Cancer Management and Research

Dovepress

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ORIGINAL RESEARCH

KDR inferred haplotype is associated with upper limb dysfunction in breast cancer survivors of mixed ancestry

This article was published in the following Dove Press journal:
Cancer Management and Research

Trevor S Mafu[1]
Alison V September[1]
Delva Shamley[2]

[1]Division of Exercise Science and Sports Medicine, Department of Human Biology, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa; [2]Clinical Research Centre, Faculty of Health Sciences, University of Cape Town, Rondebosch, South Africa

Introduction: Shoulder pain and disability are well-documented sequelae of breast cancer treatment. Angiogenesis signaling may have a role in the development of shoulder pain or shoulder disability in breast cancer survivors. The aim of this study was to determine if polymorphisms in angiogenesis-related genes are associated with shoulder pain or disability following breast cancer treatment.

Participants and methods: A cross-sectional study was conducted on 220 South African breast cancer survivors. The study aimed to evaluate associations between shoulder pain/disability and seven single nucleotide polymorphisms (SNPs) within five angiogenesis-associated genes: *KDR* (rs2305948 C>T; rs7667298 C>T), *NOS3* (rs1549758 C>T), *MMP2* (rs708269 A>T), *THBS2* (rs9766678 A>G) and *TIMP3* (rs5754312 T>A; rs715572 G>A). In addition, associations between shoulder pain/disability and inferred haplotypes for *KDR* and *TIMP3* SNPs were evaluated. Participants were grouped into no–low and moderate–high shoulder pain/disability based on total pain/disability scores: ≤30 and >30, respectively using the shoulder pain and disability index (SPADI).

Results: No independent associations with shoulder pain/disability categories were found for all SNPs. However, 1 inferred haplotype (*KDR* "TT") differed significantly (P=0.014) between the shoulder disability categories. After adjusting for participants' age, the differences in *KDR* inferred haplotype frequencies between shoulder disability categories became non-significant (P=0.052).

Conclusion: Our findings provide a preliminary suggestion of a possible association between polymorphisms in genes involved in angiogenesis and the presence of moderate–high shoulder disability among South African breast cancer survivors. A larger prospective cohort study is currently being conducted by our group.

Keywords: angiogenesis, shoulder pain, polymorphism, breast cancer therapy

Introduction

Shoulder pain and disability are well-known consequences of conventional breast cancer treatment.[1–7] Such morbidities may persist long after the recovery period for breast cancer treatment[3,4,7] and have been associated with reduced quality of life.[1,8,9] Prevalence rates of up to 68% have been reported for shoulder pain or disability beyond 6 months after primary treatment although they vary widely.[3–6,10,11] The complex etiology of breast cancer treatment-related shoulder pain and disability has long been appreciated and several risk factors have been identified including: treatment type, time after treatment, disease characteristics, age, genetic factors and the presence of co-

Correspondence: Delva Shamley
Clinical Research Centre, Faculty of Health Sciences, University of Cape Town, Anzio Rd, Observatory, Cape Town 7925, South Africa
Email Delva.shamley@uct.ac.za

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DovePress
http://doi.org/10.2147/CMAR.S191969

morbidities.[2,6,12,13] However, a large proportion of variability in the development of morbidity after breast cancer treatment still remains unexplained.[12,14] Previous findings suggest that shoulder morbidity after breast cancer treatment is bilateral[2,10,15] and it has been shown that structures unrelated to direct surgery and/or radiotherapy treatment are affected,[16] suggesting a systemic cause. These findings substantiate the need to explore the potential involvement of molecular signaling pathways.

Several molecular signaling pathways have been implicated in non-cancer shoulder complex morbidity including but not limited to angiogenesis, extracellular matrix (ECM) remodeling and apoptosis.[17–25] Such studies, investigating the role of molecular pathways, included connective tissue conditions of the shoulder such as tendon injuries or tendinopathy and rotator cuff disease (RCD).[26] Angiogenesis appears to be particularly important in shoulder complex morbidity in healing and adaption pathways. Its signaling can induce ECM remodeling and nitric oxide synthase (NOS) activity.[27,28] NOS activity has been shown to be upregulated in rotator cuff tendon injury and may play a role in the healing process.[29–31] Hypoxia-inducible factor 1α (HIF-1α), a pro-angiogenic transcription factor, is elevated in rotator cuff pathology (including impingement, tendinopathy or tears).[25] Although non-cancer shoulder conditions have a different etiology, the altered shoulder movement patterns observed in breast cancer survivors mimic those seen in known general shoulder conditions such as rotator cuff disease and adhesive capsulitis.[7] In fact, such diagnoses have been used to describe shoulder-complex morbidities in breast cancer survivors and have strongly been associated with pain.[32–34] Studies evaluating the role of molecular signaling pathways in breast cancer treatment-related morbidity have largely focused on the inflammatory pathway.[26] Evaluation of signaling factors involved in angiogenesis, ECM remodeling or apoptosis in breast cancer survivors may, therefore, increase our understanding of the pathophysiology, and contribute towards an explanation of the individual variability in the development, of shoulder pain and disability.

There is a lack of relevant studies investigating the role of angiogenesis, ECM remodeling and NOS activity in the development of shoulder pain and disability after breast cancer treatment. Previous studies evaluating gene expression profiles or genetic associations, in the context of morbidity following breast cancer treatment, have largely focused on inflammatory factors with a few exceptions.[26] Such studies did not focus, specifically, on shoulder pain/disability as a clinical end-point.[26] ECM remodeling and NOS activity are important events in angiogenesis signaling. Angiogenesis signaling through KDR (Kinase Insert Domain Receptor), its main signaling receptor, activates ECM remodeling factors such as MMPs (matrix metalloproteinases), and eNOS (endothelial NOS).[35,36] The activity of KDR can be regulated by inhibitors such as thrombospondins (THBSs) while the activity of MMPs can be regulated by TIMPs (tissue inhibitor of MMPs).[37,38] We hypothesize that shoulder pain or disability after breast cancer treatment may be associated with polymorphisms in genes involved in angiogenesis, ECM remodeling or NOS activity. The aim of this study, therefore, was to correlate DNA sequence variants of key angiogenesis-related signaling factors, including genes involved in ECM remodeling and NOS activity: *KDR, MMP2, NOS3, THBS2, TIMP3*, with the occurrence of shoulder pain or shoulder disability among female breast cancer survivors.

Methods

Study design

This is a pilot, cross-sectional study including a genetic association component. This study is a sub-study of a larger on-going project which seeks to correlate clinical disease state of the shoulder after breast cancer treatment with biomarkers of inflammation, fibrosis and angiogenesis, including their associated genetic variants.

Participants and setting

Study participants were recruited in the period August 2013 to July 2015, and relevant information regarding the study was provided upon recruitment. Participants, all women, were conveniently recruited from the waiting room of the Oncology Clinic of a tertiary public teaching hospital in South Africa. Although women of all races were recruited, only the larger "mixed-ancestry" ethnic group (Mixed-ancestry group: n=243, Black: n=43, White: n=22) was used for analysis to avoid confounding. The mixed-ancestry ethnic group, from the Western Cape region of South Africa, used in our study is composed of populations who self-identify as "Coloured." This is a unique group with a rich genetic admixture ancestrally derived from immigrants from Western Europe, West Africa, Asia and the indigenous Southern African populations. All participants agreeing to participate gave written informed consent and were included on the basis of defined inclusion and exclusion criteria (Table 1).

Table 1 Inclusion and exclusion criteria for participant recruitment

Inclusion criteria	Exclusion criteria
• >18 years old • Females • Unilateral breast cancer • ≥1 year after surgery • Mixed-ancestry	• History of shoulder or neck pathology prior to treatment for breast cancer • Diagnoses of connective tissue disorders such as rheumatoid arthritis or systemic lupus erythematosus • Diagnoses of renal insufficiency, diabetes mellitus or hyper-cholesterolemia • Diagnosed local recurrence • Diagnosed lymphoedema

Study procedures

The study was approved by the Human Research and Ethics Committee at the University of Cape Town (HREC REF: 650/2016). After providing informed consent, eligible participants completed the Shoulder Pain and Disability Index (SPADI) questionnaire and blood were drawn by venepuncture on the cubital fossa of the unaffected side. For each participant, 10 mL of blood was collected into appropriately labeled EDTA vacutainer blood collection tubes. Whole blood samples were immediately stored at −20 ºC until total DNA extraction. DNA was extracted from whole blood using the method described by Lahiri et al (1991)[39] and stored at −20 ºC. Out of 244 eligible participants, blood and/or DNA of sufficient quality (non-degraded) was available from 220 participants (90.2%). All relevant clinical variables for each participant including age, self-reported race, date of surgery, tumor grade, type of surgery, lymph node surgery, number of nodes removed and adjuvant therapy type were obtained from participants' medical records.

Patient-reported outcome: SPADI

Participants completed the Shoulder Pain and Disability Index (SPADI) questionnaire – a Patient Report Outcome Measure with 2 domains: Pain and Disability.[40,41] Using the SPADI, participants rated movement-related pain and difficulty associated with specific activities of daily living on a scale of 0 (no pain/difficulty) – 10 (extreme pain/difficulty). The pain subscale of the SPADI has 5 items whereas the disability subscale has 8 items. However, both scales are reported as percentages of possible total scores. For each subscale, the total score was divided by the number of completed items and expressed as a percentage.

Variables of interest

The primary outcome measure in this study was the shoulder pain and disability index (SPADI). Pain and disability scores were categorized into no – low pain/disability and moderate – high pain/disability based on total pain or disability scores ≤30 and >30, respectively. The development of these categories was based on reported SPADI score effects on activities of daily living[42] and reported clinical relevance of SPADI scores.[40] Exposures in this study are bi-allelic SNP genotypes from 5 candidate genes: *KDR* (*VEGF-R2*) (rs2305948 C>T; rs7667298 C>T), *NOS3* (rs1549758 C>T), *MMP2* (rs708269 A>T), *THBS2* (rs9766678 A>G) and *TIMP3* (rs5754312 T>A; rs715572 G>A). Potential covariates evaluated for association included participants' age at consent, time after surgery, type of surgery, extent of lymph node surgery, number of lymph nodes removed, tumor grade and adjuvant therapy type.

Single nucleotide polymorphism selection

SNPs within genes involved in the angiogenesis signaling pathway were selected for analysis. The selection of SNPs was based on functional significance or being located in important gene regions, having a reported global minor allele frequency >0.15 in the ENSEMBL database ([http://www.ensembl.org]), and/or previous associations with multifactorial conditions of the shoulder, as well as musculoskeletal soft tissue injuries in general. A total of seven SNPs from five genes were included (Tables 2 and 3). In order to ensure robust genetic association analyses, only SNP call rates of >95% and Hardy–Weinberg *P*-values of >0.05 were included.

Genotype determination

Genotyping was performed using TaqMan™ assays (Applied Biosystems) in 96-well plates with adherence to manufacturer's instructions in a StepOnePlus (Applied Biosystems) real-time PCR System at UCT Department of Human Biology. The reaction mix was as follows: Allele-specific TaqMan™ primer and probe mix – 0.15 µL, DNA template – 1 µL (1–10 ng), H_2O – 2.85 µL, and TaqMan™ PCR mastermix containing ampliTaq DNA polymerase Gold – 4 µL; Final reaction volume of 8 µL. Both negative (no DNA sample) and positive (DNA of known genotypes) controls were included in every plate as a quality control measure for reliability of the PCR and for the detection of potential genotyping errors. In addition, replicates

Table 2 Differences in demographic and clinical characteristics between shoulder pain categories

Characteristic	No–low pain (*n*=184)	Moderate–high pain (n=60)	*P*-value	Test
Age (years)	59.7±8.8	54.3±10.6	**<0.001**	Independent sample *t*-test (by group/ category)
Time after surgery (years)	3.7±2.4	3.4±2.5	0.372	
Nodes removed	10.7±6.3	9.2±5.4	0.105	
Total pain score	8.3±9.0	55.5±18.6	**<0.001**	
Total disability score	3.7±7.4	37.7±21.6	**<0.001**	
Side of primary				
Left	51.1 (93)	55.0 (33)	0.656	*F*
Right	48.9 (89)	45.0 (27)		
Tumor grade				
III	23.9 (39)	21.8 (12)	0.833	χ^2
II	52.8 (86)	50.9 (28)		
I	23.3 (38)	27.3 (15)		
Type of surgery				
Mastectomy	82.0 (150)	71.2 (42)	0.095	*F*
WLE	18.0 (33)	28.8 (17)		
Lymph node surgery				
ALND	85.3 (156)	81.4 (48)	0.537	*F*
SLNB	14.8 (27)	18.6 (11)		
Chemotherapy				
Yes	75.0 (135)	85.0 (51)	0.152	*F*
No	25.0 (45)	15.0 (9)		
Hormonal therapy				
Yes	77.0 (134)	81.0 (47)	0.587	*F*
No	23.0 (40)	19.0 (11)		
Hormonal regimen				
None	23.3 (40)	19.0 (11)	0.639	χ^2
Tamoxifen	60.5 (104)	63.8 (37)		
Aromatase inhibitor	8.1 (14)	5.1 (3)		
Both	8.1 (14)	12.1 (7)		
Radiotherapy				
Yes	67.4 (116)	75.9 (44)	0.252	*F*
No	32.6 (56)	24.1 (14)		

Notes: Data presented as mean ± SD or % (n). *P*-values in bold typeset indicate significance (*P*<0.05).
Abbreviations: WLE, Wide local excision; ALND, Axillary lymph node dissection; SLNBM, Sentinel lymph node biopsy; *t*, *t*-test; *F*, Fisher's exact test; χ^2,Chi-squared test.

were also included in every plate. Samples were considered successfully genotyped unless they failed twice to amplify for a particular SNP assay. More than 99% of all samples were successfully genotyped for each SNP (only 1 out of 220 samples were unsuccessfully genotyped for each of *NOS3* rs1549758, *MMP2* rs708269 and *THBS2* rs9766678. Data generated from the assays were analyzed using Thermo Fisher Cloud genotyping analysis Software Version: 3.3.0-SR2-build 21 and genotypes were automatically called.

inferred haplotype construction

KDR and *TIMP3* haplotype pairs were inferred using the genotypes at rs2305948 C>T and rs7667298 C>T, and

Table 3 Differences in demographic and clinical characteristics between shoulder disability categories

Characteristic	No – Low Disability (n=202)	Moderate – High Disability (n=42)	*P*-value	Test
Age (years)	59.1 ± 9.1	55.2 ± 10.9	**0.016**	Independent sample *t*-test (by group/category)
Time after surgery (years)	3.6 ± 2.4	3.4 ± 2.3	0.645	
Nodes removed	10.5 ± 6.2	9.4 ± 5.2	0.303	
Total pain score	11.7 ± 14.3	58.9 ± 20.5	**<0.001**	
Total disability score	4.3 ± 6.8	49.5 ± 15.2	**<0.001**	
Side of primary				
Left	51.0 (102)	57.1 (24)	0.501	F
Right	49.0 (98)	42.9 (18)		
Tumor grade				
III	22.8 (41)	26.3 (10)	0.584	χ^2
II	53.9 (97)	44.7 (17)		
I	23.3 (42)	29.0 (11)		
Type of surgery				
Mastectomy	79.6 (160)	78.1 (32)	0.834	F
WLE	20.4 (41)	22.0 (9)		
Lymph node surgery				
ALND	85.1 (171)	80.5 (33)	0.482	F
SLNB	14.9 (30)	19.5 (8)		
Chemotherapy				
Yes	75.3 (149)	88.1 (37)	0.102	F
No	24.8 (49)	11.9 (5)		
Hormonal therapy				
Yes	77.5 (148)	80.5 (33)	0.836	F
No	22.5 (43)	19.5 (8)		
Hormonal regimen				
None	22.8 (43)	19.5 (8)	0.765	χ^2
Tamoxifen	60.9 (115)	63.4 (26)		
Aromatase inhibitor	7.9 (15)	4.9 (2)		
Both	8.5 (16)	12.2 (5)		
Radiotherapy				
Yes	69.6 (133)	69.2 (27)	1.000	F
No	30.4 (58)	30.8 (12)		

Notes: Data presented as mean ± SD or % (n). *P*-values in bold typeset indicate statistical significance ($P<0.05$).
Abbreviations: WLE, Wide local excision; ALND, Axillary lymph node dissection; SLNB, Sentinel lymph node biopsy; *t*, *t*-test; *F*, Fisher's exact test; χ^2 Chi-squared test.

rs715572 G>A and rs5754312 T>A, respectively. A low haplotype frequency cut-off of 4% was used to avoid unreliable results.

Bias

Just under 10% (n=23, out of a total n=243) of participants could not provide bloodbecause they were lost after consent due to the need for further medical examination in the clinic. There could potentially be differences between participants who provided blood and those who did not. However, this is unlikely, as all participants approached consented.

Sample size

Assuming expected average baseline risks of 32% and 25% for shoulder pain and disability, respectively, calculated from previous reports,[3,4,6,9,43–48] sample size of 220 is likely sufficient to detect odds ratios of 2.0 and greater, at 80% power for allele frequencies of ≥0.2 for the log-additive genetic model (Table S2). For the same log-additive genetic model, our sample size is also sufficient to detect odds ratios of 2.5 for allele frequencies ≥0.15 (Table S2). However, for the dominant genetic model, our sample size is only sufficient to detect odds ratios of 2.5 for allele frequencies ≥0.15 (Table S2). Furthermore, our sample size is underpowered for the recessive genetic model for effect sizes of 1.5–2.5 odds ratios, and allele frequencies of 0.15–0.5. Sample size was calculated using QUANTO version 1.2.4.[49]

Statistical analysis

Demographic and clinical data were analyzed using Statistica Version 13.2.[50] Independent sample t-tests, Fisher's exact tests and Chi-square analyses were done to evaluate for differences in demographic and clinical characteristics between the shoulder pain and disability categories. Logistic regression analysis based on a generalized linear model was used to evaluate the magnitude and precision of the association between significant clinical or demographic characteristics in R version 3.3.3.[51] The genotype data were analyzed using R Studio Version 1.0.136, running R version 3.3.3.[51] Chi-squared and Fisher's exact tests were used to analyze any differences in the genotype, allele or haplotype frequencies between the clinical categories. Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) was calculated using 'genetics' Version 1.3.8.1 package.[52] Haplotypes were inferred using the R package haplo.stats.[53,54]Logistic regression analyses were performed using SNPassoc Version 1.9–2 to evaluate the association between genotypic characteristics and pain and disability category membership.[55] Significant covariates among clinical and demographic characteristics were included in the final multivariate regression analyses. The regression models were evaluated using package "modEvA" version 1.3.2 in R, using the Hosmer-Lemeshow goodness of fit test and D^{22}.[56]

Results

Differences in clinical and demographic characteristics between pain/disability categories

No significant differences (P>0.05) were noted between the no–low and moderate–high shoulder pain categories for the number of nodes removed, side of primary cancer, tumour grade, type of surgery, extent of lymph node surgery and receipt of adjuvant chemotherapy, hormonal therapy (and hormonal regimen) or radiation therapy (Table 2). However, participants in the no–low shoulder pain category were significantly (P<0.001) older (59.7±8.8) compared with those in the moderate–high shoulder pain category (54.3±10.6).

Similarly, no significant differences (P>0.05) were noted between the no–low and moderate–high shoulder disability categories for the number of nodes removed, side of primary cancer, tumour grade, type of surgery, extent of lymph node surgery and receipt of adjuvant chemotherapy, hormonal therapy (and hormonal regimen) or radiation therapy (Table 3). However, participants in the no–low shoulder disability category were significantly (P=0.016) older (59.1±9.1) compared with those in the moderate–high disability category (55.2±10.9).

No significant differences (P>0.05) were noted between shoulder pain categories, and shoulder disability categories for radiotherapy field and adjuvant chemotherapy regimen (Tables S1 and S2).

Logistic regression analyses for participants' age

In the regression analysis of participants' age as a predictor for shoulder pain, the odds of being in the moderate–high shoulder pain category decreased significantly for older participants (OR 0.94, 95% CI: 0.91,0.97; P<0.001) (Table 4). This means that older participants had lower odds, while younger participants had higher odds, of reporting moderate–high shoulder pain. The regression model predicted 5.2% of the variance in shoulder pain category membership.

In the regression analysis of participants' age as a predictor for shoulder disability, the odds of being in the moderate–high shoulder disability category decreased

Table 4 Logistic regression analysis for participants' age at consent to predict moderate–high shoulder pain

Predictor	Odds ratio	Standard error	95% CI	Z value	*P*-value
Age	0.94	0.016	0.91, 0.97	−3.67	**<0.001**

Notes: Overall model fit: χ^2=9.83, *P*=0.043, D^2=0.052. CIs (95%) in the format: lower, upper. *P*-values in bold typeset indicate statistical significance (*P*<0.05).

Table 5 Logistic regression analysis for participants' age at consent to predict moderate–high shoulder disability

Predictor	Odds ratio	Standard error	95% CI	Z value	*P*-value
Age	0.96	0.018	0.93, 0.99	−2.38	**0.017**

Notes: Overall model fit: χ^2=5.18, *P*=0.075, D2=0.025. Cis (95%) in the format: lower, upper. *P*-values in bold typeset indicate statistical significance (*P*<0.05).

significantly for older participants (OR 0.96, 95% CI: 0.93,0.99; *P*=0.017) (Table 5). This means that older participants had lower odds, while younger participants had higher odds of reporting moderate–high shoulder disability. The regression model predicted 2.5% of the variance in shoulder disability category membership.

Genotype/allele frequency distributions between shoulder pain/disability categories

For both shoulder pain and shoulder disability, no significant differences in the genotype/allele frequency distributions were noted between the no – low and moderate–high categories for all SNPs: *KDR* (*VEGF-R2*) (rs2305948 C>T; rs7667298 C>T), *NOS3* (rs1549758 C>T), *MMP2* (rs708269 A>T), *THBS2* (rs9766678 A>G) and *TIMP3* (rs5754312 T>A; rs715572 G>A) (Tables 6 and 7). The genotype distributions for the no–low category for both shoulder pain and shoulder disability were in HWE for all SNPs (*P*>0.05) (Tables 6 and 7). The genotype distributions for the moderate–high category for both shoulder pain and shoulder disability were also in HWE for all SNPs (*P*>0.05), although the *P*-values for rs7667298 and rs9766678 were <0.10. (Tables 6 and 7).

KDR and TIMP3 inferred haplotype frequency distributions between shoulder pain/disability categories

There were no significant differences in the frequency distribution of the inferred *KDR* haplotypes between the no–low and moderate–high shoulder pain categories (*P*>0.05) (Table 8). However, the frequencies of the inferred *KDR* haplotypes differed significantly between the no – low and moderate–high shoulder disability categories (*P*=0.024)(Table 9). In particular, the *KDR* "TT" inferred haplotype was significantly over-represented in the no – low shoulder disability category relative to the moderate – high disability category (*P*=0.014, 11.4% vs 0.0%) (Table 9 and Figure 1).

There were no significant differences in the frequency distribution of the inferred *TIMP3* haplotypes between the no–low and moderate–high shoulder pain categories (*P*>0.05) (Table 8). Similarly, there were no significant differences in the frequency distribution of the inferred *TIMP3* haplotypes between the no–low and moderate–high shoulder disability categories (*P*>0.05) (Table 9).

Bivariate regression analysis for inferred KDR haplotypes to predict shoulder disability including participants' age

After adjusting for age, only a trend (*P*=0.052) was observed in the distribution of inferred *KDR* haplotypes between shoulder disability categories (Table 10). In the regression analysis for inferred *KDR* haplotypes, each dose of the "TT" haplotype increased the odds of being in the no–low shoulder disability category by 100% (OR: 0.00, *P*=0.014) (Table 10).

Discussion

Our study suggests an association between functional polymorphisms within *KDR* and shoulder disability following breast cancer treatment among mixed ancestry individuals. Although the independent *KDR* SNPs were not significantly associated with shoulder disability, inferred haplotypes have implicated a genomic interval within *KDR* to be associated with shoulder disability.[57] Such an effect is especially important considering that both SNPs are functional[58,59] and one of the SNPs has previously been implicated in other forms of connective tissue pathology susceptibility.[18] To the best of our

Table 6 Genotype and minor allele frequency distributions, and *P*-values for Hardy–Weinberg (HWE) exact test of the *KDR* rs7667298 C>T, *KDR* rs2305948 C>T, *MMP2* rs708269 A>T, *NOS3* rs1549758 C>T, *THBS2* rs9766678 A>G, *TIMP3* rs715572 G>A, and *TIMP3* rs5754312 T>A polymorphisms in mixed ancestry participants with no–low pain and moderate–high pain in the shoulder following breast cancer treatment

		No–low pain (n=169)	Moderate–high pain (n=51)	*P*-value
KDR				
rs7667298	C/C	26.0 (44)	41.2 (21)	
	C/T	50.3 (85)	35.3 (18)	0.090
	T/T	23.7 (40)	23.5 (12)	
	C Allele	51.1 (173)	58.8 (60)	0.213
	HWE	1.000	0.080	
rs2305948	C/C	78.1 (132)	80.4 (41)	
	C/T	21.3 (36)	19.6 (10)	0.882
	T/T	0.6 (1)	0 (0)	
	T Allele	11.2 (38)	9.8 (10)	0.856
	HWE	0.698	1.000	
MMP2				
rs708269	A/A	51.5 (87)	49.0 (25)	
	A/T	41.4 (70)	41.2 (21)	0.820
	T/T	7.1 (12)	9.8 (5)	
	T Allele	27.8 (94)	30.4 (31)	0.618
	HWE	0.848	1.000	
NOS3				
rs1549758	C/C	72.2 (122)	64.7 (33)	
	C/T	26.0 (44)	31.4 (16)	0.493
	T/T	1.8 (3)	3.9 (2)	
	T Allele	24.3 (82)	19.6 (20)	0.280
	HWE	1.000	1.000	
THBS2				
rs9766678	A/A	44.4 (75)	45.1 (23)	
	A/G	46.7 (79)	37.3 (19)	0.192
	G/G	8.9 (15)	17.6 (9)	
	G Allele	32.2 (109)	36.3 (37)	0.473
	HWE	0.481	0.222	
TIMP3				
rs715572	G/G	69.2 (117)	68.6 (35)	
	A/G	27.8 (47)	27.5 (14)	0.945
	A/A	3.0 (5)	3.9 (2)	
	A Allele	16.9 (57)	17.6 (18)	0.881
	HWE	1.000	0.637	
rs5754312	T/T	30.2 (51)	33.3 (17)	
	A/T	53.3 (90)	49.0 (25)	0.866
	A/A	16.6 (28)	17.6 (9)	
	A Allele	43.2 (146)	42.2 (43)	0.909
	HWE	0.347	1.000	

Notes: Genotype and allele frequencies are expressed as a percentage with the number of participants (n) in parentheses. *P*-values for the exact test of Hardy–Weinberg equilibrium for each of the categories are included in the table. *P*-values in bold typeset indicate significance ($P<0.05$).
Abbreviation: HWE, Hardy–Weinberg equilibrium.

Table 7 Genotype and minor allele frequency distributions, and *P*-values for Hardy–Weinberg (HWE) exact test of the *KDR* rs7667298 C>T, *KDR* rs2305948 C>T, MMP2 rs708269 A>T, *NOS3* rs1549758 C>T, *THBS2* rs9766678 A>G, *TIMP3* rs715572 G>A, and *TIMP3* rs5754312 T>A polymorphisms in mixed ancestry participants with no–low disability and moderate–high disability in the shoulder following breast cancer treatment

		No–low disability (n=183)	Moderate–high disability (n=37)	*P*-value
KDR				
rs7667298	C/C	26.8 (49)	43.2 (16)	
	C/T	49.2 (90)	35.1 (13)	0.134
	T/T	24.0 (44)	21.6 (8)	
	C Allele	51.4 (188)	60.8 (45)	0.160
	HWE	0.882	0.163	
rs2305948	C/C	76.5 (140)	89.2 (33)	
	C/T	23.0 (42)	10.8 (4)	0.269
	T/T	0.5 (1)	0.0 (0)	
	T Allele	12.0 (44)	5.4 (4)	0.105
	HWE	0.479	1.000	
MMP2				
rs708269	A/A	51.9 (95)	45.9 (17)	
	A/T	40.4 (74)	45.9 (17)	0.799
	T/T	7.7 (14)	8.1 (3)	
	T Allele	27.9 (102)	31.1 (23)	0.574
	HWE	1.000	1.000	
NOS3				
rs1549758	C/C	71.0 (130)	67.6 (25)	
	C/T	27.3 (50)	27.0 (10)	0.459
	T/T	1.6 (3)	5.4 (2)	
	T Allele	15.3 (56)	18.9 (14)	0.485
	HWE	0.774	0.584	
THBS2				
rs9766678	A/A	43.2 (79)	51.4 (19)	
	A/G	47.5 (87)	29.7 (11)	0.077
	G/G	9.3 (17)	18.9 (7)	
	G Allele	33.1 (121)	33.8 (25)	0.893
	HWE	0.403	0.061	
TIMP3				
rs715572	G/G	68.9 (126)	70.3 (26)	
	A/G	28.4 (52)	24.3 (9)	0.671
	A/A	2.7 (5)	5.4 (2)	
	A Allele	16.9 (62)	17.6 (13)	0.867
	HWE	1.000	0.286	
rs5754312	T/T	30.6 (56)	32.4 (12)	
	A/T	51.9 (95)	54.1 (20)	0.833
	A/A	17.5 (32)	13.5 (5)	
	A Allele	43.4 (159)	40.5 (30)	0.700
	HWE	0.547	0.732	

Notes: Genotype and allele frequencies are expressed as a percentage with the number of participants (n) in parentheses. *P*-values for the exact test of Hardy–Weinberg equilibrium for each of the categories are included in the table. *P*-values in bold typeset indicate statistical significance ($P<0.05$).
Abbreviation: HWE, Hardy–Weinberg equilibrium.

Table 8 Inferred *KDR* haplotype frequency distribution for shoulder pain categories

Gene	Haplotype		Frequency (%)		HS	*P*-value	
			No–low pain (n=169)	Moderate–high pain (n=51)		Global	Specific
	rs2305948	rs7667298				0.206	
KDR	T	T	10.6	6.3	−0.96		0.337
	C	T	38.2	34.8	−0.80		0.423
	C	C	50.5	55.4	1.06		0.288
	rs715572	rs5754312				0.507	
TIMP3	G	A	30.5	25.8	−0.67		0.500
	G	T	56.6	56.5	0.50		0.619
	A	A	12.7	16.3	0.66		0.512

Notes: Haplotype frequencies are expressed as percentages. *P*-values in bold typeset indicate statistical significance (*P*<0.05).
Abbreviation: HS, Haplotype score.

Table 9 Inferred *KDR* haplotype frequency distribution for shoulder disability categories

Gene	Haplotype		Frequency (%)		HS	*P*-value	
			No–low disability (n=183)	Moderate–high disability (n=37)		Global	Specific
	rs2305948	rs7667298				**0.024**	
KDR	T	T	11.4	0.00	−2.45		**0.014**
	C	T	37.2	39.2	−0.14		0.885
	C	C	50.8	55.4	1.15		0.250
	rs715572	rs5754312				0.740	
TIMP3	G	A	30.3	24.9	−0.77		0.441
	A	A	13.2	15.7	0.37		0.709
	G	T	52.8	57.6	0.63		0.529

Notes: Haplotype frequencies are expressed as percentages. *P*-values in bold typeset indicate statistical significance (*P*<0.05).
Abbreviation: HS, Haplotype score.

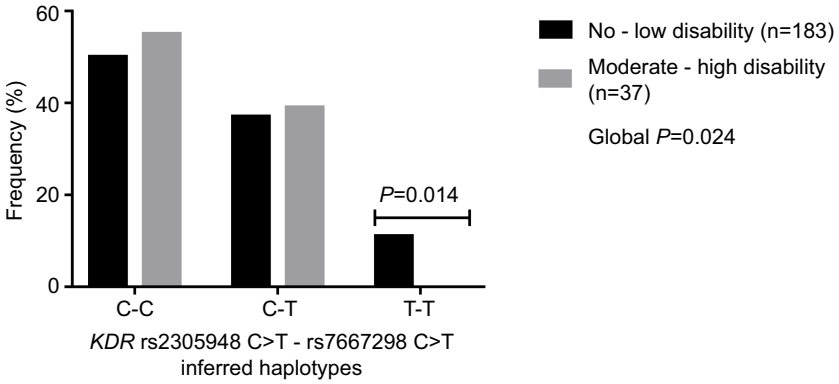

Figure 1 The frequency distribution of the *KDR* (rs2305948 C>T–rs7667298 C>T) inferred haplotype between no–low and moderate–high disability participants. Frequency distribution of inferred haplotypes constructed from the rs2305948 (C>T) and rs7667298 (C>T) variants in the no – low and moderate – high disability groups. Significant differences in haplotype frequencies between groups are depicted on the graph, with non-adjusted *P*-values. The number of participants (n) in each group is in parentheses.

Table 10 Bivariate logistic regression analysis for inferred *KDR* haplotypes to predict shoulder disability category membership, including participants' age at consent

SNP		Frequency (%)		HS	*P*-value		OR
KDR rs2305948	*KDR* rs7667298	No–low disability (n=183)	Moderate–high disability (n=37)		Global	Specific	
T	T	11.4	0.00	−2.50	0.052	**0.013**	0.00
C	T	37.2	39.2	0.08		0.936	-
C	C	50.8	55.4	1.02		0.307	1.00

Notes: Haplotype frequencies are expressed as percentages. *P*-values in bold typeset indicate statistical significance ($P<0.05$).
Abbreviations: HS, Haplotype score; OR, Odds ratio.

knowledge, this study is the first to evaluate associations between polymorphisms in genes involved in angiogenesis and shoulder pain/disability in breast cancer survivors.

In the bivariate analyses of clinical and demographic data, only participants' age at consent was significantly associated with both shoulder pain and shoulder disability following breast cancer treatment. This association is consistent with previous reports on age and persistent pain following breast cancer treatment[12,60,61] . The bivariate regression models for shoulder pain or disability explained only 5.2% and 2.5% of the variance in pain or disability category membership, respectively (Tables 3 and 4). Contrary to previous reports,[12,62] adjuvant radiotherapy was not significantly associated with shoulder pain or shoulder disability in our study. This may reflect changes in the etiology of shoulder pain and disability with long follow-up periods, which in our cohort was >3 years on average (Tables 1 and 2). Furthermore, a trend contrary to previous reports,[2,5,10,46] of higher frequency of the more aggressive mastectomy compared to the conservative wide local excision (WLE) among shoulder pain controls was noted in our study. This finding may perhaps be specific to our cohort or primary outcomes: shoulder pain and shoulder disability. As with other studies,[12,14] however, our demographic and clinical data suggest that variability in the occurrence of shoulder pain or shoulder disability is not largely explained by factors related to surgical management or adjuvant treatment.

Angiogenesis signaling has links to molecular signaling pathways that are important in shoulder complex pathology such as the inflammatory pathway, fibrogenesis and ECM remodeling.[27,28] The inflammatory pathway has potent nociceptive effects which may contribute to shoulder pain.[27,63,64] In addition, aberrant ECM production or fibrosis can potentially contribute to stiffness in the shoulder joint capsule which can lead to reductions in range of motion.[65] Our findings support the body of evidence implicating the angiogenesis pathway in non-cancer musculoskeletal conditions such as tendon injuries or tendinopathy[17,18] and rotator cuff disease (RCD).[19,20]

Our findings suggest that the "TT" haplotype for *KDR* rs2305948 C>T–rs7667298 C>T may have a protective effect on the occurrence of shoulder disability following breast cancer treatment. This haplotype was completely absent among moderate–high shoulder disability participants (Table 10 and Figure 1). *KDR* encodes VEGF-R2, the main angiogenesis signaling receptor that mediates endothelial cell survival, activation, proliferation and migration.[27,28] Both SNPs for the *KDR* "TT" haplotype have been associated with coronary heart disease[66] and notably, rs2305948 "T" allele has been associated with reduced tendinopathy risk.[18] *KDR* rs2305948 is a missense variant (Δ amino acid – valine/isoleucine) which has been proposed to be functional.[58] Although both amino acids are non-polar, the Isoleucine variant ("T" allele for rs2305948) has been reported to reduce VEGF-A binding efficiency.[58] Interestingly, the "TT" genotype of rs7667298 has been shown to increase *KDR* expression.[59] However, the biological functional significance of this haplotype on the angiogenesis pathway remains unclear. Based on the previous functional evidence,[58,59] we suggest that this "TT" haplotype may be indicating a pro-angiogenic profile. It can also be argued that the SNPs implicated in this haplotype could be in LD to other SNPs that may underlie the occurrence of shoulder disability. The frequency of the "T" allele for both *KDR* SNPs was lower for the moderate–high shoulder disability category compared

to the no–low category although this difference did not approach statistical significance (Table 7). The pathophysiology that underlies shoulder disability after breast cancer treatment may be different from that which characterizes non-cancer shoulder conditions. Unlike rotator cuff disease and rotator cuff tendinopathy which are characterized by foreshortening of the pectoral girdle muscles and soft tissues, and weakening or degeneration of tendon structure, respectively, shoulder disability in our patient group could be a result of soft tissue fibrosis.[34,67] KDR signaling through VEGF-A up-regulates MMPs which in turn may alter, and perhaps weaken, tendon structure leading to movement dysfunction.

Our study was not without limitations. Firstly, the sample size is small and underpowered (power <80%) for small effect sizes (OR=1.5) except for allele frequencies ≥0.40 (Table S3 and S4). Larger sample sizes may detect significant differences in other clinical and genotypic characteristics included in this study. Secondly, no direct measurements of protein or DNA expression were performed to provide additional data on the mechanisms that underlie the development of shoulder pain/disability. Thirdly, there was no wide score gap separating the two shoulder pain/disability categories. Therefore, close to the boundary score of 30, some individuals with similar shoulder pain/disability characteristics may be in different pain/disability categories. Future studies focusing on extreme phenotypes may increase effect sizes of these associations. Finally, ethnicity was determined by self-report which is less reliable than genomic estimates and therefore, there is a possibility of undetermined population stratification in our sample.

In conclusion, our findings provide preliminary evidence of an association between polymorphisms in genes involved in angiogenesis and the occurrence of shoulder disability in women following breast cancer treatment. Future studies in independent populations with larger sample sizes are warranted to further characterize the observations and explore the potential biological mechanisms.

Ethics

This study received ethical approval from the Human Research Ethics Committee at the University of Cape Town (HREC REF: 650/2016) and was performed in accordance with the principles of the Declaration of Helsinki, the South African Good Clinical Practice (GCP) guidelines and the laws of South Africa.

Acknowledgments

This study was funded in part by the University of Cape Town Research Council, the Medical Research Council and the National Research Foundation (NRF). TSM was funded by the University of Cape Town and the National Research Foundation.

Disclosure

Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the funders. The authors declare no conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplementary material

Table S1 Differences in radiotherapy fields and chemotherapy regimens between participants in the no-low and moderate to high shoulder pain categories

Characteristic	No – Low Pain (n=184)	Moderate – High Pain (*n*=60)	*P*-value	Test
Radiotherapy field			0.292	χ^2
None	33.1 (56)	24.6 (14)		
CW	18.3 (31)	12.3 (7)		
CW + S/C	27.8 (47)	36.8 (21)		
BCT	18.9 (32)	21.1 (12)		
BCT + S/C	1.8 (3)	5.3 (3)		
Chemotherapy regimen			0.259	χ^2
None	26.7 (43)	16.7 (9)		
AC	5.0 (8)	11.1 (6)		
AC/Docetaxel	1.2 (2)	3.7 (2)		
CAF	36.0 (58)	27.8 (15)		
CAF/Carboplatin	0.6 (1)	0.0 (0)		
CAF/CMF	0.6 (1)	0.0 (0)		
CAF/FEC	1.2 (2)	0.0 (0)		
CAF/FEC/Docetaxel	0.6 (1)	0.0 (0)		
CAF/TC	0.0 (0)	1.9 (1)		
CMF	7.5 (12)	5.6 (3)		
CMF/Docetaxel	0.6 (1)	0.0 (0)		
EC	3.7 (6)	9.3 (5)		
FEC	12.4 (20)	16.7 (9)		
FEC/Docetaxel	1.2 (2)	1.9 (1)		
FEC/Paclitaxel	2.5 (4)	3.7 (2)		
Paclitaxel	0.0 (0)	1.9 (1)		

Notes: Data presented as % (n). *P*-values in bold typeset indicate statistical significance ($P<0.05$).

Abbreviations: CW, Chest wall; S/C, Supraclavicular field; BCT, Breast; χ^2, Chi-squared test; AC, Adriamycin-Cyclophosphamide; CAF, Cyclophosphamide-Adriamycin-Fluorouracil; CMF, Cyclophosphamide-Methotrexate-Fluorouracil; EC, Epirubicin-Cyclophosphamide; FEC, Fluorouracil-Epirubicin-Cyclophosphamide; TC, Paclitaxel-Cyclophosphamide.

Table S2 Differences in radiotherapy fields and chemotherapy regimens between participants in the no-low and moderate to high shoulder disability categories

Characteristic	No – Low Disability (n=202)	Moderate – High Disability (n=42)	*P*-value	Test
Radiotherapy field			0.841	χ^2
None	30.9 (58)	31.6 (12)		
CW	17.6 (33)	13.2 (5)		
CW + S/C	29.8 (56)	31.6 (12)		
BCT	19.7 (37)	18.4 (7)		
BCT + S/C	2.1 (4)	5.3 (2)		
Chemotherapy regimen			0.133	χ^2
None	26.4 (47)	13.5 (5)		
AC	5.1 (9)	13.5 (5)		
AC/Docetaxel	1.2 (2)	5.4 (2)		
CAF	35.4 (63)	27.0 (10)		
CAF/Carboplatin	0.6 (1)	0.0 (0)		
CAF/CMF	0.6 (1)	0.0 (0)		
CAF/FEC	1.2 (2)	5.4 (2)		
CAF/FEC/Docetaxel	0.6 (1)	0.0 (0)		
CAF/TC	0.0 (0)	2.7 (1)		
CMF	6.7 (12)	8.1 (3)		
CMF/Docetaxel	0.6 (1)	0.0 (0)		
EC	4.5 (8)	8.1 (3)		
FEC	12.9 (23)	16.2 (6)		
FEC/Docetaxel	1.1 (2)	2.7 (1)		
FEC/Paclitaxel	3.4 (6)	0.0 (0)		
Paclitaxel	0.0 (0)	2.7 (1)		

Notes: Data presented as % (n). *P*-values in bold typeset indicate statistical significance ($P<0.05$).
Abbreviations: CW, Chest wall; S/C, Supraclavicular field; BCT, Breast; χ^2, Chi-squared test; AC, Adriamycin-Cyclophosphamide; CAF, Cyclophosphamide-Adriamycin-Fluorouracil; CMF, Cyclophosphamide-Methotrexate-Fluorouracil; EC, Epirubicin-Cyclophosphamide; FEC, Fluorouracil-Epirubicin-Cyclophosphamide; TC, Paclitaxel-Cyclophosphamide.

Table S3 A priori power calculation to determine adequacy of sample size

MAF	OR	N (number of participants with moderate – high pain/disability required for 80% power)								
		Dominant model			Recessive model			Log-additive model		
		Pain	Disability	AVE	Pain	Disability	AVE	Pain	Disability	AVE
0.15	1.5	347	303	325	3026	2584	2805	270	233	252
	2.0	118	102	110	1006	833	920	93	79	86
	2.5	68	58	63	571	461	516	55	46	51
0.20	1.5	306	269	288	1739	1487	1613	218	189	204
	2.0	105	92	99	579	481	530	76	65	71
	2.5	60	53	57	329	267	298	45	38	42
0.30	1.5	289	257	273	823	707	765	169	148	159
	2.0	100	89	95	275	231	253	60	52	56
	2.5	58	52	55	157	129	143	36	30	33
0.40	1.5	319	288	304	507	439	473	150	132	141
	2.0	111	101	106	171	145	158	53	47	50
	2.5	64	59	62	98	82	90	32	28	30
0.50	1.5	399	362	381	369	322	346	146	130	138
	2.0	139	129	134	125	108	117	52	46	49
	2.5	81	76	79	72	61	67	31	27	29

Notes: The number of unmatched participants in the no – low category per participant in the moderate – high category was 3 and 4 for pain and disability, respectively, based on the expected prevalence calculated from previous reports as described in the section on sample size.
Abbreviations: MAF, Minor Allele Frequency; OR, Odds Ratio; AVE, Average.

Table S4 Post-hoc power calculation to determine statistical power

SNP	MAF	OR	Power (%)			
			Shoulder Pain (n^a=51)		Shoulder Disability (n^b=37)	
			D	A	D	A
KDR						
rs7667298	0.52	1.5	58.7	96.4	58.0	96.4
		2.0	95.3	100	94.7	100
		2.5	99.7	100	99.6	100
rs2305948	0.13	1.5	66.3	76.1	66.9	77.0
		2.0	98.5	99.6	98.7	99.7
		2.5	100	100	100	100
MMP2						
rs708269	0.28	1.5	76.5	93.6	76.5	93.9
		2.0	99.5	100	99.5	100
		2.5	100	100	100	100
NOS3						
rs1549758	0.16	1.5	70.9	82.5	71.4	83.3
		2.0	99.1	99.9	99.2	99.9
		2.5	100	100	100	100
THBS2						
rs9766678	0.32	1.5	75.7	94.9	75.5	95.1
		2.0	99.4	100	99.4	100
		2.5	100	100	100	100
TIMP3						
rs715572	0.15	1.5	69.6	80.7	70.1	81.4
		2.0	99.0	99.8	99.1	99.9
		2.5	100	100	100	100
rs5754312	0.40	1.5	71.4	96.2	71.0	96.3
		2.0	98.9	100	98.8	100
		2.5	100	100	100	100

Notes: The number of unmatched participants in the no – low category per participant in the moderate – high category was 3 and 4 for pain and disability, respectively, based on the observed prevalence rates.

Abbreviations: SNP, Single nucleotide polymorphism; MAF, Minor Allele Frequency; OR, Odds Ratio; D, Dominant genetic model; A, Log-Additive genetic model; n^a, number of participants with moderate – high pain; n^b, number of participants with moderate – high disability.