ABSTRACT

The relationship between saturated fatty acids (SFAs) and bladder cancer (BC) risk has been conflicting. Our aim was to investigate the relationship between erythrocyte membrane SFAs and BC risk. Total 404 participants were enrolled in the study (including 112 cases and 292 controls). The validated food frequency questionnaire (FFQ) was utilized to assess the food intake. The constitutive composition of fatty acids in erythrocyte membrane was measured by gas chromatography (GC). After adjustment for BC risk factors, SFAs had no significant association with BC risk. However, C18:0 was positively linked with BC risk with an OR (95% CI) of 2.99 (1.37 ~6.53). In contrast, very long chain saturated fatty acids (VLCSFAs), especially C24:0, were negatively related with BC risk [OR (95% CIs): 0.28 (0.12, 0.65) for VLCSFAs and 0.33 (0.15, 0.75) for C24:0]. Higher total odd-SFAs, C15:0 and C17:0 were associated with a lower risk of BC [OR (95% CIs): 0.18 (0.076 ~0.44), 0.18 (0.068 ~0.47), 0.34 (0.14 ~0.81), respectively]. After subgroup analysis, the protective effects C15:0 and C17:0 were still remained. ROC analysis displayed that the combination of C15:0 and C17:0 indexes increased the accurate predictive rate of BC risk. Further mediation effect analysis showed that C15:0 and C17:0 could be used as partial mediation effectors for milk and dairy products and bladder carcinogenesis. In conclusion, the combination of odd-SFAs (C15:0 and C17:0) in the erythrocyte membrane could serve as a reliable mediator and predictor, indicating a relationship between high intake of milk and dairy products and a lower risk of BC.

Key Words: Saturated fatty acids, Bladder cancer, Erythrocyte membrane, Milk & dairy products

INTRODUCTION

Bladder cancer (BC) was a frequently encountered malignancy of the urinary tract, predominantly observed in males and the elderly (Antoni, Ferlay, Soerjomataram, Znaor, Jemal, & Bray, 2017). Apart from the recognized risk factors such as smoking, occupational exposure, and arsenic contamination (Lenis, Lec, Chamie, & Mshs, 2020; Witlox et al., 2020), diet has been deemed as a considerable factor in the progression of BC (Cumberbatch et al., 2018). Consumption of fresh fruits, vegetables and dairy products might present protection against BC (Edefonti et al., 2020). Nevertheless, epidemiologic evidence indicated a conflicting relationship between dietary fat and BC risk (Brinkman, Karagas, Zens, Schned, Reulen, & Zeegers, 2011; Steinmaus, Nunez, & Smith, 2000). The low fat intake might contribute to the prevention of breast cancer; however, the specific dietary components that are responsible for reducing the risks are still unknown (Brinkman, Karagas, Zens, Schned, Reulen, & Zeegers, 2011). The consumption of olive oil derived from vegetable fat was found to have a protective effect against the occurrence of BC (Steinmaus, Nunez, & Smith, 2000). The evidence regarding the role of SFAs in urothelial carcinoma has been controversial (Corn, Windham, & Rafat, 2020; Dianatinasab et al., 2022; Schulpen & van den Brandt, 2019). The majority of the SFAs-related
findings were typically measured through participant-reported dietary assessments or food frequency questionnaires (FFQs). Measurement errors were unavoidable due to the memory bias of data, subjective presence, and inaccurate assessment of dietary intake (Aglago et al., 2021). The measurement of blood SFAs levels enabled a more accurate evaluation of an individual's SFAs intake to be made. SFAs in erythrocytes exhibited longer half-lives and were characterized by objectivity, stability, and reproducibility, compared with those in serum and plasma (Sun et al., 2007; Yakoob et al., 2016; Lankinen et al., 2018). Therefore, the composition of SFAs within the erythrocyte membrane was considered to be a long-term dietary intake of lipids (Arab et al., 2002; Sun et al., 2007) and was more suitable for dietary attainment assessment.

On the other hand, SFAs with different chain lengths exhibited different metabolic and biological processes. The correlation between SFAs and the occurrence of cancer might depend on their specific types. Even-chain fatty acids (even-SFAs) (myristic, palmitic, and stearic acids) increased the risk of developing the cancer, whereas odd-chain fatty acids (odd-SFAs) decrease the risk (Nagao et al., 2000). Thus, the impact of SFAs on BC or their association was not possible to evaluate in a universal manner.

Based on our previous findings, milk and dairy products might serve as a protective factor in the development of BC (Teng et al., 2022). However, the underlined association had yet to be elucidated. This study aims to evaluate the possibilities of SFAs as the mediator and predictor for BC risk, and provide the dietary guideline for the people with high risk of BC, e.g., the elderly, smokers, etc.

**METHODS**

**Study Population**

This case-control study was conducted from October 2018 to December 2019. BC patients were recruited from the First Affiliated Hospital of Harbin Medical University, the Second Affiliated Hospital of Harbin Medical University, and Harbin Medical University Cancer Hospital, which ranked as the highest grades of hospitals in China–Grade A tertiary Hospitals. The inclusion criteria for this study comprise newly diagnosed primary urothelium cancer via either imaging, cytology, or pathology, with no prior chemotherapy or radiotherapy and no diagnosis of any other cancer. Patients between ages 25 - 80 (±5) years were eligible for participation.

The study protocol was approved by the Ethics Committee of Harbin Medical University and conducted according to Declaration of Helsinki. All participants were instructed under the condition of fully understanding the purpose and protocol of this trial. Informed consent was signed and obtained from all subjects before participation.

**Sample Size Calculation**

The sample size calculation was based on the previous study (Demidenko, 2007; Zupo et al., 2021). First, the test level was established (\(\alpha\)) and verified effectiveness (\(1 - \beta\)). Then OR value (2.5) of expected factors and the risk factor exposure rate (\(p_0 = 80\%\)) between the case and control group were determined based on previous studies (Steinmaus et al., 2000). The calculating formula was following (Demidenko, 2007; Zupo et al., 2021):

\[
P_1 = \frac{OR \times P_0}{1 - P_0 + OR \times P_0} ;
\]

\[
\bar{P} = \left( P_1 + P_0 \right) / 2 ;
\]

\[
n = \frac{(Z_{1-\alpha/2} \sqrt{2\bar{P}(1-\bar{P})} + Z_\beta \sqrt{P_1(1-P_1) + P_0(1-P_0)})^2}{(P_1-P_0)^2} .
\]

Accordingly, the number in case group should be up to 100. While considering the possible missing rate (10%), the number was reached to 110. On the basis of statistical rules for case-control study, the statistical power would be enhanced via increasing the number of controls (the biggest ratio of case to control was 1:4) under the condition of smaller size of cases (Fleiss, Levin, & Paik, 2013). Therefore, we tried our best to enroll more participants as control. The ratio of case to control was finally near to 1:3 in this study.

**Enrollment of Population According to Criteria**

A total of 130 BC patients which matching the inclusion criteria were identified. While 5 patients refused to participate, and 125 patients were interviewed and biological samples were collected. Due to the complete basic information (\(n = 10\)) and the insufficient samples (\(n = 3\)), finally 112 qualified patients were enrolled as cases. Controls (\(n = 320\)) without history of cancer were recruited from the large residential communities through advertisements, flyers, written invitations or referrals, and 310 participants agreed to participate. The refusal rate of the control group was 3.1%. The
Age of population in the control group (±5 years) was matched with BC patients. Subjects were excluded if they had heart failure, chronic bronchitis, chronic nephritis, hyperthyroidism and aplastic anemia. All participants had clear awareness and expressive ability. Taken together, 292 controls were qualified, yielding a participation rate of 91% (Figure 1).

**Basic Information and Dietary Questionnaire Collection at the Baseline**

All investigators in this trial were received unified and professional training in advance. Under the guidance of the investigators, all participants were conducted a 30-min face-to-face interview to collect demographic characteristics (including sex, age, BMI, occupation and education level), lifestyle factors (including smoking, alcohol, physical activity, energy and fat) at baseline using a structured questionnaire. Weight and height were measured by the trained nurses and then BMI (kg/m²) was calculated. The major 8 types of foods (red meat, poultry, sea fish, freshwater fish, eggs, soybeans and their products, milk and dairy products, and soybean oil) were collected through the qualified FFQ, which includes vegetables (26 items), aquatic products (4 items), fruits (12 items), grains (4 items), potatoes (1 items), eggs (1 items), milk (2 items), beans (2 items), nuts (1 items), livestock and poultry meat (8 items), beverages (3 items), and snacks (5 items). The final number of food items was 69 subcategories. The food frequency range was set into 7 items: 0 (no consume); 1 (1–3 times/month); 2 (once per week); 3 (2–3 times/week); 4 (4–5 times/week); 5 (1 time/day); 6 (2 times/day). All food varieties are classified using the above frequency range, and the corresponding frequency weights are set to 0, 0.07, 0.14, 0.36, 0.64, 1.00 and 2.00. The quantities of foods were evaluated by multiplying the weight of each food with the consumption frequency obtained from FFQ, and the contents of energy (KJ/d) and fat (g/d) in each food were calculated by the professional nutrition calculation software (Feihua Nutrition Calculator).

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**Figure 1.** Flowchart of the study population.
Quality Control

The quality of FFQ had been enhanced in several ways. First, reliability and validity of FFQ had been tested as before (Teng et al., 2022). The correlation between annual FFQ and 24h dietary recall was analyzed to test the validity of FFQ. Second, “Dietary Food Estimation Tool Atlas” with the size, weight and food photos, invented by Prof. Wang from Nanjing Medical University, and publicly recognized in China, was applied to help semi-quantify the food intake from FFQ. Third, to minimize memory bias in older adults, we allowed family companionship to assist answering FFQ. Finally, all questionnaires were input by the 2-person simultaneous entry method, and confirmed only after there was no difference.

The clinical information and sample collection had been thoroughly described in a previous publication (Teng et al., 2022). Initially, we chose study cases from 3 general tertiary hospitals. Subsequently, sample collection and questionnaire information were carried out on the day following patient admission to minimize potential survey bias. Trained investigators, who had received uniform and rigorous training, worked in pairs to enter all data.

Biological Sample Collection and Fatty Acids Profiles Determination

All participants were asked to fast overnight up to 8 h before preceding the blood draw. At 8:00 - 10:00 the next morning, 5 mL of anticoagulant venous blood was collected and immediately stored at 4°C. Then within 8 h, blood was centrifuged at 3000 rpm for 15 min to obtain serum samples (upper), and erythrocytes pellet (lower), and aliquots of each were stored at −80°C for later use.

Total fatty acids (FAs) composition in erythrocyte membrane was analyzed by gas chromatography (GC) according to the previous publication (Pala et al., 2001). First, FAs methylation products were prepared. In brief, 20 μg of the internal standard was added to 500 μL of red blood cells, and then mixed with twice volume of the chloroform-methanol mixture, followed by the full shocking and centrifugation. Concentrated sulfuric acid methanol mixture was added and heated at 80°C. After cooling, water and hexyl hydride were added to centrifuge for 10 min at 2000 rpm. Then the top clear layer was transferred to a new glass tube and dried under a stream of N2. The FAs methyl ester composition of erythrocyte membrane was analyzed and separated by high-resolution capillary gas chromatography (SP2560 L, 100 m × 0.25 mm × 0.20 μm, Sigma) and FID detector.

The chromatographic column temperature was kept at an initial temperature of 90°C, heated at 3°C/min, and terminated at 240°C for 20 min until completion of the analysis. Helium was used as a carrier, and the injection port was maintained at 300°C. The gas chromatographic peaks were analyzed soon afterward. The relative amounts of FAs were expressed as the percentage of peak area. All of the analyses were performed by the trained staff who were not aware of the status (case or control) of the samples. Matched samples from cases and controls were processed and assayed in the same batch. Erythrocyte membrane samples were analyzed in random order. Quality control samples were processed in the same way as test samples to ensure repeatability. The coefficient of variation (CV) was also calculated. The results of intra- and inter-assay were 95.2% and 94.98%, respectively.

Mediational Analysis

To evaluate the mediating effects of C15:0 and C17:0 in the association between milk and dairy products and BC, separate mediation analyses were conducted. First, bivariate correlation coefficients were calculated to test the relationship between milk and dairy products, C15:0, C17:0 and BC. In the regression analysis, we tested the variables for multicollinearity. the VIF values were all less than 5, consistent with further research. Four models were made in the hypothetical model (see Figure 2). Milk & dairy products and BC were negatively correlated with each other (Figure 2A). C15:0 mediated the relationship between milk and dairy products and BC (Figure 2B). C17:0 mediated the relationship between milk and dairy products and BC (Figure 2C). C15:0 and C17:0 showed a chain-mediated role in the relationship (Figure 2D). For the estimation process, we utilized the SPSS macro-PROCESS, specifically selecting models 4 and running 1000 bias-corrected bootstrap samples (Figure 2B&C) and selecting models 6 and running 5000 bias-corrected bootstrap samples (Figure 2D) (Preacher & Hayes, 2008).

The indirect effect estimates and confidence intervals (95%) were calculated. The presence of mediating effects was determined by estimating the significance of the predictor β coefficients. Estimates were considered significant if their confidence intervals did not include zero. Age, gender, BMI, occupation, education, physical activity, diabetes, and total energy intake were included as covariates in the mediation models.

Statistical Analysis

The distribution of individual SFAs in erythrocyte membrane was analyzed and expressed as percentages.
Percentages of FAs were considered as continuous variables. Normal distribution data were verified using a Shapiro-Wilk test. Data were expressed as mean ± SD and assessed by t-test for continuous variables. Categorical variables including demography and lifestyle characteristics were analyzed by χ²-test. The data was standardized and principal component analysis (PCA) were conducted by R Studio software. Conditional logistic regression model was established to calculated the odds ratio (OR) and 95% confidence interval (CI) for evaluating the association between individual SFA and BC risk. Restricted quadratic splines with 3 nodes (0.1, 0.5 and 0.9) was used to access potential non-linear associations (Steyerberg, Eijkemans, Harrell, & Habbema, 2001). Correlations between individual and grouped SFAs as well as SFAs with dietary factors were evaluated by Spearman correlation coefficients.

To further efficiently confirm the associations between erythrocyte membrane SFAs and the risk of BC, the percentage of SFAs were divided into 4 equal parts according to the quartile interval in the all groups. Taking the lowest interval as a reference, the binary logistic regression model was used to obtain the OR and the corresponding 95% CI after the multivariate adjustment. Potential confounding variables derived from other nondietary factors were considered by conducting univariate analyses. The variable independently associated with BC risk was then added separately to the main model. Following models with adjusting the potential confounding factors were constructed. Model 1 was adjusted for age, gender (male or female), and BMI. Model 2 was adjusted for occupation and degree of education on the basis of model 1. Model 3 was adjusted for lifestyle factors including smoking status, alcohol consumption, physical activities, energy (KJ/d) and diabetes to estimate the OR and 95% CI on the basis of model 2.

Receiver-operating characteristic curves (ROC) was used to evaluate the diagnostic accuracy of erythrocyte membrane SFAs and the risk of BC. The area under curves (AUC) of ROC was calculated in the basic factors and the model with each SFA added (as a continuous variable). Finally, a forest map was drawn to exhibit the association between SFAs and the risk of BC after stratification of demography (age, gender) and lifestyle exposure factors (smoking, drinking) using

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**Figure 2.** The mediation model for the relationship between milk and dairy products and BC. (A) Relationship between milk & dairy products and bladder cancer. (B) C15:0 as mediating variables. (C) C17:0 as mediating variables. (D) The chain mediating effect of milk & dairy products and BC. Note: A: The coefficient c was the total effect of the independent variable milk & dairy products on the dependent variable BC. B: The coefficient a1 was the effect of the independent variable milk & dairy products on the mediator variable C15:0. The coefficient b1 was the effect of the mediating variable C15:0 on the dependent variable BC. The coefficient c1 represented the direct effect of the independent variable milk & dairy products on the dependent variable BC, after controlling for the influence of the mediator variable C15:0. C: The coefficient a2 was the effect of the independent variable milk & dairy products on the mediator variable C17:0. The coefficient b2 was the effect of the mediating variable C17:0 on the dependent variable BC. The coefficient c2 represented the direct effect of the independent variable milk & dairy products on the dependent variable BC, after controlling for the influence of the mediator variable C17:0. D: The coefficient c' was the direct effect of the independent variable milk & dairy products on the dependent variable BC. The coefficient a was the effect of the independent variable milk & dairy products on the mediator variable C15:0. The coefficient b was the effect of the mediating variable C15:0 on the dependent variable BC. The coefficient b' was the effect of the mediating variable C17:0 on the dependent variable BC.
GraphPad Prism 9 software. All statistical tests were 2-sided, and \( P < 0.05 \) was considered statistically significant, and we adjusted the \( p \) value with Bonferroni. Statistical analyses were performed using SPSS 23.0 software and Stata.

**RESULTS**

**General Characteristics of Study Participants**

As shown in table 1, the patients over 60 years old accounted for 71.4%. The number of male patients was more than twice of females which was consistent with the characteristics of gender difference in BC. Obvious differences in the education degree and the occupation were also observed. BC patients consumed more energy and fats, with higher percentage of smoking and drinking. The proportion of BC patients suffering from diabetes was significantly higher than that of control people \( (P < 0.001) \).

**Unbalancing Distributions and Levels of Erythrocyte Membrane Saturated Fatty Acids**

As shown in supplementary Figure 1, there were different distribution trends of SFAs between 2 groups (Supplementary Figure 1A). Further PCAs analysis showed that the main differences were observed in odd-SFAs, C15:0, C17:0, VLCSFAs, C22:0 and C24:0 (Supplementary Figure 1B). Nine kinds of SFAs including 3 kinds of even-SFAs (C14:0, C16:0 and C18:0), 2 odd-SFAs (C15:0 and C17:0), as well as VLCSFAs (C20:0, C22:0, C23:0, C24:0) was further analyzed (Figure 3). No significance was observed in SFAs and even-SFAs between controls and cases (Figure 3A&B). While BC patients had higher levels of C14:0 and C18:0 (Figure...
3E&H), and lower levels of total odd-SFAs (Figure 3C), C15:0 (Figure 3F), C17:0 (Figure 3G), VLCSFAs (Figure 3D), C20:0 (Figure 3I) and C24:0 (Figure 3J) (P < 0.05). There was no significance in C16:0, C22:0 and C23:0 (Supplementary Figure 2A&B&C).

**Erythrocyte Membrane Saturated Fatty Acids Association Analysis**

To better visualize the changes in SFAs profiles in BC patients, *spearman* correlation analysis was performed. A heat map between SFAs was drawn in this study (Figure 4). Different SFAs in erythrocyte membrane were moderately correlated with each other. Compared with the control group (Figure 4A), C14:0, C20:0, C22:0 and C24:0 in the case group (Figure 4B) had already

![Figure 3](image-url)
showed more blue sector area, while the C18:0 and C22:0 showed smaller red sector area, indicating that the correlation between even-SFAs and others in the BC patient group became significantly stronger, while the odd-SFAs became less correlated with each group. The correlation of odd-SFAs with the individual SFA was essentially unchanged. In the meanwhile, there was no obvious significance between VLCSFAs and other SFAs.

Assessment of Bladder Cancer Risk by Erythrocyte Membrane Saturated Fatty Acids

To explore the relationship of SFAs and BC, quartile of proportions of SFAs was compared and OR value with 95% CI was shown in Figure 5 and Supplementary Table 1. There was no linear or nonlinear relationship between total SFAs and risk of BC before and after adjusting for relevant factors. Among estimated markers of exogenous intake of milk and dairy products, higher levels of odd-SFAs, C15:0 and C17:0, were associated with lower BC risk with the adjusted OR 0.18 (Q4 vs. Q1, 95% CI = 0.076 ~0.44; \( p \) for trend < 0.001), 0.18 (Q4 vs. Q1, 95% CI = 0.068 ~0.47; \( p \) for trend < 0.001) and 0.34 (Q4 vs. Q1, 95% CI = 0.14 ~0.81; \( p \) for trend < 0.001), respectively (Figure 5B). At the same time, the OR (95% CI) for a comparison of Q vs. Q was 0.28 (0.12 ~0.65) for VLCSFAs, and 0.33 (0.15~0.75) for C24:0 (Figure 5C). However, C18:0 was positively correlated with the BC risk [OR = 2.99 (95% CI = 1.37 ~6.53)] (Figure 5A). Other SFAs had not been found to be associated with the risk of BC.

Receiver Operating Characteristic Curves of Bladder Cancer Risk Model

In view of the correlation between SFAs and the BC risk, we conducted ROC curve to evaluate the accuracy of model (Figure 6). The area under the ROC curve ranged from 1.0 ~0.5. The predictive ability of BC was judged according to the AUC values, and showed that the AUC value of C22:0 (Figure 6K) was 0.474 (AUC <0.5) with no predictive value. The AUC values of SFAs (Figure 6A), even-SFAs (Figure 6C), VLCSFAs (Figure 6D), C14:0 (Figure 6E), C16:0 (Figure 6G), C20:0 (Figure 6J), C23:0 (Figure 6L) and C24:0 (Figure 6M) had AUC values between 0.5 and 0.6, which had low accuracy for the prediction of BC. The AUC values of C15:0 (Figure 5F), C17:0 (Figure 6H) and C18:0 (Figure 6I) ranged from 0.6 to 0.7, whereas the AUC values of odd-SFAs were greater than 0.7 and had a high accuracy for the prediction of BC. The values of AUC for each differential SFA as a predictor of BC were less than 0.7, which was less accurate.

To improve the accuracy of BC prediction, new ROC curves combining C15:0 and C17:0 were mapped. As shown in Figure 7, the accuracy of prediction was significantly enhanced with the higher AUC values of 0.854 (95%CI: 0.787,0.922).

Figure 4. Heat map of SFAs associations in erythrocyte membranes of participants. (A) Correlation between saturated fatty acids in the control group. (B) Correlation between saturated fatty acids in the case group. * \( p < 0.05 \); Sector size and color are used to demonstrate association strength; larger sectors and redder colors indicate greater association strength; smaller sectors and bluer colors indicate less association strength.
Associations of Saturated Fatty Acids with the Bladder Cancer Risk by Restricted Cubic Splines

Restricted cubic spline curves were employed to flexibly model and visually represent the predicted association between odd-SFAs and VLCSFAs with BC. Three nodes (10%, 50%, 90%) were selected for analysis. As a result, BC risk was obviously decreased by high intake of total odd-SFAs \( (p \text{ for non-linearity: } 0.08) \) or C15:0 \( (p \text{ for non-linearity: } 0.46) \), C17:0 \( (p \text{ for non-linearity: } 0.53) \) individually (Figure 8A&C&D). On the contrary, with the raising levels of C18:0 \( (p \text{ for non-linearity: } 0.16) \), BC risk was drastically increased (Figure 8E). Total VLCSFAs \( (p \text{ for non-linearity: } 0.04) \) and C24:0 \( (p \text{ for non-linearity: } 0.06) \) exhibited a weak protective effect (Figure 8B&F).

Subgroup Analysis between Saturated Fatty Acids and the Risk of Bladder Cancer

To further determine the relationship between SFAs and the BC risk in different subgroups, we then examined whether common risk factors (age, sex, smoking status, alcohol consumption) had any impact on the

![Figure 5. SFAs and BC risk estimated by OR proportional hazards modeling. (A) Association of SFAs and Even-SFAs with BC. (B) Association of Odd-SFAs with BC. (C) Association of VLCSFAs with BC. Crude: not adjusted; Model 1: on the basis of crude model, further adjusted for age, gender, and BMI; Model 2: on the basis of model 1, further adjusted for occupation and degree of education; Model 3: on the basis of model 2, further adjusted for smoking status, alcohol consumption, physical activities, diabetes and energy. Logistical regression model was estimated odds ratio (OR) and the corresponding 95% confidence interval (CI). SFAs, saturated fatty acids; Even-SFAs, even-chain fatty acids; Odd-SFAs, odd-chain fatty acids; VLCSFAs, very long chain saturated fatty acids. BMI, Body mass index.](image-url)
Figure 6. Receiver operating characteristic curves of model for predicting BC risk. (A) ROC curves for SFAs. (B) ROC curves for odd-SFAs. (C) ROC curves for even-SFAs. (D) ROC curves for VLCSFAs. (E) ROC curves for C14:0. (F) ROC curves for C15:0. (G) ROC curves for C16:0. (H) ROC curves for C17:0. (I) ROC curves for C18:0. (J) ROC curves for C20:0. (K) ROC curves for C22:0. (L) ROC curves for C23:0. (M) ROC curves for C24:0. AUC was the area under the ROC curve; the higher the AUC value, the higher the prediction accuracy; 95% CI, 95% confidence interval; SFAs, saturated fatty acids; Even-SFAs, even-chain fatty acids; Odd-SFAs, odd-chain fatty acids; VLCSFAs, very long chain saturated fatty acids.
risk of BC (Figure 9). The correlation between odd-SFAs (Figure 9A), C15:0 (Figure 9C), C17:0 (Figure 9D) and the occurrence of BC in all subgroups including ages (≤60, > 60), gender (male, female), smoking status (current smoker, never or former smoker) and alcohol consumption (current drinker, never or former drinker) were still consistent with the overall analysis. At the same time, when stratified by age, the OR value was found to be lower than that of the entire population group, indicating that age may act as an effective modifier in this association. However, the association of C18:0 (Figure 9E) with the risk of BC disappeared. Consistent results were also observed in the protective effects of VLCSFAs (Figure 9B) and C24:0 (Figure 9F) with BC.

**Association between Saturated Fatty Acids and Dairy Intake**

Quantity of erythrocytic SFAs were publicly recognized as a biomarker of the dietary intake of lipids in a long-term. To explore the relationship between dietary intake of lipids and erythrocytic SFAs in BC, spearman correlations were performed (Figure 10). To our surprised, little relationship was observed between total SFAs, even-SFAs and VLCSFAs and dietary intake. Whereas odd-SFAs, C15:0 and C17:0, were positively related with the intake of milk and dairy products.

**Mediating Role of C15:0 and C17:0 in the Association of Milk & Dairy Products and Bladder Cancer**

To investigate the potential mediation of the association between milk and dairy products and BC by odd-SFAs (Figure 11A), we conducted individual mediation analyses for C15:0 (Figure 11B) and C17:0 (Figure 11C).

Mediating effects of C15:0 and C17:0, along with their 95% confidence intervals (CIs) excluding zero, were presented in the Figure 11B&C and Supplementary Table 2. Hence, the findings indicated that C15:0 and C17:0 serve as mediators in the linkage between milk and dairy products consumption and BC, with both demonstrating partial mediation effects. C15:0 had an indirect effect value of −0.0008 (95% CI, −0.0015 to −0.0002), accounting for 18.1% of the total indirect effect. Similarly, C17:0 had an indirect effect value of −0.0006 (95% CI, −0.0013 to −0.0002), accounting for 13.6% of the total effect. As presented in detail in Table 2.

**The Relationship between Milk & Dairy Products and Bladder Cancer: A Test of the Chain-Mediated Effect**

Figure 11A and Supplementary Table 3 presented the results, indicating a significant negative association between milk and dairy products and BC (β = −0.13, P = 0.0067). Upon incorporating C15:0 and C17:0 into the model (Figure 11D and Supplementary Table 4), milk and dairy products exerted a significant positive predictive effect on C15:0 (β = 0.00003, P = 0.0093) and C17:0 (β = 0.00041, P = 0.042). The variable C15:0 positively predicted C17:0 with statistical significance, as indicated by the regression coefficient (β = 0.46, P = 0.00003). Furthermore, both C15:0 (β = −17.43, P = 0.034) and C17:0 (β = −8.79, P = 0.029) exhibited a statistically significant negative association with BC. Moreover, milk and dairy products showed a statistically significant predictive effect on BC (β = −0.033, P = 0.028).

The mediation effect analysis results (Table 3) indicated a mediation effect value of −0.000529 for C15:0, −0.000362 for C17:0, and −0.000122 for the chained mediation effect of C15:0 and C17:0. The 95% bootstrap confidence intervals for the 3 mediation pathways did not include zero. In addition, all 3 mediation effects were statistically significant, explaining 12.4%, 8.5%, and 2.9% of the total effect, respectively.

**DISCUSSION**

To the best of our knowledge, this case-control study was the first to demonstrate the association of eryth-
rocyte membrane SFAs and BC. Different subclasses of SFAs presented the varied and even opposite correlations with BC risk. Specifically, C18:0 showed a positive correlation, while VLCSFAs including C24:0 was negatively associated with the risk of BC. The consumption of milk and dairy products was significantly associated with higher levels of odd-SFAs in the erythrocyte membrane. Additionally, odd-SFAs, including C15:0 and C17:0, exhibited a negative association with BC. C15:0 and C17:0 in combination increased the predictive accuracy for BC risk. And C15:0 and C17:0 might act as mediators between milk and dairy products and the risk of BC.

Since the earliest reports in 1930 that fat might induce carcinogenesis (Hodge et al., 2007). SFAs had long been considered as the risk factor of cancers (Matthan, Ooi, Van Horn, Neuhaus, Woodman, & Lichtenstein, 2014; Zhuang et al., 2019). It should be noted that these data mainly focused on total SFAs intake, rather than individual SFAs with different carbon chain length. The varied types of SFAs might presented the different, even the opposite effects on the development of carcinogenesis (Liss et al., 2019). Recent results implied that odd-SFAs and VLCSFAs might decrease the likelihood of developing chronic diseases such as cardiometabolic diseases, gestational diabetes mellitus, and type 2 diabetes (Bojkova, Winklewski, & Wszydbyl-Winklewska, 2020; Hardy, El-Assaad, Przybytkowski, Joly, Prentki, & Langelier, 2003; Zhu et al., 2018). Nevertheless, higher level of even-SFAs was associated with higher risk of chronic diseases (Bojkova et al., 2020). In China, the most commonly consumed SFAs was palmitic acid with 16 carbon atoms, followed by stearic acid with 18 carbon atoms, which were commonly found in red meats and their products. For instance, organ meat such as liver increased the risk of BC due to the higher content of saturated fats (Z. Li et al., 2022). Even-SFAs, abundant in erythrocytic membrane, were derived from both exogenous consumption (rich in foods such as butter, palm oil, and red meat) and endogenous (de novo lipogenesis stimulation due to excessive carbohydrate intake) sources (Forouhi et al., 2014; Hardy et al., 2003). However, links between even-SFAs and BC were not fully illuminated. We found even-SFAs, not all SFAs, were positively associated with BC risk. One possible mechanism was that even-SFAs stimulated the synthesis of prostaglandins, which played an important role in the development of BC (Medzhitov, 2008; Szymańska, 2019).

Figure 8. Associations of erythrocytic SFAs with BC by restricted cubic splines from OR models. (A) Odd-SFs and BC risk. (B) VLCSFAs and BC risk. (C) C15:0 and BC risk. (D) C17:0 and BC risk. (E) C18:0 and BC risk. (F) C24:0 and BC risk. Model was adjusted for age, gender, BMI, occupation and degree of education, smoking status, alcohol consumption, physical activities, diabetes and energy.
Moreover, even-SFAs might increase insulin resistance and promote inflammation (Tarantino et al., 2021; Wilhelmsen et al., 2022). At the same time, chronic

![Figure 9](image_url)

Figure 9. Forest map of subgroup analysis between SFAs and BC risk. (A) Subgroup analysis of odd-SFAs and BC risk. (B) Subgroup analysis of VLCSFAs and BC risk. (C) Subgroup analysis of C15:0 and BC risk. (D) Subgroup analysis of C17:0 and BC risk. (E) Subgroup analysis of C18:0 and BC risk. (F) Subgroup analysis of C24:0 and BC risk. OR, odd ratio; CI, confidence interval; odd-SFAs, odd-chain fatty acids. VLCSFAs, very long chain saturated fatty acids.
inflammation had been confirmed to elicit bladder carcinogenesis through pro-inflammatory and lymphatic injury mechanism (Dianatinasab et al., 2021; Hodson, McQuaid, Karpe, Frayn, & Fielding, 2009; Mouillot et al., 2020). The elevated levels of lipid peroxidation products in patients with BC might induce the elevation of pro-inflammatory cytokines and pro angiogenic factors, which in turn lead to the activation of angiogenesis and tumor progression (Lankinen, Uusitupa, & Schwab, 2018). In addition, stearic acid (C18:0) was suggestively associated with ovarian cancer risk and neck squamous cell carcinomas growth (Arab & Akbar, 2002). And a significant correlation between stearic acid (C18:0) intake and increased pancreatic cancer risk was also reported in population study (Wigner, Grebowski, Bijak, Saluk-Bijak, & Szemraj, 2021).

Elevated levels of C24:0 and the VLCSFAs were significantly associated with a lower risk of BC. Numerous recent studies showed the negative associations of VLCSFAs with multiple health outcomes including cancers (Ghamarzad Shishavan et al., 2021; Zheng et al., 2017; Zhu et al., 2018). VLCSFAs were the important component of sphingomyelin and ceramide (Xu et al., 2019). In diet, VLCSFAs C20:0, C22:0, and C24:0 was only contained in the selected foods, including peanuts, peanut butter, and macadamia nuts (Chiu et al., 2018; X. Li et al., 2020). VLCSFAs had been reported to be associated with the resolution of inflammation (Kihara, 2012), making them beneficial for cancer therapy. Other studies had also indicated a potential correlation between VLCSFAs and increased peroxisome proliferator receptor α (PPARα) activity, which may stimulate the synthesis of VLCSFAs. (Fretts et al., 2019; Venn-Watson, Lumpkin, & Dennis, 2020). When PPARα was activated, the transcription of inflammation-related genes decreases, inflammatory reactions were inhibited, and the synthesis of new fats was also reduced. This process could be achieved by inhibiting
Figure 11. Intermediation effect test model path. (A) Total effect between milk and dairy products and bladder cancer. (B) C15:0 as mediating variables. (C) C17:0 as mediating variables. (D) C15:0 and C17:0 as chained mediating variables. For reasons of clarity and comprehensibility, this was a simplified version of the computational model. Measurement models that did not show potential constructs and residual correlations.

Table 2. Analysis of the parallel mediating effect

<table>
<thead>
<tr>
<th>Effect Value</th>
<th>SE</th>
<th>Bootstrap 95% CI</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total effect</td>
<td>-0.0044</td>
<td>0.0011</td>
<td>-0.0089 - 0.0009</td>
</tr>
<tr>
<td>Direct effect</td>
<td>-0.0036</td>
<td>0.0014</td>
<td>-0.0063 - 0.0008</td>
</tr>
<tr>
<td>Indirect effects</td>
<td>-0.0008</td>
<td>0.0003</td>
<td>-0.0015 - 0.0002</td>
</tr>
</tbody>
</table>

Table 3. Analysis of the chain mediating effect

<table>
<thead>
<tr>
<th>Effect Value</th>
<th>SE</th>
<th>Bootstrap 95% CI</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total effect</td>
<td>-0.00426</td>
<td>0.0018</td>
<td>-0.0087 - 0.0025</td>
</tr>
<tr>
<td>Direct effect</td>
<td>-0.000325</td>
<td>0.000144</td>
<td>-0.000672 - 0.000428</td>
</tr>
<tr>
<td>Indirect effects</td>
<td>-0.000101</td>
<td>0.000414</td>
<td>-0.000291 - 0.000368</td>
</tr>
<tr>
<td>Total effect</td>
<td>-0.000529</td>
<td>0.000303</td>
<td>-0.001256 - 0.000103</td>
</tr>
<tr>
<td>Total effect</td>
<td>-0.000362</td>
<td>0.000256</td>
<td>-0.00052 - 0.000006</td>
</tr>
<tr>
<td>Total effect</td>
<td>-0.000122</td>
<td>0.00083</td>
<td>-0.00133 - 0.000011</td>
</tr>
</tbody>
</table>
the production of endogenous even-SFAs, thereby helping to suppress the inflammatory process (Fretts et al., 2019; Siler, Neese, & Hellerstein, 1999; Venn-Watson et al., 2020; Zheng et al., 2017). Therefore, the negative links between VLCSFAs (24:0) and BC risk reported here might be related to its anti-inflammation.

The current results suggested that a higher composition of odd-SFAs was inversely associated with the occurrence of BC. Quite a few literatures suggested that foods containing odd-SFAs (such as milk fat) was closely related with the low risk of metabolic diseases including cancer (Brevik, Veierod, Drevon, & Andersen, 2005; Larsson, Andersson, Johansson, & Wolk, 2008; Pakiet, Kobiel, Stepnowski, Sledzinski, & Mika, 2019; Wolk, Vessby, Ljung, & Barrefors, 1998). To date, anti-cancer effects of odd-SFAs had been proposed (Ediriweera, To, Lim, & Cho, 2021; Matejcic et al., 2018). Intake of oddSFAs including C15:0 and C17:0 was associated with reduced risk of pancreatic cancer and breast cancer (Matejcic et al., 2018; To, Nguyen, Moon, Ediriweera, & Cho, 2020). The beneficial function of odd-SFAs was believed to be associated with their protective role against dysregulated lipid metabolism and low-grade inflammation (Venn-Watson et al., 2020). In addition, odd-SFAs could prevent the migration, diffusion and proliferation of cancer cells via incorporation into cell membrane remodeling. Odd-SFAs was shown to inhibit the proliferation of lung cancer cells (Xu et al., 2019). Although a handful of odd-SFAs such as C17:0 was found in beef fat and fish, they were mainly considered as a sign of exogenous milk fat intake (Venn-Watson et al., 2020). Some literatures showed that odd-SFAs rich in milk had been considered as the protective factors in cancer (Mozaleeb, Aledavood, & Farzad, 2012; Wigner et al., 2021). While it should be noted that the endogenous sources of odd-SFAs (C17:0) could not be neglected and required to be further examined (Larsson et al., 2008; Yakoob et al., 2016; Zhang, Dai, Liang, Zhang, & Deng, 2019). Several protective factors such as calcium, lactic acid bacteria, etc. were existed in dairy products (Bermejo, Lopez-Plaza, Santurino, Cavero-Redondo, & Gomez-Candela, 2019; Pakiet et al., 2019; Riboli et al., 1991). Here in, intake of milk and dairy products was positively correlated with odd-SFAs (C15:0 and C17:0), which was also observed in others (Ghamarzad Shishavan et al., 2021). Although the possible interactions between odd-SFAs (C15:0 and C17:0) and a variety of bioactive substances in milk and dairy products were not fully clear, research on dairy product consumption still supported evidence of negative correlation between odd-SFAs and chronic diseases, including cancer. Despite the steady rise in Chinese consumption of milk and dairy products, it was likely to remain lower than the global average (in other countries of the world) (Du et al., 2018). Specific milk and dairy products intake might vary depending on individual preferences and different regions. In our previous study, the mean consumption milk and dairy products in BC patients was around 65.6 mL/d, which was much lower than the intake of 114.3 mL observed in normal controls (Teng et al., 2022).

In the previous studies, quantities of consumption were mostly based on FFQ, rarely to explore the potential biomarkers of milk and dairy products intake, as well as their relationship with the risk of BC. The biomarkers exploration which implies the specific SFAs types in blood, particularly in the erythrocyte membrane (Sun, Ma, Campos, Hankinson, & Hu, 2007), appeared to be a more effective method for evaluating long-term intake of SFAs and mitigating memory bias. Our findings showed the strongest correlation between erythrocyte odd-SFAs including C15:0, C17:0 and milk and dairy products, and odd-SFAs could be possibly developed as the potential biomarkers of milk and dairy products intake for the prediction of BC risk. Milk & dairy product had independent predictive effects on BC development, and odd-SFAs played a role in the association between milk and dairy product and BC. The role of odd-SFAs as a mediator between milk and dairy product and sea fish and BC. Odd-SFAs and C15:0 and C17:0 had a mediating role in milk and dairy product and BC carcinogenesis, and odd-SFAs might only be part of the complex mechanism of action between milk and dairy product and BC. In the prevention of BC, bladder carcinogenesis could be investigated by combining milk and dairy product and odd-SFAs.

In summary, to our knowledge, this is the first study to reveal the relationship between SFAs and BC risk in Northeast China. The distribution of odd-SFAs in erythrocyte membrane might indicate the exogenous intake of milk and dairy products, and it might be of great significance to predict the risk of BC.

Limitations and Strengths

This study had several strengths. First, we measured the SFAs profiles of erythrocyte membrane in all the enrolled participants, which reflects the relatively long-term average intake levels of SFAs from diets and was not vulnerable to biological changes and measurement errors (including reporting errors). Our results were depended on the centralized analysis of erythrocyte membrane samples stored under the best possible conditions in professional laboratories. The use of this optimization method enables us to obtain highly accurate results. Second, we adjusted the model for further statistical analysis, and minimize the impact of limiting residual hybridization. While our research also
had some limitations. The dietary FFQ had memory bias, therefore, we adjusted the statistical analysis to minimize the potential impact of these differences. The gender of participants was mismatched due to the withdraw of some participants, although the multivariable regression analysis was conducted to avoid the bias. Quantities of SFAs here were expressed as the relative percentage of each FA to the sum of FAs, which may be inaccurate, although this measurement was commonly used. Although the blood sample was collected at −80°C for the first time, it might have some impact on sample determination. Other limitations such as the influence of BC itself on the metabolism of FAs, the reverse causality and residual confounding due to case-control study design should be considered. In addition, the results were only obtained from residents in Northern-East of China, which is possibly unsuitable to extend due to the huge differences in living styles such as diet, physical activities and environments, etc. Although our study was a single-center case-control study with a small sample size, we tried to control the bias associated with the confounding factors as much as possible, and performed rigorous screening on the selection of the target population and controls, as well as controlling the relevant data collection and statistical analyses, and thus might still provide valuable preliminary evidence. This study design might provide a useful theoretical basis for larger, more rigorous studies. A large sample of multi-center research was required for further verification.

Conclusion

We observed the distinct SFAs profiles of erythrocyte membranes in BC patients and normal population. C15:0 and C17:0 combination in erythrocyte membrane could be a reliable mediator and predictor between high milk and dairy products fat intake and low risk of BC.

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Conflict of Interest The authors declare that they have no competing interests.

Ethics Statement: - Approval of the research protocol by the Ethics Committee of Harbin Medical University. - Informed Consent was signed and obtained from all subjects before participation - Registry and the Registration No. of 202011T031. - Animal Studies. N/A.


REFERENCES


