the mammograms collected were from 2 to 7 months before diagnosis. Two of these patients were postmenopausal, current HRT users at time of diagnosis, and for these two patients, we thus lacked information on HRT status at mammography.

Assessment of MD

To measure MD, we used a computer-assisted threshold technique, Cumulus (Byng *et al*, 1996a). First, the edge of the breast is demarcated from the background, as well as from the thoracic wall (the pectoralis muscle). Second, the observer sets the threshold distinguishing between dense and non-dense tissue. Cumulus then identifies all pixels as bright as, or brighter than, the threshold level, and the absolute dense (AD) area, non-dense area, total breast area, and PD (AD area/total breast area) are thus measured.

Two independent observers (ISS and VM) carried out the density measurements blinded to the characteristics of the patients and their tumours. Both observers measured all of the images and a random repeat sample of 10% of the images. There was good inter- and intra-observer reliability with Pearson's correlation coefficients of 0.82 and 0.93, respectively, for absolute density. For our analysis, the density measurements from both observers were averaged to minimise random measurement error.

RNA preparation and microarray profiling

Details on RNA preparation and microarray profiling have previously been described elsewhere (Pawitan *et al*, 2005). Briefly, frozen tumour was cut into minute pieces and transferred into test tubes with RLT buffer (RNeasy lysis buffer, Qiagen, Hilden, Germany), followed by homogenisation. Proteinase K was then added. After this step, total RNA was isolated using Qiagen's microspin technology. DNase was added to some samples to further increase RNA quality. The quality of RNA was assessed by measuring the 28S:18S ribosomal RNA ratio.

Preparation of *in vitro* transcription products and oligonucleotide array hybridisation and scanning were performed according to the protocol of Affymetrix (Santa Clara, CA, USA). The molecular subtypes have been validated previously on a larger cohort of patients (Calza *et al*, 2006). As in the original publication (Sorlie, 2007), it was not possible to assign a unique subtype for all samples. Consequently, $n=18$ patients were excluded, leaving $n=110$ patients for the analyses.

Comparing the 110 individuals included in analyses with the 524 subjects from the source population reveals the following: included individuals are almost of the same mean age as the source population (57 compared with 58 years), but have larger tumours (23 and 20 mm, respectively) and more often present with lymph node metastasis (38% and 26%, respectively). In other words, a selection bias is introduced after the exclusion of women lacking frozen tumour, but the exclusions thereafter do not change the characteristics of the study population.

Our research was conducted under permission from the local institutional review board.

Statistical analyses

Our main variable of interest pertaining to MD was the AD area (measured in cm^2). The association between AD and breast cancer risk is equivalent in magnitude to the association between PD and breast cancer risk (Vachon *et al*, 2007; Stone *et al*, 2009). We chose to analyse AD rather than PD, owing to the lack of information on BMI and as PD is highly, inversely correlated with BMI (through BMI's strong association with the non-dense area), whereas AD has been shown to be only weakly associated with BMI (Maskarinec *et al*, 2002), if at all (Haars *et al*, 2005). We analysed AD both as a dichotomised (above/below median) and continuous variable after transformation. In the latter case, we used the square-root transformation to make the density distribution more symmetric. The transformed density values were then standardised by subtracting the mean and dividing by the s.d. (Z scores), to be able to interpret the risk estimates in terms of the inherent variability of the density values, see below.

P-values for the association between AD and patient characteristics (age, HRT use, menopausal status, and family history) were based on Kruskal–Wallis tests. Descriptive statistics for tumour characteristics (i.e., tumour size, lymph node involvement, stage,

hormone receptor status, and HER-2 status) and molecular subtypes were compared across high- and low-density groups using χ^2-tests of association and Student *t*-tests.

The relationship between AD and molecular subtypes was modelled via multinomial logistic regression. The multinomial model is an extension of the logistic regression model that allows for more than two categories in the response variable (i.e., subtype). The luminal A subtype was set as reference category and we report risk estimates as relative risk ratios (RRRs) for the standardised, square-root-transformed AD values. The RRRs reported in Table 3 measure the change in odds for a tumour falling into any of the reported categories relative to the reference category that is associated with an increase of square-root-transformed AD by one s.d. (or somewhat less than 25% of the range of densities).

We fitted both an age-adjusted model as well as a fully adjusted model, which took into account known correlates of MD and breast cancer risk (age, menopausal status, HRT, family history, and oral contraceptive use) (Vachon *et al*, 2007; Boyd *et al*, 2011b), and tumour size. The latter adjustment was made to try to account for the masking bias and its possible influence on molecular subtypes. We have no prior knowledge of whether the factors adjusted for influence molecular subtypes, so we chose a conservative approach. Significance testing was conducted via likelihood ratio tests. Confidence intervals and *P*-values for individual parameters are based on Wald statistics. All tests were conducted at a nominal significance level of $\alpha = 0.05$.

Analyses were performed using the R statistical software environment, version 2.10.0 (R Development Core Team, 2008), and STATA, version 11.2 (StataCorp, 2009).

RESULTS

Table 1 shows summary statistics of patient characteristics for the whole cohort, and separately for women with low (below median) and high (above median) AD, respectively. AD was lower in older than younger women; women in the lowest age group ($\leqslant$46 years) had a median AD of 49.2 cm^2 as compared with women in the highest age group ($\geqslant$69 years) whose median was 11.1 cm^2 ($P<0.001$). Women who were current or former users of oral contraceptives had an almost twice as large dense area as never users of oral contraceptives (38.1, 40.6, and 22.7 cm^2, respectively, $P=0.022$), probably also reflecting differences in parity and age at first birth, information which we lacked. Postmenopausal women had lower AD than premenopausal women (43.6 cm^2 compared with 18.1 cm^2, $P<0.001$; Table 1). In postmenopausal women, current HRT users had higher AD than past and never users (23.6, 13.2.0, and 13.2 cm^2, respectively, $P=0.029$; Table 1).

Table 2 shows the distributions of clinical parameters and molecular subtypes in the study population, and separately for women with low AD and those with high AD. No statistically significant associations were seen. However, in comparison with tumours diagnosed in low AD breasts, tumours diagnosed in high AD breasts tended to more often be ER-positive (69% compared with 60%, $P=0.065$) and PR-positive (76% compared with 62%, $P=0.099$). The luminal A and normal breast-like subtypes were the most common subtypes in the population as a whole (27% each), whereas the luminal A subtype was the most common subtype in women with low AD breasts (36%), and the normal breast-like subtype was most common in women with high AD breasts (33%; $P=0.249$).

Compared with the luminal A subtype (taken as the reference category), the relative risk of the luminal B, ERBB2, and normal breast-like subtypes increased with increasing AD both in the age-adjusted (RRR 1.19, 95% CI 0.58–2.45; RRR 1.88, 95% CI 0.79–4.48; and RRR 1.51, 95% CI 0.78–2.92, respectively, for an increase in

Table 1 Patient characteristics at time of diagnosis and AD, reported as median (1st quartile to 3rd quartile)

	n	AD	*P*-value
Age (years)			<0.001
32–46	26	49.2 (38.3–67.4)	
47–55	26	37.5 (19.3–53.2)	
56–68	29	27.1 (14.9–40.6)	
69–86	29	11.1 (3.9–19.9)	
Oral contraceptive use			0.022
Current	4	38.1 (33.7–42.9)	
Never	71	22.7 (10.5–48.2)	
Former	25	40.6 (29.4–42.2)	
Menopause			<0.001
Premenopausal	39	43.6 (35.3–67.4)	
Postmenopausal	69	18.1 (9.5–33.1)	
Unknown	2	20.5 (11.6–29.4)	
HRT use			0.029
Current	23	23.6 (15.5–41.7)	
Never	34	13.2 (5.5–29.0)	
Former	8	13.2 (7.4–21.6)	
Family history			0.231
Yes	24	27.0 (10.6–39.7)	
No	84	31.7 (15.5–47.4)	

Abbreviations: AD = absolute dense area; HRT = hormone replacement therapy. *P*-values are based on a Kruskal–Wallis test.

Table 2 Distribution of tumour characteristics for the whole study population and separately by AD level (below/above median)

	All ($n=110$)	Low AD ($n=55$)	High AD ($n=55$)	*P*-value
Tumour size (mm)	22.9 (1.1)	22.6 (1.6)	23.1 (1.7)	0.821
Lymph node metastasis				0.379
Yes (%)	38 (36)	21 (40)	17 (31)	
No (%)	69 (64)	32 (60)	37 (69)	
ER status[a]				0.065
Positive (%)	86 (78)	39 (71)	47 (85)	
Negative (%)	24 (22)	16 (29)	8 (15)	
PR status[a]				0.099
Positive (%)	34 (31)	34 (62)	42 (76)	
Negative (%)	76 (69)	21 (38)	13 (24)	
HER2 status[b]				0.973
Positive (%)	15 (18)	7 (18)	8 (18)	
Negative (%)	70 (82)	33 (83)	37 (82)	
Stage				0.237
1 (%)	45 (42)	18 (34)	27 (50)	
2 (%)	24 (22)	14 (26)	10 (19)	
3 (%)	38 (36)	21 (40)	17 (31)	
4 (%)	0 (0)	0 (0)	0 (0)	
Molecular subtype				0.249
Luminal A (%)	30 (27)	20 (36)	10 (18)	
Luminal B (%)	19 (17)	8 (15)	11 (20)	
Basal-like (%)	19 (17)	10 (18)	9 (16)	
ERBB2 (%)	12 (11)	5 (9)	7 (13)	
Normal breast-like (%)	30 (27)	12 (22)	18 (33)	

Abbreviations: AD = absolute dense area; ER = oestrogen receptor. Summary statistics are given as mean (s.e.) and count (%), respectively. *P*-values are based on *t*-tests and χ^2-tests comparing tumour characteristics in women with low and high AD. [a]Positive if $\geqslant$0.05 fmol receptor per μg DNA. [b]Positive if score $\geqslant$2 according to immunohistochemistry.

Table 3 RRRs for specific molecular subtypes of breast cancer compared with the luminal A subtype for an increase in square-root-transformed AD by one s.d.

	Age-adjusted[a]			Fully adjusted[b]		
	RRR	**95% CI**	***P*-value**	**RRR**	**95% CI**	***P*-value**
Subtype						
Luminal A	1.00 (ref.)			1.00 (ref.)		
Luminal B	1.19	0.58–2.45	0.641	1.22	0.53–2.83	0.644
Basal-like	0.99	0.48–2.06	0.986	0.83	0.33–2.10	0.690
ERBB2	1.88	0.79–4.48	0.153	1.74	0.62–4.85	0.291
Normal breast-like	1.51	0.78–2.92	0.222	1.43	0.64–3.17	0.385

Abbreviations: AD = absolute dense area; CI = confidence interval; HRT = hormone replacement therapy; RRR = relative risk ratios. [a]Adjusted for age; $P = 0.483$ for the association between AD and subtype as a whole based on the likelihood ratio test. [b]Adjusted for age, oral contraceptive use, menopausal status, HRT use, family history, tumour size; $P = 0.651$ for the association between AD and subtype as a whole, based on the likelihood ratio test.

square-root-transformed density by one s.d.) and in the fully adjusted (RRR 1.22, 95% CI 0.53–2.83; RRR 1.74, 95% CI 0.62–4.85; and RRR 1.43, 95% CI 0.64–3.17, respectively) models (Table 3). The relative risk of the basal subtype was essentially the same as that of the luminal A subtype in the age-adjusted model (RRR 0.99, 95% CI 0.48–2.06), but decreased with increasing AD in the fully adjusted model (RRR 0.83, 95% CI 0.33–2.10) (Table 3). None of the individual associations were, however, statistically significant, nor was the association between AD and molecular subtype as a whole statistically significant ($P = 0.483$ and $P = 0.651$ for the age-adjusted and fully adjusted models, respectively).

DISCUSSION

To our knowledge, no previous studies have investigated the relationship between MD and molecular subtypes using gene expression data. We found no associations between AD and Sorlie–Perou subtypes; neither between AD and individual subtypes, nor between AD and subtype as a whole. However, our study population was relatively small and the null findings could simply be because of low power. Hence, larger studies are needed to confirm our results.

A couple of studies have previously attempted to investigate the association between MD and Sorlie–Perou subtypes using receptor status (ER-, PR-, and HER2 status) as proxies for the different molecular subtypes (Ma *et al*, 2009; Arora *et al*, 2010; Phipps *et al*, 2012). Ma *et al* (2009) studied the association between PD and the luminal A and basal-like subtypes, and found no association in case-only analyses. In case–control analyses, they observed positive associations between PD, and both the luminal A and basal-like subtypes, but as these associations were of a similar magnitude, they are likely to simply reflect the general increase in breast cancer risk associated with PD. Phipps *et al* (2012) also conducted a case–control study investigating the association between density (assessed using a visual categorical classification, BI-RADS), ER+, ER−/PR−/HER2+, and triple-negative breast cancers. They achieved the same results as Ma *et al* (2009), that is, the density was similarly, positively associated with all subtypes. They concluded that although the different subtypes are distinct biological entities, this is not a result of differences in the association with MD. We thus believe that our results are in agreement with both studies. Arora *et al* (2010) studied the association between density and the luminal A, luminal B, basal-like, and ERBB-2 subtypes, also using BI-RADS to assess density. They observed that women with extremely dense breasts had a higher frequency of luminal A tumours ($P = 0.05$). However, as only age was adjusted for in this analysis, the findings might have been affected by residual confounding.

According to a recent review, most studies have found no association between MD, tumour size, lymph node metastasis, and hormone receptor status, respectively (Boyd *et al*, 2011a). The two published studies investigating the relationship between MD and HER2 status also found no association (Yaghjyan *et al*, 2011; Heusinger *et al*, 2012) as did one of two studies on density and survival (Chiu *et al*, 2010). We find this to be in indirect support of the null association between MD and Sorlie–Perou subtypes shown in this study, as molecular subtypes are associated with both tumour characteristics and prognosis (Sorlie *et al*, 2001; Sotiriou *et al*, 2003).

In contrast to our null findings, two large, recently published studies showed a positive association between PD and ER-negative cancers (Yaghjyan *et al*, 2011), and an association between PD and decreased ER expression (Heusinger *et al*, 2012), respectively, which could indirectly point to an association with the basal subtype. However, the former study (Yaghjyan *et al*, 2011) had a possibly biased study sample with a very large amount of HRT users (76% of cases) and women with previous benign breast disease (59%). These are both associated with MD (Byrne *et al*, 2000; Vachon *et al*, 2007) and interval cancer status (Brekelmans *et al*, 1994; Kavanagh *et al*, 2000); the latter in turn associated with triple-negative disease (Gluz *et al*, 2009). Neither mode of detection nor tumour size was adjusted for to try to account for this. The latter study (Heusinger *et al*, 2012) showed a statistically significant association between PD and lower ER expression. However, the group with an ER expression of 10–69% (considered ER-positive) had the highest PD and were not statistically different from the group with 0–9% expression, referred to as the ER-negative group. Thus, although interesting, we cannot directly apply these results to the ER status.

A limitation of our study was the selection of larger tumours, and thus, tumours of higher stage than that of the source population, because of the harvest of RNA requiring a certain amount of tumour tissue. This may have given rise to a more homogeneous population pertaining to variables associated with stage, which may have influenced the null associations between AD and molecular subtypes. However, as the analysis of molecular subtypes requires RNA, this was unfortunately inevitable.

We used AD as a measure of density instead of PD, as we lacked information on BMI. The BMI is highly, inversely correlated with PD through its association with the non-dense area, whereas AD has not been shown to be associated with BMI (Haars *et al*, 2005), or to a much lesser extent (Maskarinec *et al*, 2002). Both measures are equally predictive of breast cancer risk (Vachon *et al*, 2007; Stone *et al*, 2009; Stone *et al*, 2010). Whether AD or PD is a more appropriate measure of density in relation to molecular subtype is, to our knowledge, not known. The benefit of AD compared with PD is that it is an absolute estimate of density, whereas PD is a relative estimate; a woman with x amount of dense tissue in a small breast will have a higher PD than a woman with the same amount of dense tissue in a larger breast. As our findings on the association between AD and molecular subtypes are in agreement with most studies on related outcomes (tumour characteristics and survival; Chiu *et al*, 2010; Boyd *et al*, 2011a) where density was either measured as PD or visually categorised, we do not believe that the use of AD has weakened our study. However, the fatty tissue of the breast is an important contributor of local oestrogens (Thijssen, 2004) and could thus influence tumour subtype. Hence, we also carried out analyses adjusting for the non-dense area, based on results from a study by Lokate *et al* (2011), showing that AD adjusted for the non-dense area was an even better model for breast cancer risk prediction than both PD and AD adjusted for BMI. Adjustment for the non-dense area did not, however, change the interpretation of our results (data not shown).

We used the density measurements of the breast contralateral to the tumour to avoid a distortion of measurements due to the tumour itself.

We believe that these measurements are a reliable proxy of the pre-diagnostic level of density of the affected breast, as MD has been shown to be highly correlated between the two breasts (Byng *et al*, 1996b) and mammograms were taken before any breast cancer treatment. Density assessment was carried out using a semi-automated computer software minimising exposure misclassification (Vachon *et al*, 2007).

CONCLUSIONS

Our findings suggest that although MD is one of the strongest risk factors for breast cancer, it does not seem to differentially influence molecular subtype. However, our results should be confirmed in a larger study with more comprehensive information on breast cancer risk factors.

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Conflict of interest

The authors declare no conflict of interest.

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