A Multicentre Case Control Study of Vitamin D and Breast Cancer Risk among Pakistani Women

by

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Abstract

The prevalence of Vitamin D inadequacy is high worldwide but particularly elevated among women living in Karachi, Pakistan, even though the city is located at a high latitude with year-round adequate concentrations of UVB radiation. Sun exposure is the major source of Vitamin D, but due to multiple factors in the predominately Muslim population and modern lifestyle behaviors, Vitamin D deficiency is becoming a major public health problem. There are new data emerging that highlight the potential protective effect of Vitamin D for breast cancer, but evidence varies between different studies. Therefore, we undertook this study with the overall aim to evaluate the role of Vitamin D in breast cancer among Pakistani women. To meet these objectives, this case control study was conducted in two hospitals of Karachi, Pakistan: Aga Khan University Hospital (AKUH) and Karachi Institute of Radiation and Nuclear Medicine Hospital (KIRAN) during 2015-2017. Breast cancer cases had newly diagnosed histologically confirmed primary breast cancer. Controls were women free of breast or any other cancer were matched by age (year of birth $\pm$ 5 years), residence in the same geographic area and study site from surgery, family medicine and oncology clinics of AKUH & KIRAN. An interviewer-administered detailed questionnaire was used, and venous blood was collected to measure serum Vitamin D level at the end of interview. The detailed questionnaire provided the opportunity to explore several factors related to breast cancer in this cohort.

The objectives of the present study were: 1) To determine the association of Vitamin D (serum Vitamin D (25-hydroxyVitamin D) level, Vitamin D supplementation and sun exposure with breast cancer among Pakistani women, 2) To identify other risk factors associated with breast cancer among Pakistani women, 3) To evaluate the risk factors associated with triple negative breast cancer TNBC and non TNBC subtypes (including hormone receptor status and stage of diagnosis), 4) To evaluate the association of diet with BC among Pakistani women using the modified AHEI 2010 and its component scores 5) to assess patient delay in breast cancer diagnosis, its associated factors and stage of diagnosis among breast cancer patients in Karachi, Pakistan.

Main study findings were that Vitamin D deficiency was significantly associated with increased risk of breast cancer, and intake of Vitamin D supplements was associated with decreased risk of breast cancer, supporting the hypothesis that Vitamin D may play a protective role against breast cancer. Other factors associated with increased breast cancer risk were poor socioeconomic status, poor education and lack of employment status. There was no association of any of the reproductive factors or familial risk factors with breast cancer and it is likely that environmental and lifestyle factors related to poor socioeconomic status had a major role in breast cancer etiology. There was an association of an even higher risk of the triple negative breast cancer subtype than non-triple negative breast cancer subtype, among women with both Vitamin D deficiency and poor socioeconomic status. Based on these findings' correction of Vitamin D deficiency in women is a reasonable and cost effective strategy to reduce the incidence of all subtypes of breast cancer and triple negative breast cancer like aggressive breast cancer in particular. Such an approach should be carefully interpreted and further confirmed by large prospective studies or clinical trials.

In another sub study, high intake of grains, both whole and refined, was also significantly associated with a higher risk of breast cancer in Pakistani women. Limiting refined carbohydrate intake might be a beneficial public health message as it may represent a potentially modifiable risk factor for breast cancer in our population but requires additional in-depth study.

Breast cancer diagnosis delay study reported that despite noticing a breast lump, 64.9% of women diagnosed with breast cancer delayed medical consultation by a median of 7 months. The recommendation from this study is to provide better education to women about breast cancer awareness, and methods of self-examination and what are the most likely signs of breast cancer.

In conclusion, Pakistan is a low-income group country with fifty percent of women below the poverty line. Breast cancer rates and mortality are particularly high among poor women. This study identifies several inexpensive strategies and approaches that if implemented may help reduce the incidence, delay in breast cancer diagnosis and morbidity of breast cancer in Pakistani women.

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Thesis declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in nature, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by any other person, except where due references has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, or for any other degree or diploma in any university or other tertiary institution without prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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Dr Uzma Shamsi

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List of abbreviations

25(OH)D	25-hydroxyvitamin D
1,25 $(OH)_2D$	1,25 dihydroxyvitamin D (Calcitriol)
AKUH	Aga Khan University Hospital
BC	Breast cancer
BMI	Body Mass Index
Ca	Calcium
CAM	Complementary and alternative medicine
CI	Confidence interval
DRI	Dietary Reference Intakes
DCIS	Ductal carcinoma in situ
D2	Ergocalciferol
D3	Cholecalciferol
ERC	Ethical Review committee
ELISA	Enzyme-linked immunosorbent assay
EGFR	Epidermal growth factor receptor
ER	Estrogen Receptor
FISH	Fluorescence in situ hybridization
FTP	Full term pregnancy
ETS	Environmental tobacco smoke
FFQ	Food Frequency Questionnaire
FGR	Fibroblast Growth Factor
Fup	Follow up
GH	Growth Hormone
IDC	Invasive ductal carcinoma
ILC	Invasive lobular carcinoma
IU/d	International unit per day
HRT	Hormonal replacement therapy
HER2	Human epidermal growth factor receptor 2
HR	Hazard ratio
IARC	The International Agency for Research in Cancer
IOM	The Institute of Medicine
KIRAN	Karachi Institute of Radiology and Nuclear Medicine
LCIS	Lobular carcinoma in situ
NHW	Non-Hispanic women
NHS II	Nurses' Health Study
Mg	Magnesium
NHANES	National Health and Nutrition Examination Survey
nM/l	nmol/l
OR	Odds ratio
OC	Oral contraceptives

P	Phosphate
PRL	Prolactin
PTH	Parathyroid Hormone
QC	Quality control
Rs	Rupee
RR	Relative Risk
SD	Standard deviation
Svg	Serving
SES	Socioeconomic Status
SE	Sunlight exposure
SEM-Q	Sunlight exposure measurement questionnaire
TNBC	Triple negative breast cancer
UV	Ultraviolet
UVB	Ultraviolet B rays
VDD	Vitamin D deficiency
VDI	Vitamin D insufficiency
VDR	Vitamin D receptors
WHO	World Health Organization

Chapter 1 Breast cancer and vitamin D - Introduction and literature review

1.1 Breast cancer

Breast cancer is the most common cancer among women and the aim of this dissertation was to identify potential changeable factors that could reduce the risk of breast cancer among women in Karachi Pakistan. Accordingly, the following literature review will provide relevant background on breast cancer classification and epidemiology. In addition, a detailed review of vitamin D and its relationship with breast cancer is presented as this is a particular focus of the studies presented in this dissertation. Note each chapter of the five sub studies is presented with a relevant introduction that includes further relevant literature reviews and discussion after the results.

1.2 Breast cancer epidemiology

The International Agency for Research on Cancer 2018, reports that the incidence of breast cancer has increased globally in all age groups during the past thirty years, with the highest incidence reported in the Western countries compared to Asian countries (1). The cancer registry in Iran, similar to other Asian countries, reported age standardized rate ASR of 16.2 per 100 000 person-years, much lower than in western countries (2). However, in Korea, breast cancer incidence is reported to be increasing with the highest incidence in women in the 40-49 age group (3). Similarly in India, there is a trend of increasing breast cancer rates (4). Overall, the most pronounced increase in breast cancer is witnessed among women younger than 40 years (5). According to an estimate, 266,120 new cases of invasive breast cancer and 3,960 new cases of carcinoma -in -situ breast cancer are expected to occur in in USA in 2018 by the American Cancer Society (6).

Assessment of actual incidence of breast cancer in many developing countries is difficult due to under reporting of breast cancer and lack of national cancer registries or any other high quality databases. However, it is obvious that in developing countries, breast cancer is increasing, possibly as a result of increased life expectancy, changing population pattern, urbanization, time trends and adoption of the western life styles. According to one report, there is 1.7 % increase in cancer incidence rate among Asians (7) and by 2020 , seventy percent of the world's cancer cases will be in developing countries (8). This is a major concern as mortality due to breast cancer is already highest in low to

middle-income countries, due to the lack of early breast cancer screening programs, limited access to cancer treatment, and limited experienced medical personnel and laboratory diagnostic systems within the hospitals (9). It is therefore important in developing countries to find new approaches and solutions to help reduce the incidence of breast cancer that provide an affordable way forward.

1.3 Breast cancer rates in Pakistan

The actual breast cancer statistics in Pakistan are uncertain as there is no national cancer registry with country wide cancer database and due to failure of integrating hospital based cancer registries with the few population-based registries which were developed from time to time in the past but not sustained. However, the hospital

based data suggest an increasing incidence of breast cancer, with women frequently presenting at later stages of their disease (10). No national cancer incidence data from Pakistan is available since independence of the country in 1947 and available publications are based on small institution based cancer registries. However, these available data and studies in Pakistan are underpowered, and are single institute based studies with conflicting results and of limited value. Incidence data of the multiethnic population of the Karachi South district (1.7 million) was done for the first and only time by late Dr Yasmin Bhurgi. According to this Karachi Cancer Registry KCR , it was reported that Karachi had the highest incidence of breast cancer compared to any other Asian population (11). According to this report, breast was found to be most common site of cancer, with age standardized rate ASR 51.7 per 100,000, followed by oral cavity and ovaries. In a recent meta- analysis of seven small localized studies in Pakistan, the overall pooled prevalence of breast cancer estimated was 31% but there was considerable variation in the studies which were in different localities and involved populations of varying ethnicities (12). There seems a rapid rise in incidence of breast cancer which suggests an increasing role of environmental factors.

1.4 Classification of breast cancer

1.4.1 Histological types of breast cancer

Breast cancer is broadly divided into two categories, carcinoma in situ and invasive (infiltrating) carcinoma (13).

Carcinoma i**n situ consists** of two types: lobular carcinoma in situ (LCIS) and ductal carcinoma in situ (DCIS).

DCIS can be further classified into the following histological types:1) comedo, 2) solid, 3) cribriform, 4) papillary, 5) micropapillary

Invasive (infiltrating) carcinomas are a heterogeneous group of tumors of following histological subtypes: 1) Infiltrating ductal carcinoma (IDC), 2) invasive lobular carcinoma (ILC), 3) Ductal/lobular carcinoma, 4) Mucinous (colloid) carcinoma, 5) Tubular carcinoma, 6) Medullary carcinoma, 7) Papillary carcinoma.

1.4.2 Histological grades of breast cancer

IDC is further sub-classified into three grades on the basis of tumor nuclear pleomorphism, glandular or tubule formation and mitotic index. A scoring system is used with three points in each category and total score of 3-5, 6-7, 8-9 divided into three grades as follows:

- well-differentiated, total score of 3-5 (grade 1)
- moderately differentiated, total score 6-7 (grade 2)
- poorly differentiated, total score 8-9 (grade 3)

1.4.3 Molecular classification of breast cancer subtypes based on global gene expression

Breast cancer is classified into distinct subtypes (Table 1) based on gene expression profiles using microarray technology or RNA sequencing (14, 15). Estrogen-receptor (ER) positive and negative cancers are now considered as clinically and molecularly distinct diseases (16).

Table 1. Molecular classification of breast cancer subtypes based on global gene expression

	Luminal A	**Luminal B**	**Basal like**	**HER2 enriched**	**Normal breast like**	**Claudin - low**	**Molecular apocrine**
ER	91-100%	91-100%	0-19%	29-59%	44-100%	12-33%	ER-
PR	70-74%	41-53%	6-13%	25-30%	22-63%	22-23%	PR-
HER2	8-11%	15-24%	9-13%	66-71%	0-13%	6-22%	HER2+/-
Ki 67	low	high	high	high	low/intermediate	intermediate	High

1.5 Breast cancer subtypes risk

Breast cancer is a heterogeneous disease with different subtypes (17). On the basis of hormone receptor status and the more recent gene expression patterns there are six subtypes: luminal A, luminal B, human epidermal growth factor receptor 2 (HER2) enriched, basal like, normal breast like, claudin low and molecular apocrine (table 1). All these subtypes based on Estrogen and progesterone hormone receptor (ER/PR) protein expression status and human epidermal growth factor (HER2) protein expression have distinct etiology, risk factors, histopathology and clinical outcomes (18). Studies conducted mostly in western population report association of reproductive factors with hormone receptor-positive tumors and suggest that triple-negative breast cancer TNBC and basal-like tumors may have different etiology. TNBC is the most aggressive subtype of breast cancer and is more common in younger women (<50 years) (19).

The etiopathogenesis of breast cancer is still not fully understood. Breast cancer arises from genetic changes in mammary cells that drive abnormal cell division. Such genetic changes may be germ-line, (for example BRCA1 and BRAC2) or acquired (for example

p53 mutations). External environmental factors can modify oncogenesis at a number of concentrations, for example by inducing genetic changes or by altering the cellular and molecular environments within the breast. Identification of many genetic variants for breast cancer have been identified but the majority of published epidemiological studies are in western populations. However, these genetic studies does not ensure personalized prevention of breast cancer in most women (20). In addition, there are a number of life choices of a women that also can modify breast cancer risk. Identification of environmental factors and behavioral factors that influence breast cancer are critical as these provide possible approaches to reduce the incidence improve survival. It is important to undertake such studies in a variety of ethnic groups and in resource poor countries like Pakistan since there will be unique environmental and genetic factors that may modify breast cancer risk and outcomes. It will also help in identifying cost effective methods to reduce the burden of breast cancer in Pakistan.

In Pakistan the resources do not exist to enable genetic testing to identify major genes or SNPS associated with women at reduced or elevated risk of breast cancer. There are only very limited molecular and genetic studies in the Pakistani population to identify SNPs associated with breast (21, 22). Genome wide association studies in collaboration with the GWAS database, to explore and identify the roles of these genetic variants in breast cancer in the Pakistani population, are needed. A published study in African American shows that healthy lifestyle choice can reduce the incidence of breast cancer (23). On this basis, the focus of the research presented in this thesis is the identification of epidemiological and clinical factors that provide an opportunity to be modified for breast cancer prevention.

Age, nulliparity, late age at first child birth, lack of breast feeding, age at menopause, lifestyle factors like diet, lack of physical activity, use of oral contraceptives, smoking, body mass index, positive family history of breast cancer have been identified as risk factors for breast cancer (18). Delayed age at first live birth may be a contributing factor to the observed increase in breast cancer among younger women while there was a protective effect of high parity which was more significant among women with first live birth before the age of 20 years. Women who were of younger ages at the time of their

first and last births of babies, were associated with reduced breast cancer risk, with more association with maternal age at last birth (24).

Besides reproductive and familial risk factors, exposure to environmental factors also play important roles though such factors are not well resolved. One of the best documented is the increased rates of breast cancer among those residents of Hiroshima and Nagasaki who survived the atomic bombs in 1945 (25). The biological basis for the increase in breast cancer was during puberty rising concentrations of hormones cause increased proliferation of stem cell at the terminal end buds resulting in rapid expansion of breast epithelium and these cells are particularly sensitive to radiation (39). The exact molecular mechanism of carcinogenesis by radiation exposure is unknown but is related to changes in stem cell regulation following radiation exposure. Further details are mentioned in Chapter 3 on risk factors of breast cancer including genetic factors.

1.6 Vitamin D

1.6.1 Role of Vitamin D

Vitamin D is a fat-soluble steroid hormone and it has important roles in the regulation of calcium and bone homeostasis and also influences the absorption of phosphorus. Classically, low concentrations of vitamin D are associated with poor bone development, for example Rickets. Vitamin has important role in both skeletal and non skeletal functions (26). In recent years documented roles have also been demonstrated in other physiologic processes and diverse biological effects related to carcinogenesis are being investigated. Deficiency of vitamin D is now being associated with many diseases, for example cardiovascular diseases, osteoporosis, cancer, diabetes, and immune disorders (2). Vitamin D, has been ascribed as an antiproliferative and immunoregulatory agent in breast cancer involved in both incidence and progression and is a current area of active research interest. CYP27B1 is the enzyme responsible for the 1α-hydroxylation and is positively regulated by PTH, calcitonin, sex hormones, Prolactin PRL and Growth Hormone (GH), Fibroblast Growth Factor FGR and negatively associated with 1,25 OH_2 D, calcium, phosphate, thyroid hormones, glucocorticoids (27). These enzymes and

minerals involved in metabolism of vitamin D in the body and breast tissue may provide innovative targets for prevention and treatment of breast cancer.

1.6.2 Sources of vitamin D

The main source, approximately 80%, of Vitamin D in the body is produced via sun exposure through UVB light on the skin (28) Diet generally makes only a minor contribution and predominantly is derived from mainly from fatty fish like salmon, mackerel, tuna ,and in very small amounts from eggs, and liver of beef (29). Vitamin D fortification of diet has become common practice in many countries with foods like milk and oil being supplemented (30). In addition, cheap Vitamin D supplements in tablets and other forms are usually readily available and increasingly utilized (31).

Vitamin D, obtained from diet or from UVB sun exposure is converted to the circulating pro-hormone of 25-hydroxyvitamin D (calcidiol) by hydroxylation of Vitamin D in liver. In the kidney and other sites like the colon, prostate, and breast, 25-OH vitamin D is further hydroxylated to the most active form, 1,25dihydroxycholecalciferol (calcitriol) (32). Calcitriol binds and activates the Vitamin D receptor (VDR) present in all these sites and acts as a transcription factor to modify gene expression, affecting cell cycle proliferation, cell cycle arrest, differentiation, and apoptosis (32).

1.6.3 Potential mechanisms of action of Vitamin D in breast cancer

It has been suggested that 1,25 $(OH)_2$ D has potential anticarcinogenice effects, through stimulating apoptosis, inhibiting and slowing cell growth factors and improving cell cycle regulatory factors (33). Further, 1, 25 (OH) D may inhibit angiogenesis and down-regulate estrogen receptors (34). Activated Vitamin D exerts has its antitumor effects through Vitamin D receptor and expression of target genes such as c-fos, p21, p27, and c-myc (35). That is why, Vitamin D based therapeutics may be beneficial, for the treatment of breast cancer (36). Another biological plausible mechanisms of Vitamin D is through its important role in both innate and adaptive immune system (37, 38). Vitamin D analogs have potential for the treatment of cancer, like in other diseases (psoriasis, autoimmune diseases, and osteoporosis) (39).

1.6.4 Serum vitamin D concentrations

The Endocrine Society defines Vitamin D deficiency as 25(OH) D below 20 ng/mL and Vitamin D insufficiency as 25(OH) D of 21–29 ng/mL (34). Serum level above 30 ng/ml is sufficient, serum level of 40-60ng/ml is ideal and up to 100ng/ml is considered safe (40, 41). The Institute of Medicine (IOM) classifies concentrations of >20 ng/ml to be sufficient for the general population and it is on the basis of Vitamin D's effects on bone and minerals (42). However, there is ongoing debate that definition of Vitamin D deficiency be changed from 20 ng/ml to 30 ng/ml (43, 44). There is also seasonal variation in serum Vitamin D level with lower concentrations in winters.

The ideal and recommended serum Vitamin D level for cancer and other diseases prevention is still unknown and needs further clarification (45). Low Vitamin D <20ng/ml level is associated with multiple types of adverse musculoskeletal diseases and non-musculoskeletal health problems like cardiovascular diseases (46, 47), hypertension, type 2 diabetes mellitus (48) and cancers, osteomalacia, rickets, hypophosphatemia, osteoporosis, autoimmune diseases (e.g. psoriasis), infectious diseases(27). At present Vitamin D analogs are being used for treatment even for depression and chronic kidney disease (to treat secondary hypoparathyroidism) (29). There are multiple factors associated with Vitamin D deficiency (VDD) like less sun exposure during winter season, lack of awareness about UVB irradiation benefits, latitude, old age, dark skin, nutritional deficiency and sun avoidance lifestyle cause Vitamin D deficiency (49). Vitamin D concentrations are higher in summer with abundant UVB irradiation and lowest in late winter with lack of cutaneous synthesis of Vitamin due to less solar UVB (50). Vitamin supplements are given for both skeletal and non skeletal disease (51).

1.6.5 Metabolites of Vitamin D

Vitamin D is metabolized and the serum concentrations of the vitamin D metabolites are important in calcium homeostasis. Out of fifty metabolites of Vitamin D 1, 25 $(OH)_2$ D and 25(OH) D are the most important forms of Vitamin D and its assay methods (17).

Table 2 Two most important metabolites of Vitamin D

	25 (OH)D)	**1,25 $(OH)_2$D**
1	prohormone that is hydroxylated to 1,25 OHD	active hormone
2	The major form of serum vitamin D Serum concentrations three times higher than 1,25(OH)D	low serum concentrations
3	long half-life of 3 weeks	a short half-life of 4–6 hours
4	Not tightly regulated by thyroid hormone	tightly regulated in the kidneys and other tissues by serum calcium intake, PTH, cortisol,estradiol, calcium
5	Influenced by sun, season, fish intake,	Influenced by immobility,

Due to the short half-life and tight regulation by PTH and secondary hyperparathyroidism, assay of 1, 25 $(OH)_2$ D concentrations does not provide a consistent method for assessing Vitamin D status (table2). The concentrations of 1,25D are controlled by the rapid induction of the protein CYP24A1 which rapidly inactivates the active hormone. The accepted method is measurement of serum 25(OH)D and this is a more reliable biomarker for Vitamin D status (52).

1.6.6 Epidemiology of Vitamin D deficiency VDD

Due to less sunlight exposure with modern lifestyle factors, Vitamin D deficiency/insufficiency is becoming a major public health problem, causing many acute and chronic diseases. An important reason of Vitamin D deficiency is due to a lack of awareness that proper sun exposure in moderation is the major source of Vitamin D and is not harmful.

In the USA, despite widespread fortification of foods in USA with vitamin D at a national scale, 41.6% of women have vitamin D deficiency (<20ng/mL) (53). The prevalence of Vitamin D deficiency in Australians is also high with 40 % of women considered to be

deficient (54). Vitamin D deficiency is also high (42.1%) among people living in the sunny tropical Singapore (55). The prevalence of asymptomatic Vitamin D deficiency in healthy people is reported to be high (70-97%) in Pakistan and this is more common in people living in cities (56-58). In a Pakistani hospital based study, vitamin D deficiency was present in almost all (95.6%) women with breast cancer females (59).

1.7 Review of Epidemiological studies of Vitamin D and breast cancer

Despite the biological plausibility that Vitamin D has an anticancer role, the literature on the relationship between breast cancer and vitamin D concentrations remains controversial. While results from a number of cell line experiments, mouse studies, ecological studies, observational studies and some clinical trials indicate that Vitamin D has anticancer activity, other such studies fail to report any association between breast cancer and Vitamin D (60). Vitamin D functioning as a transcription factor through the vitamin D receptor has shown to play an important role in mammary gland development and function, is shown to be necessary and sufficient for tumor suppression in vitro experiments and in vivo models of mammary tumor cells extracted from mice (61, 62).

As the role of Vitamin D on incidence of breast cancer has been studied in many types of ecological, case control, cohort, cross sectional studies and few clinical trials, the literature review is divided on the basis of study designs and measurement of Vitamin D status by different measures.

1.7.1 Geographical ecological studies

Ecological studies on large population living in diverse countries including Europe, China, Australia, France Japan and the USA have consistently shown the protective effect of increased UVB sun exposure and latitude on breast cancer incidence and mortality (63-66). Such studies with strong support of the protective role of sun exposure on breast cancer incidence and mortality are shown in Table1. However, due to ecologic fallacy which is a bias in all ecologic study designs, due to data available at population level and not at individual level.

Table 3. Ecologic studies of breast cancer and Vitamin D

Reference	Author	Location	Measure of Vitamins D status	Result	Comments
(64)	Gorham et al 1990	USSR	Sun exposure	Protective effect on breast cancer incidence	(R = -0.75 p = 0.001).
(63)	Grant et al 2002	USA	Sun exposure	Protective effect on breast cancer mortality	
(65)	Ho, A.et al 2014	USA	Season and latitude	Decreased breast cancer in summer incidence	HR= 0.940, 95% CI= 0.93 to 0.94 P=0.002
(66)	Oh Y et al 2010	USA	Season and sun exposure	Protective effect on breast cancer incidence	High Vitamin D in summers

1.7.2 Observational studies

Results from case control, cohort and nested case control studies are summarized (Tables 4-8) according to the type of measure used for Vitamin D assessment. All these studies have different results with case control studies reporting association of Vitamin D and breast cancer more frequently than cohort studies. Prospective (cohort) studies investigating the role of Vitamin D in breast cancer have mixed results. However, both study designs have their own advantages and disadvantages. Bias of reverse causation is inherent in case control studies, for example, due to the timing of blood collection for assay of Vitamin D concentrations after breast cancer diagnosis the presence of breast cancer cells and catalytic enzymes may affect the assays. On the other hand, in cohort studies, the duration of follow-up is variable and may not be sufficient to observe any long-term effects of blood concentrations of Vitamin-D and cancer risk.

1.7.3 Studies showing association between serum 25(OH)D and breast cancer

These studies showing association of serum 25(OH)D and breast cancer incidence are shown in Table 4.

As mentioned before, serum 25(OH)D is the best biomarker of Vitamin D status. However, Bertone-Johnson et al. analysed both 25 (OH)D and $1,25(OH)_2D$ in a cohort study of Nurses' Health Study NHS II and reported that high concentrations of both metabolites of serum Vitamin D were protective against post-menopausal breast cancer and protection was greater in women of 60 years and above age. However, the results did not reach statistical significance. Overall the study findings showed role of modest association on breast cancer prevention (RR = 0.73, 95% CI = 0.49–1.07) (67). The negative association between serum Vitamin D concentrations and risk of breast cancer were confirmed in two studies in UK and USA. The findings of the UK study supported the hypothesis that low serum 25(OH) D concentrations increased the risk of breast cancer and there was a 50% less risk of breast cancer in women with a serum Vitamin D level of greater than 47 ng/ml. In the other study by Crew et al , serum Vitamin D concentrations were associated with 45% (OR = 0.56, 95% CI=0.41-0.78) decrease in breast cancer risk and this reduction was greater among postmenopausal women (68, 69). A population-based case-control study of 289 cases and 595 matched controls of premenopausal women in Germany, showed a significant inverse relationship between breast cancer risk and serum 25(OH)D level (70). In Japan, the Public Health Center-based Prospective Study showed higher serum 25(OH)D level was associated with lesser risk of all cancers, especially liver cancer but also including breast cancer in premenopausal women (71). Results of the French E3N cohort study also support a decreased risk of breast cancer with high serum 25(OH) D serum concentrations in women of younger age group (72). There was a stronger association of more aggressive subtype of breast cancer risk with Vitamin D deficiency among women of African ancestry (73) . Another study suggest that women with high serum 25(OH)D concentrations in summer season may have a decreased breast cancer risk though there was no overall association observed between breast cancer risk and concentrations of Vitamin D (74). There also a number of other studies supporting the association of breast cancer and Vitamin D status assessed by serum 25(OH)D: in US based study by Neuhoser a protective effect of high serum 25(OH)D concentrations was observed as an independent factor but results were inconclusive when adjusted for other lifestyle related factors while in another study while there was a similar association between serum

25(OH)D concentrations and breast cancer risk but there was an interaction with CYP enzymes. In a study in Spain, there was a significant protective effect between increasing serum 25(OH)D concentrations and lower breast cancer risk in dose-response manner. All these studies support the protective role of serum 25 (OH) D in breast cancer (75-80). Overall there are challenges in assessing breast cancer relationship with Vitamin D due to limitations like seasonal variation, different study designs, and complex metabolism of vitamin D.

Table 4 Observational Studies of serum 25(OH) D level and breast cancer

Ref.	Author	Type of study	Location	Number of participants	Comparison (Vitamin D measure)	Results	Comments
(67)	Bertone Johnson et al 2005	Nested Case control study	USA	701 cases 724 controls	Serum 25 OHD Quintile 5 vs. 1 1,25 OHD Quintile 5 vs. 1	RR= 0.73 p trend=0.06 RR= 0.76 p trend=0.39	For both metabolites, the association was stronger in older women >60 years of age
(68)	Lowe et al 2005	Case control study	UK	179 Cases 179 controls	Serum 25(OH)D Low vs. normal	OR=6.82 95% CI= 2.31-14.7	VDR also tested

Ref.	Author	Type of study	Location	Number of participants	Comparison (Vitamin D measure)	Results	Comments
(69)	Crew et al 2009	Nested CCS	USA	1026 cases 1,075 controls	25 OHD concentrations > 40 ng/ml vs. < 20ng/ml	OR=0.56 95% CI= 0.41-0.78.	decreased breast cancer risk
(70)	Abbas et al 2009	Case control study population based	Germany	289 cases 595 controls matched	Serum 25(OH)D 30-45 nmol/L 45-60 nmol/L >or=60 nmol/L	OR=0.68 OR= 0.59 OR= 0.45 p(trend) = 0.0006	Premenopausal women only
(71)	Budhathoki et al 2018	Nested Case control study	Japan	3301 cases 4044 controls	Serum 25(OH)D Quartile 4 vs. 1	HR = 0.81 P for trend <0.001	higher vitamin D level associated with lower risk of BC in Premenopausal women & total cancer
(72)	Engel et al 2010	Nested Case control study	France	Cases 636. Controls 1,272	Serum 25 OHD high vs. low	OR = 0.73 95% CI = 0.55-0.96 P trend = 0.02	PTH Ca, estradiol also tested
(81)	Yao et al 2013	Nested case control study	USA	579 cases 574 controls	Serum 25(OH)D ≥30 vs. 20 ng/ml	OR = 0.37 95%CI =0.27–0.51	More significant association with TNBC subtype
(74)	Eliason et al 2016	Nested case control study	USA	1,506 cases 1,506 controls	serum 25 OHD high vs. low	OR=1.20 95% CI=0.88–1.63	No overall association (summer time only)
(82)	Yousef et al 2013	Case control study	Saudi Arabia	120cases 120 controls	Serum25OHD (<20vs≥20 ng/ml)	OR=6.1 95% CI= 2.4, 15.1	Mean level cases= 9.4ng/ml controls=15.4 ng/ml

Ref.	Author	Type of study	Location	Number of participants	Comparison (Vitamin D measure)	Results	Comments
(75)	Palmer et al 2016	Cohort	USA	59000	Serum 25(OH)D Quartile 1 vs. 4	RR = 1.23 95 % CI= 1.04, 1.46	22% VDD in the 1454 cases
(76)	O' brien et al 2017	cohort	USA	1,611 cases / of 50,884	Serum 25(OH)D Quartile 4 vs. 1	HR= 0.79 95% CI: 0.63, 0.98	Supplements also decreased risk of BC among women (35-74yrs)
(78)	Mohr et al 2013	Case control study	USA	600 cases 600 controls	serum 25(OH)D Quintile 1 vs. 5	OR = 3.3 p trend = 0.09	only for blood sample within 90 days preceding BC diagnosis
(77)	Bilinski et al 2013	Case control study	Australia	214 cases 852 controls matched	Serum 25(OH)D severely deficient vs. deficient vs. insufficient vs. Normal	OR=2.3 95 % CI =1.3-4.3 OR= 2.5 95 % CI 1.6-3.9 OR= 2.5 95 % CI 1.6-3.8	Variables like sunlight exposure, vitamin D intake from food and supplements, BMI and skin pigmentation not addressed;
(79)	Kim et al 2014	Nested case control study	USA	707 cases 707 controls matched	Serum 25(OH)D3 and 25(OH)D High vs. low	OR= 0.28 95% CI= 0.14-0.56 OR =0.43 95% CI= 0.23-0.80	among whites population only, reside in low latitude regions
(80)	Shirazi et al 2016	Nested Case control study	Sweden	764 cases 764 controls	Serum 25(OH)D Tertile 3 vs. 1	OR=0.97 95%CI=0.75–1.25	women with low concentrations of 25(OH0D, compared to women in the middle tertile, had a high risk of breast tumours
(83)	Neuhoasr et al 2012	Case control study	US	1,080 cases, 1,080 controls	Serum 25(OH)D Low vs. high	OR=1.33 95% CI= 1.02, 1.72	unadjusted not adjusted OR

Ref.	Author	Type of study	Location	Number of participants	Comparison (Vitamin D measure)	Results	Comments
(84)	Colston et al 2006	Case control study	UK	179 cases 179 controls	25OHD < 50 nM vs. 25OHD > 50 nM	OR=3.54, CI= 1.89-6.61, p < 0.001	Only Caucasian
(85)	Lope V et al 2018	Case control study	Spain	546 cases 558 controls	Serum 25(OH)D High vs. low	OR =0.88 95%CI=0.82-0.94)	Dose response trend positive
(86)	Rejnmark et al 2009	Nested case–control	Denmark	142 cases 420 controls	Serum 25(OH)D Tertile 3 vs. 1	RR=0.52 95% CI= 0.32-0.85	No protective effect with dietary or Vitamin D supplements

1.7.4 Sun exposure (UVB irradiation) and Vitamin D status

Geographic locale of Karachi, with its latitude (24.8N), altitude (13 ft.) and longitude (67°02′E) provides abundant sunshine throughout the year and is ideal for studying fundamental aspects of the relationship between UVB radiation and breast cancer (87). Karachi weather is also sunny throughout the year, suitable for endogenous production of Vitamin D but vitamin D deficiency is still high. Sun exposure can also be used as a surrogate measure for Vitamin D status rather than directly assessing serum Vitamin D concentrations. The intensity of UVB exposure is less at higher latitude and also serum 25(OH)D concentrations are lower in winter than in summer (88). One important reason of Vitamin D deficiency vitamin D deficiency is air pollution which affects the ozone layer, and therefore decreases cutaneous production of Vitamin D (50). Among the white Caucasian population, an exposure to sunlight of 13 minutes to 35% skin surface area in summer is enough to attain insufficient Vitamin D status of 20-30 ng/ml (89). While a study in brown populations showed that in urban Indian men, >1 hour of sunlight exposure daily was required to maintain serum 25(OH)D concentrations above 50 nmol/L (90). In the elderly, the Vitamin D status is generally low compared with younger people and it also varies less with season (91).

1.7.5 Sun exposure (UVB irradiation) and breast cancer risk

Studies showing association of Sun exposure (UVB irradiation) and breast cancer are summarised in Table 5. Ecological studies as mentioned before have shown a protective effect of sun exposure against breast cancer (60). In the large Agricultural Health Study, results suggest that sun exposure may be protective against risk of Estrogen Receptor ER positive breast cancer (92). In the Women's Health Initiative Observational Study (WHIOS), women who spent less time outside in sunshine had a higher breast cancer risk (93). Another nation-wide breast cancer case–control study in USA showed reduced cancer risk with sunlight exposure during teen years, however, genetic background was also important (94). In a multiethnic population, a high sun exposure was associated with reduced risk of advanced breast cancer only among women with a light skin tone (OR =0.53, 95% CI = 0.31- 0.91) (95). Moreover, women diagnosed with breast cancer in summer or autumn have been shown to have improved survival rates compared with those diagnosed in winter showing a possible role of Vitamin D in breast cancer survival (96).

There was a small decrease in breast cancer risk observed with an average sun exposure ≥ 1 hour per day (versus < 1 hour per day) by Knight et al and this inverse relationship was more profound if the sun exposure was during early life time (97). Similar findings of breast cancer protections due to sun exposure was reported by Engel et al (92) and Anderson et al (98). A past cohort study confirmed the protective effect of SE and dietary intake of Vitamin D for breast cancer (99). A review paper has also showed the protective effect of sun exposure against breast cancer (100). Studies on the breast cancer risk by latitude and UVB radiation report an inverse association between 25(OH)D level and breast cancer and a strong negative association was observed between sunlight exposure and breast cancer incidence in the USSR (64). In conclusion, region of residence and geographic solar irradiance were found to be associated with risk of breast cancer. Overall, the measurement of sun exposure, timing and duration of UVB exposure and recall make it difficult to accurately correlate sun exposure with breast cancer and all studies have these limitations. Most of the studies do show protective effect of sun exposure on breast cancer risk except one by Vrieling

which also shows improved survival and decreased mortality due to breast cancer, however, these studies have limitations of observational studies, such as selection bias, information bias (besides recall) and the role of confounding.

Table 5 Studies showing association of sun exposure (UVB irradiation) and breast cancer

Ref.	Author	Type of Study	Location	No. of participants	Comparison (Vitamin D measure)	Results	Comments
(92)	Engel et al 2014	Nested Case control study	France	293 cases 586 controls	Sun exposure	HR = 0.8, 95% CI= 0.6, 1.0	Agricultural Health Study. VDR
(93)	Millen et al 2009	Cohort	USA	2,535cases/71,662 women	hours spent outside in daylight average <30 minutes versus >2hrs	increased risk of breast cancer 20% (95% CI, 2-41%; P(trend) = 0.001)	WHI 4 yrs. Follow up
(94)	Fuhrman et al 2013	Case control study	USA	282cases, 845controls	Two polymorphisms in CYP24A1 associated with increased BC,rs34043203, rs2762934 with reduced BC rs1570669	P(trend)=0.03; P(trend)=0.005 p(trend=0.048	Sunlight protection from BC depends on time and genes
(95)	John et al 2007	Case control study	USA	1,788 cases, 2,129 controls	A high sun exposure index (reflectometry)	OR= 0.53, 95% CI= 0.31, 0.91	No association with VDR FokI, TaqI, BglI
(96)	*Vrieling et al 2011	Cohort study	Germany	2,177 incident cases	women diagnosed with BC in summer or autumn had improved survival rates compared with those diagnosed in winter	RR= 1.72,95% CI=1.00,2.96	Serum 25(OH)D levels were associated with overall mortality

Ref.	Author	Type of Study	Location	No. of participants	Comparison (Vitamin D measure)	Results	Comments
(97)	Knight et al 2007	Case control study	Ontario Canada	972cases, 1135 controls	Time spent outdoor, (quartile 4 vs1)	OR= 0.65, 95% CI =0.50-0.85	Population based CCS showed exposure in earlier life more protection
(98)	Anderson et al 2011	Case control study	Canada	3,101 cases, 3,471 controls	Time spent outdoors, >21 vs. </=6 hours/week	OR = 0.71, 95% CI= 0.60, 0.85 teenage years, OR = 0.64, 95% CI= 0.53, 0.76, 20s-30s years RR = 0.67-0.85	OR = 0.74, 95% CI: 0.61, 0.88 40s-50s years OR = 0.50, 95% CI: 0.37, 0.66 60s-74 years)
(99)	John et al 1999	Cohort	USA	190cases/ 5009 women	Sun exposure, High vs low	RR = 0.67-0.85	NHANES, Small sample

* Only study with survival as outcome

1.7.6 Vitamin D supplementation and breast cancer

Studies showing association of Vitamin D supplementation and breast cancer are summarised in Table 6. Vitamin D supplementation is economical, effective, and safe intervention that needs more research to confirm its role in breast cancer prevention. There are some data indicating a possible benefit from Vitamin D supplementation, but these are far too sparse to support a definite conclusion. A French Cohort study showed that current use (not past use) of calcium and Vitamin D supplements were associated with a lower risk of developing breast cancer in postmenopausal women, but not in pre-menopausal (101). In another study, Vitamin D supplement intake > 400 IU/d compared with no intake was found to be independently associated with reduced breast cancer (OR= 0.76, 95% CI= 0.59- 0.98) (102). The Iowa Women's Health Study has reported a small decrease in risk of breast cancer with Vitamin D intake of >800 IU/d among postmenopausal women only. The protective effect of Vitamin D was highest in the first 5 years after baseline assessment of total intake but this protective effect decreased over time (103). A French cohort study showed the protective effect of high dietary Vitamin

D against breast cancer (104). Vitamin D in diet had protective effect in breast cancer risk (P for trend = 0.002) among women with normal weight in Taiwan (105). Two other case control studies by Rollison et al and Rossi et al also supported the protective effects of Vitamin D supplements (106, 107).

Table 5 Studies showing association of Vitamin D supplementation and breast cancer

Ref.	Author	Type	Location	No. of participants	Comparison (Vitamin D measure)	Results	Comments
(101)	Cadaea et al 2015	Cohort	France	2482 cases /57,403 women	Vitamin D supplements use (combined with ca) vs no use	HR: 0.82 95% CI: 0.69- 0.97	No mention of vitamin D supplement dose and duration Supplement intake was self-reported
(102)	Anderson et al 2010	Case control study	Canada	3101 cases 3471 controls	Vitamin D supplements vs no use	OR= 0.76 95% CI: 0.59- 0.98	Population based CCS
(103)	Robien et al 2013	Cohort	USA	34,321	Vitamin D >800 IU/day vs <400 IU/day	OR = 0.89 95% 0.77–1.03	postmenopausal women only
(104)	Engel et al 2011	Cohort	France	2,871 cases/67,721	Dietary Vitamin D intake high vs low supplemental intake high vs low	HR = 0.68 95% CI: 0.54-0.85 HR = 0.57 95% CI: 0.36-0.90	No mention of vitamin D supplement dose
(105)	Lee et al 2011	Case control study	Taiwan	200 cases 200 controls matched	dietary Vitamin D intake (Q2-Q4 vs Q1)	OR= 0.46 95% CI, 0.23-0.90 p for trend = 0.002	Reduced breast cancer risk in normal weight women only
(106)	Rollison et al 2012	Case control study	USA	1,527 NHW & 791 Hispanic cases 1,599 NHW & 922 Hispanic controls	Vitamin D supplement 10+ mug/day vs. none	OR = 0.79 95% CI = 0.65-0.96 p (trend) = 0.01	Matched CCS

Ref.	Author	Type	Location	No. of participants	Comparison (Vitamin D measure)	Results	Comments
(107)	Rossi et al 2009	Case control study	Italy	Cases=2569, controls= 2588	Dietary Vitamin D intake High vs. low	OR= 0.79 95% CI 0.70-0.90	Intake of vitamin D >3.57 µg or 143 IU was protective against breast cancer
(97)	Knight et al 2007	Case control study Population based	Ontario Canada	972cases 1135 controls	cod liver oil use milk >or=10 glasses /week vs none	OR= 0.76 95% CI= 0.62-0.92 OR= 0.62 95% CI =0.45-0.86	Exposure in earlier life offered more protection

1.7.7 Studies showing no association of Vitamin D with breast cancer

Studies showing no association of Vitamin D with breast cancer are summarized in Table 7. Due to diversity between types of studies, populations, variation in serum Vitamin D concentrations, not all observational studies have shown the protective effect of Vitamin D with breast cancer (108-110) and shown no association shown between breast cancer and dietary Vitamin D in a prospective study in Europe (109). In a cohort study from 1965-1976, it was found that there was no difference in the prediagnostic concentrations of $1,25(OH)_2D$ between breast cancer cases and their matched control subjects (110). Those studies showing no protective association of Vitamin D with breast cancer did not address a possible protective effect of serum Vitamin D at a time more proximal to breast cancer diagnosis (78). A possible reason for no association in such studies could be that a protective effect of Vitamin D on breast cancer has been more often associated with breast cancer in studies with shorter time intervals between Vitamin D level measurement and breast cancer diagnosis (111). A nested case–control study of the Nurses' Health Study II (NHSII), does not support any association of serum level of Vitamin D and breast cancer risk (112) or Vitamin D binding protein DBP with breast cancer risk in premenopausal women (113). However, $1, 25(OH)_2D$ due to short half- life is not a good biomarker for Vitamin D status. Other studies also show no association with breast

cancer (113-117). A study by Kuhn in Europe and another by Scarmo in USA did not support any association of serum Vitamin D and breast cancer (118, 119). There was no role of Vitamin D observed in recurrence of breast cancer in a study done by Jacobs et al (120).

Table 7. Studies showing no association of Vitamin D with breast cancer

Ref	Author	Location	Type of study	Comparison (Vitamin D measure)	Comments
(121)	Almquist et al 2010	USA	Nested case–control study	serum 25 (OH)D Quartile 4 vs 1	764 cases and 764 controls 10-15 yrs. f up. Adjusted for PTH, Cr, P Ca was associated with BC Calculated both total 25 (OH) D and 25OHD2& D separately
(108)	Freedman et al 2008	US	Nested case–control study	Serum 25 (OH)D 1, 25(OH) D_2	Short mean f up of 3.9 yrs. only
(109)	Abbas et al 2013	Europe	Cohort study	Dietary intake Vitamin D & Ca (quintiles)	Follow up of 8.9 yrs.10 European countries Serum vitamin D, sun exposure, supplements intake not measured Dietary intake of vitamin D not a good measure of vitamin D status
(110)	Hiatt et al 1998	US	Nested case–control study	prediagnostic concentrations of $1,25(OH)_2D$	follow-up was 15.4 yrs. 25 (OH) D not measured
(112)	Eliason et al 2011	USA	Nested case–control study	Serum 25 (OH)D	613 cases, 1218 controls large sample size of premenopausal women

Ref.	Author	Location	Type of study	Comparison (Vitamin D measure)	Comments
(114)	Amir et al 2012	US, Canada, Australia, Puerto Rico, Ireland	Nested case–control study	Serum 25(OH)D	C-reactive protein The National Surgical Adjuvant Breast and Bowel Project (NSABP)
(115)	Edvardsen et al 2011	Norway	Cohort	Sun exposure SE Vitamin D intake	984 cases/41,811 8.5 years F up
(116)	McCullough et al 2009	USA	Nested case–control study	Serum 25 (OH)D	Matched CCS but unconditional logistic regression
(117)	Chlebowski et al 2008	USA	RCT	Serum 25 (OH)D vitamin D supplementation	Primary outcome hip fracture Follow up of 7 yrs. No association with calcium too -
(118)	Scarmo et al 2013	USA	Nested case control	Serum 25 (OH)D	Premenopausal women
(119)	Kuhn et al 2013	Europe	Nested case control	Serum 25 (OH)D Quartile 4 vs 1	inverse association between 25(OH)Dlevels and breast cancer only in women taking hormone replacement therapy
(120)	Jacobs et al 2011	USA	Nested case–control study	serum 25 (OH)D /diet/supplements	No association with recurrence

1.7.8 Studies of Vitamin D and breast cancer done in developing countries

The few studies done in India, Iran Pakistan and Jordon are shown in Table 8. In a study in India there was a significant association of low serum 25(OH)D concentrations with breast cancer (122).Two hospital based studies in Iran Vitamin D deficiency was

associated with increased risk of breast cancer (123, 124). Two studies done in Pakistan had only descriptive analysis in one (125) and small sample size in another study (126). The study done in Jordon had small sample but showed association of breast cancer with vitamin D deficiency (127).

Table 8. Studies of Vitamin D and breast cancer done in developing countries

Ref	Author	Type of study	Location	Number of participants	Comparison (Vitamin D measure)	Results	Comments
(122)	Sofi et al 2018	Case control study	India	100 cases 100 controls	serum 25 OHD low vs normal	OR= 2.5 p<0.005	Dietary vitamin D also measured
(123)	Salarabidi et al 2015	Case control study	Iran	45 cases 105 controls	Sun exposure dietary intake	lack of sun exposure and low Vitamin D intake were significantly associated with premenopausal breast cancer risk	Small sample size
(124)	Bidgoli et al 2014	Case control study	Iran	60 cases 116 controls	Sun exposure high vs low dietary intake	OR=10.1 P =0.007	OR=0.232 CI 95%= 0.06-0.80 egg intake
(125)	Imtiaz et al 2014	Case control study	Pakistan	100 cases 100 controls	Serum 25(OH)D	99% VDD among cases vs 90% VDD among controls	Only descriptive results
(126)	Shaukat et al 2017	Case control study	Pakistan	42 Cases 52 controls	Serum 25(OH)D Low vs High	OR =7.8 CI=1.99 - 30.58	small sample Mean age 40.1years
(127)	Atoum et al 2017	Case control study	Jordan	122 cases 100 controls	Serum 25(OH)D Low vs high	OR= 22.72 95% CI= 10.06-51.29	VDR TaqI genotypes

1.7.9 Randomized controlled trials

There is dearth of well-designed and conducted large rrandomized controlled trials (RCTs) to assess the relationship between serum Vitamin D concentrations and the incidence of breast cancer. Of the positive trials, an RCT by Lappe et al in 2007

showed a protective effect of calcium plus 1100 IU Vitamin D in all cancer types (including BC) among postmenopausal women(or= 0,232, p <0.005 (87). In the Women's Health Initiative (WHI) study, the use of Vitamin D supplements showed a decreased risk of breast cancer among women using Vitamin D compared with women not taking Vitamin D supplements (128). However, overall there remains the same problem that some clinical trials support the results of association studies, while some RCTs do not support the association studies regarding cancer prevention by Vitamin D. Some of the limitations of such RCTs are inadequate sample sizes and limited time of follow-up (40). A very large sample of women would need to be randomized to various doses of Vitamin D and followed for many years to get enough breast cancer cases which would be feasible only in large existing cohorts.

1.7.10 Meta-analysis

Meta-analyses provide the opportunity to combine a number of independent studies to increase the overall power of studies. In a meta-analysis of prospective studies of serum 25(OH)D suggested there was a protective effect of serum 25(OH)D among postmenopausal women with a step-wise inverse association between breast cancer and serum 25(OH)D observed beyond 27 ng/ml serum 25(OH)D level in a dose response manner (129).

There have been several meta-analyses of case controlled studies. One study combining data from case control studies of seven countries showed the beneficial effect of high serum serum 25(OH)D against breast cancer (130). There was no more protective effects above 35 ng/ml in dose-response meta-analysis of prospective studies (131). Another meta-analysis by Chen et al showed that vitamin D and calcium have a protective effect against breast cancer (132). According to Chen et al, a link existed between certain cancers and geographic latitude, with an association between increased sunlight exposure and low breast cancer incidence and mortality. While one meta-analysis showed the protective role of Vitamin D in breast cancer (133), another meta-analysis failed to show any protective association from diet and supplements but suggested that an inverse association may be possible at higher intakes of Vitamin D (134). In a meta-analysis by Hong et al, a serum level of 30 ng/ml of Vitamin D was

associated with decreased risk of breast cancer (135) The protective association has also been observed in postmenopausal but not in premenopausal women in another meta-analysis (136). A recent Lancet review paper did not provide any association of Vitamin D with breast cancer but it excluded cross sectional and case control studies along with ecological studies in the review (137). In another meta-analysis, there was no association between Vitamin D supplements and breast cancer (138). A meta-analysis of 14 studies including 9110 breast cancer cases and 16,244 controls showed protective effect of serum Vitamin D on breast cancer risk (RR=0.84, 95 % CI=0.750–0.951) (139). It also reported that a 3.2 % reduction of breast cancer risk with every 10 ng/ml increase in serum Vitamin D level ($p<0.001$) (139). A meta-analysis by Wang et al reported no protective effect of 25(OH)D on breast cancer risk (139). Overall most of the meta-analyses results provide evidence of a chemopreventive role of Vitamin D against breast cancer but there is a need of further RCTS.

1.7.11 Critical appraisal of the literature review

There were some methodological issues, for example, type of study design, methods and timings of vitamin D exposure measurement, status of pre or postmenopausal women, and lack of variability in vitamin D levels:

Study designs

Ecological studies show a strong association of 25(OH) D and UVB irradiation with breast cancer prevention. There were more case control studies findings with positive association of vitamin D deficiency and increased risk of breast cancer compared to cohort studies. Prospective (cohort) studies investigating the role of Vitamin D in breast cancer have mixed results. However, both study designs have their own advantages and disadvantages. Bias of reverse causation is inherent in case control studies, for example, due to the timing of blood collection for assay of Vitamin D concentrations after breast cancer diagnosis the presence of breast cancer cells and catalytic enzymes may affect the assays. On the other hand, in cohort studies, the duration of follow-up is variable and may not be sufficient to observe any long-term effects of blood concentrations of Vitamin-D and cancer risk. There are limited numbers of RCTs and

the main problem with many of the current published RCTs is insufficient study length relatively to breast cancer-related outcomes. New randomized trials should be organized with better methodological tools and a focus on vitamin D metabolism and biological activities combined with role of calcium and other biomarkers like 1, 25 OH D, PTH which may help in determining more conclusive results of the chemopreventive role of vitamin D. Moreover, the sample size of the studies needs to be larger, more consistency in the baseline levels of vitamin D in the enrolled subjects, better compliance of the enrolled subjects with the intervention of vitamin D supplements, avoidance of contamination of the placebo group and longer follow-up periods. The latter factor presents a particular difficulty as the time lag between start of vitamin D supplementation and any effect on breast cancer incidence is unknown and it is possible maybe decades.

Measurement of vitamin D exposure

Main source of vitamin D is sunlight exposure, however, the accurate measurement of such exposure for a human body over time is an extremely difficult. Questionnaires as used this study are approaches but are not free from biases. Dietary intake of vitamin D measured by using a food frequency questionnaires (FFQ), is also not a good measure to assess vitamin D as both are confounded by season and lifestyle factors such as vitamin D supplements intake, genetic polymorphisms, complex vitamin D metabolism and comorbidities such as osteoporosis. Measurement of circulating 25(OH)D is the best biomarker of Vitamin D exposure.

Timing and frequency of vitamin D exposure assessment

Another important issue is that most studies reported a single serum 25(OH) D concentration measured at time of enrollment to assess vitamin D status. But there are changes in the serum level over time which may affect the findings and single measurement approach and this may underestimate the benefit of vitamin D

The majority of the studies identified in the literature review are case control studies with vitamin D measurement after the diagnosis of breast cancer and the all lack causality or temporality. In cohort studies vitamin D is measured prediagnostically but

there was variation in time interval between the time of vitamin D measurement and diagnosis of breast cancer.

Lack of systematic assessment of vitamin D and seasonal variation

Standardization of the commercial assays for serum 25(OH) D concentration was lacking in few studies. The adjustment for season to capture the seasonal variability in vitamin D exposure was not reported in many studies.

Comorbidities affecting vitamin D metabolism

The presence of comorbidities like osteoporosis, diabetes, renal diseases (nephrotic syndrome), malabsorption (celiac disease or inflammatory bowel diseases) that may affect vitamin D were not considered and discussed in many studies.

Menopausal status of study participants

Reporting of menopausal status varied in different studies with some studies not taking this into account while some studies suggested different association of vitamin D deficiency with breast cancer in pre- and postmenopausal women.

Variability of the vitamin D exposure and breast cancer in different study population

Some studies done in Asian populations report low levels of vitamin D in their study populations with lack of variability. This may contribute to non-significant associations between vitamin D levels and disease. Those studies that reported vitamin D exposure as quartile or tertiles had different results than studies using pre-defined categories for Vitamin D levels where a larger proportion of women in the lower vitamin D categories resulted in either null associations or associations by chance.

Measure effect

Some studies reporting inverse associations had very small measures of association between breast cancer and extremes of vitamin D concentrations, whether this small

difference in concentration could be causal to breast cancer is unclear. Not all studies addressed all potential confounders, including factors that influence vitamin D status.

Hormonal receptor status of breast cancer

Only few studies examined the association between vitamin D levels with breast cancer stratified by receptor status.

Other biomarkers like PTH, calcium, phosphate, magnesium, albumin, creatinine, and alkaline phosphatase levels & 1, 25$(OH)_2$D

Only few studies adjusted for calcium intake, and reported different strength of the association between vitamin D and breast cancer with different calcium levels. Serum 25(OH) D should be considered in combination with clinical information and serum calcium, phosphate, magnesium, albumin, creatinine, and alkaline phosphatase levels and a 24-h urine calcium excretion. Further studies are warranted to determine whether adjustment for calcium and PTH levels is needed when reporting associations between vitamin D and breast cancer. In women diagnosed with breast cancer, the changes in the breast tissue microenvironment, including vitamin D signaling pathway, may not necessarily be restricted to the immediate affected area and could and could influence vitamin D metabolism in normal ipsilateral and contralateral breast tissue. Thus, the findings in women with a history of breast cancer might not be directly applicable to healthy (cancer-free) women.

Other factors

In addition, the levels of vitamin D during the times of most intensive breast tissue development, such as at puberty and pregnancy, maybe critically important. Further studies are warranted to determine causal relationship between the vitamin D and breast cancer and to understand the timing of exposure that might be important for breast cancer prevention.

Interpretation

Some published studies demonstrated a potential benefit for high levels of vitamin D as protective for breast cancer while in other studies there was no consistent or strong

evidence of protective effect of vitamin D against breast cancer, whether measured through circulating concentrations, supplement intake dietary intake or sun exposure as proxy measures. There is need of further research as there is a biologic plausibility of an antitumor breast cancer effect of vitamin D but there are, as discussed above, limitations of the existing studies. In conclusion, based on the current evidence, one cannot reach to a definitive conclusion for or against the role of vitamin D in influencing the incidence of breast cancer.

Conclusion

In conclusion, the role of vitamin D in breast cancer prevention is supported by biological mechanisms and animal studies but the human epidemiologic associations remain inconsistent. The published studies are heterogeneous in their study designs, methods and results from different ethnic groups and countries. Further high quality large population based studies with international collaborations are warranted to investigate the associations while addressing the methodological issues discussed in this review. More recent advances in laboratory sciences may also help in better application of modern analytical techniques to the assessment and measurement of Vitamin D levels directly in the breast tissue, thus allowing investigation of the relationship between the tissue Vitamin D metabolites and breast density. Considering the high prevalence of vitamin D deficiency more funding should be diverted for cost effective interventions to increase vitamin D levels with recommendations to women to consider dietary vitamin D supplementation or sensible sun exposure to achieve optimal levels. There is a need to design and conduct a large international collaborative prospective cohort study of high income, middle-income, and low-income countries with sufficient study length to provide definitive evidence of association of breast cancer with vitamin D and dose adequacy of vitamin D.

1.8 Rationale

Given the growing burden of breast cancer in Pakistan and lack of studies examining the association between vitamin D and breast cancer risk in Pakistani women, this study was undertaken to evaluate evidence for the potential preventive effect of vitamin D on the

risk of breast cancer among the Pakistani population. In addition, this study investigated if various other were associated with breast cancer in Pakistani women. The long-term aims of this study are to identify factors that can be easily and cheaply applied to reduce the incidence and burden of breast cancer in Pakistan.

The, overarching aim of this study was to improve the understanding of the relationship between different sources of vitamin D and breast cancer among women in Pakistan.

Details of introduction and rationale of objectives sub studies 1, 3, 4 & 5 are mentioned in the relevant chapters.

1.9 Objectives of five sub studies

The objectives of the five sub-studies of Pakistani women which form the basis of this thesis are:

Objective 1. To assess risk factors for breast cancer.

Objective 2. To evaluate the association of serum vitamin D (25(OH) D) level, supplementation of vitamin D and sun exposure with breast cancer.

Objective 3. To evaluate the risk factors associated with triple negative breast cancer (TNBC) and non-TNBC subtypes.

Objective 4. To evaluate the association of diet with breast cancer using the modified assessment tool AHEI 2010 and its component scores.

Objective 5. To evaluate patient delay in breast cancer diagnosis, its associated factors and stage of diagnosis.

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Chapter 2: Materials and Methods

2.1 Study Setting

Karachi is the 5th most populous city in the world. Its estimated population is 24 million in 2015. Karachi is growing at a rate of around 5% per year, due to rural to urban migration in the area every month from all over in Pakistan. The city spreads over 3,527 km2 (1,362 square miles) in area(140). Oncology and surgery clinics of the two major public and private hospitals were included:

- Aga Khan University Hospital (AKUH), Karachi

In 2000, AKUH became the first teaching hospital in Pakistan to be completely ISO 9002 certified. It serves not only Karachi, but also, patients from outside Karachi with a mix of urban and rural patients.

- Karachi Institute of Radiation and Nuclear Medicine Hospital (KIRAN), Karachi

It is a leading referral and cancer treatment public hospital, which is under Strategic Planning Department (SPD) of Pakistan Army and provides financially supported diagnosis and treatment facilities to more than 75% of its patients, who come from all over the country, on welfare basis.

2.2 Study Design

A matched case control study was conducted in tertiary care hospitals of Karachi; Aga Khan University and KIRAN cancer hospital. This study design was chosen because it is the best for investigating the relationship of Vitamin D and multiple other factors with breast cancer, which has usually a long latency period, within a short time period in a cost effective manner.

Case-control study has certain disadvantages like recall bias, lack of temporality and selection bias (141), which are weaknesses of this study design. Further details of addressing relevant biases are in results chapters.

Under the guidance of thesis supervisors, Dr Uzma Shamsi designed this study and data collection methods, coordinated data collection and lab analyses, managed and analysed the data and interpreted the results.

2.3 The Pilot Study and Pretesting

In the first phase, a pilot study was undertaken to pretest the complete questionnaire and the Food frequency questionnaire (FFQ) in Jinnah Postgraduate Medical Center JPMC and AKUH. In this phase, data collection was carried out on a sample of 50 subjects prior to the main study data collection in order to ensure standardization and reliability of the questionnaire. It comprised of patients attending the outpatient department of JPMC and AKUH between Aug 2014-Jan, 2015. Those subjects were not included in the main study. The questionnaires were then revised and finalized based on the pretest results. These were minor changes that involved rephrasing of some questions to increase clarity and omitting redundant questions to shorten the interview time. Other changes were logistics relating to data collection, remote desktop access, medical records registry, and tracking of laboratory reports of radiology, histopathology and biochemistry and extraction of medical records which were evaluated for missing information.

2.4 Pretesting of FFQ

It was also done on 50 subjects to generate a food list used in our population. Some additional questions were added relating to Vitamin D containing foods. The modified version of FFQ based on this pretesting was used for conducting face-to-face interviews with the study participants. Interviews for cases were conducted in hospitals while waiting in breast cancer clinics, whereas controls were interviewed at outpatient departments. Index date for cases was the date of diagnosis of breast cancer and for controls; it was the date of interview. Information on Vitamin D intake was ascertained by including foods with Vitamin D and questions were also aimed at assessing portion sizes to calculate nutrient analysis, fat intake and general dietary habits.

2.4.1 Data collection Period

Feb 2015- July 2017

2.4.2 Case definition

All women who were newly diagnosed with a first, primary breast cancer between Feb 2015-July 2017 were eligible as cases (International Classification of Diseases code 174 and code C50 from the Ninth Revision and Tenth Revision, respectively).

2.4.3 Eligibility criteria;

- Above 20 years of age at the time of diagnosis
- No history of BC or any other cancer.
- Hospital-reported cases of histologically confirmed invasive cases of breast cancer were identified directly from the hospital records.

2.4.4 Exclusion criteria

- Women who were extremely sick and unable to complete the interview.
- Women who had been living outside Pakistan for more than a year
- Women who were diagnosed with breast cancer more than 6 months before the enrolment to avoid preclinical bias and reverse causality.
- Women who were receiving adjuvant or neoadjuvant chemotherapy or radiation.
- Those who were lost to follow up during their metastatic and lab work up.
- Those women who had nonepithelial breast tumors (ICD-O histology code 8800)
- Those who did not give consent.

2.4.5 Control definition

For every patient, two controls free of any cancer diagnosis were matched by age (year of birth $\pm$ 5 years), region of residence in the same geographic area and study site from Surgery, and general medicine clinics of AKUH & KIRAN. Control subjects were eligible if they had not had a diagnosis of either breast cancer or any other cancer. The controls were enrolled from surgery who came for follow up of mammography screening, with benign breast symptoms. Controls sourced from general medicine clinics controls had a range of medical conditions, for example diabetes, hypertension, headaches and anxiety. Controls were matched with cases from the same hospital to minimize selection bias since they generally were of similar ethnic and social economic backgrounds.

2.5 Sample size

In total, 411 Breast cancer case patients and 784 control subjects were enrolled.

2.5.1 Sample-size calculation

The number of enrolled cases and controls subjects was decided based on certain assumptions. We assumed the prevalence of deficiency of Vitamin D and other risk factors amongst the control group to be in the range of 10-90%. In an audit of blood samples, the vitamin D deficiency noted was 66.1% (142). In order to be able to detect an odds ratio of at least 2 with a power of 80%, at a significance level of 5% and considering a 1: 2 ratio between cases and controls, we calculated a sample size of 400 cases and 800 controls. However, in the final achieved sample, few stratas had 1:1 case control ratio.

Study subjects' recruitment methods

This research project comprised of five sub-studies, which are specifically outlined below.

2.6 Case ascertainment

Women (a mix of urban and rural) with breast cancer were recruited from surgery and oncology clinics and were all evaluated by a surgeon/oncologist. Detailed physical examinations were carried out for any lumps or suspicious areas, texture, size, and relationship to the skin and chest muscles. If breast symptoms were suggestive of breast cancer, patients were usually referred for a radiological examination of the breast and axillae. A baseline bilateral mammogram and an ultrasound scan of the breast were ordered and used as a reference for all future breast imaging. In cases where an abnormality was visible on mammogram and ultrasound, a core biopsy was carried out by the consultant radiologist or surgeon. Furthermore, all newly diagnosed patients were tested for the markers ER, PR/, and HER2/*neu*. In a limited number of cases, the proliferative rate of the tumor was evaluated by the Ki-67 antigen.

In this hospital based case control study, the control participants come from the same source population as the cases. Control women for the study, therefore, were recruited from those attending in- and outpatient services for general medical, and surgical

departments of the participating hospitals of Karachi. The research assistant identified those patients meeting the eligibility criteria for controls. They were similarly given the patient Information Sheet and consent form and informed consent was sought.

Patients who were potentials cases and controls were identified on the basis of inclusion criteria from the patients list. This was a multistep process consisting of clinical examination, radiological examination and histological examination as described above. The follow up data of consecutive patients was analysed by research assistants who reviewed their medical records from AKUH, KIRAN and outside on their consecutive visits until completion of their diagnosis and metastatic work up. The medical records were also accessed through the SAHL database (AKUH Clinical Systems Ltd) at AKUH, which contains information on clinical, radiological and pathological data for patients attending AKUH. Some breast cancer patients who participated in study were later excluded from the study if they did not comply with all the investigations and were lost to follow up.

The STROBE* (*The Strengthening the Reporting of Observational Studies in Epidemiology) standardized reporting guidelines for case control studies were followed to ensure standardized conduct and reporting of the study.

2.7 Data Collectors

The data collectors hired in the study were all medically qualified doctors and were designated as research assistants. They were recruited from medical personnel with the qualification of a MBBS medical degree consisting of at least 5 years' undergraduate medical education, and had interest in medical research and two of them were was interns at AKUH. A one-day training workshop was also arranged and conducted by PhD candidate Dr Uzma Shamsi for the research assistants to ensure that the interviews would be conducted in a uniform fashion. All the questions were explained in detail. The informed consent and interview process was also explained with emphasis on the conduct of interview in a polite, sensitive and time efficient manner. Primary surgeon /oncologists/s had no role in the selection process other than only informing the patients about the study and their right for voluntary participation.

2.8 Questionnaire

All participants were interviewed face-to-face using a structured questionnaire assessing various sociodemographic, clinical, lifestyle, and dietary characteristics (see appendix A). Demographics, reproductive history (including breastfeeding), medical history (including hormone use and body size), family history of malignancy, and lifestyle factors like lifetime tobacco consumption and lifetime physical activity were included. Physical activity was assessed using the International Physical Activity Questionnaire (IPAQ) index that has been validated within the Pakistani population (143). Subjects were asked to recall the number of days and hours or minutes, they engaged in physical activity of different intensities for at least ten minutes, vigorous exercise and moderate exercise like mopping etc. and walking. Smoking was assessed by participants "Have you ever smoked?" type of tobacco usage, duration of tobacco usage and environmental tobacco use. Questions related to exposure to passive smoking and use of antiperspirants were also added and asked in a sub sample. The time frame of all exposures asked was the period of one year prior to the disease among cases and one year prior to the index year among controls.

All efforts were made done to avoid selection bias by

- proper selection of cases and controls
- controls were selected independently of the exposure status, and reflected the exposure of the population which gave rise to the breast cancer cases,
- high response rate and good participation in both cases and controls,
- precise case definition and exposure definition
- explicit eligibility criteria preventing participation bias
- best methods of measurement of all the variables using validated questionnaire and ELISA assay
- blind assessment of laboratory personal testing for vitamin D ensured comparability of information in both cases and controls.

- standardized, and uniform data collection procedures.

2.9 Data collection method for objective one (chapter3)

Objective: To evaluate risk factors associated with breast cancer

For this purpose, the oncologists/surgeons of the hospitals were informed about the starting date of data collection in their respective hospitals. It was ensured that the research data collection would not intervene with the clinics pace. Following a confirmed diagnosis of BC, potential cases of BC were identified by trained research assistants at the subsequent hospital visits. Those patients, who met the inclusion criteria, were approached by research assistants during their waiting in period in the Medicine, Oncology and Surgery clinics. Informed consent was obtained using the Patient Information Sheet and Consent form (Appendix B), approved by both the Human Research Ethics Committee of the University of Adelaide and the three Karachi hospitals. Participants were clearly informed about the objectives and procedures of the study, their rights and commitments, and the benefits and risks involved. If the patients agreed to participate they were asked to sign the consent paper. The consent form of the study was provided both in English and the local language of Urdu. If unable to read the consent form, the form was read out to participants and verbal consent to participate was obtained. From others signatures were taken. Participation in the study was voluntary. The study participants had the right to withdraw from the study at any point in time, without any harm or prejudice to their treatment. After consent, participants were interviewed in a separate room for privacy.

The control women for the study were recruited from those attending in- and out-patient services for general medical, surgical and oncology departments of the participating hospitals of Karachi. These control subjects were women with no previous diagnosis of breast cancer or any other cancer. Informed consent was obtained if they met the eligibility criteria. All consenting control participants were similarly interviewed in a separate room to ensure privacy using the same structured questionnaire as cases with breast cancer.

The questionnaire was divided into different sections of sociodemographic history, information about diet, Vitamin D supplements, and past medical and obstetrical history, family history, anthropometric measurement

Reproductive / medical history

A detailed reproductive, gynecological and medical histories, were asked of all participants and confirmed from medical records. Respondents were asked about their age at start of menstrual periods (menarche), their number of pregnancies (including live births, stillbirths, miscarriages, abortions, tubal and other ectopic pregnancies as well as this pregnancy if they were currently pregnant), number full term pregnancies, their age at birth of first full term pregnancy, their last full term pregnancy, and their last pregnancy. Reproductive history also included history of preeclampsia, menopausal status, age and mode of menopause. Menopause was defined as permanent cessation of menstruation for a year or more after the last menstrual cycle on the basis of menopause definition in the Oxford handbook (144). Menopausal status was divided into premenopausal and menopausal status. Women were also asked if their menstrual periods have stopped completely, naturally in a normal way or after surgery.

The total number of weeks or months of breastfeeding for each baby was recorded and added to calculate lifetime breastfeeding. Women were also asked about use of oral contraceptive pills (OCPs) emergency morning after pills or birth control injections for birth spacing, and duration of usage in months or years, if their menstrual periods had stopped completely (naturally or after surgery), the age when menstrual periods stopped and any use of HRT. Participants were asked about the number of mammograms undertaken during the last 10 years. Finally, any past history of a benign breast lesion or any breast biopsy, removal of a lump, or an aspiration of any cyst abscess, were recorded. A detailed medical history including any other health problems and time since diagnosis was also recorded. A question on consanguineous marriage of the parents was also included (Pakistan has one of the highest rates of consanguinity in the world).

Information was also collected about socio demographic and reproductive factors as:

- ✓ Ethnicity, level of education, marital status, place and type of residence, number of dependants and current employment status.
- ✓ Education was treated as an ordinal variable and categorized as <8, 8-12, or > 12 years of full-time study. Education as a proxy for socioeconomic status is very important.
- ✓ Socio-economic (SES) factors included level of education, place and type of residence, crowding index, home ownership, and number of rooms, total household members and monthly income. Crowding index was also calculated as number of household members divided by number of rooms. It was further categorized as <1, 1-2, >2
- ✓ Factor analysis was used to identify the important variables for socioeconomic status and a composite variable was calculated for socioeconomic status which was further categorized into upper, middle and poor.
- ✓ Participants self-reported their family history of breast cancer as none (no first-degree family history), or one first degree relative and age at diagnosis of breast cancer, or multiple affected first degree relatives or second degree relatives and ages at breast cancer diagnosis.
- ✓ Other variables assessed included body mass index (BMI), comorbidity (defined as being treated for cardiovascular disease, diabetes mellitus, asthma, depression, thyroid, kidney, liver, bone disease etc.), history of benign breast disease, use of hormones (contraceptives or hormone replacement therapy) during the year before diagnosis. BMI cut off values used were based on WHO criteria and The International Association for the Study of Obesity and the International Obesity Task Force (145, 146)

Invasion and Metastasis

One of the most fatal aspects of breast tumours is their tendency to spread into the surrounding mammary tissue and invade other sites in the body away from the primary site in the form of metastasis. Combining the results of physical examination and biopsy, plus the results of surgery (called the pathologic stage) the pathologist–oncologist

described the stage of breast cancer of the patients, based on the American Joint Committee on Cancer (AJCC) TNM system. The TNM staging system classifies breast cancer based on its T, N and M stages. The letter T followed by a number from 0 to 4 describes the tumor's size, the letter N followed by a number from 0 to 3 indicates whether the cancer has spread to lymph nodes near the breast and, if so, how many lymph nodes are affected, and the letter M followed by a 0 or 1 indicates whether the cancer has spread to distant organs. Once the T, N, and M categories had been determined, this information would be combined in a process called stage grouping where stage is expressed in Roman numerals from stage I (the least advanced stage) to stage IV (the most advanced stage). Information was also recorded for the investigations up to the date of diagnosis (month and year) for case patients. The presence of lymph node metastasis was evaluated by histologic examination of sentinel lymph nodes biopsy, obtained at mastectomy in patients. Bone metastasis was evaluated by bone scan. CT scan and PET scan reports for whole body were also recorded.

Anthropometric characteristics

Body weight (in kilograms) and height (in meters) were recorded at the time of the blood draw (in addition to the assessment at the initial interview) and body mass index calculated as weight (in kilograms) divided by standing height (in meters squared). Overweight and obesity were defined as body mass index 23–25 kg/m ^{2} and >26 kg/m^2, respectively. All other potential confounders were those collected prior to the diagnosis date among cases or prior to the date of interview among controls.

2.10 Data collection method for objective two (chapter 4):

To determine the association of Vitamin D (serum Vitamin D (25-hydroxyVitamin D) level, Vitamin D supplementation and sun exposure) with the risk of breast cancer, all these variables of serum Vitamin D, Vitamin D supplements, and sun exposure score were used.

Measurement of serum Vitamin D concentrations

All enrolled subjects were asked to provide a blood sample for assessment of vitamin D.

Sample collection & transport

Venous blood samples (2.5 ml) was collected in yellow topped gel tubes by research assistants, from breast cancer patients within 30 days of diagnosis of cancer and prior to chemotherapy, and from control, at the end of the questionnaire interview. The collected blood was transported on the same day to Section of Clinical Chemistry, Department of Pathology & Laboratory Medicine, at AKUH.

Sample processing & analysis:

Samples were coded and then centrifuged, serum separated, aliquots were prepared and stored at -80˚C until analysis. Serum 25(OH) D concentrations was measured by a kit from Diametra, Via Garibaldi, 18-20090 SEGRATE (MI) Italy, using solid phase enzyme-linked immunoassay (ELISA) based on the principle of competitive binding. Low and high Vitamin D control samples provided with the kit were run with each batch for validity of the assay. For quality control, maximum consistency between methods, protocol and personnel was ensured. The AKUH laboratory participates in proficiency testing held by College of American Pathologists. A single measurement of Vitamin D was determined.

Blood test results were generally available after 4-6 weeks. Among breast cancer patients, participation rate was high (90%), however, there were some refusals and some opted for blood test at later follow-up visits. Such cases were not pursued for a subsequent blood test if they had delayed the test and had already started chemotherapy or were unable to provide a sample before the end of the study. Vitamin D testing participation among controls was higher (100%) than cases. Optimal Vitamin D concentrations were taken as 30–100 ng/ml while concentrations of 21–29 ng/ml as Vitamin D insufficiency and < 20 ng/ml as Vitamin D deficiency.

Total 25-Hydroxyvitamin D
<20 ng/ml = Vitamin D deficiency
21-29 ng/ml = Vitamin D insufficiency/ mildly deficient
>30 ng/ml = Vitamin D sufficiency/ optimal

Vitamin D supplementation

The use of Vitamin D supplement (both injections and oral), regular once-a-day multivitamins, and calcium (alone or in combination), were assessed in relation to the index year. Doses of calcium, and multivitamin name and dose were also determined. Vitamin D supplementation was assessed by asking participants if they had been taking Vitamin D supplements regularly, occasionally or not at all in their lifetime, with the mode of administration identified as either injections, oral tablets or ampoules. Similar information was used to assess use of multivitamin, name of the multivitamin, and calcium supplementation. Herbal medications which possibly increase Vitamin D inactivation was also taken into consideration. Medication history included intake of fertility drugs (clomiphene), or other hormone replacement therapy (HRT). In order to determine if there were possible drug interactions, participants were asked whether they had taken anti-hypertensive, anti-epileptic, anti-inflammatories and endocrine drugs.

Sun exposure measurement questionnaire

The geographic location of Karachi served as a surrogate measure for ultraviolet B (UVB) exposure. The latitude (24.51'N), and longitude (67°02′E) and elevation (8 metres) with abundant sunshine throughout the year influence UVB exposure and provides a good biological model for studying fundamental aspects of the relationship between UVB radiation and breast cancer.

To measure sun exposure among cases and controls, the validated long term sun exposure measurement questionnaire (LT SEM-Q) was used. This questionnaire determines the exposure over a typical week in the previous year for summer and winter seasons

separately. The questionnaire was validated against gold standard Ultraviolet (UV) dosimeters, and was purchased and analysed at the University of Southern Queensland, Queensland, Australia (147). A major strength of this questionnaires is that it tries to estimate retrospective sunlight exposure throughout a patient's lifetime, which is especially relevant for chronic diseases like breast cancer that develop over many years.

The questionnaire determines personal and atmospheric factors that affect UVB radiation exposure and Vitamin D synthesis such as UVB intensity, exposure duration to the sun, skin tone of the individual, use of sunscreens and other cosmetics, other sun avoidance behaviour like seeking shade under trees/building, clothing, hats, and glass/windows. Other factors such as atmospheric pollution, and latitude depend on the urban or rural setting and province of the enrolled subjects were addressed by matching cases and controls on region of residence.

Skin tone of the participants was assessed against a shade card by matching shade of the skin on the inner side of the forearm (unexposed part) of the participant and forehead

(exposed part) with the shade on the card, according to LT SEM-Q, to match the skin tones of Asian population (table 1).

Table 1 skin tone card and scoring

10 scale	skin tone	Type
1-2	Pale white to white	I
3-4	White to light brown	II
5-6	Moderate brown	III
7-8	Dark brown	IV
9-10	Very dark brown	V

Scoring for estimation of sunlight exposure (SE) of individuals takes into consideration all factors listed in the questionnaire (see Appendix A: section H) The final scoring algorithm of sun exposure score in summers and winters was created by multiplying the

time (minutes) spent in the sun by the proportions of the different variables as given in Table 2.

Table 2. Weights given to sun exposure variables

Variable/item	**Weights given**
Part of the body exposed based on attire used outside	1 if exposed (100% UVB absorption) 0 if covered (0 UVB absorption) 0.5 if partially covered(50% UVB absorption)
Use of sunscreen on different parts of body	1 if no use (100% UVB absorption) 0.08 if sunscreen (8% UVB absorption)
Sun avoidance behavior	1 if no protection practices (100% UVB absorption) 0.4 if seeking shade under trees/building etc. (40% UVB absorption)
Weather outdoors	1 if sunny (100% UVB absorption) 0.5 if cloudy (50% UVB absorption) 0.75 if sunny/cloudy(75% UVB absorption)
Skin tone	0.8 if Type 1 (80% UVB absorption) 0.675 if Type II (67.5% UVB absorption) 0.55 if Type III (55% UVB absorption) 0.425 if Type IV (42.5% UVB absorption) 0.3 if Type V (30% UVB absorption)

Further details of sun exposure scores are given in chapter 4 in the relevant section of sun exposure and breast cancer.

2.11 Data collection method for objective three (chapter 5):

To evaluate the risk factors associated with triple negative breast cancer TNBC and non TNBC subtypes. For this sub study we included 321 patients who had complete clinical profiling of the markers ER, PR and HER 2/*neu* status. In addition to accessing results from medical records, and using SAHL a remote desktop access system from the clinical laboratories of AKUH and KIRAN for some patients' results were also sourced from

laboratories external to these two hospitals. There were missing values for receptor status from 192 breast cancer patients and these were not included in this sub-study. Cases with HER2 results of 0, 1+, or 2+ from IHC testing and/or a negative result on FISH testing <2 were considered HER2 negative (HER2−); conversely, HER2 results of 3+ on IHC testing were considered HER2 positive (HER2+). Patients who had a 2+ HER2 immunohistochemistry result without a FISH result were considered to have an inconclusive and, thus, unknown HER2 status. 798 controls were used as referents to evaluate risk factors associated with tumor subtypes.

Breast tumor subtypes were then classified into four groups: ER+ and/or PR+/HER2−; ER+ and/or PR+/ HER2+; ER−/PR−/HER2+; and ER−/PR−/HER2−. However, our analyses focussed primarily on comparisons between TNBC and non TNBC tumours, therefore, ER+ and/or PR+/HER2−, ER+ and/or PR+/ HER2+, ER−/PR−/HER2+ were merged as non TNBC group. These groups were examined in conjunction with the potential confounders and sociodemographic characteristics which were identified from the main study.

2.12 Data collection method for objective four (chapter 6):

To evaluate the association between the modified AHEI 2010 and its component scores with breast cancer risk, dietary intake assessment methods were used.

Dietary intake assessment

Dietary intake assessment was by individual interviews of 1124 cases and controls, conducted by trained doctors using the validated food frequency questionnaire FFQ (148) (see Appendix A: section I).

It was pretested on 50 subjects before the actual study and certain additional foods items possibly related to breast cancer were included following a literature search. Index date for cases was the date of diagnosis and for controls it was the date of interview. For both cases and controls, dietary history was the usual diet in the one year prior to the date of diagnosis or interview date. Prior to the interview, informed consent was obtained from all study participants. Completion of the FFQ took an average of 35-40 minutes.

Each participant was asked about their dietary intake of 53 food items over the past year. In the case of breast cancer patients, it was the year prior to diagnosis to reduce any bias due to possible changes in diet after cancer diagnosis. Intake frequency was categorised into 7 groups ranging from "never" to "5-6 times per day" for foods and for beverages. The selected frequency category for each food item was converted to a daily intake. For example, a response of "one serving/week" was converted to 0.14 servings/day by dividing 1 by 7. Each participant was also asked about their average portion size/ common serving size of the food. A common serving (svg) size of food item or beverage was specified on the FFQ (e.g. 1 plate of pulses or 1 cup of milk or one egg, or natural unit such as one apple). The intake frequencies were multiplied by standard portion size to calculate servings per day of all food items. However, consumption in grams per unit or macronutrient intakes or calories from each food was not calculated.

Modified Alternate Healthy Eating Index scoring criteria

The eating indices for dietary assessment tools were used to measure diet quality holistically and full details are in chapter 6. Briefly, the Healthy Eating Index-2010 is a dietary assessment tool used to measure diet quality and provides an objective, quantitative measure of diet quality (149). The Healthy Eating Index-2010 has twelve components: nine that assess nutrient adequacy (fruits, vegetables, grains, dairy, total protein foods and plant proteins) and three that address moderation (refined grains, sodium and empty calories). Total score is the sum of component scores and higher scores indicate greater compliance with the 2010 Dietary Guidelines for Americans. The Alternate Healthy Eating Index (AHEI) 2010 for this sub study was modified according to dietary behaviors particular to our adult Pakistani population. After discussion with a nutrition researcher and a biostatistician to categorize specific dietary groups, we used 6 components contributing 0–10 points to the total score (10 indicated highest score of recommendations that were met, zero was the lowest that they were not). Intermediate intakes were scored proportionally between 0 and 10. The 6 components included were fruits, vegetables, dairy, grains, white to red meat ratio, and plant proteins. Alcohol, multivitamins, sodium, fat components were excluded from the original AHEI 2010. Alcohol was excluded since being strictly prohibited in Pakistan, it is not consumed at

all by women. As nutrient analysis was not done in this study, therefore, sodium and fat components were also excluded.

The modified AHEI score was calculated from each completed FFQ. Food items listed on the FFQ were assigned to their appropriate food groups. Modified AHEI variables and scoring decisions were made and modified as in the case of plant proteins where a >2 serving per day was considered as ideal instead of 1 serving per day as in the original AHEI 2010. Full details of criteria for scoring the AHEI 2010 are also mentioned in chapter 6. Briefly, the six highest intake components of AHEI-2010 are considered to be ideal (vegetables, fruit, grains, white to red meat ratio, dairy, and plant proteins (nuts and legumes). The rationale for including each component and the criteria for assigning the minimum and maximum scores are described in the Table 1 of the Annexure 1.

2.13 Data collection method for objective five (chapter 7):

To evaluate patient delay in breast cancer diagnosis, its associated factors and stage breast cancer at the time of diagnosis among breast cancer patients, certain variables related to delay were defined. Patient delay was the primary outcome which defined as the time between the appearance of any of the first symptom of breast cancer, for example, a lump or nipple discharge, and the date of initial consultation for diagnostic mammography, ultrasonography, or medical consultation for breast symptoms. No delay was defined as patients seeking medical advice for their breast cancer symptoms in a month of finding any symptoms. Delay was defined as any time greater than one month for patients seeking medical help for diagnosis after noticing possible symptoms of breast cancer. Diagnosis time is defined as the date of the first symptoms to the date of final breast cancer diagnosis based on histopathological examination (including needle biopsy or excisional biopsy) or on FNAC (fine needle aspiration cytology)**.** The initial consultation date is defined as the date of diagnostic mammography or diagnostic ultrasonography or the date of a consultation for breast symptoms.

In this sub study, we evaluated how Pakistani women present with breast cancer, the frequency and magnitude of delay in diagnosis, the factors associated with delays, and the relationship between delays and disease stage in breast cancer and all this information was obtained from 514 newly diagnosed breast cancer cases (Appendix A:sec J).

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Patient delay was measured in months before the first medical consultation. Stage of disease was categorized according to TNM staging. In order to minimize recall bias, the study participants were asked to remember the onset of symptoms and the day of first consultation. The respondents were reminded of events in the calendar year, such as such as religious and national occasions, school holidays, festival celebrations, Independence Day, or birth dates, to help them remember important dates relative to their medical history. An agreement was decided after discussion with the respondents when there were conflicting dates of events.

The initial sign or symptom of breast cancer was determined for each study participant as:

- The appearance of any breast lump
- Nipple discharge
- Breast pain
- Skin changes
- Changes in breast shape
- Breast ulcer
- Arm edema
- Axillary lymph nodes
- Others

Mode of detection was determined for each study participant as:

- Breast self-examination for a lump, nipple change, or any other breast abnormality which is self-detected accidentally.
- Clinical breast examination which is the detection of an abnormality by a clinician

- "Imaging-detected" by a screening mammogram or other imaging test.
- "Detected based on systemic symptoms" for example weight loss or fatigue.

Patients were asked about the action taken by them at the appearance of breast cancer symptoms and who was the first person they contacted to evaluate and diagnose their symptoms.

The following reasons for delay were asked as Yes/No questions:

- Fears related to breast cancer diagnosis and treatment,
- Possible shame and embarrassment,
- Misconceptions and myths related to breast cancer,
- Family commitments,
- Denial,
- Lack of awareness,
- False negative tests (a false-negative mammogram or fine needle aspiration or core biopsy that was reported normal even though breast cancer was present)
- Use of traditional methods of treatment,
- Financial constraints,
- Fear of husband's rejection
- Provider delay defined as the delay by the providers in breast cancer diagnosis, ignoring the symptoms of breast cancer when patients consulted them.

Information was collected on breast tumor characteristics including:

- Histopathology, type, and grade of the tumor
- Stage of disease categorized according to Tumor-Node-Metastasis (TNM) staging sourced from pathology reports (150)
- Receptor status of the tumor as ER/PR/Her 2 *neu*.

Risk

As it was an observational study, there was no intervention needed and no risks involved other than some psychological discomfort in some cases. All the patients were under care of their treating clinicians to take care of their diseases and related issues. Each interview took not more than 45 minutes. In spite of breast cancer diagnosis nearly all the cases were willing to participate and were supportive of the research project. As mentioned before, all the research assistants /data collectors were female medical doctors with ample undergrad training of medical history taking with female patients and to deal any emotionally distressed patient during the interview. Participating patients appreciated their efforts in comforting and reassuring them and answering to their queries regarding breast cancer. The phlebotomy done on all patients were without any problem.

Benefit

The study participants were informed of their serum Vitamin D concentrations free of any cost and at the end of interview they were educated about the sources and role of Vitamin D in health.

2.14 Ethical approval

Ethical approval of this study was taken from Human Research Ethics Committee (HREC) of the University of Adelaide, the Ethical Review Committee (ERC) of Aga Khan University Hospital (AKUH), and ERC of KIRAN cancer hospital (Appendix C).

2.15 Data management and confidentiality

The questionnaires were kept in a secure locked filing cabinet at AKUH, only accessible to the study team. For anonymity, all participants were assigned a unique 6-digit study identification number and confidentiality was maintained by using the study ID number in the study database. Additional logic checks, data cleaning and derivation of all variables was conducted by the PhD candidate. Follow-up, by contacting the laboratories and hospitals outside AKUH, was also undertaken for the few patients transferred elsewhere, in order to obtain the detailed pathology reports. The data will be kept for 4 years after the end of the study.

2.15.1 Data editing

The data collector undertook field editing of the questionnaires at the end of each interview. All the variables were double checked using the original hard copy questionnaires. Laboratory results were confirmed using remote desktop access of SAHL at AKUH. In some cases, patients were directly contacted to provide missing information. In particular, this included missing information on the clinical markers ER and PR, and HER-2/ *Neu.*

2.15.2 Validation of data entry

Data was double entered in the Epi data version 3.1 using two independent data entry operators. The entered data was checked according to Community Health Sciences Information Support Unit policy (CHS ISU, AKUH). A consistency check of the two data sets was also performed.

2.16 Statistical analysis

Normally distributed continuous variables are presented as mean values ± standard deviation, non-normally distributed variables as median and quartiles and categorical variables as frequencies. Associations between categorical variables are tested using chi-square tests. Comparisons between normally distributed continuous variables were performed using Student's t –test and in the case of non-normally distributed variables, associations were evaluated using the non-parametric U -test suggested by Mann and Whitney. Correlations between continuous variables were evaluated using the Pearson's r or Spearmen rho coefficients. Odds ratios of having breast cancer were determined using matched conditional logistic regression analysis with goodness-of-fit using the Hosmer–Lemeshow statistic.

All reported p-values are based on two-sided tests. SPSS 22.0 software (IBM Statistics, Armonk, New York, USA) was used for all the statistical calculations. Potential confounders considered for different sub studies and analyses included BMI, SES, physical activity, breastfeeding [continuous variable for total number of weeks breastfed; and categorical variable with categories (breastfed/parous, never breastfed/parous, nulliparous)], total number of full-term pregnancies (continuous variable for completed pregnancies >26 weeks), family history of breast cancer in a mother, sister or daughter

(first degree family history: yes/no), education (less than high school graduate, high school graduate, some college or technical school, college graduate) and menopausal status (pre/post-menopausal). Further details of statistical analysis done, are mentioned in each relevant chapter.

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Chapter 3: Breast cancer risk factors among women in multicenter case control study in Karachi Pakistan

3.1 Background

Lifetime risk of developing breast cancer in women all over the world in general is 12% (1 in 8 women) (7). Breast cancer shows wide variation from country to country. It is highest in Belgium with age-standardized rate (ASR) per 100,000 of 131.1, while in USA it is 84.9. Historically, Japan had the lowest ASR but this is now increasing and is expected to approach a similar rate to the USA (151). This suggests that there are important environmental and genetic factors, influencing the risk of breast cancer. According to the SEER (Surveillance, Epidemiology, and End Results) database of USA, breast cancer incidence is highest in the white population and lowest in Hispanics (152). Over the last few decades breast cancer incidence has been noticed to be stable in developed countries but on rise in developing countries including Asian American women in California (153). In Karachi Pakistan breast cancer has been too reported to be increasing and the current ASR is reported to be 51.7 per 100,000 with morbidity and mortality also reported to be elevated (11). This increase may be due to the changing prevalence of established risk factors associated with changing lifestyle behaviors and environmental factors among Pakistani women.

Etiology of breast cancer is complex due to an interaction of genetic, biologic, lifestyle, environmental, and sociodemographic factors with substantial literature evaluating the roles of these modifiable and non-modifiable factors for breast cancer (154). The major reproductive and lifestyle factors identified are ageing, age at first birth and menopause, early menarche, lack of breast feeding, obesity, unhealthy diet, alcohol and tobacco use, radiation, and family history of breast cancer that influence breast cancer risk and survival (155). There are certain other well-established risk factors related to prolonged exposure to higher estrogen concentrations like age at menarche and menopause (156), age at first live birth (157), parity and use of hormones (157). It is noteworthy that temporal changes in the reproductive behavior like parity and late age at first birth may partially explain the increasing breast cancer incidence. Ethnicity influences breast cancer risk and this is likely to be due to both differences in environmental factors and genetic factors (both major and minor gene mutations). Environmental factors that have been linked with breast cancer include diet, physical activity, infant birth weight and early lifestyle factors during adolescence (158). Identifying modifiable risk factors

provide opportunity to reduce the incidence of breast cancer in a population. For example a study at the Brazilian National Cancer Institute Hospital (159) suggest 17% of postmenopausal breast cancer could be prevented by adopting a healthy lifestyle of physical activity, weight control, healthier diet and smoking cessation.

Reproductive history and breast cancer age at breast cancer diagnosis

Relationship between age and breast cancer is complex due to the lifetime variable estrogen-receptor concentrations within and between individual women both before and after menopause.

The average age at presentation of breast cancer in Pakistan is at least 10 years earlier than in the western population. In a hospital-based study in Karachi, Pakistan, the mean age at breast cancer diagnosis was 46 (SD 10.1) years , and about two-thirds of women with breast cancer were younger than 54 years (160). The mean age of breast cancer in patients in USA and Europe is approximately 63 years with lesser percentage of patients younger than 50 years (161).

Age at first birth (AFB)

Studies show that parity is protective for breast cancer and the risk is modified by age at first live birth and possibly by breastfeeding (162). In one million Norwegian women, younger ages at first and last births both were associated with reduced risk of breast cancer, with age at last birth being slightly stronger (24). In the Women's Contraceptive and Reproductive Experiences Study, young AFB (< 20 years) had a 36% reduced risk of lobular breast cancer compared with women who started their families at an older age (> 30 years). This protective effect was observed only in ERPR-positive breast cancer cases (163).

Breastfeeding

Breastfeeding is an important modifiable risk factor, and overall studies suggest that breastfeeding is protective against breast cancer. In an Italian study, breastfeeding either less or more than 1 year, compared to no breastfeeding, was protective (HR 0.24; 95% CI 0.09-0.66; p = 0.005) & (HR 0.25; 95% IC 0.08-0.82; p = 0.022 respectively (164).

In other studies, longer breastfeeding duration among Italian and Korean women reduced the odds of breast cancer (165, 166). In the extensive Nurses' Health Study NHS of 1976 among 121,700 women and the NHSII of 1989 among 116,430 women in United States, breastfeeding was found to be protective against basal-like tumors, an aggressive subtype of breast cancer (167). Possible explanations for this protective effect are that breastfeeding reduces the lifetime exposure to estrogens by inhibiting menstruation and also structural changes in the breast that follow lactation and weaning (168).

Family history of breast cancer

Family history of breast cancer is an important established risk factor of the disease attributed to shared genetic factors and environmental exposures. The genome-wide association studies (GWAS) have identified about 25 genetic loci linked to breast cancer (169). The risk of breast cancer among women with a BRCA1 or a BRCA2 mutation was up to 87% in a multiracial population of Malaysia (170).

Familial breast cancer is associated with a number of high to low-penetrance susceptibility genes, for example, BRCA1, BRCA2, PTEN, and TP53. Other genes implicated are involved in DNA repair, for example, CHEK2, ATM, BRIP1 (FANCJ), PALB2 (FANCN) and RAD51C (FANCO). However, the majority of Genome-wide association studies (GWAS) were done in Western populations, and such studies are necessary in other ethnic groups as disparities in allele frequencies and gene-environment interaction exist (171). Results of the National Cancer Institute of Naples, Italy, showed that the first-degree relative and second degree relative of a woman with breast cancer both have an increased risk of breast cancer (172). In NHS it was also confirmed that a family history of breast cancer before the age of 50yrs was associated with a higher breast cancer (relative risk RR =1.69, 95% CI 1.39-2.05) and for age 50yrs and above (RR= 1.37 (1.22-1.53) (173). Similarly, those who were users of oral contraceptives with positive family history had a higher risk of breast cancer (174).

Previous benign breast disease (BBD)

BBD has three major histologic types: non-proliferative lesions, atypical proliferative lesions, and atypical hyperplasia. The latter two types have been shown to increase the

risk of breast cancer in some studies (175) . On the other hand, no association was observed between BBD and breast cancer in the Nurse health study cohort study (173). In the Mayo Clinic study of breast cancer and BBD, no increased risk was found among women with no family history of breast cancer and non-proliferative BBD findings while positive association was only found in women with a family history of breast cancer (175). Another study of breast cancer risk in women with BBD suggests use of HRT in women with atypical hyperplasia was associated with an increased risk of breast cancer, while the risk was reduced in postmenopausal women with BBD (176). The risk of HRT also depend on breast density which increases risk of breast cancer(177). Overall one weighs the benefits vs risks while deciding for use of HRT.

Exposure to oral contraceptives (OC) and hormone replacement therapy (HRT)

Numerous research studies including Collaborative Group on Hormonal Factors in Breast cancer study have confirmed the increased breast cancer risk with hormone replacement therapy (HRT) use (178, 179). In another study, the impact of HRT use on breast cancer risk varied depending on ethnicity, BMI, and breast density (180). In Denmark, there was 20% higher risk among women who were current users of OCs (181). However, a systematic review of 10 studies and one pooled study of 54 studies showed no association of increased breast cancer risk with use of OCs (182).

Diet

Role of diet and breast cancer has been evaluated and discussed in detail in Chapter 6.

Physical activity

Several studies have suggested increased physical activity is associated with a reduced risk of breast cancer. A population-based cohort study in China supports the protective of high physical activity or household chores on risk of breast (adjusted HR: 0.73, $P<0.05$) (183). Moderate physical activity had also a protective effect in a nested case-control study of non-BRCA-related breast cancer risk among French-Canadian women (184). A cohort study of Finnish females confirmed that life-long physical activity reduced the risk of breast cancer (185). Another prospective study of 47,649 Sister Study participants observed a reduced risk of breast cancer with occupational physical activity (186).

Birth weight

It has been reported in new studies that giving birth to an infant with high birth weight was associated with higher risk of breast cancer in later life (187, 188).However, this association between birth weight and overall breast cancer risk is controversial and such findings are not consistently reported in other studies (189).

Body mass index (BMI)

BMI has been reported to influence breast cancer risk but there are different effects in pre-menopausal and post-menopausal women, also varying with tumor subtype. In a population based case control study in the Seattle-Puget Sound metropolitan area, women with a BMI increase of 10 kg/m^2 or above doubled their risk of breast cancer (190). In another study, BMI >/= 24 kg/m^2 was positively correlated with PR+ breast cancer among post-menopausal breast cancer cases only (adjusted OR=1.420, 95% CI= 1.116-1.808 (191). It has been suggested that the influence of obesity varies in the different breast cancer subtypes through complex pathways of carcinogenesis (192). These findings, however, are not replicated in other studies as in a meta-analysis with 3,318,796 participants from 31 articles, a high BMI was not associated with decreased risk of breast cancer in pre-menopausal women (193).

Height and breast cancer

Reports on possible relationships between anthropometric characteristics especially height and breast cancer risk among women are controversial. A meta-analysis of data from 159 prospective cohorts provided strong evidence of association of breast cancer with height (pooled RR=1.17, 95% CI = 1.15 -1.19) per 10 cm increase in height. This association was found in both premenopausal and postmenopausal women but observed only in hormone receptor–positive breast cancer. The same study also identified height-associated variants at eight new loci associated with breast cancer risk, including three loci at 1q21.2, DNAJC27, and CCDC91 ($p < 0.001$). It confirms that certain genetic factors and biological pathways involved in adult height, have important role in the etiology of breast cancer (194). On the other hand, a population-based, case-control study

in the Seattle-Puget Sound metropolitan showed that height at age 18 years was not related to the risks of any breast cancer subtype (195).

Smoking

It is well established fact that active smoking increases the risk of not only lung cancer but also breast cancer. An increased risk of breast cancer in smokers was confirmed in a USA study (196). Exposure to passive smoking has also been shown to increase the risk for breast cancer, as shown in studies of non-smoking Israeli Arab (197). In Japan, husbands smoking was associated with breast cancer risk in their wives due to passive smoking (RR= 1.98, 95% CI= 1.03-3.84) (198). In another study among Caucasian women, lifetime exposure to passive smoking was associated with the risk of breast cancer with the strongest association in postmenopausal women (199). This study showed increased risk was associated with longer duration to environmental tobacco smoke ETS among women exposed to it at work (OR= 1.01, 95% CI= 0.72-1.41), at home (OR=1.88, 95% CI= 1.38-2.55), and at both home and work (OR= 2.80, 95% CI= 1.84-4.25). Many carcinogens like polycyclic aromatic hydrocarbon, found in tobacco smoke, may contribute to the development of breast cancer in humans (200).

Radiation

Several studies have shown an increased risk of breast cancer following radiation exposure Breast cancer risk increases among female survivors of Hodgkin's lymphoma BC risk who receive radiation dose (201). In another study, a 30 years' follow-up study among population of Taiwan exposed to low-dose-rate radiation in residential and school buildings also showed increased risk of breast cancer (202). Occupational X-ray radiation exposure was also reported to be associated with increased breast cancer risk (203).

Overall

3.2 Rationale of this sub study

In Pakistan, breast cancer is reported as the most common site of cancer among females, accounting for one-third of female cancers (ASR 51.7 per 100,000) (11). Research studies of possible influence of lifestyle and genetic factors on breast cancer risk among

Pakistani women are limited. Using data from this case-control study, which incorporated a detailed questionnaire to assess lifestyle factors, provides the opportunity to investigate the prevalence of risk factors for breast cancer among women in Karachi, Pakistan. The overall research question was to identify possible risk factors for breast cancer among women in Karachi Pakistan.

3.3 Materials and Methods

The study design is discussed in detail in chapter 2. Briefly, it was a matched case-control study consisting of 411 cases and 784 controls matched on ± 5 years' age group, hospital and residence in the same geographic area. The responses of 1195 women collected in the questionnaire (see questionnaire appendix A) forms the basis for the data presented in this chapter.

3.4 Statistical analysis

Normally distributed continuous variables are presented as mean values ± standard deviation, and categorical variables as frequencies and percentages. Univariable analysis was used to estimate unadjusted OR and 95% CI. Variables <0.25 were included and entered in multivariable logistic regression in a step-wise fashion, and a parsimonious final model (tab 4) was created. Conditional logistic regression was applied for analyzing matched case-control using the Hosmer–Lemeshow statistic (204). All reported p-values are based on two-sided tests. SPSS 21.0 software (IBM Statistics, Armonk, New York, USA) was used for all the statistical calculations.

3.5 Results

Table 1. Distribution of sociodemographic characteristics among breast cancer patients, and controls in multicentre case-control study (n=1195) in two major cancer hospitals of Karachi, Pakistan.

Variable	Category	Breast cancer cases n= 411		Controls n=784		OR	95% CI		p value
Education level									<0.001
	<grade 8	153	37.4	170	21.7	3.26	2.33	4.58	
	8-12 grade	143	35.0	247	31.5	2.09	1.52	2.89	
	>grade12	113	27.6	367	46.8	1(Ref)			
Mother tongue									<0.001
	Sindhi	60	14.6	72	9.2	2.04	1.39	3.00	
	Punjabi	44	10.7	82	10.5	1.35	0.9	2.02	
	Pashto	16	3.9	22	2.8	1.85	0.92	3.71	
	Balochi	14	3.4	18	2.3	1.93	0.93	4.00	
	Others	89	21.7	126	16.1	1.77	1.27	2.46	
	Urdu	188	45.7	464	59.2	1(Ref)			
Marital status									0.699
	Single/Widow/ Divorced	83	20.2	151	19.3	1.01	0.73	1.39	
	married	328	79.8	633	80.7	1(Ref)			
Employment status						0.56	0.40	0.77	<0.001
	Yes	60	14.6	185	23.6	1(Ref)			
	No	351	85.4	599	76.4				
Socioeconomic Status									<0.001
	Lower	162	39.4	195	24.9	5.57	3.32	9.34	
	Middle	220	53.5	458	58.4	2.49	1.58	3.92	
	Upper	29	7.1	131	16.7	1(Ref)			
Consanguineous marriage									0.005
	Yes	125	31.9	186	24.1	1.42	1.08	1.88	
	No	267	68.1	586	75.9	1(Ref)			

*p values generated from Chi-square test
*OR= odds ratio; CI= confidence interval

The sociodemographic characteristics of the study population (n=1195) stratified by case and control status are shown in Table 1. 51.2% of women enrolled were from AKU and 48.8% were from KIRAN. Mean age of cases was 46.1 years (SD $\pm$ 11.7 years), and due to 5yrs age range matching, control's age was 46.5 years (SD 11.8 years). Compared with controls, cases were significantly less educated with 37.4% (vs. controls 27.1 %)

having studied less than grade 8, significantly less likely to be employed (85.4% unemployed consisting of mostly housewives) and significantly more likely to belong to the lower SES group (39.4 %) compared with controls (24.9%).

Education

A significant association was observed between breast cancer and education level of women (p-value < 0.001). Breast cancer was higher among women with education level of less than grade 8 (OR= 3.26, 95% CI= 2.33- 4.58) and women with education level of grades 8-12 (OR= 2.09, 95% CI= 1.52-2.89), compared to women with education level higher than grade 12.

Mother tongue

Breast cancer was higher among Sindhi speaking women compared to Urdu speaking women OR = 2.04, 95% CI=1.39, 2.89).

Socioeconomic status (SES)

Breast cancer among those with a lower socioeconomic status and middle SES was significantly higher (OR= 5.57, 95% CI= 1.58, 3.92) and (OR = 2.49, 95% CI= 3.32, 9.34) respectively, compared to those with higher SES (p<0-001).

Employment status

Employed females had less odds of breast cancer than non-employed women (OR =0.56, 95% CI= 0.40- 0.77, p <0.001).

Parental consanguineous marriage

Similarly, patients whose parents had a consanguineous marriage had more risk of breast cancer (OR= 1.42, 95% CI= 1.08-1.88, p=0.005).

Table 2 Reproductive, clinical, characteristics among breast cancer patients, and controls in multicenter case-control study of breast cancer risk factors (n = 1195) in two major cancer hospitals of Karachi, Pakistan

Variable	Category	Case		Control		OR	95% CI		P value
		n	%	n	%				
Parity									**0.20**
	Nulliparous	52	12.7	118	15.1	0.73	0.49	1.08	
	≤3	183	44.5	369	47.1	0.83	0.64	1.08	
	>3	176	42.8	297	37.9	1(Ref)			
History of abortion									**0.73**
	No abortion	230	56	442	56.4	1.12	0.72	1.74	
	<3abortion	149	36.3	272	34.7	1.18	0.74	1.86	
	≥3abortion	32	7.8	70	8.9	1(Ref)			
Age of mother at first full term pregnancy									**0.76**
	≤30years	306	86.7	582	88.3	1.38	0.91	2.09	
	>30years	47	13.3	77	11.7	1(Ref)			
Breastfeeding									**0.63**
	Yes	338	95.8	635	96.4	1(Ref)			
	No	15	4.2	24	3.6	1.15	0.59	2.25	
Family planning									**0.008**
	No FP	310	86.4	532	79.0	1.15	0.62	2.15	
	≤24months	31	8.6	103	15.3	0.62	0.30	1.29	
	>24months	18	5.0	38	5.6	1(Ref)			
Age at menarche									**0.20**
	<12yrs	37	9.7	90	11.9	0.87	0.31	2.43	
	12-13yrs	224	58.9	406	53.6	1.71	0.95	3.10	
	>14yrs	119	31.3	262	34.6	1(Ref)			
Menopausal status									**0.04**
	Post-menopausal	226	55.5	384	49.4	1.80	1.23	2.63	
	Pre-menopause	181	44.5	394	50.6	1(Ref)			
History of benign breast disease									**<0.001**
	Yes	38	9.4	218	28.0	0.28	0.20	0.41	
	No	365	90.6	561	72.0	1(Ref)			
History of diabetes mellitus									**0.55**
	Yes	63	15.4	111	14.2	1.10	0.77	1.57	
	No	345	84.6	673	85.8	1(Ref)			

***OR, odds ratio; 95% CI, 95% confidence interval**

Variable	Category	Case		Control		OR	95% CI		P value
History of any comorbid									**<0.001**
	Yes	159	39.0	415	53.0	0.49	0.37	0.65	
	No	249	61.0	368	47.0	1(Ref)			
Family history of breast cancer									0.002
	Yes	77	18.8	211	26.9	0.63	0.47	0.85	
	No	333	81.2	573	73.1	1(Ref)			
Family history of any type of cancer									0.06
	Yes	139	34.1	309	39.5	0.77	0.59	1	
	No	269	65.9	473	60.5	1(Ref)			
Body mass index									0.008
	>26	236	62.1	510	71.2	0.7	0.50	0.99	
	23-25	67	17.6	93	13.0	1.1	0.71	1.71	
	<23	77	20.3	113	15.8	1(Ref)			
Height **		154.9	6.6	156.5	6.1	0.96	0.94	0.98	

[a] Restricted to women who ever had a full-term pregnancy (a pregnancy was considered as full-term if it resulted in a live birth or lasted 7 or more months)
[b] Restricted to postmenopausal women (women were classified as postmenopausal if their cycles ended naturally or from surgery in which both the uterus and ovaries were removed, or from surgery in which only uterus was removed)
[c] Body mass index (BMI) was calculated as weight divided by the squared height (kg/m2).
**Mean (SD)

Family history of breast cancer

Unexpectedly, there was a significantly lower risk of breast cancer among women with a family history of breast cancer (OR= 0.63, 95% CI= 0.47- 0.63, p =0.002).

Menopausal status

Postmenopausal status had higher risk of breast cancer compared to premenopausal status (OR=1.80, 95%CI=1.23- 2.63, p=0.04).

Benign breast disease

Women with benign breast disease did not have an increased odds of breast cancer but had a decreased odds of breast cancer (OR=0.28, 95%CI= 0.20, 0.41, p<0.001) compared to controls.

Body mass index

High BMI was protective against breast cancer (OR=0.70, 95%CI= 0.50- 0.99, p=0.01)

Height

Taller height was protective against breast cancer (OR = 0.96. 95 % CI =0.94- 0.98, p= 0.04)

Table 3. Distribution of lifestyle-related personal habits among breast cancer cases and controls in multicenter case-control study (n=1195) in two major cancer hospitals of Karachi, Pakistan

Variable	Category	Case		Control		OR*	95% CI*		p value
Tobacco use		**n**	**%**	**n**	**%**				**0.90**
	Yes	57	14.1	109	14.0	1.02	0.71	1.47	
	No	347	85.9	672	86.0	1(Ref)			
Form of tobacco									0.18
	Smoking	15	25.9	15	13.8	1.33	0.30	5.96	
	Chewing**	43	74.1	94	86.2	1(Ref)			
Environmental tobacco smoke ETS									0.18
	Yes	82	20.4	187	24.0	0.82	0.61	1.10	
	No	320	79.6	593	76.0	1(Ref)			
Use of talcum powder									0.22
	Yes	10	27.8	39	31.7	0.15	0.01	1.68	
	No	18	50.0	51	41.5	0.14	0.01	1.34	
	Not sure	8	22.2	33	26.8	1(Ref)			
Use of tight brassiere									0.10
	Yes	7	20.6	34	27.9	1	0.22	4.56	
	No	27	79.4	88	72.1	1(Ref)			
History of vigorous exercise									0.002
	Yes	12	2.9	46	5.9	0.48	0.25	0.91	
	No	397	97.1	738	94.1	1(Ref)			
History of moderate Exercise									0.01
	Yes	194	47.3	318	40.6	1.46	1.10	1.94	
	No	216	52.7	466	59.4	1(Ref)			
History of walking									0.07
	Yes	156	38.0	338	43.1	0.8	0.62	1.02	
	No	254	62.0	446	56.9	1(Ref)			

*OR= odds ratio; CI= confidence interval **includes oral tobacco in different forms

Table 3 shows distribution of lifestyle related personal habits among breast cancer cases and controls with OR and CI. Although the percentage of tobacco users was similar in both breast cancer cases and controls, almost twice as many breast cancer cases than controls were smokers (chewing tobacco). To address the common myths found among masses, related to breast cancer, it was found that there was no association use of talcum powder and tight brassieres with breast cancer. All the variables analyzed showed no significant association with breast cancer except for the measures of exercise where moderate physical activity, consisting of mostly household activities, was more common among both cases and controls than vigorous exercise and walking. Vigorous exercise, though it was less common among all women, but was protective against breast cancer (OR=0.48, 95%CI=0.25, 0.91).

Table 4. Multivariable conditional logistic regression analyses model of the association of statistically significant variables with breast cancer among women (n=1195) in two major cancer hospitals of Karachi, Pakistan

Variable	Category	OR	95% CI		p value*
Socioeconomic Status					**<0.001**
	Lower	**3.48**	**2.00**	**6.07**	
	Middle	**2.02**	**1.27**	**3.22**	
	Upper	**1(Ref)**			
Education level					**<0.001**
	<grade 8	**2.17**	**1.49**	**3.16**	
	8-12 grade	**1.62**	**1.15**	**2.29**	
	>grade12	**1(Ref)**			
Employment status	**Yes**	**0.70**	**0.49**	**0.99**	**0.04**
	No	**1(Ref)**			

*Abbreviations: OR= odds ratio, CI= confidence interval

**Adjusted for all reproductive risk factors (age at menarche, parity, abortions, age at first full-term pregnancy, breastfeeding, and menopausal status), consanguineous marriage, family history of breast cancer, BMI, physical activity

Multivariable conditional logistic regression analyses identified three factors that were associated with the risk of breast cancer among women in Karachi (Table 4). Breast cancer was highest among women with education level of less than grade 8 (OR= 2.17, 95% CI= 1.49- 3.16, p <0.001) compared to women with education level higher than grade 12. Breast cancer among those with a lower socioeconomic status and middle SES

was significantly higher (OR= 3.48, 95% CI= 2.00- 6.07) and (OR = 2.01, 95% CI= 1.27-3.22) respectively, compared to those with higher SES (p<0-001). Employed females had less odds of breast cancer than non-employed women (OR =0.70, 95% CI= 0.49-0.99, p <0.001).

3.6 Discussion

This multicenter case-control hospital-based study examined a number of variables that in other published studies have been shown to influence the risk of breast cancer and allowed us to investigate the associations between those breast cancer risk factors among Pakistani women. The mean age of women with breast cancer in the current study was 46.1 years (SD $\pm$11.7 years). This mean age of women with breast cancer is similar to that in our neighboring country India which was 45yrs (SD $\pm$ 9 years, while it is significantly lower than the mean age of breast cancer in a study in Iran which was 58.2 years $\pm$7.2yrs (205).

This study showed that poor SES, less education level and employment status were variables that significantly contributed to breast cancer risk in this cohort of Pakistani women. Although low SES is commonly associated with lung cancer and poor nutrition (206)but higher incidence of cancers was also reported in socially deprived areas of Germany (207). An association of low SES and an increased risk of breast cancer has been found in other published studies. For example, similar findings were reported in the California Teachers Study where Hispanic women residing in low SES neighborhoods had an increased risk of developing and dying from breast cancers (208). According to this study, SES is an important social determinant of health with poor access to healthy foods, lack of immunity, healthy physical activity and with an increased vulnerability to environmental carcinogens all contributing to an increased breast cancer risk. The association of SES with breast cancer is similarly seen in another study among African American women, where poor women had increased risk of breast cancer of the triple negative breast cancer subtype (209). Similarly, higher SES in early life was protective against breast cancer in the Wisconsin Longitudinal Study compared to poor women due to early-life social environment factors. However, in this particular study a woman's higher adult SES and associated delayed childbearing resulted in increased breast cancer prevalence (210). In a similar study, women belonging to poor SES had advanced breast

cancer at the time of diagnosis (211). On the contrary, a study in Demark linked all SES groups to breast cancer with larger increase in among low SES (212). Overall a lower SES, poorer standard of education and lack of employment were all more frequent in women with breast cancer than in the age-matched controls. These three factors are all somewhat inter-related and are in turn associated with an overall poor quality of living standards both in housing, environment, and diet. Other factors which are possible explanation of the positive association between low socioeconomic status and breast cancer risk include differences in reproductive and lifestyle factors.

Our results of a borderline significant decrease in breast cancer risk for women with employment status is consistent with several other studies that have reported a 15–25% lower risk with occupational activity (185, 186, 213). It may be attributed to more active lifestyle and better SES.

It is important to discuss here those interesting variables that were significant in the univariable analysis and though insignificant in the final model but were associated with breast cancer. Parental consanguineous marriage, predominately first cousin marriage, was significantly associated with an increased risk of breast cancer. Consanguineous marriage is particularly frequent in the Pakistani population and was significantly higher in breast cancer patients. These findings are consistent with an independent study in two major cancer hospitals located in Lahore in the province of Punjab, Pakistan, namely: the Institute of Nuclear Medicines of Oncology, Lahore (INMOL) and Shaukat Khanum Memorial Cancer Hospital (SKMCH), Lahore consisting of Punjabi ethnicity of Pakistan (214). These findings suggest an underlying genetic contribution to breast cancer risk in this population. However, there are other studies among Arab population with high consanguinity, including meta-analysis, which report no association between consanguinity and breast cancer (215-217). Results of another study showed that BRCA1 and BRCA2, and other undiscovered tumor gene carrier rates tend to decrease in consanguineous marriages, compared with non-consanguineous ones. This is due to a major drift in the carrier rate of the mutations (217). In a study in Lahore, Pakistan, the prevalence of BRCA1 or BRCA2 mutations was 42.8% for families with family history of breast cancer in multiple family members; its prevalence was 11.9% for cases of early-onset breast cancer ($\leq$30 years) (218).

In our data, a family history of breast cancer was found to be more common in the control population. This is certainly at odds with the well-accepted research data that a family history of breast cancer is associated with an increased risk of breast cancer. The possible explanation for the finding in this study is that women with a positive family history of breast cancer are more likely to have a better understanding and awareness of breast cancer and actively seek screening examinations and mammography. This was also observed in a Brazilian study where women with positive family history reported more often mammogram screening (159). Similarly, in additional reports, a positive breast cancer family history was associated with increased mammography screening (OR 2.09, 95% CI= 1.45–3.00) relative to a negative family history (219) and women with positive family history showed healthier lifestyle behaviors more often than those without such a history (220). No family history among cases in this study is another important evidence of potential role of some environmental factors causing breast cancer in our women. Many of such carcinogenic environmental factors are not confirmed but may include a range of products from everyday cosmetics to insecticides, to industrial waste exposure. There is need of more research in this area and focus on prevention by identifying and reducing carcinogenic environmental exposures.

In this study, high BMI was protective (OR= 0.70, 95% CI= 0.50- 0.99) and this is the first time, to our knowledge, that high BMI was found to be a protective factor for breast cancer. Our contrasting results to other studies (191, 195) could be due to ethnic differences in our study populations, and the lower cut-off values for BMI used for Asian populations where overweight is defined as 23.0–24.9 kg/m^2 and obesity as 25.0 kg/m^2 or greater (221). However, another explanation could be that breast cancer cases had lost weight due to the disease itself. This is likely to be particularly noteworthy as women with breast cancer in this study presented at advanced stage of disease compared with those typically seen in western populations (see Chapter 7). Our finding of high BMI as protective is similar to a study of 758,592 premenopausal women, where increased BMI at ages 18-54 years, was found to be inversely associated with breast cancer risk (222). Among premenopausal women BMI $\geq$25 kg/m^2 had a lower risk of breast cancer than lean women with BMI <25 kg/m^2 whereas the reverse was observed among postmenopausal women (156). The biological basis of the protective effect for obesity may be explained by increased anovulatory cycles among obese women, which reduces

exposure to lifetime estrogen and results in reduced risk for breast cancer (64). The Nurses' Health Study II (follow-up 1997) showed that low BMI during early life is a risk factor for breast cancer among both inactive and active girls (158). Irrespective of BMI, taller height was protective against breast cancer in this study which is similar to a meta-analysis study showing strong evidence of association between adult heights in the etiology of breast cancer (194).

Some of those variables that were non-significant in this study were smoking, family planning, HRT, because of low prevalence of these variables and also it reflects that these factors had a relatively small influences on breast cancer risk in our population.

There was also a paradoxical protective effect of benign breast disease in breast cancer cases. However, the benign breast disease included in the current study, included both proliferative and non-proliferative types. Non-proliferative lesions are not risk factor for breast cancer. As we could not collect data on histologic types of BBD, it is a limitation of the study. Similarly, history of any comorbidity was also more common among controls. Before drawing out conclusion about BBD and history of any comorbidity, it is important to point out the bias caused by choosing hospital-based noncancer controls who come with complaints of higher comorbid conditions than general population. Berkson's bias make it difficult to generalize as people seen in any hospital-based case-control study are different in their clinical states from general population.

Postmenopausal women had a higher risk of breast cancer compared to premenopausal women (OR=1.80, 95%CI=1.23- 2.63, p=0.04). It is noteworthy that mean age of menopause in our women was 46.4 years which is much younger than the average age of menopause in Western women (51 years) (156). Women with a delayed menopause after the age of 50 years leads to an increase in the number of ovulatory cycles and subsequently higher endogenous estrogen concentrations, over a woman's lifetime (223). However, there was no association of age at menopause and breast cancer.

There are few other studies evaluating the association between reproductive factors and breast cancer risk in Asian and Pakistani women. However, the results of these limited studies regarding the role of established reproductive factors for breast cancer have been inconsistent. In Japan, increase of breast cancer is related to changing reproductive

behavior like decline in fertility, late age at first birth and lack of breastfeeding (224). In a hospital-based case-control study among women younger than 45 years of age in Lahore, Pakistan, women with BMI >30, with a family history of breast cancer , later age at menarche, and at first full-term pregnancy, high parity and a history of abortion had higher risk of breast cancer (214). In another case-control study of 296 breast cancer cases and 580 controls, family history of breast cancer (OR=1.72; 95%CI= 1.10- 2.80), single marital status (OR=1.55; 95%CI= 1.10- 2.39), older age at menopause (OR=3.92; 95%CI= 2.52- 6.18) conferred an increased risk of breast cancer for women. Increasing parity was protective and decreased the risk of breast cancer (OR=0.90; 95%CI= 0.85, 0.97 for each live birth) (160).

Breastfeeding was common in our cohort of women, compared to the west where lower parity with lack of or short duration of breastfeeding is more frequent and a major contribution to the high incidence of breast cancer in those countries (225). The mean duration of lifetime breastfeeding among both cases and controls was 52 months (median 44 months) compared to the mean duration of lifetime breastfeeding of 34.8 weeks in developed countries (168). Lifetime breastfeeding duration of >12 months was 86.1% among cases and 85% among controls which according to a published meta-analysis would be predicted to be protective effect against breast cancer (168). The lifetime duration of breastfeeding of 4 months (p = 0.037, OR= 0.7, CI 0.5-0.98) was also protective among Iranian women (226). Another study in Korea also showed that longer average breastfeeding of more than or equal to 13 months per child can reduce their breast cancer risk by about 50% (227). It is apparent that the mean duration of lifetime breastfeeding was higher in rural areas of Asia during the 1990s but in recent years this has now decreased in Pakistan (228). For example, in a 2013 study the mean duration of lifetime breastfeeding was 60 months among cases and controls in 2013 study (160), but in this study, it is reported 52 months in our study. It is important to encourage breastfeeding, and this can be achieved by targeted campaigns since this is an easily modifiable risk factor that is protective against breast cancer (229).

The exact reasons of lack of association of reproductive factors (breastfeeding, parity, age at first full-term birth, hormone replacement therapy, late age at menopause, and early age at menarche) with breast cancer in our women are unknown. Compared to other

studies, our population consisted of women who were permanent residents of Pakistan. It is suggested that these reproductive factors are not evident due to the overwhelming influence of low SEF and poor education. Environmental factors such as exposure to carcinogens among poor Pakistani women are likely to be significant contributors to breast cancer risk and need further investigation. Our data suggest that there are other lifestyle and environmental factors related to poverty in Pakistani women and deeper insight into these factors may explain the increased breast cancer risk in Karachi women. Overall it has been reported that the causes of increasing breast cancer incidence in low-income countries are complex, and vary from country to country with country-specific solutions needed (230). Healthy lifestyle is protective for breast cancer (231). Demographic and lifestyle determinants of breast cancer were assessed, but genetic determinants by ethnicity specific of our population could not be studied as it was beyond the scope of this study.

Very few studies have evaluated the role of use of antiperspirant and breast cancer. We attempted to evaluate it and found that there was no association between underarm use of talcum powder and breast cancer in our population which is similar to another observational study finding (232).

3.7 Conclusion

The observed association of low education and poor SES with breast cancer in this study is an important finding as breast cancer treatment and management is non-affordable to the majority of such women here in Karachi. The influence of SES status on breast cancer risk is likely to be multifactorial with environmental factors such as exposure to carcinogens in poor environment and poor diets as possible major contributing factors. Further research into pinpointing the exact nature of these factors responsible for high breast cancer among poor and less educated women is needed, and this could provide new opportunities for the development of risk reduction strategies to decrease the incidence and mortality of breast cancer in Pakistani women. Understanding the biological mechanism underlying this association would have important implications for breast cancer prevention. It is also important that the poor women benefit from frequent screening but at the moment these facilities are not available free of cost at national level. Overall, some of these lifestyle and environmental risk factors are potentially modifiable

and could, therefore, reduce the incidence of breast cancer. However, more research is needed to fill the gaps to identify those environmental toxicants and their interactions with social factors, biologic pathways for environmental and behavioral factors in the complex etiology of breast cancer.

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Chapter 4: The Association of Vitamin D with Breast Cancer among Women in Karachi, Pakistan

4.1 Background

Cancer rates and the financial burdens of cancer treatments are increasing worldwide including both developed and developing countries (230, 233). Cancer burden is of significance especially in developing countries where health systems are not sufficiently well resourced to provide the optimum facilities for cancer diagnosis and cancer management. Expensive targeted cancer treatments continue to advance the treatment for breast cancer in more affluent countries. In developing economies, the exploration of more affordable cancer treatment and management is a necessity and there is comparatively limited research and strategies towards less expensive breast cancer prevention research.

There is substantial evidence supporting the theory that hormonal mechanisms play a vital role in the etiology of breast cancer. Epidemiological studies and laboratory findings indicate that Vitamin D has potential anticancer activity (60). As discussed in detail in chapter 1, the enzyme 25-hydroxylase synthesizes 25- hydroxyvitamin D (referred to as 25(OH) D) in the liver. Then 25(OH) D is converted to the active form of 1, 25 dihydroxyvitamin D (calcitriol) in the liver by 1α-hydroxylase (CYP27B1). The concentrations of pro hormone form of Vitamin D 25(OH) D can be measured in the serum (Vitamin D concentrations in this thesis refers to the serum concentrations of total 25(OH) D. There are nuclear Vitamin D receptors (VDRs) present in many sites including breast and Vitamin D mediates its actions via these vitamin D receptors. An individual's Vitamin D concentrations are principally determined by the level of the skin's ultraviolet (UVB) radiation exposure from the sun and skin pigmentation can influence this exposure. There are also dietary sources of Vitamin D. Since Vitamin D deficiency and insufficiency are associated with poor skeletal development, for example rickets, there are national programs that vary between countries to provide dietary Vitamin D fortified foods(30). In addition, easily affordable Vitamin D supplements are available.

With the findings that low concentrations of Vitamin D are associated with an increase in the risk of some cancers, Vitamin D representing D2, D3 or both, is being investigated as possible preventive agents as well as therapeutic agent with adjuvant chemotherapy in breast cancer management. However, observational studies,

particularly with regard to breast cancer, have shown inconsistent results regarding the role of Vitamin D concentrations in breast cancer incidence (see Chapter 1 for detailed discussion).

Rationale for this sub study

There are many published studies evaluating the relationship between low Vitamin D concentrations and increased risk of breast cancer. The Pakistani population is reported to have low concentrations of Vitamin D and therefore is ideal for such studies. Vitamin D has been evaluated as having anticancer activity against cancers especially colorectal, lung and liver cancers, while evidence for breast cancer is inconsistent. Moreover, evidence on this topic from Asian populations especially Pakistan is limited. This chapter presents a case control study in the Pakistani population examining the association between women's serum Vitamin D concentrations and breast cancer risk. In addition, other sources of Vitamin D like sun exposure and intake of Vitamin D supplements are also evaluated in this population. It is important to identify and validate Vitamin D deficiency, as this is a modifiable risk factor for BC among Pakistani women. Another important issue addressed in this study is that it has included all sources of Vitamin D. The majority of published observational studies, have assessed the effects of dietary sources of Vitamin D and Vitamin D supplements intake, but have not attempted to measure in the same study, the other major source of Vitamin D, like sun exposure and relate this to serum Vitamin D 25(OH) D level. It is important to correlate serum Vitamin D level with both exogenous Vitamin D sources and endogenous cutaneous synthesis through UVB sunlight exposure. Therefore, additional analysis with Vitamin D was also conducted on specific breast cancer subtypes (chapter5). The main focus of the study was serum 25(OH) D as it is a best biomarker to measure Vitamin D status compared to assessment by diet, Vitamin D supplements and sunlight exposure (94).

Objective

To determine the association of Vitamin D (serum Vitamin D (25-hydroxyvitamin D) level, Vitamin D supplementation and sun exposure) with the risk of breast cancer among Pakistani women.

4.2 Methods

4.2.1 Measurement of serum Vitamin D concentrations

Serum level of Vitamin D 25(OH) D is the most reliable method for assessing Vitamin D status (40). After drawing venous blood, all the blood samples were collected in yellow-topped gel tubes, left to clot and then centrifuged for 15 minutes. Serum in aliquots were immediately stored at -80^0C. The plasma Vitamin D concentrations were measured by an ELISA technique (Diametra, Italy DCM146-2) according to the standard procedure in the main laboratory of AKUH (College of American Pathologists CAP accredited). Samples were analysed in batches using eight kits of the assay, as described previously in chapter on materials and method chapter 2. Chemical pathologists at AKUH laboratory used quality controls to assess inter-assay accuracy and precision for monitoring assay performance and quality control (QC). During each run, 5 quality control (QC) samples were run together with the study samples. Charts were maintained to follow the performance of each kit. Results were expressed in ng/ml. Laboratory personnel were blinded as to case or control status of the enrolled subject

(In the chapter, Vitamin D refers to 25(OH) D unless mentioned otherwise).

4.2.2 Vitamin D supplements intake

In relation to the index year, for all participants a questionnaire was used to determine Vitamin D supplementation. It included both Vitamin D injection, oral tablets, multivitamins, and calcium tablets. Frequency of calcium and multivitamin intake and the brands of tablets were also determined. Vitamin D supplementation was assessed by asking participants if they had been taking Vitamin D supplements regularly, occasionally or not at all in their lifetime, with the mode of administration identified as either injections, oral tablets or ampoules.

4.2.3 Sun exposure measurement

Details are given in the Materials and Methods chapter 2. Briefly, the sunlight exposure measurement questionnaire (SEM-Q) was used to obtain detailed information regarding UVB exposure (234). The questionnaire estimated skin sun exposure to various parts of the body, use of sunscreen, style of dress and skin tone. The time spent outdoors during 10 am and 4 pm in a routine week or day in summers and winters was assessed to estimate the amount of time in minutes per day and per week each participant was exposed to UVB radiations.

As mentioned in chapter 2, questions related to sociodemographic history, obstetrical history, and family history, laboratory reports of side of tumor, histopathology and complete molecular profiling of tumors of breast cancer cases were retrieved from medical records. Data relating to tumor size (T), nodes involved (N), and presence of metastasis (M), and receptor status were also extracted from pathology reports and all this information was combined as stage grouping where stage was expressed in Roman numerals from stage I (the least advanced stage) to stage IV (the most advanced stage). Questions asked about comorbidities included osteoporosis but use of DXA for confirmed diagnosis of osteoporosis was rare. Therefore, based on limited published data available on osteoporosis, in this study it was assumed that osteoporosis was equally common in both cases and controls

Statistical analysis

All collected variables were described using both descriptive and analytical statistics. Categorical variables were described as counts and percentages. Continuous variables sun exposure score was described as mean, median and range. Serum 25(OH) D concentrations were categorized into Vitamin D deficiency (VDD) as <20 ng/ml (<50 nmol/L), Vitamin D insufficiency VDI as 20-30 ng/ml (50.0-75 nmol/L) and sufficiency >30 ng/ml (>75.0 nmol/L). The cut-off used to define vitamin D deficiency is the point where parathyroid hormone (PTH) starts to rise (235). Conditional logistic regression with matched sets as strata was used to compute odds ratios (ORs) and 95% confidence intervals (CIs) to evaluate the association of 25(OH) D, sun exposure, and Vitamin D supplements with breast cancer. Univariate and multivariate analysis tests were applied

to report the association between 25(OH) D concentrations and some demographic and clinical characteristics including BMI, tumor grade, hormonal, and menopausal status. SPSS 21.0 software (IBM Statistics, Armonk, New York, USA) was used where a p-value< 0.05 was accepted as significant for all statistical tests.

4.3 Results

Table 1 presents the median, minimum, maximum and mean with SD for continuous data of serum Vitamin D concentrations. Individual Vitamin D concentrations encompassed a broad range from highly deficient (0.3 ng/ml) to exceptionally high (165.5 ng/ml). Origins of these high values, which can result in clinical side effects of hypercalcemia, are discussed later. The median Vitamin D concentrations among breast cancer cases was lower (15.3 ng/ml) compared to controls (16.7 ng/ml).

Table 1 Serum level of Vitamin D among cases and controls

	Median	**Minimum**	**Maximum**	**Mean**	**SD**
Cases	15.3	0.3	165.5	20.1	21.3
Controls	16.7	0.9	149.0	23.0	20.3

To enable a more rigorous statistical analysis cases and controls were categorized into three different concentrations of Vitamin D, broadly defined as deficient, insufficient and sufficient (Table 2). In this analysis, deficient group was further sub divided into two groups of <12 ng/ml and 12-19 ng/ml categories (Table 2). The median level of serum level of Vitamin among cases was 15.3 ng/ml and 16.7 ng/ml among controls.

Table 2 Number and percentage of breast cancer cases and controls for different serum Vitamin D concentrations

		case		**control**		**All participants**		**p value***
		n	**%**	**n**	**%**	**n**	**%**	
Vitamin D (ng/ml)								0.016
Severely deficient	<12	112	38.9	202	32.9	314	34.8	
Deficient	12-19	83	28.8	146	23.8	229	25.4	
Insufficient	20-30	43	14.9	114	18.6	157	17.4	
Sufficient	>30	50	17.4	152	24.8	202	22.4	

* p values generated from Chi-square test

Table 2 shows that Vitamin D deficiency was significantly more frequent in women with breast cancer. 38.9% of breast cancer cases had severe vitamin D deficiency compared to 32.9 % of controls, while 28.8% of breast cancer cases had vitamin D deficiency compared to 23.8 % of controls. Vitamin D sufficiency was higher among controls (24.8%) compared to cases (17.4%).

Figure 1. Distribution of serum Vitamin D concentrations in breast cancer cases and controls.

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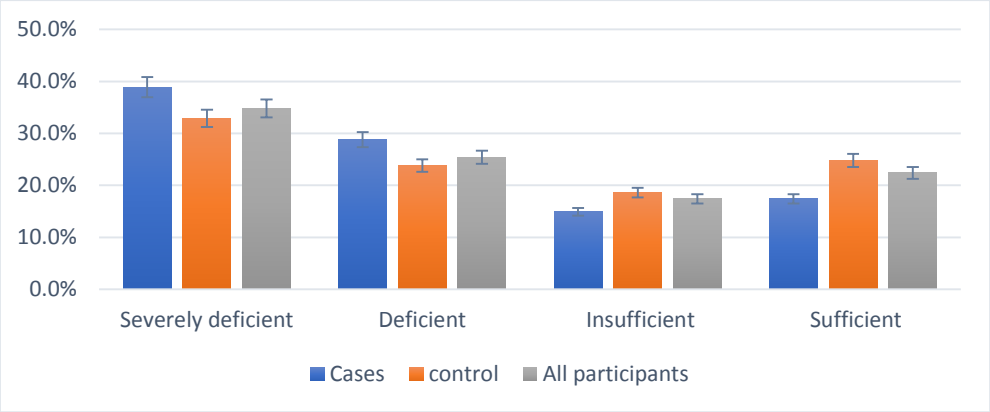

Fig 1 shows that severe deficiency of serum Vitamin D <12 ng/ml was reported among 38.9% of women. This is despite of the climate of Karachi providing ample sunlight throughout the year required for adequate Vitamin D concentrations. Consequently, the number of women with insufficiency and sufficient concentrations of Vitamin D were lower in the cancer cases (Fig. 1).

Table 3. Sociodemographic and reproductive characteristics stratified according to serum Vitamin D concentrations

Variable	category	Serum vitamin D level (ng/ml)						p value*
		Deficient		Insufficient		Sufficient		
		<20		20-30		>30		
		Count	%	Count	%	Count	%	
Age groups (years)								<0.001
	<35	100	18.4	19	12.1	22	10.9	
	35-45	209	38.5	55	35	54	26.7	
	46-54	128	23.6	36	22.9	52	25.7	
	55 & above	106	19.5	47	29.9	74	36.6	
Education								0.57
	<grade8	140	25.8	31	19.7	49	24.3	
	grades 8-12	178	32.8	56	35.7	63	31.2	
	>grade12	224	41.3	70	44.6	90	44.6	
Socioeconomic status (SES)								0.002
	upper	62	11.4	34	21.7	35	17.3	
	middle	308	56.7	84	53.5	122	60.4	
	lower	173	31.9	39	24.8	45	22.3	
Parity								0.02
	nulliparous	85	15.7	14	8.9	30	14.9	
	≤3	232	42.7	85	54.1	105	52	
	>3	226	41.6	58	36.9	67	33.2	
Breastfeeding								0.11
	no	12	2.6	9	6.3	7	4.1	
	yes	443	97.4	133	93.7	164	95.9	
Menopause								<0.001
	menopause	247	45.7	83	53.2	123	61.8	
	pre menopause	293	54.3	73	46.8	76	38.2	
Body mass index**								0.66
	<23	83	16.3	21	14.4	39	20	
	23-25	71	14	23	15.8	25	12.8	
	>26	354	69.7	102	69.9	131	67.2	

*p values generated from Chi-square or Fisher Exact test
**Weight (kg)/height $(m)^2$

Table 3 shows differences between different serum concentrations of Vitamin D with respect to demographic and reproductive characteristics, with significant p value. Vitamin D deficient women, compared with Vitamin D sufficient women, were significantly more likely to be in the 35-44-year age group, premenopausal, and of lower

SES. Those women with Vitamin D deficiency on average had a greater number of children. This is likely to be related to their lower SES status.

Table 4. Association between serum Vitamin D concentrations and the tumor characteristics among breast cancer cases

Variable	category	Deficient (<20)		Insufficient (20-30)		Sufficient (>30)		p value*
		n	%	n	%	n	%	
Histopathology	IDC	160	85.6	36	87.8	40	80.0	0.39
	lobular	7	3.7	1	2.4	5	10.0	
	Others	20	10.7	4	9.8	5	10.0	
ER	positive	111	67.7	19	63.3	32	74.4	0.57
	negative	53	32.3	11	36.7	11	25.6	
PR	positive	98	59.8	15	50	32	74.4	0.09
	negative	66	40.2	15	50	11	25.6	
grade of tumor	III	74	41.6	13	35.1	11	23.9	0.08
	I/II	104	58.4	24	64.9	35	76.1	
tumor size	<5cm	98	55.4	20	57.1	24	55.8	0.98
	>5cm	79	44.6	15	42.9	19	44.2	
nodes	NoN1	121	72.9	29	82.9	29	69.0	0.37
	N2	30	18.1	2	5.7	7	16.7	
	N3	15	9.0	4	11.4	6	14.3	
Metastasis	no metastasis	130	81.8	24	80	31	81.6	0.97
	metastasis	29	18.2	6	20	7	18.4	
TNM Stage	Stage 1	23	15.5	10	34.5	5	13.9	0.24
	Stage 2	53	35.8	8	27.6	11	30.6	
	Stage 3	43	29.1	5	17.2	13	36.1	
	Stage 4	29	19.6	6	20.7	7	19.4	

P value generated from Chi-square or Fisher Exact test

Table 4 presents the distribution of tumor characteristics according to the three different serum Vitamin D concentrations. There was no significant association of Vitamin D concentrations with any of these tumor characteristics although there was trend of a higher percentage of Vitamin D deficiency (41.6 %) among women with grade III tumors.

Table 5. Use of different forms of Vitamin D supplementation and multivitamin with breast cancer cases and controls

Vitamin D supplementation	Category	Case (411)		Control (784)					
		n	%	n	%	OR	95% CI		p value*
Vitamin D tablets									<0.001
	Yes	27	6.7	154	19.6	0.28	0.18	0.44	
	No	375	93.3	630	80.4	Ref(1)			
Oral Vitamin D Drops									<0.001
	yes	15	3.7	67	8.5	0.42	0.23	0.75	
	No	387	96.3	717	91.5	Ref(1)			
Vitamin D calcium tablets									<0.001
	Yes	83	20.6	264	33.7	0.47	0.35	0.64	
	No	319	79.4	520	66.3	Ref (1)			
Injection Vitamin D									<0.001
	Yes	69	17.2	254	32.4	0.41	0.3	0.56	
	No	333	82.8	530	67.6	Ref (1)			
Multivitamin									<0.001
	Yes	100	24.9	300	38.3	0.52	0.39	0.68	
	No	302	75.1	484	61.7	Ref (1)			

OR = odds ratio, CI= confidence interval

Table 5 shows the various forms of Vitamin D supplementation intake among women. Overall, 50.6 % of women were using some form of Vitamin D supplementation. For each form of supplementation, the breast cancer group had a significantly lower usage than the control group.

Table 6. Vitamin D level and intake of Vitamin D supplements among breast cancer cases & controls

Serum Vitamin D ng/ml	Vitamin D supplements	case		control		p value*
		n	%	n	%	
Vitamin D deficiency (<20)						0.001
	non user	139	72	176	50.6	
	user	54	28	172	49.4	
Vitamin D insufficiency (20-30)						0.001
	non user	25	59.5	31	27.2	
	user	17	40.5	83	72.8	
Vitamin D sufficiency (>30)						0.001
	non user	22	44.9	31	20.4	
	user	27	55.1	121	79.6	

*p values generated from Chi-square Exact test

Intake of Vitamin D supplementations was stratified based on serum Vitamin D concentrations (table 6). There was a significant association between serum Vitamin D concentrations and intake of Vitamin D supplements. 72 % of breast cancer cases in Vitamin D deficiency group were non-users of Vitamin D supplementation compared to controls (50.6%).

Table 7. Distribution of sun exposure variables among the BC cases and controls in the multicenter case control study

		case				control			
	Sun exposure variables	Mean	Median	Range	SD*	Mean	Median	Range	SD*
IA	Total sun exposure score								
	Score of Sun exposure in Summer per week	36.5	3.6	689	79.1	32.2	1.6	1545	99.6
	Score of Sun exposure in Winter per week	56.4	19.4	716.3	97.6	48	11.5	1545	117.6
IB	Components of sun exposure score								
	Sun exposure in summer(days/week)	2.7	1	7	2.9	2.6	1	7	2.9
	Sun exposure in winters(days/week)	3.6	3	7	3	3.3	3	7	3
	Sun exposure in summers (minutes/ day)	20.7	7	260	37.5	18	5	600	44.5
	Sun exposure in winters (minutes/day)	29.8	15	300	44.1	25	10	600	51.5
	Sun Exposure during summer in minutes/ week	108.8	20	1820	233.7	93.6	10	4200	281.7
	Sun Exposure during winter in minutes/ week	166.2	60	2100	290.1	137.8	35	4200	328.5

* SD standard deviation

Table 7 shows the descriptive analysis of total sun exposure score, and individual components of sun exposure score among cases and controls in summer and winter and time spent outdoors in minutes per week and minutes per day. There was no significant difference between cases and controls in the total sun exposure score and individual sun exposure variables.

Table 8. Sun exposure variables including body parts exposed and sun avoidance behavior with breast cancer in cases and controls

	Variables		**case**		**control**					
			n	**%**	**n**	**%**	**OR**	**95% CI**		**p value**
IA	**Total sun exposure score (mean SD) ***									
	Score of Sun exposure in Summer per week		32.2	99.5	36.48	79.1	1	1.01	1.02	0.98
	Score of Sun exposure in Winter per week		56.4	97.6	48	117.6	1	0.99	1	0.9
IB	Individual components of sun exposure score									
	Head Covered	yes	332	82.4	572	73	1.97	1.41	2.74	<0.001
		no	71	17.6	210	27	1 (Ref)			
	Face Covered	yes	97	23.8	161	20.5	1.29	0.94	1.76	0.11
		No	306	76.2	621	79.5	1 (Ref)			
	Neck Covered	yes	255	63.3	426	54.5	1.53	1.18	1.99	0.001
		No	148	36.7	356	45.5	1 (Ref)			
	Full Arm Covered	yes	299	74.2	537	68.7	1.37	1.03	1.82	0.03
		No	104	25.8	245	31.3	1 (Ref)			
	Hands Covered	yes	11	2.7	14	1.8	1.78	0.74	4.29	0.19
		no	392	97.3	768	98.2	1 (Ref)			
	Full Legs Covered	yes	389	96.5	760	97.2	0.77	0.35	1.66	0.76
		No	14	3.5	22	2.8	1 (Ref)			

	Variables		**case**	**control**	**OR**		**p value**

			n	%	n	%		95% CI		
	Sun avoidance behavior	yes	361	89.6	660	84.4	1.73	1.14	2.62	0.01
		no	42	10.4	122	15.6	1 (Ref)			
	Skin Tone Forehead (mean SD) *		5.7	1.5	5.3	1.4	1.19	1.08	1.31	<0.001
	attire outside	chadder #	146	36.3	214	27.4	2.28	1.62	3.22	<0.001
		burqa**	177	44	320	41	1.89	1.35	2.66	
		others	79	19.7	247	31.6	1 (Ref)			

*Mean SD # covering head, full arms and full legs **covering head, face, neck, full arms and full legs

There was no difference in total sun exposure score between cases and controls and no association with breast cancer risk (table 8). However, component variables of sun exposure score were analyzed separately. Compared to controls, women covering their head, neck, full legs and full arms with more sun avoidance behavior had increased risk of breast cancer because it was associated with increased Vitamin D deficiency.

Table 9. Multivariable analysis showing the association of Vitamin D related variables with breast cancer risk among women in multicenter case control study (n = 1195).

Variable	Category	OR	95% CI		p value*
Serum 25(OH)D level(ng/ml)					0.032
	<20	1.65	1.10	2.50	
	20-30	1.17	0.68	2.01	
	>30	1 (Ref)			
Use of Vitamin D supplementation	yes	0.32	0.24	0.43	<0.001
	no	1 (Ref)			
attire outside	Chadder**	1.80	1.25	2.59	0.006
	burqa***	1.50	1.05	2.16	
	others	1 (Ref)			

*Adjusted for socioeconomic status, and education

** A big cloak covering head, neck, full arms and full legs

***A big outer coat with scarf and veil covering head, face, neck, full arms and full legs

According to multivariable analysis (table 9), the following variables were significantly associated with breast cancer risk:

Serum Vitamin D level

Compared to patients with sufficient serum Vitamin D (>30 ng/ml), women with serum Vitamin D deficiency (<20ng/ml), had a higher risk of breast cancer (OR =1.65, 95%CI: 1.10, 2.50).

Use of Vitamin D supplements

Women with past history of intake of Vitamin D supplements had significant protective effect against breast cancer (OR= 0.32, 95% CI: 0.24, 0.43).

Attire outdoors

Women whose most body parts remaining covered outdoors by wearing chadder and burqa had higher breast cancer compared to women without those attires (OR =1.80, 95% CI: 1.25, 2.59) and (OR =1.50, 95% CI: 1.05, 2.16) respectively.

Overall, these findings are consistent with a diverse literature that higher concentrations of Vitamin D are associated with a reduction in the incidence of breast cancer. This finding is further reinforced by the reduced incidence of breast cancer and increased serum Vitamin D level in women taking Vitamin D supplements.

4.4 Discussion

The median level of serum Vitamin D in our study among cases was 15.4 (SD 21.31) ng/ml and among controls 16.7 (SD 20.3) ng/ ml. This was on average somewhat lower than the median level of serum Vitamin D (25(OH)D) of 18.8 ng/ ml reported in a population based study of Pakistani population in Karachi in 2011 (236). However, it is slightly higher than the reported median serum Vitamin D level of 13.5 ng/ml in a clinical laboratory audit of Karachi Pakistan in the same year of 2011(142). In spite of the low latitude of Karachi and regular monthly sunshine throughout the year, Vitamin D deficiency was found in 60.2% of the study participants with severely Vitamin D (<12ng/ml) in 34.8%. Similar findings were reported in neighboring countries of India and China (237, 238). In a study in Saudi Arabia, mean serum Vitamin D level was 13.1ng/ml (239). This compares to concentrations in the United States western population where Vitamin D deficiency was reported to be 28.9%. In the National Health

and Nutrition Examination Survey (NHANES) 2001–2010 (240) and in a separate study of National Health and Nutrition Examination Survey (NHANES) 2005-2006, it was 41.6% (12). In the US study the incidence of Vitamin D deficiency varied in different ethnic groups being highest among blacks (82.1%), followed by Hispanics (69.2%) (53).

Among the participants studied, unusually high serum Vitamin D concentrations of greater than 100ng/ml was found in 8 women in our study. Such high values have the potential to be associated with clinical side effects of hypercalcemia and symptoms of gastrointestinal disorders (241). In these cases, the high values were confirmed following retesting of blood samples to exclude laboratory error. All eight women, the high serum concentrations of Vitamin D were associated with high usage of Vitamin D injections in the past which typically involved injectable Vitamin D with oral dose at higher than normal dosage.

Serum Vitamin D level and breast cancer

In this hospital-based case-control study, women with Vitamin D deficiency had higher risk of breast cancer (OR =1.65, 95%CI: 1.10, 2.50) compared with women reporting sufficient Vitamin D level. This is consistent with the findings of a number of similar observational studies in different populations reporting that women with serum Vitamin D concentrations below 20 ng/ml have a higher risk of breast cancer among different ethnic groups including populations in India (237) and China (238). The results of another case control study in China and meta-analysis of 21 independent studies that Vitamin D also suggest that may have a chemo-preventive effect against breast cancer (242). Findings of another meta-analyses also support that low Vitamin D values are associated with an increased risk of breast cancer, colorectal and prostate cancer (243). Similarly, a study of breast cancer patients in Jordan showed an inverse association between Vitamin D serum level and breast cancer (127). A study among Caucasian population in UK reported that low concentrations of circulating 25(OH) D may increase risk of breast cancer (68). In a Nurses' Health Study cohort, high concentrations of serum Vitamin D was associated with non- significant reduced risk of breast cancer in older women (67). A population-based case-control study among premenopausal women of 289 cases and 595 matched controls, showed a significant inverse association between

breast cancer risk and plasma 25(OH)D. Compared with <30 nmol/L of Vitamin D the ORs (95% CI) for the upper categories of 30–45, 45–60, ≥60 nmol/L were 0.68 (0.43–1.07), 0.59 (0.37–0.94) and 0.45 (0.29–0.70), respectively (70). In a recent study among Spanish women, there was decreasing ORs of breast cancer with increasing level of serum Vitamin D (OR per 10nmol/L=0.88; 95%CI=0.82–0.94) (85). In a Prospective Observational Mediterranean Study, deficient Vitamin D concentrations were associated with node-positive high grade breast cancer (244). In a study of Indian women, low serum Vitamin D concentrations (<20ng/ml) were associated with an increased risk of breast cancer (245).

Although the studies mentioned above consistently show low concentrations of Vitamin D are associated with an increased risk of breast cancer, several large studies do not show such an association. These include the Northern Sweden Mammary Screening Cohort study (246) and a case–control study nested within the Nurses' Health Study II (NHSII) (112). An additional study found that Vitamin D binding protein DBP was not associated with breast cancer risk in premenopausal women (113). Further, in a cohort study from 1965-1976, it was found that there was no difference in the pre diagnostic concentrations of 1,25 $(OH)_2D$ between cases of breast cancer and their matched control subjects (110).

Use of Vitamin D supplements and breast cancer

This study provides evidence that women with a past history of intake of Vitamin D supplements had both increased serum concentrations of Vitamin D and a significantly protective effect against breast cancer (OR= 0.32, 95% CI:0.24, 0.43). There are published studies that also report an inverse association of Vitamin D supplementation intake and breast cancer risk (101, 103). For example, an observational study in France showed regular use of Vitamin D supplementation was associated with a decreased postmenopausal risk of breast cancer in hormonal therapy MHT users (101). In a population-based case-control study, Vitamin D supplement intake > 400 IU/day compared with no intake was associated with a 24 % decreased risk of breast cancer (102). In another population based case control study, there was an inverse association between breast cancer and Vitamin D intake (OR 0.58; 95% CI 0.47 -0.70) (247). Overall, the efficacy of Vitamin D supplementation and actual doses needed for reducing breast cancer incidence and mortality remains uncertain.

The data presented in this study of Karachi women shows Vitamin D deficiency is highly prevalent in women with breast cancer, and treatment with Vitamin D supplements is predicted to be beneficial in reducing the risk of breast cancer. Some studies have shown Vitamin D supplementation is beneficial (248). However, there are other studies demonstrating no association between Vitamin D status and breast cancer risk. Postmenopausal women enrolled in a Women's Health Initiative clinical trial were randomly assigned to elemental calcium and 400 IU of Vitamin D daily or placebo for a mean of 7.0 years but showed no evidence of breast cancer prevention (117). Potential problems with such studies in more affluent countries is that overall Vitamin D concentrations are higher in the population and women frequently use supplements with Vitamin D. Our study shows an association of Vitamin D supplements, venous Vitamin D concentrations and breast cancer in a cohort of Pakistani women where there are severe concentrations of Vitamin D deficiency. Further research is required to investigate the optimum dose and delivery method of Vitamin D and for breast cancer prevention

Sun exposure and breast cancer

Sun exposure is the major source of Vitamin D ((34) and this is reviewed in detail in Chapter 1.) Women whom cover the majority of their body outdoors as a result of wearing the chadder and burqa had the highest incidence of breast cancer compared to women without these attires (OR =1.80, 95%CI: 1.25, 2.59, and OR =1.50, 95%CI: 1.05, 2.16 respectively). Being a Muslim community, Pakistani women cover most of their body for cultural and religious reasons in a similar manner to Arab women who also have reduced concentrations of Vitamin D (34). However, 54.5% of women in Singapore also had vitamin D deficiency, although they do not wear outer cloak and scarf like Muslim women (55). This may suggest Asian women have a predisposition to low concentrations of Vitamin D although it should be noted that many women in Singapore tend to avoid exposure to the sun.

The sites of sun exposure of women in this study was mainly the hands and face and sun block was not used. However, most women also had a frequent habit of sun avoidance by always standing under a shade when outdoors. The skin pigmentation tone of most participants in this study was 7 (compared with xx for European women) and will also

result in limiting the effects of sun exposure. However, there was no association of total sun exposure score (a proxy measure of Vitamin D concentrations) with serum Vitamin D concentrations or breast cancer risk in our study, which may not be sufficient proxy measures for sunlight/Vitamin D exposure. Similarly another study showed no protective effect of UVB against breast cancer (249). Serum Vitamin D is a useful biomarker for measuring an individual's recent exposure to sun exposure but may not correlate with lifetime sun exposure in different seasons. The absence of an association between total sun exposure score and Vitamin D and breast cancer could also be due to inaccuracies in the collection of this type of data, in particular the recall of past sun exposure time. Moreover, it should be noted that Karachi is the 4th most polluted city in the world with the presence of heavy smog and air pollution associated with rapid industrialization. This further has the potential to affect access to sun exposure and its UV-B light, causing decreased penetration of ultraviolet radiation from the sun. Numerous studies, particularly those at higher latitudes, have shown a strong positive association between sunlight exposure concentrations and Vitamin D concentrations and negative association with various cancers including breast. For example in the USSR (64). Similarly, among a white population living in low latitude regions a multi-ethnic cohort nested case-control study, showed high concentrations of Vitamin D were associated with a reduced risk of postmenopausal breast cancer (79). In a study of data from the Ontario Cancer registry, breast cancer risks were reduced among women who had increasing sun exposure at earlier life (ages 10-19 years) (97). These studies emphasizing of the importance of natural sources of Vitamin D though sun exposure. The duration and timing of the sun exposure is difficult to define as this will depend on latitude, weather conditions and concentrations of air pollution, skin coloration and amount of skin exposed.

Unavoidable weaknesses of this study include the recall bias in determining the extent of sun exposure and causing some exposure misclassification of non-differential type. Measurement of serum Vitamin D concentrations was by a single venous blood sample collected at one point in time. Vitamin D concentrations may fluctuate over time. However, findings from an important clinical trial suggest that serum Vitamin D level at a single time point may be representative of long-term Vitamin D status over a five-year

period (250). Moreover, this type of misclassification would be non-differential to both cases and controls.

A large sample size and high response rate were the strengths of this study. Measurement of Vitamin D from all sources especially serum level of 25(OH) D which is the gold standard for assessing Vitamin D status.

4.5 Conclusion

In this case control study, lower serum Vitamin D level was associated with higher risk of breast cancer. These findings support the hypothesis that Vitamin D may play protective effects against cancer. Deficient concentrations of serum Vitamin D may contribute to the process of carcinogenesis among the breast cancer patients. Vitamin D status is a possible modifiable risk factor for breast cancer, and optimizing its level is safe and affordable to prevent breast cancer. It was demonstrated that Vitamin D supplementation has the potential to both increase the concentrations of Vitamin D and reduce the incidence of breast cancer. However, additional studies, for example a prospective cohort study, are needed to clarify this association in a dose-response relationship and the optimal concentrations of serum Vitamin D for breast cancer prevention. Public awareness about the sources of Vitamin D and its benefits must be encouraged. Policy makers must formulate public health policies to prevent vitamin D deficiency.

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Chapter 5: Factors associated with breast cancer risk according to tumor subtypes: TNBC vs non TNBC among breast cancer cases in Karachi, Pakistan

5.1 Background

Research shows that breast cancer is a heterogeneous disease (251, 252). Estrogen and progesterone hormone receptor (ER/PR) protein expression status and human epidermal growth factor (HER2) protein expression or gene amplification are important biomarkers with variable risk factors, clinical & pathologic outcomes (253). The integration of these biomarkers of breast cancer with epidemiological research enable us to identify additional risk factors for breast cancer and to better understand the role played by recognized risk factors in different breast cancer subtypes. On the basis of hormone receptor status and gene expression pattern, breast cancer can be classified into four major intrinsic subtypes: luminal A, luminal B, human epidermal growth factor receptor 2 (HER2) enriched, and triple negative breast cancer (TNBC). Expression of estrogen receptor (ER), progesterone receptor (PR), and HER2-*neu* (HER2) alone are usually used to differentiate between these subtypes in clinical settings. It is also observed that there are possible drifts in molecular subtype throughout breast cancer progression (254).

The most common breast cancer subtypes are ER -positive (luminal A and B) subtypes), while TNBC is one of the most aggressive subtypes. This latter clinically challenging subtype of breast cancer is of particular interest in breast cancer research and is characterized by absence of expression of the ER, PR, and HER2 receptors. TNBC, also now considered as heterogeneous disease, can be further sub-classified into three or four sub types, with the basal-like pattern of gene expression being the most frequently observed (75%) (255). It is aggressive also because of lack of any targeted therapy available due to absence of hormonal receptors. Increasing number of genes are differentially expressed both down regulatory and up regulatory genes in different stages of TNBC (256). Most of the studies reporting risk factors for TNBC are from developed countries. The etiology of TNBC remains understudied in developing countries.

Risk factors according to breast cancer subtypes

All these breast cancer subtypes are associated with different risk factor due to different etiologies. The association of parity differs in different tumor subtypes with parity as

having negative association with ER+/PR+ breast cancers, and positive association with TNBC subtype (257). Older age at first pregnancy is found to be positively associated with HER2 positive subtype in research literature (258). The luminal A subtype is associated with reproductive risk factors like age at menarche, and age at first birth (258). There is also increased risk of Luminal A subtype and TNBC with hormone replacement therapy (258). Family history of breast cancer is differentially associated with breast cancer subtypes. In a study in Spain, family history of breast cancer was related to an increased risk of ER-&PR- breast cancer among younger Spanish women (259). In a study in Wisconsin, late age at first birth was associated with increased risk of lobular and ERPR-negative breast cancer (163). Increasing parity and Vitamin D intake was protective for breast cancer in a case control study done in Karachi Pakistan (160).

TNBC has no hormonal markers, and is usually high grade of poorly differentiated type. It has usually poor prognosis with higher risk of recurrence and high five-year mortality rates (260, 261). Compared to well defined risk factors for ER/PR + breast cancer, factors associated with TNBC are less well defined and vary in different studies (262). High parity and lack of breastfeeding are usually associated with TNBC compared to Luminal A subtype (225, 262, 263). The protective association of breastfeeding duration with TNBC is a possible modifiable risk factor for this most aggressive subtype.

Overall review of TNBC confirms it as a breast cancer of different molecular subtype with distinct risk factors. Moreover it is associated with BRCA1 mutation status, and poor survival (264, 265). TNBC is associated with race of African American and premenopausal status age (265).

5.2 Rationale of this sub study

Several studies conducted in western population have confirmed association of reproductive factors with hormone receptor-positive tumors only and found different risk factors for TNBC. However, studies of breast cancer subtypes conducted among Asian populations' especially Pakistani women are extremely limited. Also, there is a lack of information on how Vitamin D deficiency influences the risk of different molecular subtypes of breast cancer. There are contradictory findings of relationship between Vitamin D and breast cancer could be related to tumor heterogeneity, which suggests that effects of Vitamin D may only be exhibited in specific subtypes of breast cancer.

Therefore, additional analysis with Vitamin D was conducted on specific breast cancer subtypes.

5.3 Objective

To evaluate the association of breast cancer risk with molecular subtypes of breast cancer (TNBC vs non TNBC).

5.4 Data collection method for this objective:

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Source population

The source population for this study is from the multi-center case–control study of breast cancer and Vitamin D study among women visiting two hospitals of Karachi Pakistan, which has previously been described in material & methods (chapter 2). There were 321 patients who had complete molecular profiling compared with 798 controls. ER, PR, and HER2 status of participants were recorded as cases were retrieved from medical records of the medical files, and SAHL, a remote desktop access system. Cases with HER2 results of 0, 1+, or 2+ from IHC testing and/or negative results on FISH testing <2 were considered HER2 negative (HER2−); conversely, HER2 results of 3+ on IHC testing were considered HER2 positive (HER2+). Patients who had a 2+ HER2 immunohistochemistry result without a FISH result were considered to have an inconclusive and, thus, unknown HER2 status. In addition to basic information on breast cancer diagnosis, information on tumor histology was extracted. Breast cancer subtyping was based on immunohistochemical (IHC) staining which was part of routine diagnostics and performed according to the College of American pathologists (CAP) Clinical Practice Guidelines (266, 267). On the basis of these receptors, breast cancer subtypes were classified into four groups: ER+ and/or PR+/HER2−; ER+ and/or PR+/ HER2+; ER−/PR−/HER2+; and ER−/PR−/HER2−. Missing values for ER and PR status were minimized by accessing the patients' records and getting their information from labs outside AKUH and KIRAN. Finally, breast cancer subtypes were broadly divided as TNBC (ER−/PR−/HER2−) and non TNBC subtypes (ER+ and/or PR+/HER2−; ER+ and/or PR+/ HER2+; ER−/PR−/HER2+ subtypes were merged). These factors were

examined in conjunction with the potential confounders identified from the previous sub studies.

5.5 Statistical analysis

All analyses were conducted using SPSS package for Windows 21.0 (SPSS, IBM, Armonk, NY, USA (268). Descriptive statistics were computed for all variables. Frequencies, mean and standard deviations were obtained for continuous variables, while categorical variables were assessed by percentages. To facilitate analysis, variables with multiple categories were collapsed to fewer categories in a meaningful way. Chi square and Fischer exact tests were used to assess categorical variables. To identify the factors associated with breast cancer subtype, univariable analysis of each variable of interest, crude odds ratio and their 95% confidence intervals, along with p values, were calculated. The reference group for each risk factor was generally determined by the category with the minimal level of risk for breast cancer. Risk factors were included in the multivariable analysis if they were significant at p-value <0.25 (269) or had biological significance. All statistical tests were two-sided, with P less than 0.05 used as the cut off for statistical significance.

In multivariable analysis, multinomial logistic regression was performed to identify factors associated with breast cancer subtypes, while adjusting for other variables. All independent variables with univariate analyses p-values less or equal to 0.25 were included in the model. Analysis was done by the purposeful selection method and all the variables that were selected from the univariable analyses were entered in the model simultaneously to adjust for confounding and to identify interactions between the independent variables. Confounders were identified as any variable that changed the OR of the exposure variable by more than 10% when added to the model. Finally, any variable with a p-value >0.05 that was not a confounder or did not interact with other variables was removed from the model to obtain a parsimonious and biologically meaningful model that best explains factors associated with breast cancer subtype. It is important to mention that the statistical power of our analyses is limited by the inclusion of only 73 triple negative breast cancer and 28 HER-2-overexpressing breast cancer cases. We also had to exclude192 potentially eligible cases whose reports for molecular subtypes were not available to be classified into any subtype.

Separate case to case analyses was carried out for 4 sub types of breast cancer using polytomous logistic regression but not included in the main chapter. The relatively low number of HER 2 enriched breast cancer cases in our study limited the power of some variables in this subtype with inconsistencies in results limiting our understanding of reproductive risk factors relationship to risk for the non-luminal breast cancer subtypes.

5.6 Results

Out of all cases, there were 321 cases with complete molecular profiling (table 1). Luminal A subtype (156/321, 48.6%) was the most prevalent followed by triple-negative (73/321, 22.7%), luminal B (64/321, 19.9%), and HER2-overexpressing (28/321, 8.7%) (Figure 1). However, case to case analysis comparing three subtypes to luminal A and case to control analysis were done**,** but not included in the main chapter as small numbers in HER2 overexpressed subtype (n= 28) limited power of the study for this subtype and precluded definite conclusions. The main focus of the study is comparing non TNBC (n=307) cases versus controls, and TNBC (n=73) cases versus controls.

Table 1. Breast cancer molecular subtypes

Intrinsic subtype	**Clinicopathologic surrogate definition**	**n=321**	**%**
Luminal A	ER+ PR+ >20%	156	48.6
Luminal B	'Luminal B–like (HER2 -)' ER+ PR+ <20% Luminal B–like (HER2 +)' ER +	64	19.9
The oncogene c-erbB2/HER2 enriched	HER2 overexpressed or amplified	28	8.7
TNBC	ER and PR - ER and PR - HER2 -	73	22.7

ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor; TNBC, triple negative breast cancer.

Table 2: Sociodemographic, reproductive and clinical characteristics of breast cancer cases according to molecular subtypes of breast cancer among women in two major cancer hospitals of Karachi, Pakistan.

Variable	Category	Control		non-TNBC		TNBC		p-value*
		n= 798	%	n= 307	%	n= 73	%	
Hospital								0.001
	Private hospital	420	52.6	198	64.5	42	57.5	
	Public hospital	378	47.4	109	35.5	31	42.5	
Age at diagnosis (years)								0.001
	< 35	132	16.5	37	12.1	11	15.1	
	35-44	245	30.7	79	25.7	22	30.1	
	45-54	231	28.9	75	24.4	22	30.1	
	55 & above	190	23.8	116	37.8	18	24.7	
Education level								<0.001
	< grade 8	180	22.6	91	29.7	30	41.7	
	8-12 grade	250	31.3	107	35	24	33.3	
	> grade 12	368	46.1	108	35.3	18	25	
Marital status								0.57
	Single/widow/ divorced	151	18.9	65	21.2	12	16.4	
	Married	647	81.1	242	78.8	61	83.6	
Employment status								0.005
	Yes	184	23.1	51	16.6	8	11	
	No	614	76.9	256	83.4	65	89	
Socioeconomic status (SES)								<0.001
	Upper	131	16.5	25	8.3	5	7	
	Middle	463	58.4	188	62	34	47.9	
	Lower	199	25.1	90	29.7	32	45.1	
Consangiuos marriage								0.49
	Yes	191	24.3	82	27.9	18	25.4	
	No	594	75.7	212	72.1	53	74.6	
	Nullipara	121	15.2	38	12.4	8	11	
Parity								0.47
	1-3 children	376	47.1	138	45	38	52.1	
	> 3 children	301	37.7	131	42.7	27	37	
Abortion								0.2
	No abortion	450	56.4	164	53.4	45	61.6	
	< 3 abortions	277	34.7	126	41	24	32.9	
	> 3 abortions	71	8.9	17	5.5	4	5.5	
Breast feeding history								0.38
	No	145	18.5	51	16.9	9	12.3	
	Yes	640	81.5	251	83.1	64	87.7	

Variable	Category	Control		non-TNBC		TNBC		p-value*
Lifetime months of breast feeding								0.75
	No breast feeding	145	18.5	51	16.9	9	12.5	
	< 12 months	95	12.1	39	12.9	10	13.9	
	> 12 months	545	69.4	212	70.2	53	73.6	
Family planning (FP)								0.13
	No FP	535	78.9	190	85.6	47	81	
	< 24 months	103	15.2	20	9	6	10.3	
	> 24 months	40	5.9	12	5.4	5	8.6	
Age of mother at first live birth (years)**								0.92
	< 30	609	91.6	240	90.9	60	92.3	
	>30	56	8.4	24	9.1	5	7.7	
Menopausal Status								0.001
	Premenopause	440	55.7	131	43.2	35	50	
	Post menopause	350	44.3	172	56.8	35	50	
Age at menarche (years)								0.23
	< 12	92	12.2	27	9.7	5	7.7	
	13-14	410	54.2	152	54.5	44	67.7	
	> 14	255	33.7	100	35.8	16	24.6	
Family history of breast cancer								0.1
	Yes	212	26.7	68	22.2	13	17.8	
	No	581	73.3	238	77.8	60	82.2	
								0.41
Family history of any cancer	Yes	312	39.3	107	35.3	30	41.1	
	No	481	60.7	196	64.7	43	58.9	
Serum Vitamin D level (ng/dl)								0.039
	> 30	163	25.4	50	23.4	5	10	
	20-30	115	17.9	29	13.6	11	22	
	< 20	364	56.7	135	63.1	34	68	
Body mass index ***								0.074
	< 23	115	15.6	55	19.5	11	16.9	
	23-25	144	19.6	67	23.8	19	29.2	
	> 26	476	64.8	160	56.7	35	53.8	

*p values generated from Chi-square or Fisher's exact tests

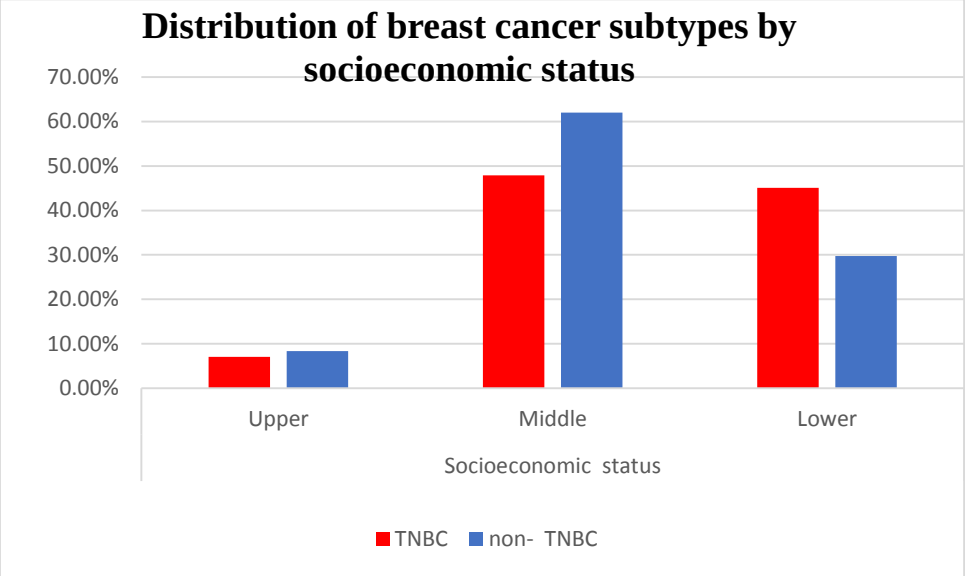
** Restricted to women who ever had a full-term pregnancy (a pregnancy was considered as full-term if it resulted in a live birth or lasted 7 or more months) ***BMI, body mass index; BMI was categorized according to the WHO classification for Asian as underweight/normal weight (<23 kg/m2), overweight (23-25 kg/m2) or obese (>26 kg/m2).

We analyzed 73 cases of triple negative breast cancer (TNBC) vs 307 cases of non-TNBC subtypes. We evaluated association of sociodemographic and reproductive and other factors with TNBC and non TNBC subtypes (tab 2). Sociodemographic factors included age group, education, marital status (single/divorced/widow vs married), employment status and SES. Reproductive factors included age at menarche (≤12, 13-14 yrs., >14years), parity (nulliparous/ parous). Parous women were categorized as 1 -3 and >3 children and age at first birth as ≤30years vs >30 years.

Compared with non-TNBC, TNBC cases tended to be younger less than 35 yrs. (15.1%), less educated with 41.7% having studied less than grade 8. More TNBC cases belonged to the lower SES group (45.1 %) compared with Non-TNBC case group (29.7 %) and controls (25.1%). Middle SES group had more cases of non-TNBC (62%) compared with TNBC (47.90 %) and controls (58.4%). Postmenopausal women had more cases of non-TNBC (56.8%) as compared to controls (44.3%) and TNBC (50%). Vitamin D concentrations were more likely to be deficient (<20 ug/dl) in TNBC cases (68%) and non-TNBC cases (63.1%) as compared to controls (56.7%). Women with sufficient concentrations of Vitamin D (> 30ug/dl) had the least number of TNBC cases (10%).

Although not statistically significant, a greater number of non-TNBC cases (41%) had a history of abortions as compared to the TNBC group and controls, whereas more women diagnosed with TNBC had a history of family planning with hormonal contraceptives for more than 24 months (8.6%). Women with menarche between ages 13 to 14 years had more cases of TNBC (67.7%) compared to controls and non-TNBC, whereas menarche age more than 14 years had fewer cases of TNBC (24.6%). BMI was high among all three groups.

Figure 1 Distribution of breast cancer subtypes by socioeconomic status



TNBC cases were higher in women of age groups 35-44 and 45-54 years of age and non TNBC higher in patients of 55 years and older age group. TNBC group was more likely to be less educated as 41.7% women had education level below grade 8. Socioeconomic status showed least number of TNBC cases in upper SES (7 %) and higher numbers in lower SES group (45.1%) when compared to non TNBC cases (Fig. 1). Premenopausal women had the highest number of non TNBC (55.7%). Cases with a positive family history of breast cancer had more luminal A subtype (24.5%). Vitamin D deficiency (<20 ng/dl) had highest percentage of TNBC cases (68%).

Table 3: Distribution of histopathology, grade, TNM stages and tumor characteristics of molecular subtypes of breast cancer among women in two major cancer hospitals of Karachi, Pakistan

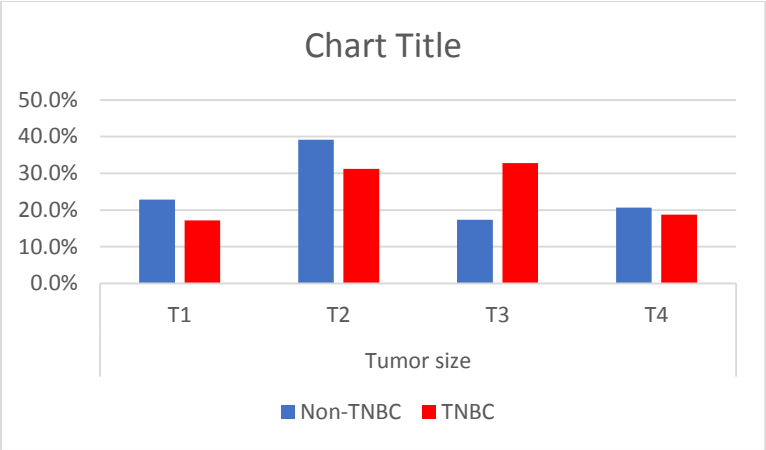
Variable	Category	Non-TNBC		TNBC		p-value
		n	%	n	%	
Side of tumor	Right	157	52.0	32	44.4	0.27
	Left	142	47.0	38	52.8	
	Both	3	1.0	2	2.8	
Tumor type	Invasive Ductal carcinoma (IDC)	272	90.1	68	94.4	0.69
	Invasive lobular carcinoma (ILC)	12	4.0	2	2.8	
	Others	18	6.0	2	2.8	
Grade of tumor	III	96	32.8	44	64.7	<0.001
	I/II	197	67.2	24	35.3	
Tumor size	T1 (T $\leq$ 2.0 cm)	62	22.9	11	17.2	0.05
	T2 (2.0 - 5.0 cm)	106	39.1	20	31.3	
	T3 (T > 5.0 cm)	47	17.3	21	32.8	
	T4 (Extension to the chest wall)	56	20.7	12	18.8	
Nodal involvement	N0/N1	198	73.6	43	69.4	0.68
	N2	39	14.5	13	21.0	
	N3	32	11.9	6	9.7	
Metastasis	No metastasis	207	72.9	48	71.6	
	Metastasis	42	14.8	9	13.4	0.85
	Unknown	35	12.3	10	14.9	
TNM Stage	Stage 1	41	16.8	6	11.3	0.77
	Stage 2	88	36.1	20	37.7	
	Stage 3	73	29.9	18	34.0	
	Stage 4	42	17.2	9	17.0	

p values generated from Chi-square or Fisher's exact tests

Table 3 represents the distribution of histopathology, grade, TNM stages and tumor characteristics among TNBC and non TNBC subtypes of breast cancer patients. Right-sided breast cancer was 52 % in non TNBC (52%) and left sided breast cancer was 52.8% in TNBC cases. out of 72 TNBC cases, 94.40% had invasive ductal carcinoma, 2.8% had invasive lobular carcinoma and 2.8% had other histological types of breast cancer. There was a statistically significant difference in grade of tumor among TNBC cases with 64.7% as grade III and poorly differentiated, compared to the grade III in non TNBC

cases (32.8%). T1 was more common among non-TNBC cases (22.9%) compared to TNBC cases (17.2 %). T3 was more common in TNBC cases (32.8%) compared to non-TNBC cases (17.3%).

Figure 2 tumor sizes in TNBC and non TNBC



T1 was most common in Luminal B (27.3%) compared to the rest. TNBC cases had higher numbers of T3 (328%) compared to the non TNBC group (fig.2) which is similar to other studies (270). However, there was no difference in TNM staging and metastasis.

Univariate multinomial logistic regression analyses were used to calculate odds ratios (ORs) and their associated 95% confidence intervals (CIs), to compare different subtypes of breast cancer with a common control group**.** This approach is helpful in performing a series of simple binary logistic regression models for different tumor subtypes-control comparisons.

Table 4. Univariate multinomial logistic regression analyses of the association between sociodemographic characteristics and breast cancer subtypes among women in two major cancer hospitals of Karachi, Pakistan

Variable	Category	Non-TNBC				TNBC			
		OR	95% CI		p value	OR	95% CI		p value
Age					<0.001				0.74
	55 & above	2.18	1.41	3.35		1.14	0.52	2.49	
	45-54	1.16	0.74	1.81		1.14	0.54	2.43	
	35-44	1.15	0.74	1.79		1.08	0.51	2.29	
	<35	1(Ref)				1(Ref)			
Education level									
	< grade 8	1.72	1.24	2.4	<0.001	3.41	1.85	6.28	<0.001
	8-12 grade	1.46	1.07	1.99		1.96	1.04	3.69	
	> grade 12	1(Ref)	.			1(Ref)	.	.	
Marital status					0.6				0.4
	Single/widow/divorced	1.15	0.83	1.6		0.84	0.44	1.61	
	Married	1(Ref)	.	.		1(Ref)	.	.	
Employment status					0.02				0.02
	No	1.5	1.07	2.12		2.43	1.15	5.17	
	Yes	1(Ref)	.	.		1(Ref)	.	.	
Socioeconomic status					0.01				0.04
	Lower	2.37	1.44	3.89		4.21	1.6	11.09	
	Middle	2.13	1.34	3.37		1.92	0.74	5.02	
	Upper	1(Ref)	.	.		1(Ref)			
Consanguineous marriage					0.23				0.84
	Yes	1.2	0.89	1.63		1.06	0.6	1.85	
	No	1(Ref)	.	.		1(Ref)	.	.	
Parity					0.12				0.46
	Nullipara	0.72	0.48	1.1		0.74	0.33	1.67	
	1-3 children	0.84	0.64	1.12		1.13	0.67	1.89	
	> 3 children	1(Ref)	.	.		1(Ref)	.	.	
History of abortion					0.2				0.33
	No abortion	1.44	0.82	2.52		1.68	0.58	4.81	
	< 3 abortion	1.77	1	3.13		1.43	0.48	4.26	
	> 3 abortion	1(Ref)	.	.		1(Ref)	.	.	
Age at first live birth (years)					0.74				0.83
	< =30	0.92	0.56	1.52		1.1	0.43	2.86	
	> 30	1(Ref)	.	.		1(Ref)	.	.	
History of breast feeding					0.54				0.19
	No	0.9	0.63	1.27		0.62	0.3	1.28	
	Yes	1(Ref)	.	.		1(Ref)	.	.	

		TNBC				Non TNBC			
		OR	**95% CI**		**p value**	**OR**	**95% CI**		**p value**
Family planning					0.58				0.22
	no FP	1.18	0.61	2.3		0.7	0.27	1.87	
	< 24 months	0.65	0.29	1.45		0.47	0.14	1.61	
	> 24 months	1(Ref)	.	.		1(Ref)	.	.	
Menopausal status					0.001				0.35
	Premenopause	0.61	0.46	0.79		0.8	0.49	1.3	
	Postmenopauseb	1(Ref)	.	.		1(Ref)	.	.	
Age at menarche (years)					0.24				0.78
	< 12	0.75	0.46	1.22		0.87	0.31	2.43	
	12 to 14	0.95	0.7	1.27		1.71	0.95	3.1	
	> 14	1(Ref)	.	.		1(Ref)	.	.	
Family history of breast cancer					0.12				0.09
	Yes	0.78	0.57	1.07		0.59	0.32	1.1	
	No	1(Ref)	.	.		1(Ref)	.	.	
Family history of cancer					0.22				0.77
	Yes	0.84	0.64	1.11		1.08	0.66	1.75	
	No	1(Ref)	.	.		1(Ref)	.	.	
Serum Vitamin D level (ng/dl)					0.31				0.02
	< 20	1.21	0.83	1.76		3.05	1.17	7.93	
	20-30	0.82	0.49	1.38		3.12	1.05	9.22	
	> 30	1(Ref)				1(Ref)			
Body mass index c					0.06				0.4
	< 23	1.42	0.99	2.06		1.3	0.64	2.64	
	23-25	1.38	0.99	1.95		1.79	1	3.23	
	> 26	1(Ref)	.	.		1(Ref)	.	.	

[a] Restricted to women who ever had a full-term pregnancy (a pregnancy was considered as full-term if it resulted in a live birth or lasted 7 or more months)

[b] Restricted to postmenopausal women (women were classified as postmenopausal if their cycles ended naturally or from surgery in which both the uterus and ovaries were removed, or from surgery in which only uterus was removed)

[c]BMI, body mass index; BMI was categorized according to the WHO classification for Asian as underweight/normal weight (<23 kg/m2), overweight (23-25 kg/m2) or obese (>26 kg/m2).

OR are compared to controls

Bold values indicate statistical significance ($p<0.05$)

Odds ratios for non TNBC cases versus controls, and TNBC cases versus controls, through univariate multinomial logistic regression analyses are presented in Table 4. Age of 55 yrs and above was positively associated with risk of non TNBC (OR=2.18, 95% CI=1.41, 3.35). Premenopausal status had a protective effect only among women with

non TNBC subtype (OR= 0.61, 95% CI= 0.46, 0.79). Most of the women were unemployed being housewives and this was also associated with risk of both TNBC and non TNBC with higher OR among TNBC (OR= 2.43. 95%CI=1.15, 5.17). TNBC was higher among women with education less than grade 8 (OR=3.41, 95%=1.85, 6.28). Less than grade 8 concentrations of schooling was also associated with non TNBC (OR=1.72, 95%=1.24, 2.40). Poor SES was associated with both TNBC and non TNBC with stronger association with TNBC (OR= 4.21 95% CI=1.60, 11.09) and less strong with non TNBC (OR= 2.37, 95% CI=1.44, 3.89). Vitamin D deficiency (VDD) was associated with TNBC (OR= 3.05. 95% CI= 1.17, 7.93). Vitamin D insufficiency (VDI) was also associated with TNBC (OR= 3.12, 95%CI= 1.05, 9.22).

Table 5: Adjusted odds ratio of association of vitamin D with triple negative breast cancer (TNBC) and non TNBC subtypes of breast cancer using multinomial logistic regression analyses

		TNBC				**non TNBC**			
Variable	**category**	**OR**	**95%CI**		**p value**	**OR**	**95%CI**		**p value**
Socioeconomic status					<0.001				**<0.001**
	Lower	8.76	2.45	31.32		4.08	2.06	8.10	
	middle	2.39	0.80	7.15		3.21	1.85	5.57	
	upper	1(Ref)				1(Ref)			
serum Vitamin D (ng/ml)					0.02				0.09
	< 20	3.11	1.17	8.29		1.41	0.95	2.09	
	20-30	3.45	1.13	10.56		0.92	0.54	1.57	
	> 30	1(Ref)				1(Ref)			

Abbreviations: OR, odds ratio CI, confidence interval

OR are compared to controls

*Adjusted for hospital, and menopausal status

The multivariable multinomial logistic regression analyses showed that both TNBC and non-TNBC subtypes were associated with poor socioeconomic status and low Vitamin D concentrations with TNBC risk much higher among women of low SES (OR=8.76, 95% CI= 2.45, 31.32) and women with vitamin D deficiency (OR=3.11, 95%CI= 1.17. 8.29).

5.7 Discussion

This detailed sub study allowed us to investigate the associations between well-known risk factors among non TNBC and TNBC subtypes of breast cancer among Pakistani women. Overall TNBC typically constitutes 10-20 % of all breast cancer subtypes (271) but in our study sample it was high and constituted 22.7 % of all subtypes. The frequency of TNBC is reported to vary between different ethnic groups. In a pooled data from three population-based studies and consisting of 558 TNBC and 5,111 controls, TNBC accounted for 12% of newly diagnosed breast cancers (272). In a retrospective study of White patients in West Virginia. Hospital, TNBC occurred in 18.9% of the 620 patients being diagnosed at age <50 years (273). TNBC is reported high in multiple studies consisting of African ancestry (261, 274, 275). It was 22 % in studies among African American women (276, 277). TNBC in a study in the National Cancer Institute in Mexico City was also high i.e. 23.1% (261). In another study, TNBC comprised 17.28% of the breast cancers in Pakistani women diagnosed at the Armed forces Institute of Pakistan Rawalpindi (278). Variation in incidence of TNBC could be due to multiple factors including differences in environmental exposures or behaviors and genetic factors.

The mean age of TNBC cases was younger (46.1 SD 11.7 years) than mean age of non TNBC cases (49.4 SD 12.5 years) which is consistent with other studies (279). In a study in Saudi Arabia, the mean age of patients with TNBC was similarly relatively young 45 years (280). The median age at diagnosis of TNBC in Morocco was also young i.e. 46 year (281).

Among non TNBC cases, there was a lower risk of breast cancer in premenopausal women while among TNBC cases there was no association with menopausal status. Our result of protective effect of premenopause with non TNBC and not with non TNBC is consistent with similar results in the Women's Health Initiative study (276).

The significant association of TNBC with poor SES, as shown in this study, is similar to a study in West Virginia Hospital, where TNBC was high among socioeconomically deprived population (273). California Cancer Registry also reported poor SES as a risk factor for TNBC among white women (282). In a study by Banegas et al., women living

in a low socioeconomic status (SES) neighborhood had an increased risk of TNBC diagnosis and higher mortality due to breast cancer (208). This points towards the potential impact of SES, an important social determinant of health, on risk factors that may be etiologically important in increasing the risk of developing TNBC. It may be due to poverty related lifestyles choices of these women like eating lesser healthy foods, lack of healthy physical activity, and having exposure to higher concentrations of environmental carcinogens. Additionally, low SES may also be related to reproductive factors like younger age at first pregnancy and lack or shorter duration of breastfeeding, both of which are risk factors for TNBC. It is important to identify lifestyle choices which are modifiable, and may help decrease TNBC among poor women. Conversely TNBC was more common among high SES in The San Francisco Bay Area Breast Cancer Study(272). However, like our study, there were no associations of TNBC risk with reproductive factors like age at menarche or parity (272).

Triple-negative breast tumors are shown to be associated with a younger age, high tumor size, higher-grade tumors , and a higher rate of node positivity (265). However, in our case only high histologic grades were significantly associated with TNBC similar to previous studies (283-286). In our study 64.7% of TNBC were grade III. In a study in North Morocco, 40.4 % of TNBC them were grade III (287). In a study in India, it was more common in the left breast lump and of high grade (288). In a Chinese study, there were similarly more high grade tumors in the TNBC group than those in the non-TNBC(289). In this study TNM stage and distant metastasis were not different at the time of diagnosis in patients with triple-negative tumor and non-triple-negative group similar to findings of a study in Turkey (290).

In the Carolina Breast Cancer Study, among premenopausal patients, TNBC was found to be more common among women with a younger age at menarche, higher parity, younger age at full-term pregnancy, shorter duration of breast-feeding, and higher body mass index (BMI) (264). However, no remarkable associations of reproductive factors with TNBC or non TNBC were observed in this study. Breastfeeding and high parity was not associated with TNBC as identified in other studies (291).

These findings are consistent with the major breast cancer risk factors findings reported in Chapter 3 and showed that both TNBC and non-TNBC were not associated with any of the known traditional risk factors of breast cancer risk such as reproductive factors. As established for all breast cancers, the risk of TNBC was increased in women of low SES and those with lesser education indicating again the important role of environmental related factors. These findings emphasize the importance of the contribution of poverty to the etiopathogenesis of this aggressive subtype. This also implies that women living in conditions of poverty are exposed to unidentified carcinogenic factors in the environment that are responsible for the increased risk of TNBC. These factors were, however, not to be identified in the scope of the study objectives.

Our results confirmed the findings of previous studies that showed TNBC was associated with Vitamin D deficiency (292, 293). Epidemiological data also indicate that women with sufficient Vitamin D have lower incidence of TNBC (73). Based on these findings correction of Vitamin D deficiency in women is a reasonable and cost effective strategy to reduce the incidence of all subtypes of breast cancer, and in particular the aggressive TNBC. Larger prospective studies or clinical trials are needed to further confirm these findings.

In our study there was no genetic testing available for breast cancer cases enrolled in this study. This study due to high percentage of TNBC warrants identification of BRCA mutations in our population because TNBC is more common in women with BRCA1 mutations (294).

Limitation of the study was we did not have complete molecular profile of all cases enrolled in study. Though missing values for receptor status were minimized by accessing the patients and accessing their outside AKUH and KIRAN lab's results but still we had missing data on HER-2/ *neu* and ER/PR status on 192 breast cancer cases. Therefore, we could not analyse all four tumour subtypes separately but had to merge different subtypes as non TNBC group.

5.8 Conclusion

Correction of Vitamin D deficiency in women maybe a reasonable and cost-effective strategy to prevent TNBC like aggressive breast cancer. It should be further tested though clinical trials in our population.

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Chapter 6: Modified Alternate Healthy Eating Index-2010 and breast cancer risk in the Pakistani Population

6.1 Background

According to a recent World Health Organization (WHO) report, cancer prevalence in developing and developed countries is expected to increase by 73% and 29%, respectively from 2000 to 2020 (295). In addition to reproductive factors, sedentary lifestyle and inadequate eating habits may increase breast cancer risk by up to 40% (2). Nutrition plays an integral role in the management of multiple chronic diseases. WHO guidelines for chronic disease prevention include abstinence from tobacco and alcohol and adherence to a healthy diet and a regime of physical activity (295). The correlation of increased breast cancer incidence with rapid changes in dietary intake over the past twenty years suggests that diet may have an essential role in this increased breast cancer incidence and therefore could be a significant modifiable risk factor for reducing it.

Dietary habits are now being increasingly recognized to be important modifiable factors influencing cancer risk (296, 297) and have been estimated, together with overweight/obesity and physical inactivity, to account for a population attributable risk of 35%–38% of 12 common cancers in high-income countries (298). Recent studies have focused on the role of particular dietary factors, the harmful effects of dietary fat and fatty acids (3) and the protective effect of fruits and vegetables (4), in the etiology of breast cancer. However, overall the observational epidemiologic studies have reported inconsistent findings in relation to diet and breast cancer. A likely contributing factor to these variable findings is the challenging nature of dietary assessments which are prone to measurement error.

In Pakistan, compared to the western population, women are diagnosed with breast cancer at a younger age, and this has also been reported in other Asian countries (299). However, epidemiological studies on the relationship of diet and breast cancer risk in Pakistani women are nonexistent in spite of the fact that breast cancer is the most commonly diagnosed cancer, accounting for one-third of female cancers (age-standardized rate ASR 51.7 per 100, 000) (300). There is extensive research on the relationship between diet and cardiovascular diseases CVD and this has shown positive

associations with plausible biological bases. However, studies on the association of diet and cancer, especially breast cancer, have variable results and there are no definite biological mechanisms to provide a basis for positive findings.

6.2 Rationale of this study

To determine any relationship between diet and the incidence of breast cancer in Pakistan, it is important to utilize a standardized healthy eating index score such as Alternate Healthy Eating Index 2010. With the help of nutrition epidemiologist, the AHEI 2010 was modified to reflect the particular cultural patterns of diet in the Pakistani population. This study evaluated the association between the modified AHEI 2010 and its component scores with breast cancer risk among women visiting two tertiary care hospitals of Karachi, Pakistan.

6.3 Methods

Full details of the overall study design are presented in the materials and methods chapter 2. Briefly, this was a matched case-control study in two major hospitals of Karachi Pakistan, AKUH and KIRAN. Cases were all Pakistani women between 18 years and 75 years of age with a confirmed diagnosis of the newly diagnosed first primary in situ or invasive breast cancer. Controls were individually matched to the hospital, province and aged-matched (+/- 5 years), and were selected from the general medicine, and surgical outpatient clinics of the two hospitals.

6.4 Dietary intake assessment with a Food Frequency Questionnaire (FFQ**):**

Dietary intake assessment done by individual interviews of 1124 cases and controls, conducted by trained doctors using the validated food frequency questionnaire FFQ (148).

It was pretested on 50 subjects before the actual study and certain additional foods items possibly related to breast cancer were included following a literature search. The index date for cases was the date of diagnosis, and for controls, it was the date of the interview. For both cases and controls, dietary history was the usual diet in the one year prior to the time of diagnosis or interview date. Prior to the interview, informed consent was obtained from all study participants. Completion of the FFQ took an average of 35-40 minutes.

Intake frequency was categorized into 7 groups ranging from “never” to “5-6 times per day” for foods and for beverages. The selected frequency category for each food item was converted to daily intake. For example, a response of “one serving/week” was converted to 0.14 servings/day by dividing 1 by 7. Each participant was also asked about their average portion size/ common serving size of the food. A standard serving (SVG) size of a food item or beverage was specified on the FFQ (e.g., 1 plate of pulses or 1 cup of milk or one egg, or natural unit such as one apple). The intake frequencies were multiplied by standard portion size to calculate servings per day of all food items. However, consumption in grams per unit or macronutrient intakes or calories from each food was not determined.

6.4.1 Overview of diet-quality scores/ eating indices

The eating indices are dietary assessment tools used to measure diet quality holistically. The purpose of using eating indices is to assess diet quality in individuals using a structured scoring scale. The commonly used eating indices are the Healthy Eating Index-2010 and AHEI 2010 which provide an objective, quantitative measure of diet quality.

The Healthy Eating Index-2010 is a dietary assessment tool used to measure diet quality (149). It was developed by the United States Department of Agriculture and the National Cancer Institute and revised in 2010 to assess the extent to which diets conform to the recommendations outlined in the 2010 Dietary Guidelines for Americans (301). The Healthy Eating Index-2010 has twelve components: nine that assess nutrient adequacy (Fruits, Vegetables, Grains, Dairy, Total Protein Foods, and Plant Proteins) and three that address moderation (Refined Grains, Sodium and empty calories). The total score is the sum of component scores, and higher scores indicate greater compliance with the 2010 Dietary Guidelines for Americans.

6.4.2 Alternate Healthy Eating Index AHEI

The AHEI-2010, is also a validated questionnaire to assess healthy diet quality (302) and was created as an alternative to the HEI 2010. The AHEI is derived and modified from the original HEI, and this index was designed to incorporate foods that have been consistently associated with chronic disease risk (303), and it also provides quantitative scoring for qualitative dietary guidance (e.g., choose more white meat). This diet quality

analysis tool is based on a number of servings of food per day, whereas the Healthy Eating Index-2010 analyzes the diet by amounts of food per 1,000 calories.

AHEI 2010 includes nine components, including some elements from the original HEI, such as fruits and vegetables and the score ranges from 2.5 (lowest) to 87.5 (highest) (302). The nine components are fruits, vegetables, white to red meat ratio, trans fat, polyunsaturated-to-saturated fat (P: S) ratio, whole grains, non- meat proteins like nuts and soy, moderate alcohol consumption, and long-term multivitamin use. Each component has a maximum of 10 points, except for the multivitamin, which was assigned either 2.5 or 7.5 to avoid over-weighting. The AHEI scores reflect only overall diet quality, and not the nutrient and food specific findings of possible biological importance in carcinogenesis.

6.4.3 Modified Alternate Healthy Eating Index scoring criteria

The Alternate Healthy Eating Index (AHEI) 2010 was altered according to our dietary behaviors particular to our adult population. After discussion with a nutrition researcher and a biostatistician to categorize specific dietary groups, we used 6 components contributing 0–10 points to the total score (10 indicated the highest score of recommendations that were met, zero was the lowest that they were not). Intermediate intakes were scored proportionally between 0 and 10. The 6 components included were fruits, vegetables, dairy, grains, white to red meat ratio, and plant proteins. Alcohol, multivitamins, sodium, and fat were excluded from the original AHEI 2010. Alcohol was eliminated since being strictly prohibited in Pakistan; it is not consumed at all by women. As nutrient analysis was not done in this study, therefore, sodium and fat components were also excluded.

The modified AHEI score was calculated from each completed FFQ. Food items listed on it were assigned to their appropriate food groups. Modified AHEI variables and scoring decisions were made and altered as in the case of plant proteins where a >2 serving per day was considered as an ideal instead of 1 serving per day as in the original AHEI 2010.

6.4.4 Criteria for scoring the AHEI

Briefly, the six highest intake components of AHEI-2010 are considered to be ideal (vegetables, fruit, grains, white to red meat ratio, dairy, and plant proteins which includes nuts and legumes). The rationale for including each component and the criteria for assigning the minimum and maximum scores are described in Table 1 of the annexure 1. Each element is given a minimal rating of 0, and a maximal score of 10, with intermediate values, scored proportionally and had the potential to contribute 0–10 points to the total score. The counts for were merged and summed for all vegetables (e.g., spinach, potatoes, okra, bitter gourd, raw salad, mixed greens) to create a total vegetable serving (SVG) per day. A higher score was given for a greater intake of vegetables (10 points for 5+ SVG/day; 0 points for no SVG/day). Similarly, we summed all the reported fruits like bananas, apples, melons, mangoes and created total fruits per day (10 points for 4+ SVG/ day; 0 points for no SVG/day). Higher scores were assigned for consuming more white meat like fish and poultry than red meat. The ratio of white to red meat was scored as 10 points for 4:1 ratio; 0 points for '0'. A separate component for non-meat protein or plant sources of proteins like nuts, beans, and pulses was created (10 points for 2+SVG/day; 0 points for no SVG/day). For grains we merged and summed all food items like chappati, naan, puri, paratha, fried and boiled rice and biryani and created total grains servings per day; we gave a higher score to higher intake (10 points for 5+ SVG/day; 0 points for no SVG/day). We could not differentiate between whole grains and refined or processed grains due to the lack of information on the use of whole or processed grains in our questionnaires and captured only total number of grains. All individual component scores were summed for a total modified AHEI-2010 score ranging from 0 (lowest) to 60 (highest).

6.4.5 Assessment of other variables

For each participant of the study height and weight were recorded from medical files. Body mass index (BMI) was calculated using the formula of weight (kg) / height $(m)^2$. Socioeconomic (SES) factors included the level of education, place, and type of residence, crowding index, home ownership, number of rooms, total household members and monthly income. Crowding index was also calculated as the number of household members divided by a number of rooms. It was further categorized as <1, 1-2, >2.

Factor analysis was used to identify the important variables for socioeconomic status and a composite variable was calculated for socioeconomic status which was further categorized into upper, middle and lower SES. Menopause was defined as permanent cessation of menstruation for a year or more after the last menstrual cycle on the basis of menopause definition in the Oxford handbook (144). Menopausal status was divided into premenopausal and menopausal status. Women were also asked if their menstrual periods have stopped completely, naturally in a healthy way or after surgery.

6.5 Statistical analysis

The distribution of all the variables among cases and controls were reported in percentages for categorical variables & mean (SD) for continuous variables. Conditional logistic regression was used to calculated matched odds ratio (OR) with 95% confidence intervals (CI) to evaluate the relationship between the risk of breast cancer and the modified AHEI-2010 total and component scores, adjusting for the potential confounders, ie body mass index (kg/m2), SES and menopausal status. Modified AHEI score was analyzed as tertile, and trend test was also carried out to assess dose-response relationships between the score tertiles and breast cancer risk. The six component scores were divided into two categories of low and high scores. A two-tailed, p value of <0.05 was considered significant, and Social Sciences Statistical Software package version 21 (SPSS Inc., Armonk, NY, USA) was used for statistical analyses.

6.6 Results

A total of 1184 subjects were enrolled, of which 1124 (374 cases and 750 controls) completed the FFQ. Sixty participants were excluded as they either refused to answer or some questions were incomplete. The mean intake of different food items for cases and controls were assessed and described in Table 1. Section 1A shows the mean intake of dietary components of vegetables, fruits, plant protein, dairy and meat, and section IB shows the mean intake of grains. While the consumption of grains was significantly different between cases and controls ($p<0.001$), there were no significant differences between the two groups in the mean intakes of the other food components. A more detailed analysis of foods in the grain component (Section 1B of Table 1) showed the consumption of chapattis ($p<0.001$) and to a lesser extent naan ($p<0.01$) contributed to

this significant finding. Chapatti, nan, parathas and puri are made from wheat flour and part of traditional Pakistani breakfast and meals.

Table 1 Mean dietary intake of different food items among breast cancer cases and controls in two major cancer hospitals of Karachi, Pakistan

	Food items as serving per day	**Controls (n= 750)**		**Breast cancer cases (n= 374)**		**p value**
		Mean	**SD**	**Mean**	**SD**	
A	**Components of modified AHEI Score**					
	Grains (whole)	2.85	1.61	3.41	1.97	<0.001
	Dairy (cup)	1.63	1.49	1.66	1.47	0.93
	Red meat (plate)	0.46	0.78	0.48	0.83	0.55
	White meat (plate)	0.58	0.86	0.58	1.03	0.97
	White Meat to Red Meat Ratio	3.39	5.60	3.23	5.18	0.43
	Plant Protein (plate)	2.86	1.14	2.89	1.15	0.77
	Fruits (whole)	1.22	1.68	1.34	1.84	0.44
	Vegetables (plate)	3.00	2.54	2.99	2.59	0.95
B	**Grains**					
	Chapatti (whole)	2.20	1.35	2.63	1.65	<0.001
	Paratha (whole)	0.16	0.25	0.14	0.2	0.51
	Naan (whole)	0.32	0.72	0.47	0.91	0.01
	Puri (whole)	0.24	0.75	0.28	0.93	0.27

The overall mean modified AHEI 2010 score was 35 with a range of 8 to57. The mean score among breast cancer cases was 35.5 and controls 34.8. Tertiles were then created for the AHEI score, and Table 2 shows the association between demographic and other characteristic variables among cases and controls. The highest proportion of participants in the highest tertile were from AKUH (60.8%) while 35% of those in the lower tertile were in, the lower SES group showing poor diet quality among them.

Table 2 Characteristics of women in the case-control study according to tertiles of modified Alternate Healthy Eating Index (AHEI=2010) score

Characteristics		**Modified AHEI Score 2010 Lowest Tertile[1]**		**Modified AHEI Score 2010 Middle Tertile[2]**		**Modified AHEI Score 2010 Highest Tertile[3]**		**p value**
		Count	%	Count	%	Count	%	0.001*
Hospital	AKUH	193	47.9	180	49.2	216	60.8	
	KIRAN	210	52.1	186	50.8	139	39.2	
Age groups	<35	64	15.9	58	15.8	62	17.5	0.78
	35-44	117	29.0	110	30.1	110	31.0	
	45-54	128	31.8	104	28.4	93	26.2	
	55 & above	94	23.3	94	25.7	90	25.4	
Education	< grade 8	122	30.3	99	27	81	22.9	0.21
	grades 8-12	130	32.3	122	33.3	119	33.6	
	> grade 12	150	37.3	145	39.6	154	43.5	
Marital status	single/widow/divorced	88	21.8	78	21.3	59	16.6	0.15
	married	315	78.2	288	78.7	296	83.4	
Employed		79	19.7	86	23.5	65	18.5	0.21

Characteristics		Modified AHEI Score 2010 Lowest Tertile[1]		Modified AHEI Score 2010 Middle Tertile[2]		Modified AHEI Score 2010 Highest Tertile[3]		p value
		Count	%	Count	%	Count	%	
SES	upper	48	12.0	51	14.3	44	12.5	0.004*
	middle	211	52.6	196	54.9	226	64.4	
	lower	142	35.4	110	30.8	81	23.1	
Parity	nullipara	54	13.4	57	15.6	51	14.4	0.65
	≤ 3	187	46.4	164	44.8	176	49.6	
	> 3	162	40.2	145	39.6	128	36.1	
History of abortion	no abortion	229	56.8	212	57.9	198	55.8	0.91
	≤ 3 abortion	136	33.7	126	34.4	125	35.2	
	> 3 abortion	38	9.4	28	7.7	32	9.0	
Menopausal status	Postmenopausal	204	51.3	181	49.9	181	51.3	0.91
	Premenopause	194	48.7	182	50.1	172	48.7	
Diabetes mellitus		62	15.4	56	15.3	44	12.4	0.42
History of any comorbid		191	47.4	185	50.5	163	46.0	0.46
Serum Vitamin D level ng/mL	< 20	178	60.5	162	59.1	161	59.2	0.87
	20 - 30	52	17.7	50	18.2	43	15.8	
	> 30	64	21.8	62	22.6	68	25.0	

Modified AHEI 2010 Score Lowest tertile[1] mean (SD) 27.3(4.20),

Middle tertile[2] mean (SD) 35.5(1.6),

Highest tertile[3] mean (SD) 43.5(3.7)

Table 3 shows the multivariable odds ratio and 95% confidence intervals for breast cancer risk according to the tertile of modified AHEI-2010 scores. The modified AHEI 2010 score was significantly associated with breast cancer (p<0.001) with women with higher scores having a higher risk of breast cancer.

Table 3 Odds ratio (OR) and 95% CIs of breast cancer in women according to Modified Alternate Healthy Eating Index 2010 (AHEI 2010) scores

Modified AHEI score tertile	**OR***	**95 % CI**		**p for trend**	**p-value**
Lowest tertile	1(ref)			<0.001	0.009
Middle tertile	1.36	0.93	1.97		
Highest tertile	1.81	1.24	2.64		

*Adjusted for BMI menopausal status and SES

To better understand the individual role of each component score of the modified AHEI-2010, we examined the association between each food component and the risk of breast cancer (Table 4). High scores on the grains component were associated with a higher risk of newly diagnosed breast cancer (OR=2.53, 95% CI=1.69, 3.79). SES was also strongly associated with breast cancer risk with higher risk of breast cancer among women belonging to lower SES.

Table 4 – Association between each component of modified AHEI -2010 score and breast cancer in women in two major cancer hospitals of Karachi, Pakistan

Component of modified AHEI -2010 score**	**OR***	**95% CI**		**p-value**
Grains	2.53	1.69	3.79	<0.001
Dairy	1.1	0.79	1.52	0.63
Fruits	1.22	0.62	2.4	0.81
Vegetables	1.41	0.95	2.1	0.37
White red meat ratio	0.97	0.68	1.41	0.81
Plant proteins	1.03	0.67	1.6	0.96

*Adjusted for socioeconomic status, BMI and menopausal status

**Reference is a lower component score

6.7 Discussion

This study, assessing the role of a diet quality score with breast cancer in Pakistan, showed that the total modified AHEI 2010 score and its components did not show any protective association with breast cancer risk among Pakistani women. Contrary to the other studies where higher scores are protective, there was a positive association between higher modified AHEI score (lowest tertile vs. highest tertile OR =1.80, 95% CI= 1.23. 2.64) and one of its component grains score (OR= 2.53, 95% CI= 1.69, 3.79) with breast cancer.

There could be several possible reasons a protective association was not observed. Firstly, the original AHEI was developed on the basis of nutritional recommendations for Americans and may not be suitable for a Pakistani population. In addition, there are currently no validation studies for the modified AHEI and thus no cutoff points defining high adherence in the Pakistani society. The types of food included for some dietary components, were also different from the original AHEI, particularly with regard to grains, that is, based on total grains and not whole grains only. Evaluation of the performance of this scoring index is further limited by the types and heterogeneity of studies. It has been reported that the predictive ability of the score also varies widely across countries, indicating that if such scores are used, they should be modified and validated to local circumstances and recalibrated in particular populations (304). Measures for the performance of this index for the majority of studies have been morbidity and mortality due to chronic diseases, and survival of breast cancer patients (305). Additionally, breast cancer is a heterogeneous and multifactorial disease, including different subtypes with different etiologies. It is now recognized as more than one biological entity, with varying mechanisms of carcinogenesis (306). It is also important to note that AHEI does not accept food specific findings of likely biological importance in carcinogenesis. Moreover, different cooking styles and dietary items in our study population may also account for the positive association of modified AHEI 2010 and breast cancer in this study. Therefore, these are a few of factors making it difficult to compare our results to the literature in terms of diet quality.

In the literature, AHEI score has been observed to be a better predictor of some of the diseases like COPD and asthma compared to cancers where its role has been inconsistent,

with a weaker association of diet with all cancers combined, compared to CVD (304). Participants in the Nurses' Health Study and the Health Professionals Follow-up Study, United States showed that a high AHEI-2010 diet score (reflecting high intakes of whole grains, fruits, vegetables, nuts, and milk products, chicken and low intakes of red/, refined grains, and sugar-sweetened drinks) was associated with a lower risk of chronic obstructive pulmonary disease COPD (307). It has been inversely associated with neoplasms of gastrointestinal tract like colorectal carcinoma, pancreas, and esophagus where the etiology of those cancers is very different from breast cancer. NIH-AARP diet and health study by Bosire C show that the AHEI-2010 diet score as being protective for prostate cancer (308).

A published meta-analysis showed no association between AHEI and risk of breast cancer mortality among women with breast cancer (309). Similarly, in a cohort study of women diagnosed with stages I-III breast cancer AHEI-2010 diet was not associated with breast cancer mortality (310). In the prospective Nurses' Health Study (NHS) cohort, followed from 1984 to 2006, no association between the AHEI and risk of breast cancer by molecular subtype was found (311). On the other hand, AHEI was associated with lower risk of estrogen-receptor-negative, but not estrogen-receptor-positive, breast cancer (312).

In the current study, the positive association between the risk of newly diagnosed breast cancer and grains component of modified AHEI 2010 score was an unexpected finding and can be again explained by multiple factors. In Pakistan, grains are commonly consumed as refined grains such as refined flour, white rice, instead of whole grains and brown rice, and are, therefore, rich sources of high starch and carbohydrates. It is confirmed from the COBRA study that our Pakistani population consumes very high carbohydrate diets mainly from refined sources (148). According to Household Integrated Economic Surveys (HIES 2015-2016) per capita, monthly consumption of wheat and wheat flour was highest (7.26 kg) and for rice (0.99 Kg) (313). A similar association of high starch /carbohydrate intake (more than about 60% of energy), especially from refined sources and total mortality, was found in a large prospective cohort study from 18 countries in five continents (314). This is in line with the results from several studies which identified refined grains to be associated with increased risk of stomach,

colorectal, and other cancers. Results of a study in Jordan shows that higher consumption of refined grains is associated with higher colorectal cancer CRC risk (315). Whole-grain consumption, on the other hand, particularly whole-grain wheat, has been reported inversely associated with risk of esophageal cancer (316), several neoplasms including GIT and breast (317) and pancreatic cancer while increased consumptions of refined grains were not associated with a decreased risk (318). Similarly, in some other studies, for example, the National Institutes of Health NIH-AARP Diet and Health Study and the HELGA study, increased whole-grain consumption was associated with a modestly reduced risk of neoplasms such as colorectal cancer (319-321). On the contrary, the results are different in some studies as in a systematic review of 43 longitudinal studies conducted in Europe and North America demonstrating a null association between total grains and colon cancer (322) and a similar null association was reported between whole grains and colon cancer in a prospective study (323).

Similar protective effect of whole grains has also been observed with breast cancer risk with a study where high whole grain food intake was associated with lower breast cancer risk before menopause (324), and in a case-control study where whole grain consumption more than 7 times/week was consistently associated with reduced risk of breast cancer (325). However, in the Nurses' Health Study II, total refined grain food intake was not associated with risk of breast cancer (326). In the Iowa Women's Health Study, which was a prospective cohort study of women among postmenopausal women only, there was no association of breast cancer and refined grains (327). Similarly, another study found no reduction of risk of breast cancer for a healthy dietary index in British women (HR for maximal adherence to the diet score compared with minimal adherence: 0.94 with 95% CI: 0.67–1.32) (328). In another cohort study of breast cancer cases from California, authors reported no association of breast cancer with intake of fruits, vegetables, or grains. To summarize, although there is some evidence for a cancer protective role for a diet high in whole grains overall the results of all studies are inconsistent.

Conversely, increased intakes of refined grains indicate lower intakes of healthful whole grains. Refined grains undergo a harsh refining process that removes the bran and other key nutrients. An excess consumption of refined grains cause increased oxidative stress

and high concentrations of inflammatory biomarker concentrations leading to CVD, diabetes, and breast cancer (329). Recent studies have shown that carbohydrate quality rather than an absolute quantity of intake may be important in breast cancer risk, particularly for premenopausal women. A plausible biological explanation in relation to breast cancer is that dietary carbohydrate quality affects circulating insulin concentrations and thus promote cancer growth. Because insulin concentrations are higher in the presence of insulin resistance, it is a possibility that high carbohydrate or glycemic load intake would increase breast cancer risk (330). High-carbohydrate diets, and particularly diets high in glycemic index (GI) or glycemic load (GL), increase postprandial glucose and insulin concentrations. There are a number of studies reporting a positive association between breast cancer and high carbohydrates among Mexican women (331), South Korean women (332) and Chinese women in the Shanghai Women's Health Study (333). Biologically, a diet rich in other carbohydrates, such as sugars and refined carbohydrates, may increase breast cancer risk possibly by inducing metabolic syndrome due to altered endocrine and inflammatory responses (334). Findings from another study support the association between the incidence of premenopausal breast cancer and adolescent and early adulthood diet characterized by high intake of carbohydrates in the form of sugar-sweetened soft drinks, and refined grains (335). Carbohydrate intake was similarly associated with breast cancer among postmenopausal women with estrogen-negative tumors (RR 1.13; 95%CI, 1.02–1.25) although no differences in BMI was observed (336).

Another possible explanation for the positive association between higher scores of grains and breast cancer could be the potential pollution of grains by industrial wastes, which contaminate rice and flour with certain carcinogens like aflatoxins (337). Contaminated grains have previously been shown to be associated with a higher incidence of esophageal cancer in China (338).This issue requires further examination.

Lastly, local food preparation procedures may also be a factor as in Pakistan, grains and rice are commonly cooked locally, using excessive oil or fat. These methods of food preparation contribute to grains being exposed to the high-fat content. In a case-control study involving three hospital populations in Spain, there was a strong relationship

observed between oils and breast cancer (339), and this is also an area for further investigation.

This study already showed an increased risk of breast cancer among poor women (chapter 3). Poor SES in Pakistan is usually associated with poor lifestyle and dietary choices as well as certain reproductive factors like earlier age at marriage and at first birth. It is supported by similar study findings in California teachers study among Hispanic women residing in low SES neighborhoods (340) and another study where poor women had increased risk of breast cancer mainly triple negative breast cancers (341). A possible explanation could be that women of low SES status may have poorer dietary choices due to lesser access to and affordability of buying healthy foods, or maybe more exposure to consumption of contaminated or poor quality adulterated foods specially grains and oils used in this case. Overall, these findings warrant further environmental and nutrition studies to identify modifiable dietary factors responsible for breast cancer among poor women. It will expand our knowledge of the role of nutrition and may provide new opportunities for the development of risk reduction strategies that may decrease the incidence and mortality of breast cancer in our women.

Our study does not support an inverse association between intake of fruit, vegetable, and plant proteins and breast cancer. The European Prospective Investigation Into Cancer and Nutrition (EPIC) study has also shown that total or specific vegetable and fruit intake is not associated with risk for breast cancer (342) however in a larger population-based case-control study in Poland, vegetables and fruits were observed to protect against breast cancer (343). Specific types of fruits and vegetables may be more strongly associated with cancer, and their effects would be diluted by combining fruits and vegetables into a single score. Therefore, individual fruits were also analyzed, but none of them showed any protective association with breast cancer. Despite the conflicting results from epidemiologic studies, the public health significance and the biological plausibility for a beneficial effect of fruits and vegetable suggest that further research in the form of an RCT should be conducted, particularly with respect to breast cancer, for which few modifiable risk factors have been identified.

Strengths and limitations of the study

The main strength of this study is that all the data was collected by trained clinician and researcher that allowed investigating the research objective with adequate precision. Another advantage is that a high proportion (94%) of participants completed the FFQ.

However, several limitations should be noted. The first one is the inability of the study to differentiate between refined and whole grains intake and to adequately capture the dietary grains component. The modified AHEI component scores gave points for all grains whereas the original AHEI scores whole-wheat bread/grains. This scoring difference explains how the modified AHEI and grains score in this study were positively associated with breast cancer contrary to the negative and protective association with breast cancer. The original AHEI score is not validated in the Pakistani population thus making comparisons difficult. Type of cooking and the amount of oils and fats used was also not assessed. Measurement error, for example, self-reports of pre-breast cancer diet one year prior to diagnosis with breast cancer, could provide misclassification in exposure status, but it is likely to be non-differential. To minimize bias, patients were interviewed for dietary assessment prior to their confirmed diagnosis of breast cancer to reduce any impact of changes in diet following the cancer diagnosis, However, the effect of investigations and stress on diet cannot be excluded. Another weakness is the inability to accurately capture intake of all food items and difference in the portion sizes. Dietary measurements are still not free from measurement error, and the risk estimates may be imprecise. Like all case-control studies, there are biases related to the temporality and exposure misclassification caused by differential recall, which may be due to the cases' cancer diagnosis, although clinicians were trained to elicit information from cases and controls in a standardized way in order to minimize bias. Furthermore, the time window of exposure is also critical in epidemiologic studies of diseases with prolonged latent period such as breast cancer. To improve accuracy and precision, we used one year from diagnosis as the point of measurement in the dietary survey which is a good reproducible measure of diet and close to those obtained by dietary records (344). Individual nutrient analysis of fat and energy intake was not undertaken.

6.8 Conclusions

A higher AHEI-2010 score and higher diet grain score (reflecting high intakes of grains both whole and refined, and rice) were significantly associated with a higher risk of breast

cancer in Pakistani women. Limiting refined carbohydrate intake might be a useful public health message as it may represent a potentially modifiable risk factor for breast cancer in our population. There is a need for awareness of a healthy diet based on more of whole grains and brown rice replacement with refined grains and white rice respectively which may play a role to prevent breast cancer. These results suggest that in our population emphasis on selecting the healthiest choices within each food group, specifically high-quality grains (whole vs. refined grains) is needed. The association of breast cancer risk with use of grains needs further exploration with detailed information about different carbohydrate types including energy intake and a prospective cohort study design in order to better understand the associations between whole grains, different carbohydrates, and breast cancer.

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Chapter 7: Patient delay in breast cancer diagnosis, its associated factors and stage of breast cancer at first presentation

7.1 Background

Although breast cancer incidence was reported to be highest in developed countries, its incidence is rising rapidly in low and middle-income countries (LMICs), such as Pakistan. In Pakistan, breast cancer is a disease with major public health significance due to its increasing incidence and mortality. The incidence of breast cancer amongst females in Pakistan has previously been shown to be 2.5 times greater than that in India and Iran (345). Pakistan to this date does not have any national screening or awareness programs that contribute to the presentation of the disease at a more advanced stage (346).

Early detection of breast cancer is of critical importance due to smaller tumor sizes and reduced chances of metastasis at the time of the first diagnosis and better survival later on (347). Increased involvement of lymph nodes, larger tumor size, distant metastases and poor survival following surgery are all the indicators of late detection and more adverse prognosis. An understanding of the factors influencing the delay in medical diagnosis is therefore crucial for patients, clinicians, and policymakers. Policies and strategies to shorten delays, and ultimately reduce the impact on the health care system, can be developed accordingly. A study in Thailand reported that one of the significant weaknesses of disease control was the delay in breast cancer treatment which in turn was caused by system and patient delay, specifically in a developing country (348). Pakistan is also a developing country and is in the low and middle-income category with countries such as Bangladesh, Bhutan, and India, where gross national income (GNI) per capita is US $1,555, and total expenditure on health as % of GDP is only 2.6% (349). According to a study undertaken at Aga Khan University Hospital (AKUH), the financial burden of cancer care is huge and overwhelming (350). The age standardized rate of breast cancer (ASR per 100,000) is 51.7 which is the highest in Asia (351). Pakistan is the fifth most populous country in the world with Karachi, as the most populous city of Pakistan. It has an estimated population of 24 million which includes people from multiple ethnicities. However, there is a dearth of well-equipped cancer hospitals: nearly 320,000 new cases of cancer are expected every year in Pakistan, but the entire country has only 20 cancer

hospitals. Moreover, there are no palliative care facilities or hospice for advanced cancer patients (352). In a study of delayed breast cancer diagnosis in Malaysia, complementary alternative medicine (CAM), negative information about breast cancer and treatment such as side effects of chemotherapy, perceived lack of cure of breast cancer, and fear of divorce or remarriage of the husband were the significant factors leading to a delay in breast cancer diagnosis and treatment (353).

Patients with breast cancer in Pakistan commonly present with advanced disease which remains a dilemma for the treating oncologists and surgeons. According to a local study in Pakistan, 69.9% of breast cancer cases had stage III and IV disease at the time of the first presentation (354). Previous studies show high rates of delay in breast cancer diagnosis with 39% reporting late in Northern Pakistan and going for their first visit regarding the breast lump after a delay of 6 months since noting any symptoms (355-357). The delay in the presentation and detection of patients with breast cancer is a major contributor to their advanced stage at presentation and increased mortality rates in Pakistan (358). Delay by patients in seeking medical treatment is generally complex and multifactorial, not taking into account further delay after the first visit till the initiation of the treatment, as it was beyond the scope of the current study. Development of specific strategies by policymakers to reduce delays requires an understanding of the factors that influence this delay (359, 360). Strategies could include public education and awareness campaigns about the symptoms and signs of breast cancer, breast self-examination, and screening mammography, and encouraging women to seek medical consultation at the earliest possible time (361).

7.2 Rationale of this sub study

Because of limited human and financial resources for effective diagnosis, breast cancers frequently present at an advanced stage and consequently mortality rates are high. Delayed presentation of cancer also has a significant economic impact, since it is far less expensive to treat patients with early-stage disease and success rates are significantly increased. An understanding of the factors influencing delay in presentation is important to formulate strategies to achieve timely diagnosis and treatment. Delayed presentation

of breast cancer is a preventable problem, which if addressed, would have a significant impact on reducing the morbidity and mortality of breast cancer.

The objective of the present study was to evaluate the frequency and magnitude of patient delay in women of Karachi with a diagnosis of breast, and in addition to providing a detailed assessment of factors and reasons associated with this delay of breast cancer patients in seeking medical consultation and if there was a relationship between delays and disease stage.

7.3 Methods

The detailed methodology for this study has been described previously in Material and Methods Chapter 2. Briefly, 514 cases of newly diagnosed breast cancer were enrolled in a matched case-control study during the period from Feb 2015 to Aug 2016 (number of cases is more than the main study sample of 411 cases because it includes the unmatched cases too). All participants were interviewed face to face by research medical officers in clinics in a quiet and private place for confidentiality and privacy. The questionnaire and interview procedures were evaluated and revised earlier during a pilot study on 50 patients. Questions were asked to evaluate how Pakistani women present with breast cancer, the frequency, and magnitude of delay in diagnosis, the factors associated with delays, and the relationship between delays and disease stage in breast cancer.

Variables in the Questionnaire

Patient delay was the primary outcome which was defined as the time between the appearance of any of the first symptom of breast cancer, for example, a lump or nipple discharge, and the date of initial consultation for diagnostic mammography, ultrasonography, or medical consultation for breast symptoms. No delay was defined as patients seeking medical advice for their breast cancer symptoms in a month of finding any symptoms. Delay was defined as any time greater than one month for patients seeking medical help for diagnosis after noticing possible symptoms of breast cancer. Diagnosis time is defined as the date of the first symptoms to the date of final breast cancer diagnosis based on histopathological examination (including needle biopsy or excisional biopsy) or on FNAC (fine needle aspiration cytology)**.**

The study participants were asked to recall the onset of any symptoms which they became aware of and the day of first medical consultation. To minimize recall bias, the participants were reminded of events in the calendar year, such as religious and national occasions, school holidays, festival celebrations, Independence Day, or birth dates, to help them remember important dates relative to their medical history(362). An agreement about exact dates was reached after discussion with the participants and their family members / attendants (all unaware of the stage of cancer) when there were conflicting dates of events.

Questions were asked about the initial sign or symptom of breast cancer any breast lump, nipple discharge, breast pain, skin changes, changes in breast shape, breast ulcer, arm edema, axillary lymph nodes or any other sign or symptom. Mode of detection asked was recorded as either "self-detected" by a lump, nipple change, or other breast abnormality), "exam-detected" (i.e., a clinician detected an abnormality), "imaging-detected" (i.e., a screening mammogram, or other imaging test indicated an abnormality), or "detected based on systemic symptoms" (e.g., weight loss or fatigue). Yes-no questions of beliefs about breast cancer and treatment, fear, denial, barriers, husband/family support were included in order to determine the impact of these factors on patient delay. Information was also collected on breast tumor characteristics of hhistopathology, type, and grade of the tumor, stage of disease categorized according to Tumor-Node-Metastasis (TNM) staging sourced from pathology reports (150). Information was collected about socio-demographic and reproductive factors as mentioned in the Materials and Methods Chapter 2. Briefly, information was collected for ethnicity, level of education, marital status, place, and type of residence, number of dependants and current employment status, socioeconomic (SES), self-reported family history of breast cancer and age at diagnosis of breast cancer, or any other cancer and reproductive history including age at menarche, parity, abortion, history of preeclampsia, menopausal status, age and mode of menopause, duration of breastfeeding. Other variable assessed was body mass index (BMI).

Sample size

The sample size consisted of 514 cases of breast cancer sourced from two main hospitals of Karachi, Pakistan which were Aga Khan University Hospital (AKUH) and Karachi Institute of Radiation and Nuclear Medicine (KIRAN) cancer hospital. KIRAN caters mostly to people from low or middle-income group compared to AKUH. These cases were extracted from the matched case-control study consisting of 1200 participants.

7.4 Statistical Analysis

Statistical analysis of the data was carried out using the SPSS package for Windows 22.0 (SPSS, IBM, Armonk, NY, USA). Descriptive statistics were computed for all variables. Mean and standard deviations were obtained for continuous variables while categorical variables were assessed by frequencies and percentages. To facilitate analysis, variables with multiple categories were collapsed to fewer categories in a meaningful way. Chi-square and Fisher exact tests were used to assess categorical variables. To identify the factors associated with delay, univariable analysis of each variable of interest and unadjusted odds ratio and their 95% confidence intervals along with p values were calculated. The reference group for each risk factor was generally determined by the category with a minimal level of risk for delay. All independent variables with univariate analyses p values less or equal to 0.25 were included in the model. In multivariable analyses, logistic regression was performed to identify factors associated with delay, while adjusting for other variables. The analysis was done by the purposeful selection method, and all the variables that were selected from the univariable analyses were entered in the model simultaneously. Finally, any variable with a p-value > 0.05 was removed from the model to obtain a parsimonious and biologically meaningful model that best explains the phenomena of patient delay. The overall significance of the model was assessed by likelihood ratio test statistics (269).

7.5 Results

Descriptive analysis

Table 1. Socio-demographic and clinical characteristics of breast cancer cases in two hospitals of Karachi Pakistan (n=514)

	AKUH		KIRAN		TOTAL		
Characteristic	**(n)**	**%**	**(n)**	**%**	**(n)**	**%**	**p value**
Age of patient							<0.001
<35	29	10.4	41	17.4	70	13.6%	
35-44	66	23.7	73	30.9	139	27.0%	
45-54	69	24.8%	66	28.0%	135	26.3%	
55 & above	114	41.0	56	23.7	170	33.1%	
Education							<0.001
<grade 8	69	25.0	138	58.5	207	40.4%	
grades 8-12	96	34.8	74	31.4	170	33.2%	
>grade 12	111	40.2	24	10.2	135	26.4%	
Socioeconomic status (SES)							<0.001
Upper	30	10.9	4	1.7	34	6.7%	
Middle	213	77.7	52	22.5	265	52.5%	
Lower	31	11.3	175	75.8	206	40.8%	
Consanguineous marriage							0.03
Yes	70	25.9	86	38.9	156	31.8%	
No	200	74.1	135	61.1	335	68.2%	
Family History of breast Cancer							
YES	73	26.4%	25	10.6%	98	19.1%	
NO	204	73.6%	211	89.4%	415	80.9%	

	AKUH		KIRAN		TOTAL		
Characteristic	**(n)**	**%**	**(n)**	**%**	**(n)**	**%**	**p value**
Family History of any Cancer							
YES	124	44.9%	53	22.5%	177	34.6%	
NO	152	55.1%	183	77.5%	335	65.4%	
BMI*							<0.001
<23	42	17.0	52	25.0	94	20.9%	
23-25	35	15.0	43	21.0	78	17.3%	
>26	165	68.0	113	54.0	278	61.8%	

*Body Mass Index

*p values generated from Chi-square or Fisher Exact test

The sociodemographic characteristics of the study population (n=514) overall and stratified by the two hospitals are shown in Table 1. The proportion of cases below 35 years of age was significantly higher in KIRAN compared to AKUH. Patients in KIRAN were less educated than in AKUH: 58.5% of cases in KIRAN and 25% cases in AKUH had had education less than grade 8 {p<0.001). Regarding socioeconomic status (SES), 75.8% and 11.3 % of cases in KIRAN and AKUH belonged to the lower SES respectively (p<0.001). Consangiuos marriage was higher among cases in KIRAN (38.9%) than in AKUH (25.9%). The proportion of obese and overweight women was higher than that in the general population, and the mean BMI was 27.1 (SD ± 5.5).

Table 2. Symptoms of breast cancer and diagnosis delay in two hospitals of Karachi Pakistan (n=514)

		AKUH		KIRAN		Total		
Characteristic	**Category**	**n**	**%**	**n**	**%**	**n**	**%**	**p-value***
First symptom								0.001
	Lump	233	85.0	224	95.3	457	89.8	
	Other symptoms	41	15.0	11	4.7	52	10.2	
Mode of Detection								0.19
	Accidental	208	83.5	186	80.5	394	82.1	
	Breast self-examination BSE	24	9.6	22	9.5	46	9.6	
	Clinical examination CE	7	2.8	16	6.9	23	4.8	
	Others	10	4.0	7	3.0	17	3.5	
First consultation								0.01
	General Practitioner (GP)	82	32.7	74	33.6	156	33.1	
	Gynecologist	33	13.1	14	6.4	47	10.0	
	Surgeon/oncologist	125	49.8	111	50.5	236	50.1	
	Homeopathy/ CAM	11	4.4	21	9.5	32	6.8	
Delay in seeking care for breast cancer symptoms								<0.001
	No delay	120	45.5	55	23.4	175	35.1	
	Delay	144	54.5	180	76.6	324	64.9	

*p values generated from Chi-square or Fisher Exact test

Table 2 presents the first symptom of breast cancer experienced by cases, mode of detection, first consultation and reasons for delay among patients in two hospitals. A breast lump was the most common first symptom of breast cancer (89.8% of women) with this being an accidental finding in 82.1 % of these women. Routine breast self-examination was performed by only 9.6 % females with 90.4 % not performing BSE or receiving any routine clinical breast examinations during routine visits to care providers

and other clinics prior to experiencing their breast symptoms. Clinical breast examination services provided by trained doctors and nurses is very affordable but was not observed in our health delivery system, at least in our study sample. Only 3.5 % of breast cancer was detected by screening mammography, evidence of poor screening for early detection of breast cancer among Pakistani women. Overall 64.9 % of women had a delay (>1month) in seeking a medical consultation, and this was significantly (p>0.001) more common among cases at KIRAN (76.6%) than among cases at AKUH (54.5%). The greater delay at KIRAN is likely to reflect the higher pressures on services at this hospital but the details were beyond the scope of our study objectives.

Figure 1. Distribution of reasons of delay in KIRAN and AKUH

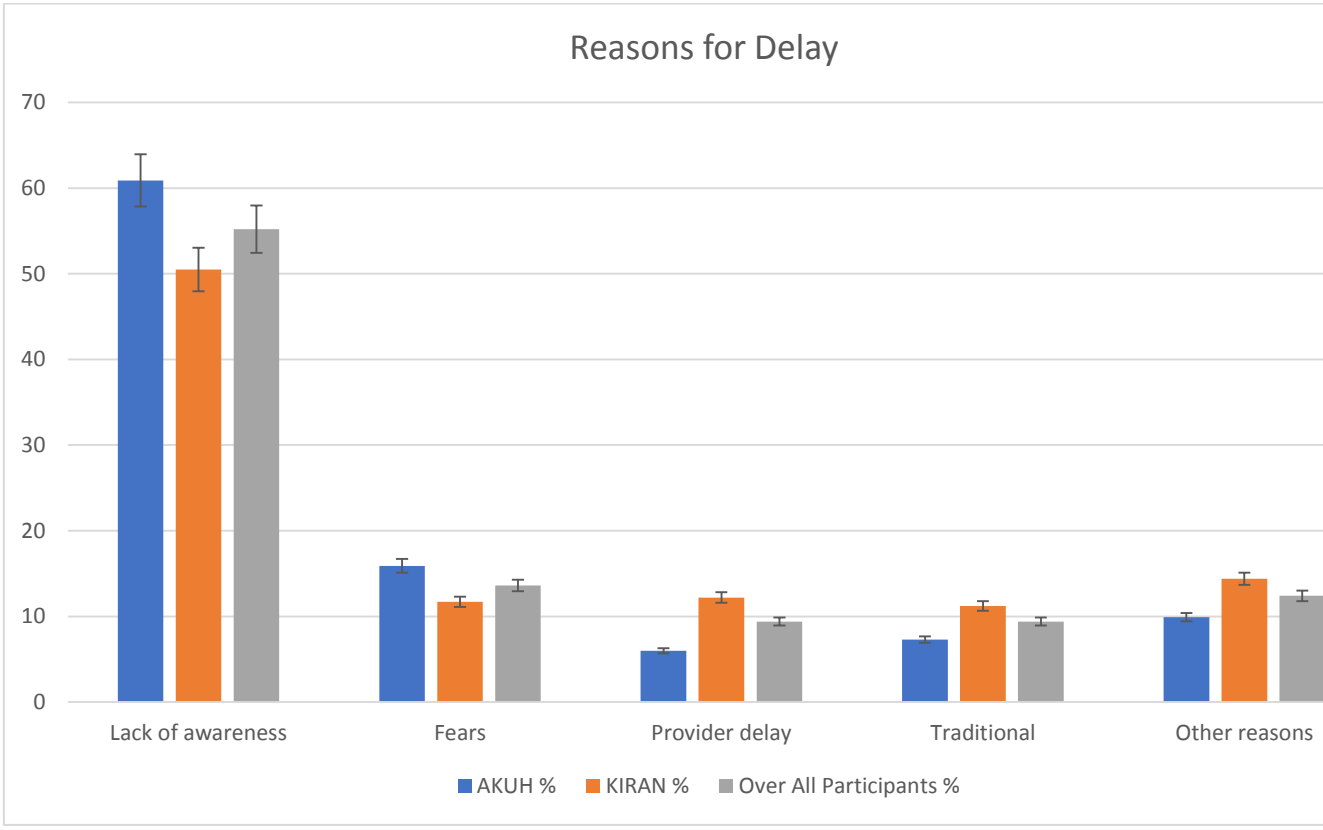

Table 3. Reasons for delay in seeking medical help for breast cancer among patients who delayed their first consultation in two hospitals of Karachi (n=314)

	AKUH		KIRAN		Over All Participants		p-value*
	n	**%**	**n**	**%**	**n**	**%**	
Reasons for Delay							0.06
Lack of awareness	92	60.9	95	50.5	187	55.2	
Fears	24	15.9	22	11.7	46	13.6	
Provider delay	9	6.0	23	12.2	32	9.4	
Traditional	11	7.3	21	11.2	32	9.4	
Other reasons	15	9.9	27	14.4	42	12.4	

*p values generated from Chi-square or Fisher Exact test

Table 3 shows the reasons for a delay greater than one month in seeking medical help in those women where symptoms were noted consistent with a breast cancer diagnosis. The most common cause of delay amongst patients was lack of awareness (55.2%), that is, they did not realize that the symptoms were significant enough to require medical consultation (Fig. 1). 13.6 % women delayed due to fear of fear of informing anyone, fear of diagnosis, fear of treatment, fear of mastectomy, fear of adverse effects on relationship with husband (husband's rejection). 9. 4 % women delayed due to opting for traditional methods of treatment for their symptoms. Traditional treatment (CAM) consisted of cheap affordable herbal and homeopathic medicines by "hakeem" and homeopathic practitioners. Provider delay was the reason of delay in 9.4 % of cases due to inability of health provider to diagnose breast cancer symptoms and false reassurance to women without recommending further tests or referring them to the specialist. Both the traditional method of treatment and provider delay as reasons of delay were higher among women in KIRAN as compared to AKUH. Not only women but a proportion of local general physicians. Despite the fact that the lump in the breast was the most common mode of breast cancer presentation, there was a poor awareness of this early breast cancer symptom both in the general population and in some cases among medical professionals who appeared to be ignorant of breast cancer symptoms and diagnosis, ignoring their symptoms when patients consulted them. Other reasons included myths

and misconceptions about breast cancer, shame and embarrassment, financial constraints, and family commitments.

Table 4. Comparison of reasons for delay according to the length of delay in months among breast cancer cases who delayed their first consultation (n=313)

	Delay < 3 months		**Delay > 3 months**		
	(n)	**%**	**(n)**	**%**	**p-value***
Reasons for delay					0.02
Lack of awareness	62	68.9	120	53.6	
Fears	11	12.2	25	11.2	
Provider delay	8	8.9	19	8.5	
Traditional	4	4.4	26	11.6	
Other factors	5	5.6	34	15.2	

*p values generated from Chi-square or Fisher Exact test

Table 4 shows the reasons of delay categorized by $\leq$ 3 months and > 3 months**.** The median time of delay in seeking consultation for their breast symptoms was seven months. Lack of awareness, as the main reason for delay, was even higher in women who delayed for less than three months. Provider delays in diagnosis were approximately equal in both the categories, resulting in a delay in starting appropriate treatment. Traditional treatment was higher in those who delayed for more than three months (11.6%).

Table 5. The association of independent variables with patient delay among women in two major cancer hospitals of Karachi Pakistan

Variables	Unadjusted OR	95%CI		p-value*	Adjusted OR	95%CI		p-value
Hospital		Lower	Upper	<0.001		Lower	Upper	
AKUH	1(Ref)							
KIRAN	2.73	1.85	4.01					
Education Level				<0.001				
Grade > 12	1(Ref)							
< Grade 8	2.97	1.86	4.75					
Grade 8-12	1.84	1.15	2.95					
Socioeconomic Status (SES)				<0.001				0.01
Upper	1(Ref)							
Middle	1.04	0.64	1.69		0.91	0.5	1.68	
Lower	4.32	2.45	7.6		2.94	1.32	6.61	
Family History of Breast Cancer				0.001				
Yes	1(Ref)							
No	2.23	1.43	3.48					
First Consultation				0.004				
General Practitioner (GP)	1(Ref)							
Gynaecologist	0.5	0.26	0.98					
Surgeon	0.73	0.47	1.12					
Oncologist	0.69	0.11	4.24					
Homeopathy/CAM	6.87	1.58	29.9					
Tumor Size								
T1	1(Ref)			0.001				
T2	3.13	1.71	5.71					
T3	4.27	2.2	8.29					
T4	7.22	3.56						
Metastasis	2.58	1.32		0.01				
Stage				<0.001				
Stage1	1(Ref)							0.004
Stage 2	3.42	1.57			3.4	1.37	8.39	
Stage 3	3.41	1.54			3.59	1.44	8.95	
Stage 4	7	2.83			6.75	2.41	18.9	

*p values generated from Chi-square test

Only statistically significant variables associated with patient delay among women on univariate analysis and multivariate binary logistic regression are shown in table 5.

The statistically significant variables with patient delay among women based on univariate analysis using simple binary logistic regression are:

Hospital

Concerning the hospitals, the analysis showed that KIRAN patients were more likely to experience delay than AKUH patients (OR= 2.73, 95% CI: 1.85- 4.01, p = < 0.001).

Education

A significant association was observed between patient delay and the education level of women (p-value < 0.001). This delay was higher among women with the education level of less than grade 8 (OR= 2.97; 95% CI: 1.86- 4.75) and women with the education level of grade 8-12 (OR= 1.84, 95% CI: 1.15-2.95), compared to women with education level higher than grade 12.

Socioeconomic status (SES)

Patient delay among those with a lower socioeconomic status was significantly higher compared to those with higher SES (OR= 4.32, 95% CI: 2.45-7.60, p = < 0.001).

The family history of breast cancer

Cases were more likely to delay their first consultation if they did not have a family history of breast cancer (OR= 2.23, 95% CI: 1.43- 3.48, p = 0.001).

First Consultation

There was a significant association between delay and women traditional methods of treatment like homeopathic treatment and “hakeems” for complementary alternative treatment (CAM) first, as compared to gynecologists, surgeons or GPs OR= 6.87, 95% CI: 1.58-29.90, p = 0.004).

Tumor size

Patient delay led to larger tumor size T4 at the time of their first presentation (OR= 7.22, 95% CI: 3.56-14.62, p = 0.001).

Metastasis

Patient delay was associated with metastasis (OR= 2.58, 95% CI: 1.32-5.06)

Stage of breast cancer

Delay was associated with advanced stage of breast cancer (p = 0.004). Delay was associated with stage 4 (OR= 7.00, 95% CI: 2.83-17.34). Similarly, delay was also associated with stages II and III; for stage II (OR=3.42, 95% CI; 1.57- 7.45), and stage III (OR =3.41, 95% CI 1.54- 7.54).

The multivariable analysis showed a strong association between lower socioeconomic status with patient delay (OR: 2.47, 95% CI: 1.29, 4.76). Those patients who delayed tended to present at a more advanced stage at initial diagnosis. Compared to stage 0 /I, the ORs for stages II, III and IV were 2.75(95% CI: 1.38, 5.47), 3.97(95%CI: 1.84, 8.54), and 8.44(3.70,19.23) respectively

7.6 Discussion

This sample of 514 women with breast cancer from two hospitals in Karachi assessed the patient delay in seeking medical care following their finding of breast cancer symptoms and identified the extent, factors and reasons behind this patient delay. The overall finding was that 64.9% of women delayed their first medical consultation by a median delay of 8.79 months, although 89.9 % had a breast lump as the first symptom of breast cancer. There were twenty-one women with a history of a breast lump for more than four years and presented with ulcerated masses due to delay. By comparison, in a study in Germany, the median delay before consulting a physician was sixteen days and 18% delayed for more than three months (363). According to this study, advanced stage diagnoses of breast cancer cases could be reduced if all patients with breast cancer symptoms would not delay coming to a doctor for more than one month.

The main factors associated with the delay were poor education, and lower socioeconomic status. These findings of a strong association between lower socioeconomic status and advanced stage of breast cancer at first consultation with patient delay are similar to other studies (364-367). Similarly, a French study showed that the differences in the diagnostic stage were more likely due to delay in diagnosis

compared to the aggressive course of tumors in lower socioeconomic status patients (368). A systematic review of factors associated with delay in Africa showed low SES as an important factor casing delay(369). These data suggest that more attention needs to be given to women of lower socioeconomic and educational status, which may improve the stage at diagnosis of breast cancer at the time of the first presentation and improve the prognosis of the disease. The long delays in lower socioeconomic patients could be due to multiple factors related to non affordability of health care services.

The most common reason given by patients for the delay in diagnosis was lack of awareness (55.2%) about the importance of the finding of an abnormality of the breast. Fears of informing anyone, of diagnosis, of treatment, of mastectomy, of adverse effects on relationship with husband (husband's rejection) were second more important reason of delay.

There was a strong influence of complementary and alternative medicine CAM, like homeopathy and "Hakeems," similar to two other studies (370, 371). Traditional healer and low education were similarly the reasons for patient delay in a study among African women (365). Alternative medicine is shown to be responsible for delays in various other studies too (370, 372) but our study results show it is less of an issue than in Malaysia where it was reported as 42% (373). Breast lump was also not correctly assessed and managed by some doctors. Our findings indicate that 9.4% of cases were due to provider delay who inappropriately reassured after the first visit to the general practitioners without referral or biopsy of their lump. It is clear from the study findings that additional training of general practitioners for breast cancer symptoms and timely referral would reduce the incidence of advanced stage. Other reasons of delay like myths, fears related to diagnosis and treatment and family commitments were also observed in other studies (374-376).

These findings are similar to other studies in developing countries and among minority groups in developed countries. All those studies have reported that lack of awareness and knowledge and poor education are strongly associated with patient delays (366, 371, 373, 377-383). Moreover as suggested by other studies, more education and awareness is needed to address misconceptions about breast cancer (374, 384). It is apparent that even in the more educated women, lack awareness of the symptoms of breast cancer was also

a major reason of the delay as the lack of awareness was higher in AKUH with women having better education level compared to KIRAN.

An interesting finding was that cases were more likely to delay their first consultation if they did not have a family history of breast cancer. This is another evidence from this study suggesting that awareness of breast cancer significantly reduce delay since it is consistent with the personal knowledge and experience of cancer in these families. This results in better recognition of breast cancer symptoms and a willingness to seek appropriate medical advice.

Of the two hospitals included in this study, AKUH is a private hospital while KIRAN is a welfare hospital. Patients at KIRAN were significantly younger in age, of lower educational and socioeconomic status. Overall KIRAN had 75.8% while only 11.3% of the patients at AKUH belonged to the lower socioeconomic status. There was a significantly higher percentage of delay among women at KIRAN (76.6%) compared to AKUH (54.5%). Compared with AKUH, KIRAN has reduced financial and staff resources that may contribute to its higher rate of breast cancer patient delay. Provider delay was greater among women coming to KIRAN compared to AKUH, and this is likely to reflect the fewer resources and greater workload of both staff and pathology services at KIRAN. It also supports the hypothesis that diagnosis delays are probably caused by problems in the first level of healthcare in such settings (e.g., waiting times to get appointments, quality of care, etc.). Additional extended periods of delay in treatment were observed among breast cancer cases who were lost to follow up after their first visit to a health care provider. However, these additional delay factors were beyond the scope of our study. Moreover, lack of sufficient knowledge, information, and awareness in our population regarding breast cancer is also correlated with variables like religious beliefs about breast cancer, but these were not addressed in this current study. These issues need to be separately assessed in future studies. In summary, the lack of education associated with delay in this study is important and needs to be considered in targeting such women as a starting point for breast cancer education campaigns. Reducing the delay in seeking specialist medical treatment will allow more successful and timely treatment options and ultimately reduce the burden of breast cancer.

Limitations of the study include patient recall of delay and symptoms but it was minimized by limiting the incident cases only and enrolling them within 6-12 months of breast cancer diagnosis. Moreover, verification of the verbal findings in some information was done by medical records and documented the findings of laboratory investigations. Moreover, delays were self- reported by patients, who might have in some cases tended to underreport patient delay because of wish bias to avoid any guilt. However, major strength of our study was the good quality of data collection of information on patient delay through in personal interviews conducted by medically qualified doctors with the qualification of a MBBS medical degree consisting of at least 5 years' undergraduate medical education, and their medical history taking experience ensured conduct of interview in a polite, sensitive and time efficient manner with confidence of patients in them. Another strength of the study was the assessment and analysis of a wide range of factors associated patient's delay in multicenter hospital based sample of breast cancer patients.

Conclusions

Lack of awareness about breast lump was commonest reason of delay. This is a preventable problem which if addressed would have a significant impact on reducing the morbidity and mortality of breast cancer in Pakistan. The major recommendations from this study are to provide better education in the general population concerning breast cancer, and, methods of self-examination and what are the most likely signs of breast cancer.

There is an immense need for an organized breast cancer screening program, at the national level, through providing low-cost mammograms, with a target population of women especially high-risk women and try to outreach into the public sector at community level including both urban and rural areas.

The implementation of an **intensive and comprehensive and positive breast cancer education campaign through structured community health awareness programs** addressing the myths and misconceptions **related to** breast cancer will reduce the burden of breast cancer in Pakistani women. **Such an** education campaign will need to be cognizant of the socio-cultural and religious values of the Pakistani society while making women 'breast aware' of their healthy breasts and any changes in the breast. Educational campaigns must also

be targeted to health care providers, clinicians, nurses, community health workers, and lady health visitors. Ideally, this will need to be supported by the Ministry of Health with the integration of other non-governmental organizations. The resource requirements to implement such a program are relatively limited and do not require expensive technology but will have a significant impact on reducing the impact of breast cancer in Pakistani women.

Delayed presentation is a preventable problem, which if addressed, would have a significant impact on reducing the morbidity and mortality of breast The recommendation from this study is to improve the awareness of women regarding breast cancer symptoms and the critical importance of seeking immediate medical diagnosis and treatment to improve their outcomes. This can be achieved by an education program to women regarding the signs and symptoms of breast cancer and in particular methods of self-examination. It is essential that health care providers and traditional healer play their role for timely referrals and necessary investigations.

Last but not the least, in Pakistan, like any other low to middle-income country, the health care access and treatment facilities for breast cancer patients should be improved as a part of breast cancer control program. For timely initiation of breast cancer treatment and quality care, only awareness and education to avoid delays will not be useful without the provision of cancer hospitals.

7.7 Conclusion

This study found that 64.9% of women diagnosed with breast cancer had previously recognized changes in their breast that were consistent with breast cancer, but delayed medical consultation (median seven months). This is a preventable problem which if addressed would have a significant impact on reducing the morbidity and mortality of breast cancer in Pakistan. The major recommendations from this study are to provide better education in the general population concerning breast cancer, and, methods of self-examination and what are the most likely signs of breast cancer.

These data suggest that more attention needs to be given to women of lower socioeconomic and educational status, which may improve the stage at diagnosis of breast cancer at the time of the first presentation and reduce mortality. The long delays

in lower socioeconomic patients could be due to multiple factors related to no affordability of health care services.

There is an immense need for an organized breast cancer screening program, at the national level, through providing low-cost mammograms, with a target population of women especially high-risk women and try to outreach into the public sector at community level including both urban and rural areas.

The implementation of an intensive and comprehensive and positive breast cancer education campaign through structured community health awareness programs addressing the myths and misconceptions related to breast cancer will reduce the burden of breast cancer in Pakistani women. Such an education campaign will need to be cognizant of the socio-cultural and religious values of the Pakistani society while making women 'breast aware' of their healthy breasts and any changes in the breast. Educational campaigns must also be targeted to health care providers, clinicians, nurses, community health workers, and lady health visitors. Ideally, this will need to be supported by the Ministry of Health with the integration of other non-governmental organizations. The resource requirements to implement such a program are relatively limited and do not require expensive technology but will have a significant impact on reducing the impact of breast cancer in Pakistani women.

Last but not the least, in Pakistan, like any other low and middle-income country, the health care access for breast cancer patients should be improved as a part of breast cancer control program, for timely initiation of breast cancer treatment and quality care, as only awareness and education will not be useful without the provision of cancer hospitals.

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Chapter 8: Conclusions

This presents the summary of the results of this thesis followed by concluding remarks. Also discussed are the limitations and difficulties confronted during meeting the objectives of this study and in particular the assessment of all variables including serum Vitamin D measurements.

According to a 2008 report by the Pink Ribbon Campaign, approximately 90,0000 new breast cancer cases are detected annually in Pakistan and its incidence rate is rising over time, with higher occurrence at a younger age compared with women in the West (385). The prevalence of Vitamin D inadequacy is also very high among women living in Karachi, in spite of abundant solar UVB irradiation throughout the year which is the major source (80-95%) of Vitamin D. The main aim of the study was to explore all risk factors of breast cancer risk with main focus on association of Vitamin D with breast cancer.

It was a hospital based multicenter case control study in two hospitals of Karachi Pakistan. Ethical approval for this study was received from University of Adelaide, Australia; Aga Khan University Hospital AKUH, and the KIRAN cancer hospital Ethics Review Committees. 411 breast cancer cases and 784 controls were enrolled in the study after meeting the eligibility criteria. Face to face in person interviews were conducted using a detailed questionnaire and blood samples for serum Vitamin D were collected at the end of the interview. The questionnaire included questions related to sociodemographic, medical, reproductive information. In addition to this, sun exposure questionnaire and FFQ were also included. Family history and drug history was also asked. Breast cancer patients were age matched to two controls (except in 19 cases where there was only a single control).

This is the comprehensive study to assess vitamin D related factors associated with breast cancer with inclusion of lifestyle factors among Pakistani women,

The thesis was divided into five sub-studies based on the five objectives of the study.

The first objective was to identify all risk factors associated with breast cancer among Pakistani women (Chapter 3). The major findings of this sub study were that low

socioeconomic status and poor education were the main factors associated with increased breast cancer risk. Most of the women were housewives, and employed females had less odds of breast cancer than non-employed women (OR =0.70, 95% CI: 0.49, 0.99, p <0.001)**.** Though the family history of breast cancer was not associated with breast cancer risk but parental consanguineous marriage was associated with breast cancer patients as an independent factor. However, we did not have any genetic testing to identify the role of genetics in detail.

We also evaluated the role of all conventional risk factors such as reproductive factors, hormone exposure (both endogenous and exogenous hormones), medical history, family history, obesity, and physical activity but contrary to the western research literature and overall literature, most of these reproductive and hormonal factors were not associated with breast cancer in our study data. Only postmenopausal status had higher risk of breast cancer compared to premenopausal status (OR=1.80, 95%CI=1.23, 2.63, p=0.04). The findings of even null associations are important and do generate the hypothesis that breast cancer may have a biologically distinct etiology among Pakistani women.

As there was no association of any of the reproductive factors with breast cancer it is likely that environmental and lifestyle factors related to poor socioeconomic status play a major role in breast cancer etiology in Karachi women. These findings suggest an underlying unidentified environmental and genetic contributions to breast cancer risk in this population. It is of importance that high parity was also not associated with reduced breast cancer risk which was significantly associated with protective effect in a previous study in a similar hospital based case control study (160). These results are in accordance with the findings of a study in Lahore, Pakistan in 2009 where early menarche, late menopause, family history of breast cancer breastfeeding had no effect on the risk of breast cancer and only nulliparity and late age at first birth were major reproductive risk factors (386). These findings are consistent with another independent study in two major cancer hospitals located in the province of Punjab, Pakistan, namely: the Institute of Nuclear Medicines of Oncology, Lahore (INMOL) and Shaukat Khanum Memorial Cancer Hospital (SKMCH), Lahore consisting of Punjabi ethnicity of Pakistan (214). However, another study of risk factors among Pakistani women less than 45 years of age

showed association of few reproductive factors with breast cancer (214). Studies among Pakistani women have presented with conflicting results on the role of family history in breast cancer risk. Earlier study by the same author as of this current study had reported that family history of breast cancer was associated with breast cancer among Pakistani women and had 1.5 times the odds of breast cancer compared to women with no family history of breast cancer, contrary to the current finding of no association of breast cancer with family history of breast cancer (160). However, the sample size of that study was smaller than the current study.

The aim of the second sub-study (Chapter 4) was to clarify the association between risk of breast cancer and vitamin D status in a multiethnic female population of Karachi Pakistan. Uniquely, for each woman all sources of vitamin D, including sunlight exposure and various types of vitamin D supplementation, were assessed and gold standard of their serum concentrations of vitamin D as the best assessment tool of Vitamin D status was used to evaluate the association of Vitamin D with breast cancer. The major findings were Vitamin D deficiency was significantly associated with an increased risk of breast cancer, and intake of Vitamin D supplements was associated with decreased risk of breast cancer. This supports the hypothesis that Vitamin D may play a protective role against breast cancer. These findings add to the increasing body of literature that the there is a significant contribution of low Vitamin D concentrations to breast cancer risk. In a previously published smaller study of Pakistani women, there was a similar association of vitamin D deficiency with increased risk of breast cancer (59). Another recent pooled cohort showed that serum Vitamin D level > 60 ng/ml lowered the risk of breast cancer by 80 % compared to women with serum Vitamin D <20ng/ml (387). Lowe et al also showed that there was higher risk of breast cancer among women with serum Vitamin D less than 20 ng/ml (68).

Solar UVB is the major source of Vitamin D in the body. A sun exposure score was used to determine and compare sun exposure between women. However, there was no significant relationship between the overall sun exposure score with Vitamin D status or incidence of breast cancer.

The majority of observational studies regarding the relationship between vitamin D and breast cancer have been undertaken in Western population and results are inconsistent. It is suggested that the association between serum Vitamin D concentrations and risk of breast cancer, as demonstrated in this thesis, increases at particularly low concentrations of Vitamin D <20ng/ml. Western populations do not have such low concentrations of vitamin D and thus the relationship with risk of breast cancer may be more difficult to establish.

There is some data supporting that dietary Vitamin D is associated with increased concentrations of vitamin D and lower risk of breast cancer. A study on the association between dietary vitamin D and calcium to mammographic breast densities also suggested that Vitamin D decreased breast density and breast cancer risk (388). However, in this thesis the intake of dietary Vitamin D was not analyzed as Vitamin D containing foods consumption was very low small numbers prevented us from assessing its relationship with breast cancer.

The third sub-study of the thesis was to evaluate the risk factors associated with triple negative breast cancer (TNBC) and non-TNBC subtypes (chapter 5). There were limited Pakistani studies in the available literature assessing risk factors according to the different tumor subtypes of breast cancer. The source population for this study was from the main case–control study and 321 patients had complete clinical marker profiling, compared with 798 controls. Multinomial logistic regression, where the control group was the referent, showed women of younger age group, relative to women above 55 years of age, had a protective effect against non-TNBC.

TNBC in the study sample accounted for 22.7% of all the newly diagnosed invasive breast cancers, which is similar to the high percentage among African American women and Indian women (19, 272, 389, 390). The mean age of TNBC cases was younger (46.1 SD 11.7 years) than mean age of non TNBC cases (49.4 SD 12.5 years) which is consistent with other studies (279). Both TNBC and non-TNBC were associated with poor socioeconomic status and low Vitamin D concentrations but this association was

significantly higher in the TNBC subtype. Therefore, Vitamin D deficiency is associated with an increased risk of the more aggressive TNBC subtype of breast cancer. Among non TNBC cases, there was a lower risk of breast cancer in premenopausal women while among TNBC cases there was no association with menopausal status. Breastfeeding and high parity was not associated with TNBC as identified in other studies (291).

These findings are consistent with the major breast cancer risk factors findings reported in Chapter 3 and showed that both TNBC and non-TNBC were not associated with any of the known traditional risk factors of breast cancer risk such as reproductive factors. As established for all breast cancers, the risk of TNBC was increased in women of low SES and those with lesser education indicating again the important role of environmental related factors. These findings emphasize the importance of the contribution of poverty to the etiopathogenesis of this aggressive subtype. This also implies that women living in conditions of poverty are exposed to unidentified carcinogenic factors in the environment that are responsible for the increased risk of TNBC. These factors were, however, not to be identified in the scope of the study objectives.

Based on these findings correction of Vitamin D deficiency in women is a reasonable and cost effective strategy to reduce the incidence of all subtypes of breast cancer, and in particular the aggressive TNBC Larger prospective studies or clinical trials are needed to further confirm these findings.

The fourth study objective was to evaluate the association of diet with breast cancer among Pakistani women using the modified AHEI 2010 and its component scores (chapter 6). This study also highlights the importance of using diet indices like AHEI 2010 related to inflammation when evaluating the association between diet and breast cancer. The study results showed that a higher modified AHEI-2010 score, and higher diet grain score (reflecting high intakes of grains both whole and refined), was significantly associated with a higher risk of breast cancer in Pakistani women. It also indicates that there may be a link of diet and socioeconomic status. Limiting refined carbohydrate intake might be a beneficial public health message as it may represent a potentially modifiable risk factor for breast cancer in our population but requires additional in depth study.

The fifth study objective was to assess patient delay in breast cancer diagnosis, its associated factors and stage of diagnosis among breast cancer patients in Karachi, Pakistan (Chapter 7). Delay in breast cancer by patients have been reported and evaluated in various ethnic groups and multiple reasons have been established. In this cross-sectional sub study, 514 breast cancer cases were examined with analysis of their role in breast cancer diagnosis delay and factors associated with this delay. It was found that in this cohort of women with breast cancer from Karachi, that although 89.9 % had a breast lump as the first symptom of breast cancer 64.9% delayed medical consultation by a median of 7 months. Routine breast self- examination practice was very low (9.6 % of all breast cancer cases) and breast lumps were usually discovered accidentally (82.1%). This delay in presentation resulted in these women having significantly more advanced disease and it is well established that this results in greatly increased morbidity and mortality.

Our study findings clearly show that lack of awareness about breast cancer and its symptoms was the main determinant of delay by women. The reason of delay in 9.4% cases of women was due to affordable and cheap traditional methods of treatment for their symptoms. As well as a lack of awareness among women about the significance of breast symptoms, 9.4% of women reported that their medical professionals initially failed to advise proper investigations for their breast lump. Delayed presentation is a preventable problem, which if addressed, would have a significant impact on reducing the morbidity and mortality of breast cancer in Pakistan. The recommendation from this study is to improve the awareness of women regarding breast cancer symptoms and the critical importance of seeking immediate medical diagnosis and treatment to improve their outcomes. This can be achieved by an education program to women regarding the signs and symptoms of breast cancer and in particular methods of self-examination. It is essential that health care providers play their role for timely referrals and necessary investigations. A previous study of Pakistani women with breast cancer also reported that extended periods of delay in treatment can occur since a further period of delay occurred

after their first visit to a health care provider and additionally women are lost to follow (ref 15). However, these additional delay factors were beyond the scope of our study.

Limitations of the study

As discussed in previous chapters, patient recall was minimized by limiting the incident cases only and enrolling them within 6-12 months of breast cancer diagnosis. Also difference in recall was avoided between cases and controls by ensuring adoption of identical study procedures in cases and controls, use of validated food frequency questionnaire, and careful training of data collectors and instructions to improve accuracy of reporting exposures. Moreover, verification of the verbal findings in some information was done by medical records and documented the findings of laboratory investigations.

A single measure of 25(OH) D within one month of BC diagnosis to define Vitamin D status does not reflect Vitamin D status before the disease. However, single measurement may be a reliable indicator of Vitamin D status in epidemiological studies (391). The availability of biochemical parameters like parathyroid hormone, calcium, phosphorus, 1,25-dihydroxyvitamin D, could have allowed us to verify vitamin D deficiency-related hyperparathyroidism, and related changes in calcium, phosphorus, and the active form of vitamin D, 1,25-dihydroxyvitamin D but were not included in the study due to budget constraints. Breast tissue expresses vitamin D receptor and both vitamin D status and genetic variations in vitamin D receptor can affect the risk of developing breast cancer (392). However, due to budget and time constraints, we did not have this objective of vitamin D receptor and Vitamin D polymorphisms and risk of breast cancer included in the study. Limited statistical power in certain variables like hormonal replacement therapy HRT may make our analysis prone to spurious associations as well as false negatives.

Missing values for receptor status were minimized by accessing the patients and accessing their outside AKUH and KIRAN lab's results but still we were unable to have complete molecular profile of all cases enrolled in the tumour subtype analysis and had complete molecular data on HER-2/ *neu* and ER/PR status on 321breast cancer cases. Therefore, we had small numbers in certain tumor subtypes and we could not analyse all four tumour subtypes separately but had to merge different subtypes as non TNBC group.

As mentioned before , case-control study has disadvantage of selection bias (141). The cases and controls in this study are not representative of the general population and we cannot establish temporality and avoid reverse causation in these associations.

Strengths of study

This was an international multidisciplinary research project across disciplines of oncology, surgery, epidemiology, public health, endocrinology and basic sciences. The study involved two mentioned major Hospitals in Karachi, Pakistan and two Universities in South Australia (University of Adelaide & University South Australia). This fostered the development of sustainable programs in collaborative breast cancer research between Pakistan and Australia, and enabled transfer of expertise to a developing country for breast cancer research. This collaborative study provided a platform of national and international research collaborations and improved our understanding of the potential role of cost effective Vitamin D supplementation in reducing breast cancer incidence in a developing and limited resources country like Pakistan.

This study helped to clarify the role of supplemental Vitamin D intake and influence on vitamin D status of women and association with breast cancer risk. In addition, a relationship between low Vitamin D concentrations and increased risk of the aggressive TNBC was established. By undertaking this study in Pakistani women, associations between Vitamin D concentrations and breast cancer risk could be clearly identified since, in contrast to more affluent societies, there were extremes in the serum concentrations of vitamin D. A major strength of this study was the analysis of detailed tumour characteristics. Survival bias was minimal because and women were recruited, soon after their confirmed diagnosis of breast cancer. Overall it was comprehensive study to examine all major personal, environmental, and genetic factors related to vitamin D and breast cancer

Future research directions

To confirm a direct relationship between low Vitamin D deficiency and increased risk of breast cancer risk, evidence-based interventions or large randomized clinical trials are

required to be undertaken in a population where the existing concentrations of Vitamin D allow significant findings to be easily assessed. This will contribute and help explore further the role of Vitamin D as a chemopreventive agent for breast cancer. The Vitamin D signaling pathway is complex and has interaction with other signaling pathways, with concentrations of PTH, Mg, Ca, P, that may or may not contribute to the development of breast cancer (393). Their effects on cellular differentiation, proliferation, and apoptosis are not yet fully understood (394)). It is, therefore, important to evaluate the interaction of deficiency of Vitamin D in combination with concentrations of all these related biomarkers of PTH, Mg, Ca, P along with Vitamin D receptors and genetics in an integrated manner in future studies. In this study an increased risk of breast cancer, and in particular the TNBC subgroup, was associated with conditions of poverty, therefore, Population based research studies with larger sample sizes and cohort study designs are required to validate factors associated with breast cancer and also to identify and define possible environmental carcinogens. More funding should be diverted for cost effective interventions like vitamin D. There is a need to design and conduct a large international collaborative prospective cohort study of high income, middle-income, and low-income countries with sufficient study length to provide definitive evidence of association of breast cancer with vitamin D and dose adequacy of vitamin D.

Public health policy implications

This dissertation addressed an important area of breast cancer research that evaluates the role of Vitamin D status in relation to risk of cancer. The findings of this dissertation therefore have important implications, in regard to the role of serum Vitamin D and Vitamin D supplements in breast cancer, risk factors associated with breast cancer and different subtypes of breast cancer, diet and breast cancer and assessment of factors associated with delay in breast cancer diagnosis and factors that may reduce their presentation at advanced stage of breast cancer through breast cancer awareness. An effective and low cost strategy for Pakistani women to reduce in the incidence of breast cancer is to prevent Vitamin D deficiency and insufficiency. This can be achieved through sensible sun exposure, consumption of foods that contain Vitamin D,

fortification of food items with Vitamin D and use of Vitamin D supplements. In Pakistani women where Vitamin D deficiency is common, raising and maintaining serum 25(OH)D level at the population level is a safe and affordable strategy to reduce the incidence of breast cancer. It is highly recommended to formulate such public health policies to prevent vitamin D deficiency based on these recommendations including lifestyle changes for appropriate sun exposure among women. In the long-term, the implementation of these recommendations will ensure all women have the required concentrations of Vitamin D and the incidence of breast cancer may be reduced.

Further research into pinpointing the exact nature of these factors responsible for high breast cancer among poor and less educated women is needed, and this could provide new opportunities for the development of risk reduction strategies to decrease the incidence and mortality of breast cancer in Pakistani women. Understanding the biological mechanism underlying this association would have important implications for breast cancer prevention. It is also important that the poor women benefit from frequent screening but at the moment these facilities are not available free of cost at national level. Overall, some of these lifestyle and environmental risk factors are potentially modifiable and could, therefore, reduce the incidence of breast cancer. However, more research with cohort study design, larger sample size and longer follow up is needed to fill the gaps to identify those environmental toxicants and their interactions with social factors, biologic pathways for environmental and behavioral factors in the complex etiology of breast cancer.

This study also determined the cultural and social factors that cause delayed presentation of women with breast cancer to clinics, resulting in women with late stage and untreatable disease. An understanding and evidence of these factors will help policy makers develop public health strategies to encourage earlier presentation when the first indications of breast cancer are discovered. This study underpinned new public health measures that can be feasibly implemented to reduce the incidence and mortality of breast cancer in Pakistani women. Considering the lack of awareness concerning breast cancer, appropriate awareness campaigns should be Public health priority by the Ministry of

Health. Breast cancer rates and mortality are particularly high among poor women. This study identifies several inexpensive strategies and approaches that if implemented would reduce the incidence and morbidity of breast cancer in Pakistani women.

The implementation of an intensive and comprehensive and positive breast cancer education campaign through structured community health awareness programs addressing the myths and misconceptions related to breast cancer will reduce the delay by patients with breast cancer in Pakistani women. Education campaign which are cognizant of the socio-cultural and religious values of the Pakistani society, will help in making women 'breast aware' of their healthy breasts and any changes in the breast. Educational campaigns must also be targeted to health care providers, clinicians, nurses, community health workers, and lady health visitors to encourage breast self examination and utilization of screening mammography. Integration of other non-governmental organizations with the Ministry of Health to implement such programs will have a significant impact on reducing the problem of patient delay in breast cancer diagnosis among Pakistani women.

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Appendices

Appendix A: Questionnaires

English

Case ☐ control ☐

STRATA		Hospital	

A Multicentre Case Control Study of Vitamin D and Breast Cancer Risk among Pakistani women

Section A; Sociodermographic data				
S no.	**Question**	**Code**	**SKIP**	**Answer**
1	What is your age? (In completed years)			_____
2	What is your educational status?	1. Illiterate (cannot read & write 2. Can read and write without 3. Formal school education. 4. 1-5 years of education 5. 6-8 years of education 6. 9-10 years of education 7. 11-12 years of education 8. >12 years of education Other (Specify)		
3	What is your mother tongue?	1. Urdu 2. Sindhi 3. Punjabi 4. Pashto 5. Balochi 6. Saraiki 7. Other (Specify)		
4	What is your marital status?	1. Unmarried 2. Married 3. Widowed 4. Divorced		
5	Type of Residence?	1. Apartment 2. Town house 3. Independent bungalow 4. Quarter settlement 5. Squatter settlement 6. Any other (specify)		
6	House is owned or rented?	1. Own 2. Rented 3. Other(specify)		
7	Where do you live?	1. Rural 2. Urban 3. Small town		
8	Are you employed?	1. Yes 2. No	If no, skip to Q 10	
9	Occupation?			
10	Is your husband employed?(married women)	1. Yes 2. No	If no, skip to Q 12	
11	Occupation?			

Case ☐ Control ☐

STRATA		Hospital	

12	What is your total house hold income per month in rupees? (Please include income from all members who contribute to the house hold)			
13	How many people including you are supported by monthly income?			
14	Have you ever been pregnant?	1. Yes 2. No	If no skip section B	

Section B;
Reproductive /Breast feeding History
Nulliparous _____ **Gravida** _____ **Para** _____

	15.1 Year of pregnancy (Start with first pregnancy)	15.2 Outcome 1.Alive birth 2.stillbirth 3. Preterm birth 3.induced abortion 4.spontanous abortion 5.Ectopic pregnancy	15.3 Maternal age at the time	15.4 Breast feeding duration(after each live birth)	15.5 Duration of family planning OCPs/Injections used after each pregnancy/birth
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					

16	What was your age at menarche?			_____
17	Are you still having menstrual periods?	1. Never menstruated 2. Still having menstrual periods	If 1,2 or 3, skip to Sec	

Case ☐ Control ☐

STRATA		Hospital	

		3. Not sure, periods are irregular 4. Menstrual periods have stopped permanently	C	
18	How old were you when your menstrual periods stopped permanently?			
19	How did your periods stop?	1. Stopped on their own 2. Stopped after surgery 3. Stopped after radiations/chemotherapy		
Section C; Medical history				
20	Have you ever been told by a doctor that you have diabetes, high blood sugar?	1. Yes 2. No 99. Don't know	If no or don't know, skip to Q22	
21	How long have you had it? (years)			
22	Is there a medical history of any other disease?	1. Yes 2. No 99. don't know	If no skip to Q 24	
23	Specify the disease with duration in years.	1. High blood pressure (hypertension) 2. Diabetes 3. High Cholesterol 4. Heart disease 5. Stroke (Paralytic attack) 6. Kidney disease 7. Any bone disease 8. Liver disease 9. Malabsorption disorder	_____	_____ _____ _____ _____ _____ _____ _____ _____ _____
24	Is there a medical history of benign breast disease?	1. Yes 2. No 99. don't know		
25	Past history of preeclampsia(high BP with proteinuria during any pregnancy	1. Yes 2. No 99. don't know		
26	Have you ever had a mammogram?	1. Yes 2. No	If no, skip to Sec D	
27	How many mammograms have you had in the past?	1. None 2. ----------		
Section D; Family History of cancer				
28	Is there a family history of BC among first blood relations?	1. Yes 2. No 99. don't know	If no, skip to Q 30	
29	Relation & age?	1. Mother 2. Daughter 3. Sister 4. Others (specify)		_____ _____ _____ _____

Case ☐ Control ☐

STRATA		Hospital	

30	Is there a family history of any other cancer among first blood relations?	1. Yes 2. No 99. Not sure	If no, skip to Section E	
31	What type of cancer?	1. Ovarian 2. Uterine 3. cervical 4. Lung 5. Colon/rectum 6. Others		
32	Name the relation with you?	1. Mother 2. Sister 3. Daughter 4. Others		

Section E; Drug History
Any drugs taken 1 year prior to breast cancer diagnosis (cases) or enrolment (controls)

33	Past Medications	a. Usage 1. Yes 2. No 3. occasionally	b. How often did you take/use them? (daily/weekly/monthly)	c. For how many years/months have you taken/used it regularly?
1	Vitamin D/cod/liver oil • Injection • Oral • With calcium (Calci-D, Ossopan, Chewcal)			
2	Multivitamin • Optilets M • Supradyn N • Centrum • others			
3	Vitamin C			
4	Vitamin E			
5	Calcium alone			
6	Aspirin/NSAIDs			
7	Others			

Section F;
34. Use of smoking /smokeless tobacco

1	Have you ever used tobacco in any form (smoking, chewing, snuff, etc)	1. Yes 2. No		
2	In what form you consume tobacco?	1. Smoking 2. chewed form 3. *any other form*		

Case ☐ Control ☐

STRATA | | Hospital | |

		(snuff)		
3	How many days a week? How much time during a day?			_____ _____
4	Total years of tobacco use			
5	Are you exposed to tobacco smoke by other people at home or at workplace regularly*? *At least once a day in a week.	1. Yes 2. No		
6	How many days a week? How much time during a day?			
7	How many days a week? How much time during a day?			
8	Total years of passive smoking exposure			

Section G ;

35. Physical Activity

We will ask you about the time you spent being physically active during a routine **7 days** (a year before breast cancer diagnosis among cases and enrolment among controls respectively). Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you did at work, as part of your house work, to ge from place to place, and in your spare time for any exercise.

First think about any **vigorous** activities that you did on an average 7 days before breast cancer diagnosis .Vigorous physic activities refer to activities of more than 10 minutes duration that take hard physical effort and make you breathe much harder than normal.

35.1	On how many days/week did you do **vigorous** physical activities like aerobics or jogging?	___ days/week ___ No vigorous physical activity
35.2	How much time did you usually spend doing **vigorous** physical activities on one of those days?	____ hours/ day ____ Minutes/day ____ Don't Know

Moderate

Think about all the **moderate** activities that you did in a routine **7 days** period. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time-

35.3	On how many days did you do **moderate** physical activities like swimming, carrying light loads like laundry/mopping? Do not include walking.	___ days/week ___ No moderate physical
35.4	How much time did you usually spend doing moderate physical activities on one of those days?	___ hours/day ___ minutes/day ___ don't know

Case ☐ Control ☐

STRATA		Hospital	

Walking		
Think about the time you used to spend **walking** in a routine **7 days period**. This includes at work and at home, walking t travel from place to place, and any other walking that you might do solely for recreation, sport, exercise, or leisure.		
35.5	On how many days did you **walk** for at least 10 minutes at a time?	___ No Walking ___ days/week
35.6	How much time did you usually **spend walking** on one of those days?	___ hours/day ___ minutes/day ___ don't know

Section H;

36. Sun Exposure (I would like to ask some questions about your home that will help us in ascertaining level of your exposure to sunlight)

1	Presence of Varanda/Sahan/ Lawn/Terrace	1. **Yes** 2. No		
2	Mark one of the options	1. Varanda 2. Sahan 3. Lawn 4. Terrace		
3	How many rooms are in the house?			
4	Do you keep windows of your house open?	1. Yes 2. No		
5	Do you keep curtains drawn during morning hours?	1. Yes 2. No		
6	Do you receive good amount of sunlight in your house?	1. Yes 2. No		
7	Do you turn the light on in most of the rooms during morning hours for better visibility inside home?	1. Yes 2. No		

EXPOSURE TO SUNLIGHT DURING SUMMER AND WINTER SEASON

No.	Questions & Filters	Coding Categories	Specific Answers	
37. Think of your exposure to direct sunlight 1 year prior to breast cancer diagnosis (cases) or enrolment (controls) and respond regarding a typical week			Summer: (April –Oct)	Winter: (Nov –March)

Case ☐ Control ☐

STRATA		Hospital	

1	Usually, how many days in a week did you go out in the sunlight during the daytime? (between 0 – 7days)			
2	Usually, between 9 am-4 pm, what was the average amount of time spent outdoor in the sunlight in a typical day?		____min/day	____min/day
3	Usually, what was the weather like during the time spent outdoor?	1. Always sunny (a radiant sky) 2. sometimes sunny/ sometimes cloudy 3. Mostly cloudy		
4	How much of your body was generally covered during outdoor hours (between 9 am -4 pm)? (Respond to all that apply as : Never-**N** Sometimes –**S/t** Always- **A**)	1. Head 2. Face 3. Neck covered 4. Full Arms covered 5. Half arms covered 6. Hands covered 7. Full Legs covered 8. Feet covered		
5	Were any of these a part of your attire during outdoor hours (between 9 am- 4 pm)? (Respond to all that apply)	1. Chadar 2. Hajab/scarf 3. Jelbaab/abaya/Burka 4. Niqab 5. Other (specify)		
	Use of sunscreen/cosmetics			
6	Did you usually use the following during the time spent in direct sunlight? (Respond to all that apply)	1. Did not apply anything to skin 2. Sunscreen/sun block 3. Lotion/cream 4. Moisturizer 5. Foundation 6. Face powder 7. Oil/gel/cream on hair 8. Others (specify	If 1, skip to 9	
7	If yes, how often did you apply it during the time spent in the sun?	1. < once a week 2. 1-2 times a week 3. 3-4 times a week		

Case ☐ Control ☐

STRATA		Hospital	

		4. 5-6 times a week 5. Daily 6. More than once daily		
8	What was the protection factor of the product? (SPF/ UVA/ UVB)	1. No protection facto 2. Don't know 3. _____SPF 4. UVA protective 5. UVB protective		
	Other Sun protection practices			
9	Did you use any other means to protect yourself from the sun?	1. Nothing extra 2. Wore a hat 3. Wore gloves 4. Wore sunglasses 5. Used umbrella 6. Stood under the shade 7. Others (specify)		
	Skin tone			
10	Skin tone (As matched by the skin tone card and assessed by the interviewer)			

Section I;
38. Food frequency questionnaire

I will now list various foods and I would like you to tell me with reference to one year before the disease whether you ate these foods and if so how often per month, per week or per day you ate these foods. Also mention any changes in their use related to season. (If "Never use", mark with □ in given column. For winters, write "W" and for summers, write : "S" in seasons column)

No.	Food Items	Average serving	amount	Never	Daily (tm/day)	Weekly (tm/wk)	Monthly (tm/month	Yearly	Seasona
1	Eggs								
2	"Paratha"								
3	Tandoori Naan								
4	"Halwah Puri"								
5	Milk whole (with cream or balai)								
6	Milk (Low fat)								
7	Cream or malai/Balai								
8	Milk Dessert e.g. custard, kheer, firni etc.								
9	Ice cream/ qulfi/ falooda								
10	Dahi								
11	Lassi sweet								

Case ☐ Control ☐

STRATA		Hospital	

12	Lassi Saltish								
13	Margarine								
14	Butter								
15	Whole milk Cheese								
16	Fortified products(cereals)								
17	Mutton (Salan, roasts, etc.)								
18	Beef (salan, roasts, kabab, qeema etc.)								
19	Chicken (salan, roasts, tikka etc.)								
20	Fish (salan, fry, etc.) • Tinned fish • Fatty fish • White fish								
21	Prawns, other shell fish (lobsters, crabs etc.)								
22	Organ Meats (Liver, brain etc.)								
23	Foods purchased from outside such as. Kata kat, karahi, nehari, burgers, Pizza, etc.)								
24	Cooked Vegetables								
25	Potatoes (including fried potato)								
26	Raw vegetables (e.g. salad)								
27	(Biryani/pulaoo)								
28	Beans lentils, and dal.								
29	Fruits (do not include juices)								
30	Fresh fruit juices (not including packaged ***Drinks**** like Frost, Frooto etc) [*Drinks are not jucies]								
31	Bakery products (e.g. cakes, pastries, Biscuits etc, bread is not included)								
32	Mithais, Halwas.								
33	Salty/fried snacks, such as potato chips, popcorn, samosa, pakorah kababs, nimco etc.								
34	Dry fruits e.g. nuts, peanuts, pista, almonds, chilgoza.								
36	Any soft drinks (Pepsi, Coca-Cola, Fanta, Sprite or 7up)								
37	Chocolate and other candies								
38	Green tea								

Case ☐ Control ☐

STRATA		Hospital	

39	Any other foods that you consume regularly and we have not asked about it (**do not mention names**; write actual response/ food items)									

Section J;
39. Information regarding breast cancer diagnosis and delay (for cases only)

1	First symptom during diagnosis (not exclusive)	1. Breast lump 2. Nipple problems/discharge 3. Breast pain 4. Skin changes (Peau de orange) 5. Changes of breast shape / Breast dimpling/ Gross swelling 6. Breast ulcer 7. Arm edema 8. Axillary lymph nodes 9. Supraclavicular lymph nodes 10. No symptom 11. Did not recognize any symptom 12. Others		
2	Systemic symptom/sign during diagnosis (not exclusive)	1. Loss of weight 2. Loss of appetite 3. Bony pain 4. Weight loss 5. Cough 6. Bone fracture 7. Short of breath 8. other		
3	Mode of first symptoms detection (first symptoms only)	1. Accidental 2. Breast self-examination 3. Clinical examination		
4	Action taken by the patient at appearance of the first symptoms	1. Consult the doctor immediately 2. use of alternative therapy 3. Delay treatment		

Case ☐ Control ☐

STRATA		Hospital	

39	Any other foods that you consume regularly and we have not asked about it (**do not mention names**; write actual response/ food items)									

Section J;
39. Information regarding breast cancer diagnosis and delay (for cases only)

1	First symptom during diagnosis (not exclusive)	1. Breast lump 2. Nipple problems/discharge 3. Breast pain 4. Skin changes (Peau de orange) 5. Changes of breast shape / Breast dimpling/ Gross swelling 6. Breast ulcer 7. Arm edema 8. Axillary lymph nodes 9. Supraclavicular lymph nodes 10. No symptom 11. Did not recognize any symptom 12. Others		
2	Systemic symptom/sign during diagnosis (not exclusive)	1. Loss of weight 2. Loss of appetite 3. Bony pain 4. Weight loss 5. Cough 6. Bone fracture 7. Short of breath 8. other		
3	Mode of first symptoms detection (first symptoms only)	1. Accidental 2. Breast self-examination 3. Clinical examination		
4	Action taken by the patient at appearance of the first symptoms	1. Consult the doctor immediately 2. use of alternative therapy 3. Delay treatment		

Case ☐ Control ☐

STRATA		Hospital	

5	After how many weeks did you seek help for your complaints?			
6	What were the reasons for the delay?	1. Fear of informing anyone 2. Fear of diagnosis 3. Fear of treatment 4. Fear of mastectomy 5. Shame and embarrassment 6. Misconceptions about why breast cancer occurs 7. Family/career commitments 8. Denial 9. Not recognizing a symptom as suspicious 10. False-negative diagnostic test 11. Traditional treatment 12. Financial considerations 13. Fear of adverse effects on relationship with husband (interference with sexuality, husband's abandonment) 14. Other reasons		
7	Action taken by the doctor at the first visit			
8	What do you think was the main reason for the delay in BC diagnosis?			

Thank you very much for your participation; this is the end of this questionnaire.

Would you like to know about the findings of this study?

- **Yes**
- **No**

Case ☐ Control ☐

STRATA		Hospital	

If yes, please provide us with your contact information:
Telephone No/email: _____
Mailing address: _____
_____

Urdu

Case ☐ control ☐

STRATA		Hospital	

صحت کے مختلف مراکز پر پاکستانی خواتین میں وٹامن ڈی اور چھاتی کے سرطان کے حوالے سے ایک کیس کنٹرول مطالعہ۔

Section A - سماجی وشماریاتی معلومات				
س	سوال	کوڈ	اسکپ	جواب
1	آپ کی عمر کیا ہے؟ (مکمل سالوں میں)			
2	آپ نے کہاں تک تعلیم حاصل کی ہے؟	1. (پڑھنا لکھنا نہیں آتا) انپڑھ 2. پڑھ اور لکھ سکتے ہیں 3. رسمی طور پر اسکول کی تعلیم 4. 1-5 سال تک تعلیم 5. 6-8 سال تک تعلیم 6. 9-10 سال تک تعلیم 7. 11-12 سال تک تعلیم 8. 12 سال سے زیادہ تعلیم (وضاحت کریں) دیگر		
3	آپ کی مادری زبان کون سی ہے؟	1. اردو 2. سندھی 3. پنجابی 4. پشتو 5. بلوچی 6. سرائیکی 7. (وضاحت کریں) دیگر		
4	آپ کی ازدواجی حیثیت کیا ہے	1. غیر شادی شدہ 2. شادی شدہ 3. بیوہ 4. طلاق یافتہ		
5	رہائش کی قسم؟	1 اپارٹمینٹ/فلیٹ 2. چھوٹا گھر 3. اپنا بنگلہ 4. کنارٹر میں رہائش 5. کچی آبادی/بستی میں رہائش 6. (وضاحت کریں) کوئی اور		
6	اپنا گھر ہے یا کرئے کا ہے؟	1. اپنا ہے 2. کرائے کا ہے 3. (وضاحت کریں) دیگر		
7	آپ کہاں رہتی ہیں؟	1. گاؤں میں 2. شہر میں 3. چھوٹے شہر میں		
8	کیا آپ کو ملازمت ہے؟	1. ہاں 2. نہیں	اگر نہیں، تو سوال	

Case ☐ Control ☐

STRATA		Hospital	

			نمبر9 پرجائیں	
	پیشہ			
9	کیا آپ کا شوہرملازمت کرتا ہے؟ (شادی شدہ عورت سےپوچھاجائے)	1. ہاں 2. نہیں	اگرنہیں، توسوال نمبر10 پرجائیں	
	پیشہ			
10	آپ کےگھرکی کل ماہانہ آمدنی کتنی ہے (روپیوں میں) (براہ مہربانی گھرمیں کمانےوالےتمام افراد کی آمدنی شامل کریں)			
11	کتنےافراد بشمول آپ اس ماہانہ آمدنی پرگذارا کرتےہیں؟			
12	کیا آپ کبھی حاملہ ہوئی ہیں؟	1. ہاں 2. نہیں	اگرنہیں تو section B میں جائیں	

Section B تولیدی/ دودھ پلانےکی تاریخ

زندہ بچوں کی تعداد (زچگی کےدوران) _____ حمل کی تعداد _____ اولاد نا پیدا کرسکنا _____

	13.1 حمل کےسال، پہلےحمل کےساتھ شروع کریں	13.2 نتیجہ 1.زندہ بچوں کی پیدائش 2.مردہ بچوں کی پیدائش 3. قبل ازوقت بچوں کی پیدائش 3. ارادتاً/دانستہ اسقاط حمل 4. خود بہ خود اسقاط حمل 5. بچےدانی کےباہرحمل ہونا	13.3 وقت زچگی عمر	13.4 دودھ پلانےکی مدت (ہرزندہ بچےکی پیدائش کےبعد)	13.5 خاندانی منصوبہ بندی کی مدت ہرحمل/پیدائش کےبعد استعمال کی گئی انجیکشن/OCPs
1					
2					
3					
4					
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9					

Case ☐ Control ☐

STRATA		Hospital	

10						
11						
12						
13						
14						

14	جب آپ کی پہلی ماہواری ہوئی تواس وقت آپ کی عمرکیا تھی؟			_____
15	کیا آپ کوابھی بھی ماہواری آتی ہے؟	1. کبھی بھی ماہواری نہیں ہوئی 2. ابھی تک ماہواری آتی ہے 3. اس بات کایقین نہیں، باقائدہ سےنہیں ہوتی 4. مستقل ماہواری آنا بند ہوگئی ہے	اگرجواب 1، 2 یا 3 section ہےتو Cپرجائیں	
16	اس وقت آپ کی عمرکیا تھی جب آپ کوماہواری آنا مستقل طورپربند ہوگئی تھی؟			
17	آپ کی ماہواری کیسےبندہوئی؟	1. اپنےطوربند ہوگئی 2. جراحی/آپریشن کےبعد بند ہوگئی 3. کیمائی علاج کےزریعےبند ہوگئی		
Section C	**فرد کی طبی معلومات/تاریخ**			
18	کیا آپ کوکبھی ڈاکٹرنےبتایا ہےکہ آپ کوبلندفشارخون اورزیابیطس ہے؟	1. ہاں 2. نہیں 99. معلوم نہیں	اگرنہیں یا معلوم نہیں توسوال نمبر20 پرجائیں	
19	یہ آپ کوکب سےہے (سال)			
20	کیا کیسی دوسری طبی بیماری کی شکایت تونہیں رہی؟	1. ہاں 2. نہیں 99. معلوم نہیں	اگر نہیں توسوال نمبر22 پرجائیں	
21	براہ مہربانی وضاحت کریں کہ وہ بیماری کتنی مدت تک یا کتنےسالوں تک لاحق رہی؟	1. بلندفشارخون 2. زیابیطس 3. خون میں چکناہٹ کی زیادتی 4. دل کی بیماری 5. (فالج کاحملہ) فالج 6. گردوں کی بیماری 7. ہڈیوں کےنرم پڑجانےکی بیماری 8. جگرکی بیماری	_____ _____ _____ _____ _____ _____ _____ _____ _____	

Case ☐ Control ☐

STRATA		Hospital	

		9. ہاضمہ کی بیماری(جس میں غذائی اجزأغیرمعمولی طورپرجذب ہوتےہیں)		
22	کیا آپ کوکبھی چھاتی کی بیماری کی معمولی سی شکایت رہی ہے؟	1. ہاں 2. نہیں 99. معلوم نہیں		
23	حمل کےدوران بلندفشارخون کی شکایت (کسی بھی حمل کےدوران بلند فشارخون کےساتھ پیشاب میں زردی مائل رطوبت کااخراج)	1. ہاں 2. نہیں 99. معلوم نہیں		
24	کیا آپ نےکبھی چھاتی کا ایکسرے کروایا ہے؟	1. ہاں 2. نہیں	اگرنہیں section D پرجائیں	
25	ماضی میں آپ نےکتنےایکسرے کروائےہیں؟	1. کوئی نہیں 2. ----------		
Section Dسرطان کےحوالےسےخاندان کی طبی معلومات/تاریخ				
26	کیا آپ کےخاندان میں کسی کوسرطان کی بیماری ہوئی ہے؟ (خاندان کےوہ افراد جن سےآپ کےخون کاپہلہ رشتہ قائم ہو)	1. ہاں 2. نہیں 99. معلوم نہیں	اگرنہیں توسوال نمبر29 پرجائیں	
27	تعلق اورعمر	1. والدہ 2. بیٹی 3. بہن 4. (وضاحت کریں)دیگر		—— —— —— ——
28	کیا آپ کےخاندان میں کسی دوسرےسرطان کی بیماری تونہیں رہی؟ (خاندان کےوہ افراد جن سےآپ کےخون کاپہلہ رشتہ قائم ہو)	1. ہاں 2. نہیں 99. معلوم نہیں	اگرنہیں Section E پرجائیں	
29	سرطان کی کون سی قسم	1. بیضہ دانی 2. رحم 3. گردن کےمہروں 4. پھیپڑوں 5. بڑی آنت/مقعد 6. دیگر		
30	آپ کےساتھ ان کا تعلق	1. ماں 2. بہن 3. بیٹی 4. دیگر		

Section Eادویات کےمتعلق معلومات چھاتی کےسرطان کی تشخیص سےپہلےلی جانےوالی ادویات (کیسز) یا اندراج (کنٹرولز)				
31	ماضی میں ادویات کا استعمال	a. استعمال	b. عموماً آپ ادویات کس	c. آپ

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		1. ہاں 2. نہیں 3. کبھی کبھار	طرح لیتے ہیں (مہینہ وار/ہفتہ وار/روزانہ)	نے کتنے سال/مہینے باقاعدگی سے ان کا استعمال کیا ہے؟
1	Vitamin D/cod/liver oil • Injection • Oral • With calcium (Calci-D, Ossopan, Chewcal)			
2	Multivitamin • Optilets M • Supradyn N • Centrum • others			
3	Vitamin C			
4	Vitamin E			
5	Calcium alone			
6	Aspirin/NSAIDs			
7	دیگر			

Section F
تمباکو کا استعمال/چبانے والہ تمباکو .32

1	کیا آپ نے کبھی کسی بھی قسم کے تمباکو کا استعمال کیا ہے (تمباکو نوشی، چبانے والہ تمباکو، نسوار وغیرہ)	1. ہاں 2. نہیں		
2	کس قسم کے تمباکو کا استعمال کرتے ہیں؟	1. تمباکو نوشی 2. چبانے والہ تمباکو 3. کوئی اور قسم (نسوار)		
3	ہفتے میں کتنے دن؟ ایک دن کے دوران کتنا وقت؟			____ ____
4	تمباکو کے استعمال کے کل سال			
5	کیا آپ کو باقاعدہ گھر پر یا دفتر میں دوسرے لوگوں کی تمباکو نوشی کا سامنا کرنا پڑتا ہے کم از کم ایک ہفتے میں دن میں ایک بار *	1. ہاں 2. نہیں		
6	ہفتے کے کتنے دن؟ ایک دن کے دوران کتنا وقت			
7	ہفتے کے کتنے دن؟			

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	ایک دن کے دوران کتنا وقت			
8	دوسرے لوگوں کی تمباکو نوشی کا سامنہ کے کل سال غیر فعال/			

33-جسمانی سرگرمی Section G

آپ سے اس وقت کے بارے میں پوچھیں گے جو گذشتہ 7 دنوں کے دوران چھاتی کے تشخیص سے پہلے آپ نے جسمانی طور پر فعال/سرگرم ہوتے ہوئے گذارا ہو۔ اگر آپ جسمانی طور پر کود کو سرگرم نا بھی سمجھتے ہوں پھر بھی براہ مہربانی ہر ایک سوال کا جواب دیجئے گا۔ براہ مہربانی ان کاموں/سرگرمیوں کے بارے میں سوچیں جو آپ نے کام کے دوران سرانجام دی ہوں۔ جیسا کہ آپ کے گھر کا کام، دفتر کا کام، ایک جگہ سے دوسری جگہ جانا یا فارغ اوقات میں آپ نے کوئی ورزش وغیرہ کی ہو۔

اب سے پہلے ان سخت جسمانی سرگرمیوں کے بارے میں سوچیں جو آپ نے چھاتی کے سرطان کی تشخیص سے پہلے اوسطً 7 دنوں کے دوران سرانجام دی ہوں۔ سخت جسمانی سرگرمیوں سے مراد وہ کام جن کا دورانیہ 10 منٹ سے زیادہ ہوں، جس میں زیادہ مشقت گے اور سانس کی رفتار معمول سے زیادہ ہو۔

33.1	ایک ہفتے میں آپ نے کتنے دن سخت جسمانی مشقت والے کام کئے، جیسا کہ، رسی پر کودنا، ورزش کرنا یا دوڑنا	ہفتہ/دن ___ کوئی سخت جسمانی مشقت ___ والہ کام نہیں کیا
33.2	عام طور پر ان دنوں میں سے ایک دن کے اندر آپ کتنا وقت سخت جسمانی کاموں/سرگرمیوں میں خرچ کرتی ہیں	**دن/گھنٹے** ___ دن/منٹس ___ معلوم نہیں ___

درمیانی جسمانی مشقت والے کام

تمام درمیانی جسمانی سرگرمیوں کے بارے میں سوچیں جو آپ نے گذشتہ 7 دنوں کے دوران سرانجام دی ہوں۔ درمیانی جسمانی سرگرمیوں سے مراد، وہ کام جن کا دورانیہ 10 منٹس سے زیادہ ہوں، جن میں درمیانی مشقت لگے اور سانس کی رفتار معمول سے کچھ زیادہ ہو۔

33.3	ایک ہفتے میں کتنے دن آپ نے درمیانی مشقت والے کام کئے، جیسا کہ، تیرنا، ہلکہ وزن اٹھانا، کپڑے دھونا یا جھاڑو لگانا اس میں چلنا شامل نا کریں	ہفتہ/دن ___ کوئی درمیانی مشقت والہ ___ کام نہیں کیا
33.4	عام طور پر ان دنوں میں سے ایک دن کے اندر آپ کتنا وقت درمیانی جسمانی کاموں/سرگرمیوں میں خرچ کرتی ہیں	دن/گھنٹے ___ دن/منٹس ___ معلوم نہیں ___

چلنا

وقت کے بارے میں سوچیں جو آپ معمول کے 7 دنوں میں پیدل چلنے میں صرف کرتی ہیں، اس میں وہ وقت شامل کریں جو آپ گھر پر، رمیں، ایک جگہ سے دوسری جگہ پیدل سفر کرنے میں گذارا ہو اور اس میں وہ چلنا بھی شامل کریں جو آپ صرف تفریح، کھیل، ورزش یا رغ وقت میں کرتے ہوں۔

33.5	عام طور پر کتنے دن آپ نے ایک وقت میں کم از کم 10 منٹس تک چلنے میں صرف کئے	پیدل نہیں چلی ___ ہفتہ/دن ___
33.6	عام طور پر ان دنوں میں آپ کتنا وقت ایک دن میں پیدل چلیں	دن/گھنٹے دن/منٹس ___ معلوم نہیں ___

Case ☐ Control ☐

STRATA		Hospital	

Section H 34. سورج کی روشنے/دھوپ میں کا سامنا (میں آپ کے گھر کے متعلق کچھ سوالات پوچھوں گی، وہ سوالات ہمیں یہ معلوم کرنے میں مدد کریں گے کہ آپ کتنی دیر تک سورج کی روشنی یا دھوپ میں وقت گذارتے ہیں)				
1	باآمدہ/صحن/لان/چھت یا چبوترے کی موجودگی	1. ہاں 2. نہیں		
2	کسی بھی ایک پرنشاں لگائیں	1. برآمدہ 2. صحن 3. لان 4. چھت یا چبوترہ		
3	گھرمیں کتنے کمرے ہیں؟			
4	کیا آپ گھرکی کھڑکیاں کھلی رکھتے ہیں؟	1. ہاں 2. نہیں		
5	کیا آپ صبح کے اوقات میں کھڑکیوں کے پردے بند رکھتے ہیں؟	1. ہاں 2. نہیں		
6	کیا آپ اپنے گھرمیں سورج کی روشنی مناسب مقدار میں حاصل کرتے ہیں؟	1. ہاں 2. نہیں		
7	کیا آپ صبح کے اوقات کے دوران گھرکے اندر بہتر نظر/روشنی کے لئے بیشتر کمروں میں بتی جلاتے ہیں؟	1. ہاں 2. نہیں		

موسم سرما اورگرما میں سورج کی روشنی/دھوپ کا سامنا

نمبر	Questions & Filters	Coding Categories	Specific Answers	
	کیا آپ چھاتی کے سرطان کی تشخیص سے پہلے گذشتہ ایک سال کے دوران سوارج کی روشنی سے براہ راست متاثر ہوئے کسی ایک مخصوص ہفتے کے حوالے سے سوچ کر جواب دیں		موسم گرما (May – Aug)	موسم سرما (Nov – Feb)
1	عموماً ایک ہفتے میں کتنے دن آپ باہر سورج کی روشنی/دھوپ میں گذارتے ہیں (0 سے 7 دن کے درمیاں)			
2	ایک عام دن میں صبح 9 سے شام 4 بجے تک تقریباً کتنا وقت باہر آپ سورج کی روشنی میں گذارتے ہیں؟		____min/day	_____ min/day
3	عموماً جب آپ باہر جاتے ہیں اس وقت موسم کیسا ہوتا ہے؟	ہمیشہ دھوپ ہوتی ہے 01 کبھی دھوپ کبھی ابرآلود 02 ہمیشہ ابرآلود 03		

Case ☐ Control ☐

STRATA		Hospital	

4	عام طورسورج کی روشنی میں آپ کےجسمانی اعضاء (9 سے4 کےدرمیاں) کتنے ڈھکےہوئےہوتےہیں؟ (ان ساب کا جواب دیں جن کا اطلاق ہوتا ہے) کبھی نہیں کبھی کبھار ہمیشہ	1. سر 2. چہرہ 3. گردن ڈھکی ہوئی 4. پورےبازوڈھکےہوئے 5. آدھے بازوڈھکےہوئے 6. ہاتھ ڈھکےہوئے 7. پوری ٹانگیں ڈھکی ہوئی 8. پاؤں ڈھکےہوئے		
5	مندرجہ ذیل کیا آپ کےلباس کا حصہ ہوتےہیں اس واقت جب (صبح 9 سےشام 4 کےدرمیاں) آپ گھرسےباہرہوتےہیں (ان ساب کا جواب دیں جن کا اطلاق ہوتا ہے)	1. چادر 2. حجاب/اسکارف 3. عبایا/برقہ 4. نقاب 5. دیگر(وضاحت کریں)		
	سن اسکرین اور آرائشی سامان کااستعمال			
6	باہر جاتےوقت آپ مندرجہ ذیل میں سےکونسی استعمال کرتےہیں؟ (ان سب کا جواب دیں جن کااطلاق ہوتا ہے)	1. کچھ بھی نہیں 2. سن اسکرین/سن بلاک 3. لوشن/کریم 4. نمی مہیاکرنےوالالوشن 5. فاؤنڈیشن 6. چہرےکاپاؤڈر 7. پاؤں پےلگانےوالاکریم یا تیل 8. دیگر (وضاحت کریں)	اگر00 ہےتوسوال نمبر9 پرجائیں	
7	اگرہاں توکتنی بار استعمال کرتےہیں؟	1. ہفتےمیں 1 بارسے < 2. ہفتےمیں 1-2 3. ہفتےمیں 3-4 4. ہفتےمیں 5-6 5. روزانہ 6. دن میں ایک سےزیادہ بار		
8	ان اشیاء میں سورج کی روشنی سےحفاظت کاعنصرکیاہے؟ (SPF/ UVA/ UVB)	1. کچھ نہیں 2. معلوم نہیں 3. _____SPF 4. UVA protective 5. UVB protective		

Case ☐ Control ☐

STRATA		Hospital	

	سورج کی شعاعوں سے بچنے کے لیے دیگر طریقے			
9	کیاآپ سورج کی شعاعوں سے بچنے کے لیے کچھ اور استعمال کرتے ہیں؟	1. کچھ نہیں 2. ٹوپی 3. دستانے 4. چشمے 5. چھتری 6. سائے میں کھڑے ہوجانا 7. دیگر (وضاحت کریں)		
	جلد کی رنگت			
10	کارڈ سے ملا کر نمبر لکھیں			

Sec I. کھانے کے اعداد و شمار کا سوالنامہ

میں آپ سے مختلف کھانے کی اشیاء کے بارے میں پوچھوں گی، براہ مہربانی اپنی بیماری کے ایک سال پہلے کے حوالے سے مجھے بتائیں کہ کیا آپ نے یہ کھانے کھائے؟ اگر ہاں تو آپ نے کس طرح فی مہینہ، فی ہفتہ اور فی دن یہ کھانے کھائے؟ اور موسم کے حوالے سے ان میں اگر تبدیلی آتی ہے تو اس کا بھی ذکر کریں۔ اگر کبھی استعمال نہیں کیا تو دنے گئے کھانے میں نشان لگائیں، موسم سرما کے لئے ڈبلیو اور موسم گرما کے لئے ایس لکھیں۔

No.	کھانے کی اشیاء	اوسط مقدار	مقدار	کبھی نہیں	روزانہ (tm/day)	ہفتہ وار (tm/wk)	مہینہ وار (tm/month)	سالانہ	موسمی
1	انڈے								
2	"پراٹھہ"								
3	تندوری نان								
4	"حلوا پوری"								
5	دودھ (کریم یا بالائی کے ساتھ)								
6	دودھ (کم چربی والا)								
7	کریم/ملائی/بالائی								
8	دودھ سی بنی اشیاء (کسٹرڈ، کھیر، فیرنی وغیرہ)								
9	آئس کریم، کلفی، فالودہ								
10	دہی								
11	میٹھی لسی								
12	نمکین لسی								
13	مارجرین								
14	مکھن								
15	دودھ پنیر کے ساتھ								
16	اظافی غذائی اجزا والی مصنوعات (اناج)								
17	چھوٹا گوشت (سالن، بھنہ ہوا)								
18	بڑا گوشت (سالن، بھنہ ہوا، کباب، قیما)								
19	مرغی (سالن، ٹکا وغیرہ)								
20	مچھلی (سالن، فرائے وغیرہ) ٹبہ میں پیک مچھلی فربہ مچھلی سفیدمچھلی								

Case ☐ Control ☐

STRATA		Hospital	

21	جھینگا، دیگر شیل فش مچھلی (لوبسٹر، کیکڑے وغیرہ)								
22	کلیجی، دماغ وغیرہ								
23	باہرسےخریدہ ہوا کھانا،کٹاکٹ، کڑاہی، نہاری، برگر، پیزا وغیرہ								
24	پکی ہوئی سبزیاں								
25	آلو(فرائے آلو بھی شامل ہیں)								
26	کچی سبزیاں (سلاد وغیرہ)								
27	(بریانی/پلانو)								
28	پھلیاں اوردال								
29	(پھل(جوس شامل نا کریں)								
30	تازا پھلوں والےجوس (فراسٹ یا فروٹوجیسےپیک کئےہوئے جوس شامل نا کریں) *مشروبات جوس نہیں ہے								
31	بیکری والی مصنوعات (کیک، پیسٹری، بسکٹس، ڈبل روٹی شامل نہیں)								
32	مٹھائی، حلوا								
33	نمکین، تلی ہوئی اشیاء، جیسا کہ آلوکی چپس، پوپ کارن، سموسہ، کباب، پکوڑہ، نمکووغیرہ								
34	خشک پھل جیسے، بادام، چلگوزہ، اخروٹ، پستہ وغیرہ								
36	کوئی سوفٹ ڈرنک، پیپسی کوکوکولا، فنٹا سیون اپ وغیرہ								
37	چاکلیٹ اوردیگرٹافیاں								
38	سبزچائے								
39	کوئی اورکھانےکی اشیاء جوآپ روزانہ استعمال کرتےہیں اورہم اس کےمتعلق ابھی پوچھا نہیں(نام کا ذکرنا کریں، اصلی جواب/اشیاء لکھیں)								

چھاتی کےسرطان کی تشخیص اورتاخیرکےبارےمیں معلومات (کیسزکےلئےصرف)Section J				
34.1	تشخیص کےدوران پہلی علامت (کوئی ایک مخصوص نہیں) ✓	1. چھاتی میں گانٹھ 2. چھاتی کی چوسنی/نپل کے مسئلے/مادہ خارج ہونا 3. چھاتی میں درد 4. جلد میں تبدیلی 5. چھاتی کی شکل میں تبدیلی/چھاتی میں ہلکورےپڑجانا/چھاتی کاسوج جانا 6. چھاتی کا السر/ناسور		

Case ☐ Control ☐

STRATA		Hospital	

		7. بازومیں ورم 8. بغلی غدود/گانٹھ 9. گردن/ہنسلی کےغدود 10. کوئی علامت نہیں 11. کسی بھی علامت کونا پہچاننا 12. دیگر		
34.2	تشخیص کےدوران جسم کومتاثرکرنےوالی علامات (کوئی ایک مخسوص نہیں)	1. وزن میں نقصان 2. بھوک میں کمی 3. ہڈیوں میں درد 4. وزن میں کمی 5. کھانسی 6. ہڈی ٹوٹنا 7. سانس کی کمی 8. دیگر		
34.3	پہلی علامت کاپتہلگانےکاطریقہ کار (پہلی علامات)	1. اتفاقی 2. چھاتی کاخود امتحان 3. طبی یمتحان		
34.4	پہلی علامات کےظاہرہونےپرمریض کی طرف سےکی گئی تدبیر	1. فوری طورڈاکٹرسےمشورہ کیا 2. متبادل علاج کا استعمال 3. علاج نیں تاخیر		
34.5	کتنے ہفتوں کے بعدآپ نے اپنی بیماری کی شکایات کےلئےمدد حاصل کی			
34.6	تاخیر کی وجوہات کیا تھیں	1. کسی کوبتانےسےڈرلگنا 2. ڈاکٹروں/ہسپتال کےلئےمنفی رویا رکھنا 3. بیماری کی تشخیص کاخوف 4. علاج سے ڈر/گھبراہٹ 5. پستان کی جراحی/کٹنےکاخوف 6. شرمندگی اورپریشانی		

Case ☐ Control ☐

STRATA		Hospital	

		7. یہ غلطفہمی کے چھاتی کا سرطان کیوں ہوتا ہے 8. خاندانی/ملازمت کی پابندیاں 9. بیماری کومانننے سے انکار 10. علامات کے بارے شک ہونا/صحیح سے نا پہچاننا 11. مرض کے تشخیص کے ٹیسٹ کاغلط یا منفی آنا 12. روایتی طریقے سے علاج کرانا 13. مالی مشکلات/پریشانیاں 14. شریک حیات کے ساتھ تعلقات پر منفی اثرپڑنے کاخوف (جنسی مداخلت، شریک حیات کاعلحدہ ہونا/چھوڑجانا) 15. دیگروجوہات		
34.7	ڈاکٹر کے پاس پہلی ملاقات میں تجویزکردہ علاج			
34.8	آپ کے خیال میں چھاتی کے سرطان کی تشخیص میں تاخیر کی بنیادی وجہ کیا تھی؟			

آپ کی شرکت کابہت بہت شکریہ، سوالنامہ پورا ہوگیا۔

کیا آپ اس تحقیق کے نتائج کے بارے میں جاننا چاہتے ہیں

- ہاں
- نہیں

اگرہاں، توبراہ مہربانی ہمیں اپنے رابطے کی معلومات فراہم کریں

ٹیلی فون نمبر/ای میل: _____

رابطے کا پتہ: _____

_____

Case ☐ Control ☐

STRATA		Hospital	

SECTION K Data extraction from Medical records (Cases only)		
1	Date of first hospital appointment with the surgeon/oncologist	
2	Date of Mammography	
3	Date of Biopsy/FNA	
4	Height	
5	Weight pre-op	
Lab Reports from Medical records		
6	Breast cancer (right or left)	
7	Histopathology of Tumor	
8	**Grade of Tumor** 1. Grade I well differentiated 2. Grade II moderately differentiated 3. Grade III poorly differentiated	
9	**TNM classification for staging malignancy** **Tumor size** 1. Tx Cannot be evaluated for nonspecified reasons 2. T1 Lesion < 2.0 cm in greatest dimension 3. T2 lesion 2-5 cm 4. T3 > 5 cm 5. T4 Tumor with skin changes	
10	**N (0-3):** degree of spread to regional lymph nodes 1. N0: No lymph nodes metastasis 2. N1: tumor cells spread to closest or small number of regional lymph nodes 3. N2: tumor cells spread to an extent between N1 and N3. 4. N3: tumor cells spread to most distant or numerous regional lymph nodes 5. NX; Lymph nodes, not evaluable	
11	**M** (0/1): presence of metastasis 1. M0: no distant metastasis 2. M1: metastasis to distant organs (beyond regional lymph nodes) 3. MX ;distance metastasis NA	

Case ☐ Control ☐

STRATA		Hospital	

12	serum 25-hydroxyvitamin D (mention the date)	
13	Season of blood test • April –Oct • November -March	
14	serum 25-hydroxyvitamin D from medical records with dates	

Quality control's signature and date: _____

Appendix B: Ethical Approval Forms

آغا خان یونیورسٹی
THE AGA KHAN UNIVERSITY

August 24, 2015

Dr. Uzma Shamsi
Department of Community Health Sciences
The Aga Khan University
Karachi

Dear Dr. Uzma Shamsi,

Re: 3074-CHS-ERC-14. PI - Dr. Uzma Shamsi: A Multicentre Case Control Study of Vitamin D and Breast Cancer Risk among Pakistani women

Thank you for the progress report received on August 21, 2014 requesting extension of ethical approval to the above mentioned study.

The progress report submitted by you was found satisfactory. The study is given an extension of ethical approval for further period of one year. If required, a request for further extension must be submitted after one year along with the annual report.

Any changes in the protocol or extension in the period of study should be notified to the Committee for prior approval. All informed consents should be retained for future reference. A progress report should be submitted to ERC office after six months.

Thank you.

Yours sincerely,

Dr. Shaista Khan, FRCS (Edin.)
Chairperson
Ethical Review Committee

آغا خان یونیورسٹی
THE AGA KHAN UNIVERSITY

June 24, 2014

Dr. Uzma Shamsi
Department of Community Health Sciences
Aga Khan University
Karachi

Dear Dr. Uzma Shamsi,

Re: 3074-CHS-ERC-14. PI - Dr. Uzma Shamsi: A Multicentre Case Control Study of Vitamin D and Breast Cancer Risk among Pakistani women

Thank you for your application for ethical approval received on 5/14/2014 regarding the above mentioned study.

Your study was reviewed and discussed in our meeting held on June 13, 2014 at Aga Khan University. Members made following recommendations:

1. Why does the PI want objective No: 4?
2. Selection procedure needs to be delineated example how will patients and controls be approached? Whose clinics?
3. Will the consultant whose patients are used as controls would know?
4. There should be separate Informed consent forms for patients and controls. The starting statement for patients consent should be "you have been diagnosed as breast cancer patient…" and for control "we are inviting you as control and you do not have cancer…" The consent forms should also mention of Vitamin D levels, the process and place of blood drawing.
5. If the participants are expected to come to AKU and collection units for blood withdraw then they should be compensated for travel especially the non AKU patients?
6. English questioner is missing?
7. The proposal also seems like a draft as at some places text is highlighted.
8. Permission letter from KIRAN should be submitted.

Cont'd on page 2

Your response to above recommendations should be received latest by September 24, 2014. After that the project would stand as abandoned.

Thank you.

Yours sincerely,

Salma Rattani, MScN
Vice Chairperson
Ethical Review Committee

PAKISTAN ATOMIC ENERGY COMMISSION (PAEC)
Karachi Institute for Radiotherapy and Nuclear Medicine (KIRAN)
Near Safoora Chowk, Off University Road, KDA Scheme-33, Gulzar-e-Hijri, Karachi
Ph:021-99261601, Fax: 021-99261610, Email: kiran@ntc.net.pk

No. KIRAN-Estt.2(22)/13 Dated: 23-Jun-14

Dr. Uzma Shamsi
Agha Khan University Hospital (AKUH)
Stadium Road,
KARACHI

Subject: ***REQUEST FOR ETHICAL APPROVAL OF "A MULTICENTRE CASE CONTROL STUDY OF VITAMIN D AND BREAST CANCER RISK AMONG PAKISTANI WOMEN"***

Dear Sir,

With reference to your letter dated 08-5-2014 on the subject, it is communicated that Ethical Committee of this Institute has very kindly approved your visit to KIRAN, as per following schedule:

Date & time: Wednesday, 25-June-204 at 11am
Liaison person: Admin Officer, KIRAN

Yours truly

(KHAN M. MAIMAR)
Admin Officer

THE UNIVERSITY of ADELAIDE

RESEARCH BRANCH
OFFICE OF RESEARCH ETHICS, COMPLIANCE AND INTEGRITY

SABINE SCHREIBER
SECRETARY
HUMAN RESEARCH ETHICS COMMITTEE
THE UNIVERSITY OF ADELAIDE
SA 5005
AUSTRALIA

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email: sabine.schreiber@adelaide.edu.au
CRICOS Provider Number 00123M

31 July 2014

Professor D Callen
School of Medicine

Dear Professor Callen

PROJECT NO: H-2014-111
A multicentre case control study of vitamin D and breast cancer risk among women in Karachi Pakistan

I write to advise you that the Human Research Ethics Committee has approved the above project. Please refer to the enclosed endorsement sheet for further details and conditions that may be applicable to this approval. Ethics approval is granted for a period of three years subject to satisfactory annual progress reporting. Ethics approval may be extended subject to submission of a satisfactory ethics renewal report prior to expiry.

The ethics expiry date for this project is: 31 July 2017

Where possible, participants taking part in the study should be given a copy of the Information Sheet and the signed Consent Form to retain.

Please note that any changes to the project which might affect its continued ethical acceptability will invalidate the project's approval. In such cases an amended protocol must be submitted to the Committee for further approval. It is a condition of approval that you immediately report anything which might warrant review of ethical approval including (a) serious or unexpected adverse effects on participants (b) proposed changes in the protocol; and (c) unforeseen events that might affect continued ethical acceptability of the project. It is also a condition of approval that you inform the Committee, giving reasons, if the project is discontinued before the expected date of completion.

A reporting form for the annual progress report, project completion and ethics renewal report is available from the website at http://www.adelaide.edu.au/ethics/human/guidelines/reporting/

Yours sincerely

Dr John Semmler
Convenor
Human Research Ethics Committee

THE UNIVERSITY of ADELAIDE

RESEARCH BRANCH
OFFICE OF RESEARCH ETHICS, COMPLIANCE AND INTEGRITY

SABINE SCHREIBER
SECRETARY
HUMAN RESEARCH ETHICS COMMITTEE
THE UNIVERSITY OF ADELAIDE
SA 5005
AUSTRALIA

TELEPHONE +61 8 8313 6028
FACSIMILE +61 8 8313 7325
email: sabine.schreiber@adelaide.edu.au
CRICOS Provider Number 00123M

Applicant: Professor D Callen

School: School of Medicine

Project Title: ***A multicentre case control study of vitamin D and breast cancer risk among women in Karachi Pakistan***

THE UNIVERSITY OF ADELAIDE HUMAN RESEARCH ETHICS COMMITTEE

Project No: **H-2014-111** RM No: 0000019101

APPROVED for the period until: **31 July 2017**

Thank you for the response dated 18.7.14 and 28.7.14 to the matters raised by the Committee. It is noted that this study is to be conducted by Dr Uzma Shamsi, PhD candidate.

Refer also to the accompanying letter setting out requirements applying to approval.

Dr John Semmler
Convenor
Human Research Ethics Committee

Date: 30 July 2014

Appendix C: Consent Forms

Human Research Ethics Committee (HREC)

1. I have read the attached Information Sheet and agree to take part in the following research project:

Title:	A Multicentre Case Control Study of Vitamin D and Breast Cancer Risk among Women in Karachi, Pakistan
Ethics Approval Number:	**H-2014-111**

2. I have had the project, so far as it affects me, fully explained to my satisfaction by the research worker. My consent is given freely.

3. I have been given the opportunity to have a member of my family or a friend present while the project was explained to me.

4. Although I understand that the purpose of this research project is to improve the quality of medical care, it has also been explained that my involvement may not be of any benefit to me.

5. I have been informed that, while information gained during the study may be published, I will not be identified and my personal results will not be divulged.

6. I understand that I am free to withdraw from the project at any time and that this will not affect medical advice in the management of my health, now or in the future.

7. I understand and give permission for the researchers to access my medical records.

8. I am aware that I should keep a copy of this Consent Form, when completed, and the attached Information Sheet.

Participant to complete:

Name: _____ Signature:_____ Date:_____

Researcher/Witness to complete:

I have described the nature of the research to _____
(print name of participant)

and in my opinion she/he understood the explanation.

Signature: _____ Position: _____ Date:_____

Human Research Ethics Committee (HREC)

1. I have read the attached Information Sheet and agree to take part in the following research project:

Title:	A Multicentre Case Control Study of Vitamin D and Breast Cancer Risk among Women in Karachi, Pakistan
Ethics Approval Number:	**H-2014-111**

2. I have had the project, so far as it affects me, fully explained to my satisfaction by the research worker. My consent is given freely.

3. I have been given the opportunity to have a member of my family or a friend present while the project was explained to me.

4. Although I understand that the purpose of this research project is to improve the quality of medical care, it has also been explained that my involvement may not be of any benefit to me.

5. I have been informed that, while information gained during the study may be published, I will not be identified and my personal results will not be divulged.

6. I understand that I am free to withdraw from the project at any time and that this will not affect medical advice in the management of my health, now or in the future.

7. I understand and give permission for the researchers to access my medical records.

8. I am aware that I should keep a copy of this Consent Form, when completed, and the attached Information Sheet.

Participant to complete:

Name: _____ Signature:_____ Date:_____

Researcher/Witness to complete:

I have described the nature of the research to _____
(print name of participant)

and in my opinion she/he understood the explanation.

Signature: _____ Position: _____ Date:_____

Date:July 8, 2014

Dr. Shaitsa Khan,
Chairperson,
Ethical Review Committee

Re: 3074-CHS-ERC-14
A Multicentre Case Control Study of Vitamin D and Breast Cancer Risk Among Women in Karachi Pakistan

RESPONSE TO ERC RECOMMENDATIONS:

S. No	ERC recommendations:	Investigator' Responses:
1.	Why does PI want objective no. 4?	In this collaborative multidisciplinary research project across disciplines of oncology, surgery, epidemiology, public health, endocrinology and medicine in AKUH and UofA, we want to cover objective no. 4 of immense public health importance; In Pakistan, women typically present with an advanced stage of breast cancer (BC) and this is related to the high rates of mortality. These delays by patients are clearly complex and multifactorial. This study provides us with a significant opportunity to determine the social and cultural reasons why women delay their presentation and these issues may play a role with regard to the specific BC risk factors such as undertaking activity, diet and sunlight exposure. Research on this topic is important for clinicians to have a better understanding of how to manage patients and for policy makers to implement strategies and activities to prevent delay in breast cancer diagnosis. Overall, the evidence is currently insufficient to inform the development of specific strategies to shorten delay by patients. An understanding of the factors influencing delays by patients is therefore important for policymakers so that strategies to shorten delays can be developed and encourage earlier presentation when the first indications of breast cancer are discovered. In this objective, we will evaluate how Pakistani women present with breast cancer, the frequency and magnitude of delay in diagnosis, the factors associated with delays, and the relationship between delays and disease stage and subtype of breast cancer.
2.	Selection procedure needs to be delineated example how will patients and controls be	Done For this purpose the oncologists/surgeons of the hospitals will be informed about the starting date of data collection in their

	approached? Whose clinics?	respective hospitals. Preliminary discussions with them have ensured their keen interest and support for the study. Following a confirmed diagnosis of BC, cases of BC will be identified by trained research assistants at a subsequent hospital visit. Those patients, who meet the inclusion criteria, will be approached by research assistants during their waiting in the Oncology and surgery clinics of the three major public and private hospitals. The control women for the study will be recruited from those attending in and outpatient services for general medical, antenatal, gynaecologic (noncancerous), maternity, and surgical departments of the three participating hospitals of Karachi. These control subjects will be women with no previous diagnosis of any cancer and informed consent will be obtained if they meet the eligibility criteria. All consenting participants will be similarly interviewed by the trained interviewers in a separate room to ensure privacy using the pretested questionnaire.
3.	Will the consultants whose patients are used as control would know?	Yes, after ethical approval, all the consultants will be informed about the starting dates of data collection in all clinics of JPMC, KIRAN, and AKUH.
4	There should be a separate informed consent form for cases and controls. Consent form should mention the process and place of blood drawing for vitamin D levels.	Done
5	If the patients are expected to come to AKU, for blood tests then they should be compensated for travel especially the non AKU subjects.	After the interview, venous blood samples (10ml) will be collected in gel tubes by trained phlebotomists from breast cancer patients within 30 days of diagnosis of cancer and prior to chemotherapy; and from controls at the end of the interview. The collected blood will be transported in icepacks to Aga Khan University Hospital (AKUH), Department of Pathology and Microbiology for analysis. Participants are informed of this on the consent form and will not be expected to travel to AKU or go to the AKU lab for blood tests. The cost of testing serum 25 OHD will be covered by the study funds, which have been applied for through the University of Adelaide. A system will be developed whereby the participants will get this test done free of cost while the study funds will be directly paid to the lab for the test.
6	English questionnaire missing.	Attached
7	The proposal seems like a draft at some places test is highlighted.	All highlighted text has been removed unless it is a specific response to the ERC questions

8	Permission letter from KIRAN should be submitted.	Attached

Name of responder / PI : Dr. Uzma Shamsi

Department: CHS AKUH

A Multicentre Case Control Study of Vitamin D and Breast Cancer Risk among Pakistani women

ERC Ref No----------

Consent form(cases)

My name is _____. We are conducting a research study to evaluate the role of Vitamin D related factors associated with breast cancer, among women of 20-75 years age group in Karachi. Since you are in this age group and diagnosed with breast cancer, we would like to ask you questions related to your sun exposure, diet, supplementation intake and other related information.

By participating in this survey you will not get direct benefit but the information obtained from you will help us to develop a better understanding of the risk factors associated with BC in Karachi Pakistan. We want to assure you that all the information obtained from you will be kept confidential. There is no chance of any harm to you by your participation in this study. If you don't like to answer any question you can.

No compensation/remuneration for your participation will be offered and your participation is wholly voluntary. Even after signing the consent form you can refuse to participate in the study. Even if you start the study, you can still withdraw at any time without any penalty. This questionnaire will take approximately 35-45 minutes of your time. This information sheet provides you with information about breast cancer and this study. At the end of the interview, our trained phlebotomist will collect 10 cc of your blood for vitamin D level testing. The collected blood will be transported in icepacks to Department of Pathology and Microbiology,AKUH, and tested for analysis, free of cost. You will not be required to travel to AKUH or go to the lab.

If you have read and understood this consent form, and volunteer to participate in this research study then please sign below or give verbal consent.

Participant Name and Signature:

Signature of Person Obtaining Consent:
Date:

Principal Investigator:

DR. UZMA SHAMSI , *CHS*

*E mail:*uzma.shamsi@aku.edu

Phone.

1st edited by	**Date**
Comments	
2nd edited by	**Date**
Comments	

صحت کے مختلف مراکز پر پاکستانی خواتین میں وٹامن ڈی اور چھاتی کے سرطان کے حوالے سے ایک کیس کنٹرول مطالعہ۔

ای آر سی حوالہ نمبر----------

رضامندی فارم

میرا نام---------- ہے۔ ہم کراچی میں 20-75 سال عمر کے گروپ کی خواتین میں چھاتی کے سرطان کے ساتھ منسلک وٹامن ڈی سے متعلق عوامل کے کردار کا اندازہ کرنے کے لئے ایک تحقیقی مطالعہ کر رہے ہیں۔ چونکہ، آپ اس عمر کے افراد کے زمرہ میں آتی ہیں اور آپ کو چھاتی کے سرطان کی تشخیص بھی ہوئی ہے، اسلئے ہم آپ سے آپ کی غذا، یا اضافی خوراک کا استعمال اور سورج کی روشنی سے متاثر ہونا/دھوپ میں وقت گذارنے اور دیگر متعلقہ معلومات کے متعلق کچھ سوالات پوچھیں گے۔

اس تحقیق میں شرکت کرنے پر آپ کو براہ راست کوئی فائدہ حاصل نہیں ہوگا، لیکن آپ سی لی گئی معلومات کراچی میں کی جانے والی اس تحقیق کے حوالے سے سرطان کے ساتھ منسلک خطرے کو عوامل کو بہتر طور پر سمجھنے میں ہمیں مدد فراہم کرے گی۔ ہم آپ کو یقین دلانا چاہتے ہیں کہ آپ سے لی گئی معلومات کو خفیہ رکھا جائیگا۔ اس تحقیق میں شرکت کرنے سے کسی بھی قسم کے نقصان کا کوئی امکان موجود نہیں ہے۔ اگر آپ کسی سوال کا جواب نا بھی دینا چاہیں تو یہ آپ کی مرضی پر منحصر ہوگا۔

آپ کی شرکت کے لئے کوئی معاوضہ/اُجرت کی پیشکش نہیں کی جائیگی اور آپ کی شرکت مکمل طور پر رضاکارانہ ہے۔ حتیٰ کہ رضامندی فارم پر دستخط کرنے کہ بعد بھی آپ کو یہ اختیار حاصل ہے کہ آپ تحقیق میں شامل ہونے سے انکار کر سکتی ہیں۔ تحقیق کی شروعات میں شامل ہونے کے بعد کسی بھی وقت بغیر کسی جرمانے ادا کئے آپ تحقیق سے دستبردار ہو سکتی ہیں۔ اس سوالنامے کو پورا کرنے کے لئے ہمیں آپ کے کم از کم 35-45 منٹس چاہیے۔ یہ معلوماتی پرچہ آپ کو اس تحقیق اور چھاتی کے سرطان سے متعلق معلومات فراہم کرتا ہے۔ انٹرویو کی آخر میں ہمارا تربیت یافتہ فلبا ٹمسٹ وٹامن ڈی کی سطح جانچنے کے لئے آپ کے خون کا 10 سی سی سیمپل لیگا، جمع شدہ خون برف سے بھرے پیکس میں ڈال کر پیتھالوجی اور مائکروبیالوجی کے ڈپارٹمنٹ ای کے یو میں منتقل کیا جائیگا۔ اور تجزیہ کی خاطر ٹیسٹ .مفت میں کیا جائیگا۔ آپ کو آغا خان میں آنے یا لیب میں جانے کے لئے آنا نہیں پڑیگا۔
اگر آپ نے اس رضامندی فارم کو پڑھ اور سمجھ لیا ہے اور اس تحقیق میں رضاکارانہ طور پر شامل ہونا چاہتے ہیں تو براہ مہربانی ذیل میں اپنی دستخط کریں یا زبانی رضامندی دیدیں۔

شرکت کرنے والے کا نام اور دستخط

رضامندی حاصل کرنے والے کا نام اور دستخط

تاریخ

پہلی درستگی	تاریخ
رائے	
دوسری درستگی	تاریخ
رائے	

محقق اعلی: ڈاکٹر عظمیٰ شمسی

کمیونٹی ہیلتھ سائنسز

ای میل: uzma.shamsi@aku.edu

فون نمبر

A Multicentre Case Control Study of Vitamin D and Breast Cancer Risk among Pakistani women

ERC Ref No----------

Consent form

My name is _____. We are conducting a research study to evaluate the role of Vitamin D related factors associated with breast cancer, among women of 20-75 years age group in Karachi. Since you are of the same age group and free of cancer, we are inviting you to be a cancer-free control subject in the study. We would like to ask you questions related to your sun exposure, diet, supplementation intake and other related information.

By participating in this survey you will not get direct benefit but the information obtained from you will help us to develop a better understanding of the risk factors associated with BC in Karachi Pakistan. We want to assure you that all the information obtained from you will be kept confidential. There is no chance of any harm to you by your participation in this study. If you don't like to answer any question you can.

No compensation/remuneration for your participation will be offered and your participation is wholly voluntary. Even after signing the consent form you can refuse to participate in the study. Even if you start the study, you can still withdraw at any time without any penalty. This questionnaire will take approximately 35-45 minutes of your time. This information sheet provides you with information about breast cancer and this study. . At the end of the interview, our trained phlebotomist will collect 10 cc of your blood for vitamin D level testing. The collected blood will be transported in icepacks to Department of Pathology and Microbiology, AKUH, and tested for analysis, free of cost. You will not be required to travel to AKUH or go to the lab.

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Signature of Person Obtaining Consent:
Date:

Principal Investigator:

DR. UZMA SHAMSI , *CHS*

*E mail:*uzma.shamsi@aku.edu

1st edited by	**Date**
Comments	
2nd edited by	**Date**
Comments	

صحت کے مختلف مراکز پر پاکستانی خواتین میں وٹامن ڈی اور چھاتی کے سرطان کے حوالے سے ایک کیس کنٹرول مطالعہ۔

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اگر آپ نے اس رضامندی فارم کو پڑھ اور سمجھ لیا ہے اور اس تحقیق میں رضاکارانہ طور پر شامل ہونا چاہتے ہیں تو براہ مہربانی ذیل میں اپنی دستخط کریں یا زبانی رضامندی دیدیں۔

شرکت کرنے والے کا نام اور دستخط

رضامندی حاصل کرنے والے کا نام اور دستخط

تاریخ

محقق اعلی: ڈاکٹر عظمیٰ شمسی

کمیونٹی ہیلتھ سائنسز

ای میل:uzma.shamsi@aku.edu

فون نمبر:

پہلی درستگی	تاریخ
رائے	
دوسری درستگی	تاریخ
رائے	

PARTICIPANT INFORMATION SHEET

A Multicenter Case Control Study of Vitamin D and Breast Cancer Risk among Pakistani Women

PRINCIPAL INVESTIGATOR: Prof David Callen

STUDENT RESEARCHER: Dr Uzma Shamsi

STUDENT'S DEGREE: MBBS, MSc Epi/Bio, PhD candidate Medicine

Dear Participant,

You are invited to participate in the research project described below.

The aim of the research study is to evaluate in women from three Hospitals of Karachi, the role of vitamin D related lifestyle factors on concentrations of vitamin D, and determine any association with breast cancer. There have been studies suggesting that low concentrations of vitamin D are a risk factor for breast cancer. We wish to determine if low concentrations of vitamin D contribute to the high frequency of breast cancer in Pakistani women.

This research is being conducted by Dr Uzma Shamsi at the University of Adelaide. It will form the basis for her PhD degree under the supervision of Professor David Callen and Dr Tiffany Gill. You are invited to be part of this study because you are 20-75 years of age free of breast cancer and visiting one of the three hospitals of Karachi. The study design of this research project is a case control study in which we choose cases with breast cancer and a comparison group without breast cancer (controls) and then measure their past exposure to certain risk factors of breast cancer like vitamin D related lifestyle factors.

You will be excluded from this study if you have past history of any cancer.

We will ask you questions about your reproductive, medical, drug & smoking history. There will also be questions related to your lifestyle including your diet, sun exposure and physical activity, as all of these factors may be relevant to your vitamin D concentrations and the development of breast cancer. The interview will take approximately 40-45 mins. It is your choice if you do not wish to answer any particular questions.

Following the interview, you will be asked to give a 10 ml blood sample to determine vitamin D concentrations. There is no cost for this blood test. The blood sample will be taken from a vein in your arm by a specially trained staff laboratory technician. This may cause some temporary discomfort and sometimes there can be some local bruising following completion of the blood test. It is entirely up to you whether you agree to give the sample and your medical care will not be influenced by your decision. The collected blood will be transported on icepacks to Department of Pathology and Microbiology, AKUH, and vitamin D concentrations determined. You will not be required to travel to the laboratory at AKUH.

By participating in this study you will not get direct benefit but the information obtained from you will help us to develop a better understanding of the risk factors associated with breast cancer in Karachi. We want to assure you that all the information obtained from you will be kept confidential. No compensation/remuneration for your participation will be offered and your participation is wholly voluntary. Even after signing the consent form you can refuse to participate in the study. Even if you start the study, you can still withdraw at any time and this will not affect any of the health services you receive.
The study has been approved by the Human Research Ethics Committee at the University of Adelaide (approval number H-2014-xxx). If you have questions or problems associated with the practical aspects of your participation in the project, or wish to raise a concern or complaint about the project, then you should consult the Principal Investigator. Contact the Human Research Ethics Committee's Secretariat on phone +61 8 8313 6028 or by email to hrec@adelaide.edu.au if you wish to speak with an independent person regarding concerns or a complaint, the University's policy on research involving human participants, or your rights as a participant. Any complaint or concern will be treated in confidence and fully investigated. You will be informed of the outcome.

If you have read and understood this form, and volunteer to participate in this research study then please sign the consent form or give verbal consent or a thumb impression.

Yours sincerely

Prof David Callen

Phone no.

david.callen@adelaide.edu.au

Dr Uzma Shamsi

Phone no.

uzma.shamsi@adelaide.edu.au

Appendix D: E poster presentation

Three minutes' oral presentation '' **Patient delay in breast cancer diagnosis, its associated factors and stage of breast cancer at first presentation among Pakistani women''** in WCC 2018 at e-poster abstract hub convention center, Kuala Lampur, Malaysia (chapter 7)

World Cancer Congress
Kuala Lumpur, Malaysia
1—4 Oct 2018

Strengthen
Inspire
Deliver

Kuala Lumpur, 9 October 2018

CERTIFICATE OF ATTENDANCE

This is to certify that:

Uzma Shamsi

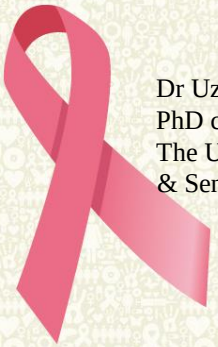
attended the 2018 World Cancer Congress held in Kuala Lumpur, Malaysia from 1 – 4 October 2018.

Sincerely,

Dr. Cary Adams
Chief Executive Officer, UICC

Professor Sanchia Aranda
President of UICC for the
World Cancer Congress

Patient delay in breast cancer diagnosis, its associated factors and stage of breast cancer at first presentation among Pakistani women
Presentation Title



Dr Uzma Shamsi
PhD candidate, School of Medicine,
The University of Adelaide, Australia
& Senior Instructor, Aga Khan University, Pakistan

World Cancer Congress
Kuala Lumpur, Malaysia
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Strengthen
Inspire
Deliver
THE AGA KHAN UNIVERSITY

Abstract code A-969-0001-00151

Track # 10 - Epidemiology

Disclosure of Interest: None declared

Annexure 1 Chapter 6 AHEI 2010 and breast cancer

Table1. Modified alternate healthy eating index-2010 (modified AHEI Scoring)

Component(serving/ day)	Criteria for minimum score of 0**	Criteria for maximum score of 10**	
Vegetables	**0**	**5**	**All vegetables on the FFQ were included, 5 servings of vegetables/Day was given highest score**
Fruit	**0**	**4**	**Fruit consumption of 4 servings/day was considered as ideal**
Grains	**0**	**5**	**Total grains consumption of 5 servings/day was considered as ideal**
Dairy products*	**0**	**2**	**Dairy consumption of 2servings/day was considered as ideal.**
Ratio of white to red meat**	**0**	**4**	**both red and white meat servings /day were used for calculating the ratio of white vs red meat. An**

			ideal score of 10 was given for ratio ≥4:1
non meat protein* **	**0**	**2**	**Nuts and vegetable protein (e.g., pulses, beans, nuts) of 1 serving/day was considered ideal.**

*Includes all milk products, such as fluid milk, milk shake, yogurt and cheese,
**White meat was defined as poultry or fish, whereas red meat was defined as beef, lamb, or mutton
***Includes daal, cholay, dry fruits, and a serving of 2 was considered ideal

There are limited major sources of vitamin D in the diet. The following individual foods rich in vitamin D were individually asked about: milk, fish (fatty or white fish), any fortified cereals, breads or other products. Questions regarding amount and frequency of intake of milk, dairy products, eggs, organ meats such as liver, other meats (beef, chicken, salmon, tuna, fish, and seafood), fruit, and vegetables were also asked. Individual food intakes were calculated as servings per day, week, or month (depending on the frequency of consumption).

Annexure 2 (Chapter 7)

Figure Reasons of delay among breast cancer cases in case control study of Vitamin D and breast cancer among Pakistani women

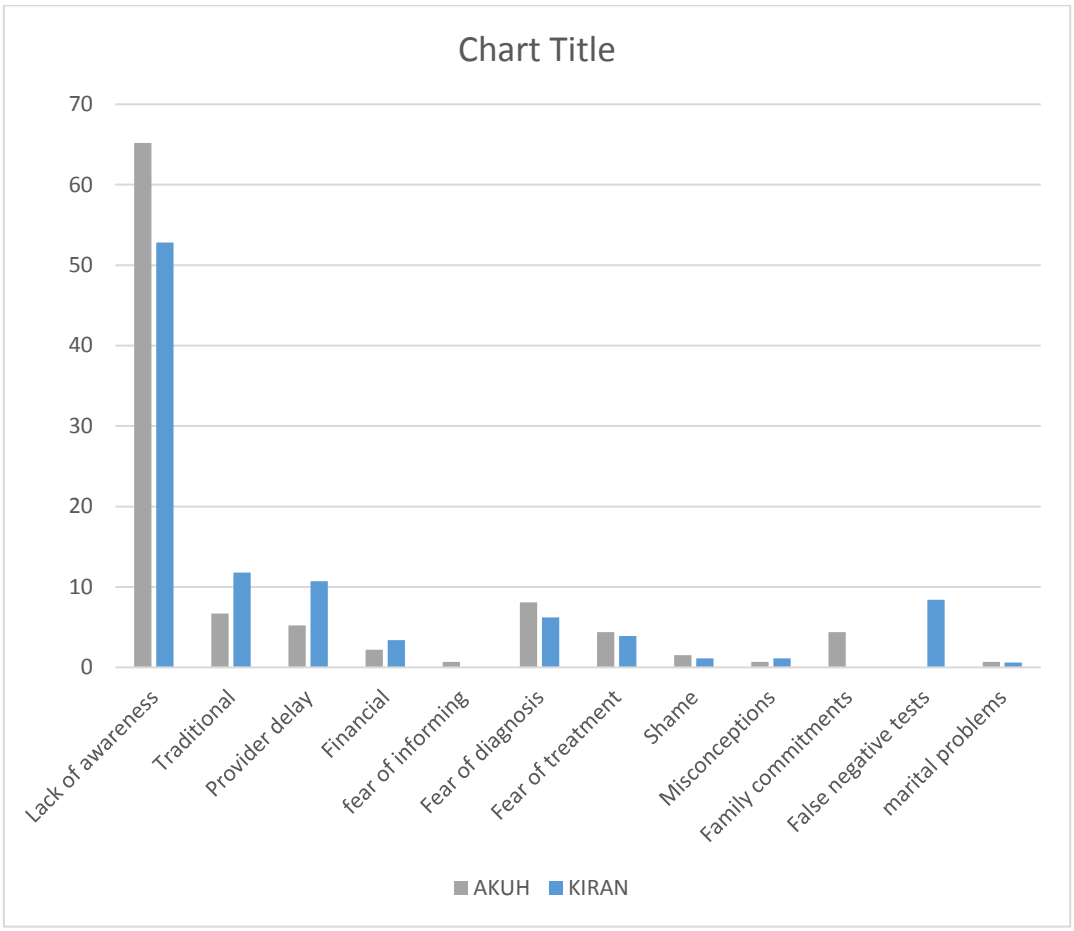
Chart Title
70
60
50
40
30
20
10
0
Lack of awareness
Traditional
Provider delay
Financial
fear of informing
Fear of diagnosis
Fear of treatment
Shame
Misconceptions
Family commitments
False negative tests
marital problems
AKUH
KIRAN