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Use of membrane lipidome, body weight, and composition in stratification of early breast cancer patients

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Fat quality and quantity have a strong impact on cancer metabolism, however, in oncology practice, only body mass index (BMI) is evaluated. The observational prospective study performed at Fondazione Policlinico Gemelli explored the combination of membrane lipidome, BMI, and body composition, together with nutritional information, as evaluation criteria of fifty newly diagnosed early breast cancer patients (BRECALIP study). The fatty acid content of red blood cell membrane phospholipids, dividing patients by the BMI, individuated normal weight subjects for their molecular signatures different from the other groups, pointing to increased membrane fluidity and inflammation (saturated fatty acid decrease, omega-6 fatty acid increase), known to sustain cancer proliferation. Fat mass (FM% ≥30) and phase angles (PA° ≥ 5.6) in the normal weight group correlated with specific pro-inflammatory fatty acid modifications. Such patient stratification, confirmed by large and longitudinal studies, can better individuate nutritional/metabolic risks of inflammatory implications in breast cancer.

There is a growing interest in the causal links between body weight, body composition, and cancer onset and progression due to the global cancer burden and the high incidence of overweight and obesity in the worldwide population[1–4]. Body mass index (BMI) has been significantly associated with several solid cancers, including postmenopausal breast cancer (BC)[4–7], but in the clinical setting, this parameter seems to have some caveats and limitations. Lipidomic research highlighted new aspects of cancer metabolism, correlated with the fatty acid pool and their indispensable role in cell proliferation, both for membrane formation and as precursors of bioactive lipids (BAL) for immune and inflammatory signals[8–10]. Considering the interdependence between dietary and metabolic contributions for the cell membrane phospholipids formation (Fig. S1 in Supplementary Information), it is worth underlining the need for an appropriate balance among saturated, monounsaturated, and polyunsaturated fatty acids (SFA, MUFA, PUFA) to form the variety of body tissues[11]. Membrane lipidomics analysis verifies such a balance in the cells of the tissues, providing molecular signatures of the metabolic and nutritional status of the individual. In particular, the SFA formation by the de novo lipogenesis (DNL) and the desaturation step transforming SFA into MUFA can be combined with the effects of the exogenous supply of essential PUFAs from dietary intakes. PUFAs must be supplied by the diet since in humans they cannot be formed from MUFA by enzymatic pathways[11]. The functional omega-6/omega-3 balance from the diet has a well-known and decisive impact on health due to the consequent pro- and anti-inflammatory cell signaling[11–13].

Membrane lipidome is the *conditio sine qua non* for all cells, whether healthy or tumoral, can be formed. In cancer, the need for a complete and varied fatty acid pool is accelerated to sustain cancer growth[13–17]. Clinical application of the membrane lipidome profiling is allowed by the analysis of the mature red blood cell (RBC) since the membrane lipids of this cell have a well-recognized ability to represent the lipid pool available for the cells of all body districts[11–14]. Early molecular signatures of enhanced lipid recruitment can be individuated in a personalized way, potentially impacting primary prevention and cancer patient dietary management[18–21]. Indeed, cancer onset and invasiveness are associated with a) the increase of enzymatic activities such as fatty acid synthase (FAS)[22,23] and desaturase (stearoyl-CoA desaturase)[24,25], b) augmented enzymatic transformations of omega-6 precursors into long chain-PUFA, in particular of arachidonic acid (AA) with the formation of related eicosanoids involved in angiogenesis, tumoral cell replication, and aggressiveness[26].

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As regards the influence of body weight in cancer patients, in a recent meta-analysis, obesity was associated with increased overall and cancer-specific mortality in breast, colon, and uterine cancers. Renal cell carcinoma, lung cancer, or melanoma did not show this association[27]. It is intriguing that weight gain and obesity present fatty acid biomarkers that are, in principle, similar to those of a cancer condition, such as the increases of oleic and palmitoleic acids (MUFA), and omega-6 PUFA such as AA[22,28–30]. Moreover, obesity is discussed as a paradox in cancer: on one hand, it is a risk factor that can increase overall cancer mortality[27], and on the other hand, considering weight loss as a sign of worseness, less mortality risk is reported in early-stage breast and colorectal cancers for overweight and mildly obese rather than normal-weight patients[5].

This scenario highlights the complexity of lipid metabolism in cancer and the need for a more personalized approach to better explain the exact lipid turnover according to the BMI condition and the early cancer phase.

Previous information on fatty acids and cancer comes from the Nurses' Health Study, exploring the association between BC incidence with BMI and fatty acids in RBC membranes. From a predictive point of view, the negative correlation of SFA to BC risk in normal weight (NW) women emerged; on the other hand, several SFA, trans fatty acids (TFA), and dairy-derived fatty acids correlated positively with the cancer incidence in overweight/obese (OW/OB) women, and negatively in case of the omega-3 alpha-linolenic acid[31]. Once again, it emerges that the common features between cancer and obesity concern inflammatory signatures and lipogenesis, which are well indicated by the fatty acid contents in cell membranes, overcoming the debated relationship with food intakes[32].

Based on these premises, we decided to perform an observational study examining a cohort of newly diagnosed nonmetastatic BC patients, awaiting surgery, by combining dietary habits information, BMI and anthropometric values, and body composition, with the fatty acid analysis of the mature RBC membrane lipidome. We anticipate that this data panel, obtained by easily performed and minimally invasive methods, will give interesting novel aspects of the BMI-related patient stratification, highlighting molecular and metabolic signatures in NW subjects, who are generally poorly considered for lipid unbalances and dietary control.

Results

Membrane lipidome analysis and patient stratification

After enrollment of the 50 patients diagnosed with early-stage BC (I-II), undergoing upfront surgery and with no previous neoadjuvant systemic treatment, following inclusion and exclusion criteria as summarized in the Consort diagram (Fig. 1), the clinical, immune-hematological, anthropometric, body composition, and histopathological data were recorded, and are reported in Table 1. The age range was 29–64 years, and patients were divided, according to BMI, into three groups: NW (BMI = 18.5–24.9) $n = 26$; OW (BMI = 25–29.9) $n = 11$; OB (BMI = 30–39.9) $n = 13$.

A cohort of 15 NW healthy subjects aged 22–54 (BMI = 18.5–24.9) recruited among the Hospital's employees was included in the study as geographically matched control (CTRL H in Supplementary Table 1). Moreover, from an anonymized database of a healthy Italian population, a group of NW age-matched women ($n = 26$) was also selected as a non-geographically matched control group (CTRL DB in Supplementary Table 1); the two groups were merged to form one control group (denominated CTRL or Healthy) of 41 NW subjects, such as for reporting the membrane fatty acid values in Fig. 2 and Supplementary Table 3. Finally, RBC membrane fatty acid and index intervals, described for healthy populations[12,14,33], were used as benchmark values.

All recruited subjects underwent an interview with nutritionists about their food frequencies, and foods were grouped into categories based on their lipid contents, for statistical evaluation of the fatty acid types usually consumed by the subjects (Supplementary Table 1). No significant differences in food consumption were found among the three BC groups (Supplementary Table 2); it is important to note the high variability of intakes recorded in these groups that could have influenced the statistical significance.

The fatty acid-based membrane lipidome of mature RBC from both the patients' cohort and control group is obtained by a known protocol. Plasma is carefully removed since circulating lipids (in plasma and serum) are known to be strongly influenced by recent dietary habits; instead, membrane phospholipids are connected with stabilized nutritional habits and individual metabolism[12,30,34–38]. It is worth noting that the blood workup protocol must ensure the chemical integrity of the PUFA components, being the most sensitive molecules that can be damaged during manipulation[39]. Gas chromatography (GC) is the gold standard for fatty acid separation, identification, and quantification[30,34–38]. The representative cohort of ten SFA, MUFA, and PUFA components of the RBC membrane phospholipids and two trans fatty acid isomers of oleic and arachidonic acids are the basic fatty acid building blocks involved in the membrane formation, reactivity, and remodeling of all body tissues, as described in Supplementary Fig. 1[12,33]. Each fatty acid value is quantified over the total quantity (100%) of the identified fatty acids and reported as a % relative quantitative (% rel. quant.).

The following lipid indexes are calculated from these values: (1) the omega-3 index (%EPA + %DHA), also reported in populations as

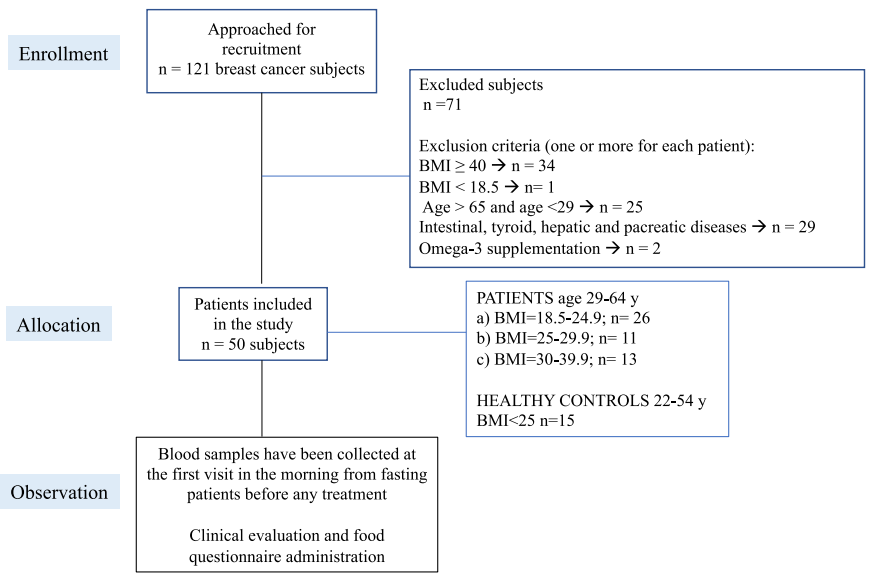

Fig. 1 | Consort diagram of the study. Inclusion and exclusion criteria and patients evaluated in the study.

Table 1 | Clinical, immune-hematological, anthropometric, body composition, and histopathological data of the studied cohorts; all cancer patients (ACP, *n* = 50) divided into 3 groups: normal weight, NW (BMI = 18.5–24.9) *n* = 26; overweight, OW (BMI = 25-29.9) *n* = 11; obese, OB (BMI = 30–39.9) *n* = 13, with the statistical significance of the clinical and immune-hematological values

Clinical and Immuno-hematological status[§]	ACP Mean ± sd	NW Mean ± sd	OW Mean ± sd	OB Mean ± sd
Menarche (age)	12.49 ± 1.44	12.67 ± 1.40	12.17 ± 1.46	12.00 ± 1.53
Pregnacies (n°)	1.27 ± 0.95	1.08 ± 1.00	1.40 ± 1.02	1.36 ± 0.77
Systolic pressure (mmHg)	120 ± 7.07	117.54 ± 12.97	123.64 ± 16.25	124.50 ± 8.20
Diastolic pressure (mmHg)	74.17 ± 14.14	72.92 ± 8.65	77.45 ± 9.53	74.00 ± 6.24
Glycemia (mg/dL)	93.4 ± 12.48	85.58 ± 9.76	93.91 ± 7.65[a]	104.25 ± 14.57[b,d]
Cholesterol (mg/dL)	187.34 ± 30.52	185.96 ± 32	199.27 ± 28.56	179.83 ± 26.90
Triglycerides (mg/dL)	95.4 ± 45.12	80.73 ± 29.57	110.91 ± 56.72[a]	116.83 ± 48.12[c]
Lymphocytes (mil/mm^3)	2.00 ± 0.54	1.97 ± 0.43	1.80 ± 0.32	2.33 ± 0.72[d]
Neutrophils (mil/mm^3)	4.40 ± 1.56	4.36 ± 1.27	4.09 ± 1.70	4.90 ± 1.83
	n (%)	*n* (%)	*n* (%)	*n* (%)
Familiarity	20 (40)	14 (54)	3 (27)	3 (23)
Menopause	22 (44)	8 (30)	6 (54)	8 (62)
Smoke	11 (22)	5 (19)	3 (27)	2 (15)
Comorbidities	20 (40)	9 (33)	3 (27)	9 (69)
Anthropometric and body composition measures	Mean ± sd	Mean ± sd	Mean ± sd	Mean ± sd
Weight (kg)	72.2 ± 18.64	59.75 ± 6.10	73.37 ± 5.91	99.18 ± 14.57
Height (m)	1.63 ± 0.06	1.63 ± 0.05	1.61 ± 0.05	1.66 ± 0.08
Body Mass Index	27.23 ± 6.39	22.49 ± 1.95	28.43 ± 1.43	36.09 ± 4.97
Waist circumference (cm)	88.03 ± 15.63	77.00 ± 7.15	91.55 ± 5.51	107.73 ± 13.58
Hip circumference (cm)	104.26 ± 11.66	96.44 ± 4.88	104.68 ± 4.28	120.19 ± 9.47
WHR	0.84 ± 0.08	0.80 ± 0.06	0.88 ± 0.06	0.91 ± 0.08
Resistance at 50 kHz– (Ohm)	512.16 ± 79.40	548.85 ± 78.36	480.91 ± 55.62	461.85 ± 60.34
Reactance at 50 kHz (Ohm)	45.02 ± 7.14	46.78 ± 8.07	44.05 ± 5.15	42.28 ± 5.76
Phase angle (°)	5.08 ± 0.62	4.90 ± 0.67	5.31 ± 0.53	5.27 ± 0.49
Fat mass (kg) Fat mass (%)	26.01 ± 14.09 33.53 ± 9.58	16.03 ± 5.07 26.31 ± 6.22	27.59 ± 3.21 37.27 ± 2.43	45.36 ± 11.72 45.17 ± 4.93
Fat free mass (kg) Fat free mass (%)	46.71 ± 5.56 66.47 ± 9.58	43.68 ± 2.59 73.70 ± 6.22	47.97 ± 3.58 62.73 ± 2.43	53.82 ± 5.05 54.83 ± 4.93
Total body water L Total body water %	36.53 ± 6.09 52.05 ± 8.34	33.16 ± 4.36 56.62 ± 7.73	37.18 ± 4.55 50.80 ± 5.88	42.96 ± 5.00 43.55 ± 2.57
Histopathological status	*n* (%)	*n* (%)	*n* (%)	*n* (%)
Grade G1	6 (12)	3 (12)	2 (18)	1 (8)
Grade G2	20 (40)	9 (35)	5 (46)	6 (46)
Grade G3	15 (30)	10 (38)	2 (18)	2 (15)
Grade N.A	9 (18)	4 (15)	2 (18)	4 (31)
Estrogen receptor positive (ER (10%)	37 (74)	21 (81)	7 (64)	9 (69)
Estrogen receptor negative (ER−)	4 (8)	1 (4)	3 (27)	0 (0)
Estrogen receptor (ER) N.A– *n* (%)	9 (18)	4 (15)	1 (9)	4 (31)
Progesterone receptor positive (PR+) (10%)	34 (68)	19 (73)	7 (64)	8 (61)
Progesterone receptor negative (PR−)	7 (14)	3 (12)	3 (27)	1 (8)
Progesterone receptor (PR) N.A− *n* (%)	9 (18)	4 (15)	1 (9)	4 (31)
Human epidermal growth factor receptor 2 positive (HER2+)	2 (4)	1 (4)	1 (9)	0 (0)
Human epidermal growth factor receptor 2 negative (HER2−)	38 (76)	21 (81)	8 (72)	9 (69)
Human epidermal growth factor receptor 2 (HER2) N.A. *n* (%)	10 (20)	4 (15)	2 (18)	4 (31)
High Ki-67 (H-Ki-67)	21 (42)	12 (46)	4 (36)	5 (38.5)
Low Ki-67 (L-Ki-67)	19 (38)	10 (39)	4 (36)	5 (38.5)
N.A. Ki-67	10 (20)	4 (15)	3 (27)	3 (23)
Luminal A (ER+/PR+/HER2−/L-Ki-67)	18 (36)	10 (38)	2 (18)	5 (38)
Luminal B (ER+/PR+/HER2−/H-Ki-67)	17 (34)	10 (38)	2 (18)	4 (31)
Luminal B (ER+/PR+/HER2+/H-Ki-67)	0 (0)	0 (0)	0 (0)	0 (0)

Table 1 (continued) | Clinical, immune-hematological, anthropometric, body composition, and histopathological data of the studied cohorts; all cancer patients (ACP, *n* = 50) divided into 3 groups: normal weight, NW (BMI = 18.5–24.9) *n* = 26; overweight, OW (BMI = 25-29.9) *n* = 11; obese, OB (BMI = 30–39.9) *n* = 13, with the statistical significance of the clinical and immune-hematological values

Clinical and Immuno-hematological status[§]	ACP Mean ± sd	NW Mean ± sd	OW Mean ± sd	OB Mean ± sd
HER2+ (ER−; PR−; HER2+)	3 (6)	1 (4)	2 (18)	0 (0)
Triple negative (ER−/PR−/HER2−)	2 (4)	1 (4)	1 (9)	0 (0)
N.A – *n* (%)	10 (20)	4 (15)	4 (36)	4 (31)

[§]Significance (unpaired *t*-test). [a]OW vs NW $p \leq 0.0398$. [b]OB vs NW $p < 0.0001$. [c]OB vs NW $p = 0.0062$. [d]OB vs OW $p \leq 0.046$.
Estrogen Receptor positive (ER+) ≥10% positive staining for ER; Estrogen Receptor negative (ER−) <10% positive staining for ER; Progesterone Receptor positive (PR+) ≥10% positive staining for PR, Progesterone Receptor negative (PR−) <10% positive staining for PR, HER2 positive: IHC (immunohistochemistry) score 3+ or Her2 gene amplification, HER2 negative: IHC score 0-1 or score 2 with no Her2 gene amplification.

cardiovascular risk[40], (2) the ω-6/ω-3 (omega-6/omega-3) ratio, a well-known parameter used for evaluating the pro- and anti-inflammatory balance with influence from the diet (see Supplementary Fig. 1)[13], (3) the saturation indexes, SI (a, C18:0/9c–C18:1 ratio)[41,42], and SI (b, C16:0/9c-C16:1 ratio), that indicate the endogenous SFA-MUFA conversion[28], (4) the two desaturation indexes, indicating the omega-6 metabolic pathway, i.e., delta-6 desaturase index [C18:2/C20:3 ratio, that includes the elongase enzymatic path] and delta-5 desaturase index [C20:4/C20:3 ratio], (5) the unsaturation and peroxidation indexes (UI and PI) indicating the contribution of unsaturated fatty acids (MUFA and PUFA) for the membrane fluidity and peroxidizability, respectively[43,44]. The separation and quantification of trans fatty acid (TFA) isomers is an important feature of this analysis, indicating the exposure of membrane lipids to free radical insults, connected to the intensity of oxidative stress and the level of antioxidant defenses[45–47].

The RBC membrane fatty acid levels of the three groups of early-stage BC patients with different BMIs are summarized in the graphs of Fig. 2, and Table 2 reports the statistically significant fatty acids with their p-values, comparing each patient group with the control group of healthy NW women ($n = 41$). For the full data set of fatty acids, see Supplementary Table 3 (data reported as % rel. quant., mean ± sd).

The focus of this study is the changes in the membrane fatty acid composition due to the cancer condition, individuated by (a) the "endogenous" SFA-MUFA transformations, (b) the PUFA balance resulting from nutritional contributions, and the omega-6 and omega-3 metabolic transformations. As shown in Table 2 (full data in Supplementary Table 3), the early BC NW group has more changes in the membrane profile compared to healthy controls than all patients with different BMI, with the number of significant fatty acid changes decreasing within the BMI-increasing patient groups. In particular, the BC NW group showed a decrease of two SFA (palmitic and stearic acids, C16:0 and C18:0, respectively) and one MUFA (palmitoleic acid, C16:1), together with the increase of one omega-6 PUFA (arachidonic acid, C20:4), and a decrease of one omega-3 PUFA (eicosapentaenoic acid, C20:5). Consequently, the total SFA decrease and total PUFA increase are significant, the latter due to the omega-6 increase which contributed to the increases in the unsaturation and peroxidation indexes (UI and PI, respectively) (Table 2).

The BC OW group showed a few significant changes concerning the diminution of one SFA (stearic acid, C18:0) and the increase of two omega-6 PUFA (dihomo-gamma linolenic and arachidonic acids, C20:3 and C20:4, respectively), which determine the corresponding decrease of total SFA and increase of total omega-6 PUFA in this group (Table 2). The sub-division of early BC patients into two groups with BMI < 25 ($n = 26$) and BMI ≥ 25 ($n = 24$) (Supplementary Table 4) confirmed the significant SFA decrease and PUFA increase as characteristics of the profile of NW BC patients.

The BC OB group showed increased arachidonic acid and delta-6 desaturase + elongase enzymatic index, confirming the omega-6 cascade activation. However, this group differentiated from the BC NW patients for the membrane profile with increased SFA and PUFA values (Fig. 2 and Table 2, with full data in Supplementary Table 3), making the UI increase also significant.

In Fig. 2, a gray zone shows the interval values described for the RBC membrane benchmark[12,14,33]. The omega-6 PUFA level of almost all BC patients is higher than the benchmark range.

Clinical, immune-hematological, anthropometric, body composition, and histopathological data are reported in Table 1, whereas food frequency data are reported in Supplementary Table 1. They were registered for BC patients and controls of the Hospital (CTRL H) at the recruitment, using well-trained procedures, as described in other studies[30,38]. There is no significance of the food frequencies among cancer patients of different BMIs, as reported in Supplementary Table 2, as well as with the control group of the Hospital (CTRL H).

The RBC membrane fatty acid data of BC patients with different BMI were examined for their correlations with food frequencies; Fig. 3 shows the results as heatmaps for the three groups (heatmaps a-c), as well as for the group of healthy controls of the Hospital (ctrl). The details of correlation parameters are reported in Supplementary Table 5.

Interestingly, the food-fatty acid correlations found for NW diminished in number for OW and OB patients (Fig. 3). Only legumes correlated with fatty acid data in all BC groups, but with different food type combinations in the three BMI groups. For example, in the OB group legumes have a positive correlation with palmitic acid (C16:0; $r = +0.70$; $p = 0.006$) and a negative correlation with arachidonic acid (omega-6 C20:4; $r = -0.575$; $p = 0.041$), whereas in the OW group legumes correlated positively with the omega-6 C18:2 (C18:2; $r = +0.665$; $p = 0.035$). Remarkedly, only in the controls and in NW BC patients did the frequency of fish consumption positively correlate with the levels of omega-3 DHA ($p = 0.05$ and $p = 0.01$, respectively, in Supplementary Table 5).

A snapshot of the RBC membrane fatty acid profiles can be obtained from the spider maps in Fig. 4, representing the membrane fatty acid assets in the three BMI-grouped patients.

Fatty acids and body composition in normal-weight patients

In the assessment panel of the BC patients, the fat mass percentage (FM%) and phase angle (PA°) using Bioelectrical Impedance Analysis (BIA) were also included. The NW BC patients ($n = 26$) formed two sub-groups based on their FM% and PA° results, and we checked the significant correlations with their membrane fatty acid profiles. For the first time, the BIA data are combined with molecular information of the cell membrane compartment in breast cancer patients. The results for the significant fatty acid levels are shown in Table 3 for FM% and in Table 4 for PA°. The full data are reported in Supplementary Tables 6, 7, respectively.

Regarding FM% values, three groups were identified [FM% ≦ 24.9 ($n = 12$), FM% = 25–29.9 ($n = 6$), FM% ≧ 30 ($n = 8$)]. Table 3 shows that a) the patients with FM% = 25–29.9 had significantly lower levels of omega-6 C18:2 (linoleic acid) compared to the FM% ≦ 24.9 ($p = 0.021$), b) the group with FM% ≧ 30 had a significant decrease of trans fatty acid levels (TFA, 9trans C18:1, mono-trans arachidonic acid and total trans, with $p = 0.04$, $p = 0.04$ and $p = 0.0018$, respectively).

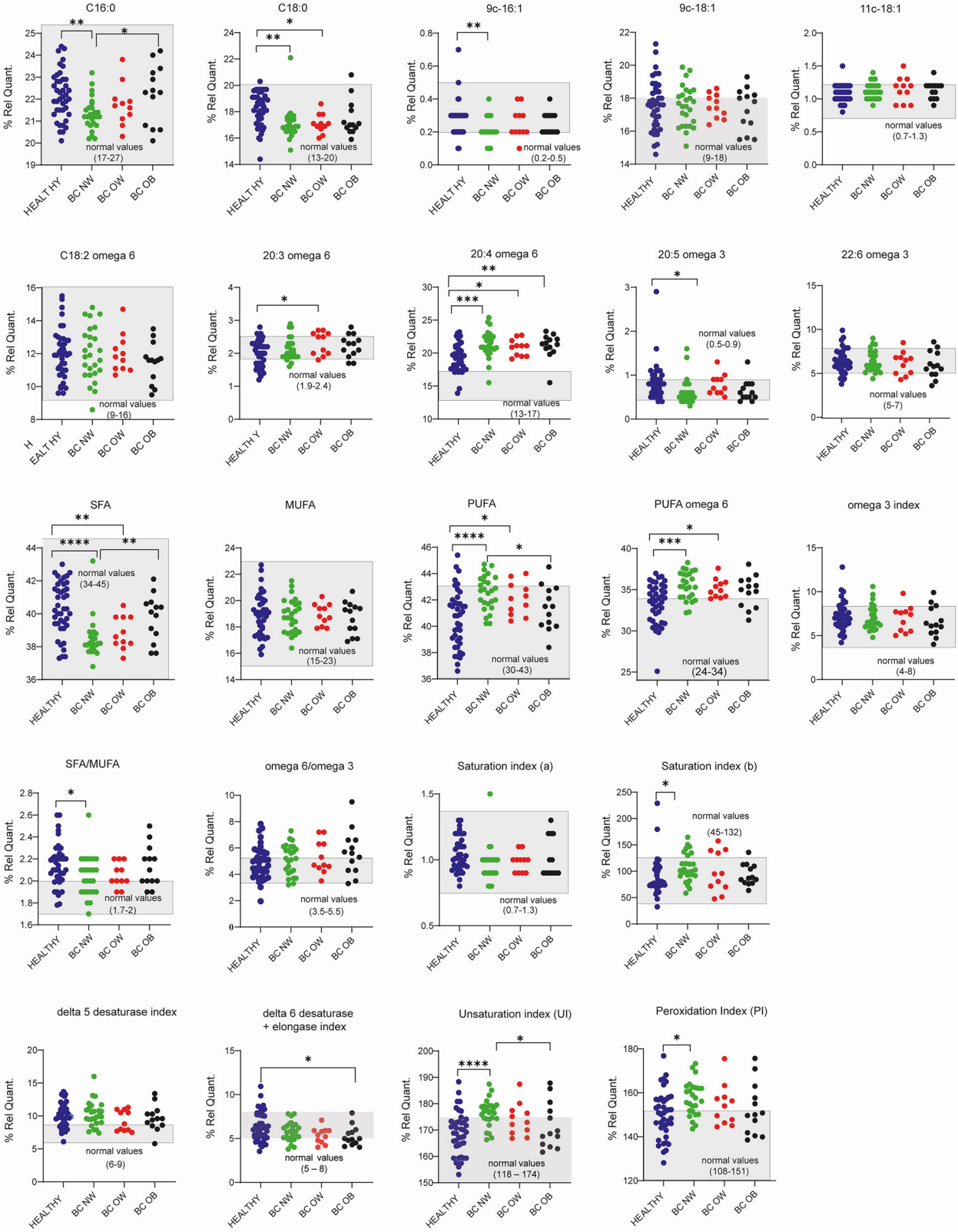

Fig. 2 | Scattered dot plots of the red blood cell (RBC) membrane fatty acid data and indexes obtained from the subjects of the study. Graphs show the results of the three groups of early-stage breast cancer (BC) patients ($n = 50$) divided by BMI: normal weight, NW (BMI = 18.5–24.9) $n = 26$; overweight, OW (BMI = 25–29.9) $n = 11$; obese, OB (BMI = 30–39.9) $n = 13$, in comparison with healthy controls (HEALTHY, $n = 41$). The controls comprise healthy NW women aged 22–54 recruited among the Hospital's employees (CTRL H, $n = 15$) and age-matched healthy NW women (CTRL DB, $n = 26$) taken from an anonymous Italian lipidomic analysis database. In the plots, the gray areas correspond to the interval values reported for the healthy RBC membrane fatty acids benchmark[12,35]. Significances (unpaired t-test): $^{*}p \le 0.04$; $^{**}p \le 0.0093$; $^{***}p = 0.0002$; $p \le 0.0001$. Significant changes are detailed in Table 2.

For the phase angle (PA°), two NW BC groups were individuated with PA° ≦ 5.5 ($n = 19$) and PA° ≧ 5.6 ($n = 7$). Table 4 shows that the subjects with PA° ≧ 5.6 had significantly decreased levels of the omega-3 docosahexaenoic acid (DHA) and the total omega-3 PUFA level (both $p \leqq 0.045$), together with the significant increase of the omega-6/omega-3 ratio ($p = 0.045$).

Table 2 | Significance (*p* value) of the fatty acids and lipid indexes of the early-stage breast cancer (BC) patients of this study compared among BMI groups [normal weight, NW (BMI = 18.5–24.9) *n* = 26; overweight, OW (BMI = 25–29.9) *n* = 11; obese, OB (BMI = 30–39.9) *n* = 13] and to the healthy normal weight control group (Healthy, *n* = 41)

Fatty acids and Indexes	BC NW vs Healthy *p* value	BC OW vs Healthy *p* value	BC OB vs Healthy *p* value	BC OB vs BC NW *p* value
C16:0 palmitic acid	0.0010	-	-	0.011
9c-C16:1 palmitoleic acid	0.0068	-	-	-
C18:0 stearic acid	0.0017	0.014	-	-
C20:3 omega-6 (dihomo-gamma linolenic acid)	-	0.002	-	-
C20:4 omega-6 (arachidonic acid)	0.0002	0.025	0.0093	-
C20:5 omega-3 (eicosapentaenoic acid)	0.02	-	-	-
SFA	≤0.0001	0.0049	-	0.0038
PUFA	≤0.0001	0.03	-	0.023
PUFA omega-6	0.0002	0.02	-	-
SFA/MUFA	0.05	-	-	-
Saturation index (b)	0.03	-	-	-
Delta-6desaturase +elo index	-	-	0.03	-
Unsaturation index UI	≤0.0001	-	-	0.04
Peroxidation index PI	0.004	-	-	-

Full data are reported in Supplementary Table 3, and corresponding graphs are shown in Fig. 3.

Discussion

Based on the evidence of increased DNL and inflammatory signaling in cancer onset and progression[22,24,25,48–50], it is clear that lipid metabolism in BC patients should be properly analyzed, not only to personalize the traits of the cancer metabolism but also to highlight metabolic features of each cancer subtype and identify the most appropriate intervention[51,52].

The present study reports the results of an assessment panel combining RBC membrane lipidome profile[30,35–37,53], nutritional data, BMI, and body composition parameters, such as fat mass percentage (FM%) and phase angle (PA°), pointing to the fatty acid metabolism and formation of the cell membrane (see Supplementary Fig. 1). This approach covers molecular-disease implications that are still missing but can have a high potential in clinical practice for personalized diagnostics and interventions in newly diagnosed early BC patients[7,18–21].

In our BC cohort, divided into three groups according to BMI, the assessment panel realized a new stratification in early BC patients, highlighting NW patients. The RBC membrane lipidome profile of NW BC patients had significantly higher PUFA levels and lower SFA values than healthy NW controls and OB BC patients (Fig. 2, Table 2, and Supplementary Table 3). The membrane lipidome profile of the OW BC group kept the same characteristics as the NW BC one but with less statistical significance for PUFA and PUFA omega-6 increases ($p = 0.03$ vs $p \leq 0.0001$, $p = 0.02$ vs $p = 0.0002$, respectively, Table 2). When all BC patients were separated by BMI < 25 and BMI ≥ 25 (full data in Supplementary Table 4), the SFA decrease and PUFA omega-6 increase were again characteristics of the profile of normal-weight individuals. Such a membrane lipidome profile of NW patients expresses a higher membrane fluidity status, strictly related to proliferation signaling[22,24] and an enhanced response to carbohydrate intakes via insulin receptor functions[54,55]. The role of PUFA in membrane fluidity regulation is thought-provoking: higher levels of MUFA, endogenously formed by desaturase enzyme (see Supplementary Fig. 1), are well-known in cancer and affect cell membrane structure and signaling, boosting cell proliferation and invasiveness[22–24]. In our early-stage BC cohort, the unsaturation increase comes instead from the PUFA pathway, specifically from omega-6 fatty acids. These data point attention to PUFA levels in the early stages of the disease. On the other hand, the early-stage BC patients with an obesity condition show a significant SFA increase, which suggests an influence on the membrane fluidity in the opposite direction compared to NW patients ($p = 0.0038$ in Table 2; see also Supporting Tables 3 and 4). It is reasonable to hypothesize that, in these patients, the obesity condition

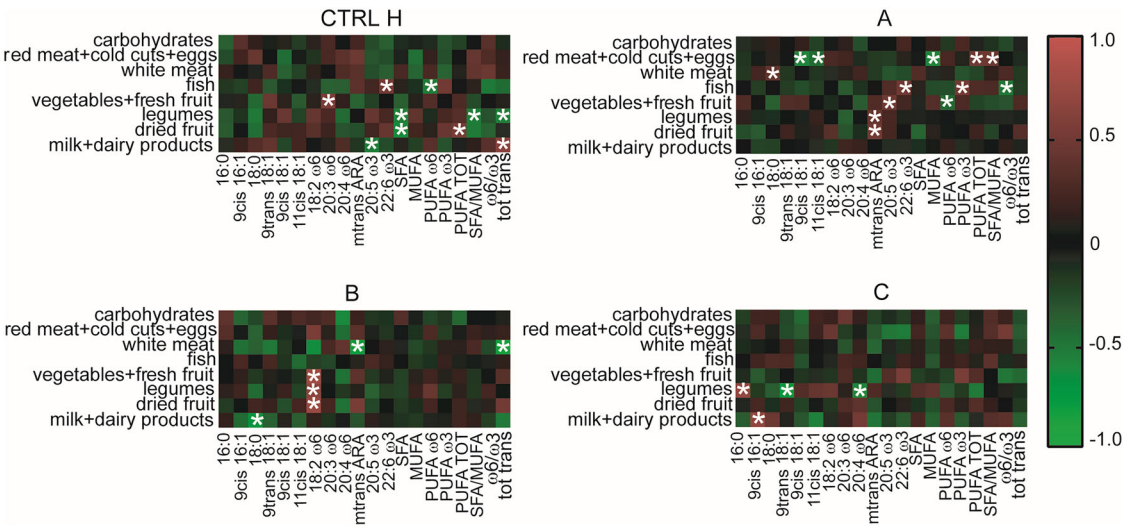

Fig. 3 | Heatmaps of the fatty acid-food frequency correlations. The RBC membrane fatty acid data and indexes (for full data see Supporting Table 3) were correlated to food frequency (for full data see Supporting Table 1) for the three breast cancer (BC) cohorts: **A** normal weight (NW, BMI = 18.5–24.9; $n = 26$); **B** overweight (OW, BMI = 25–29.9; $n = 11$); **C** obese (OB, BMI = 30–39.9; $n = 13$). In the Figure the CTRL is referred to CTRL-H group, i.e., age-matched healthy NW women recruited among the Hospital's employees ($n = 15$). The asterisks indicate significant correlations, and the *p* values are reported in Supporting Table 5.

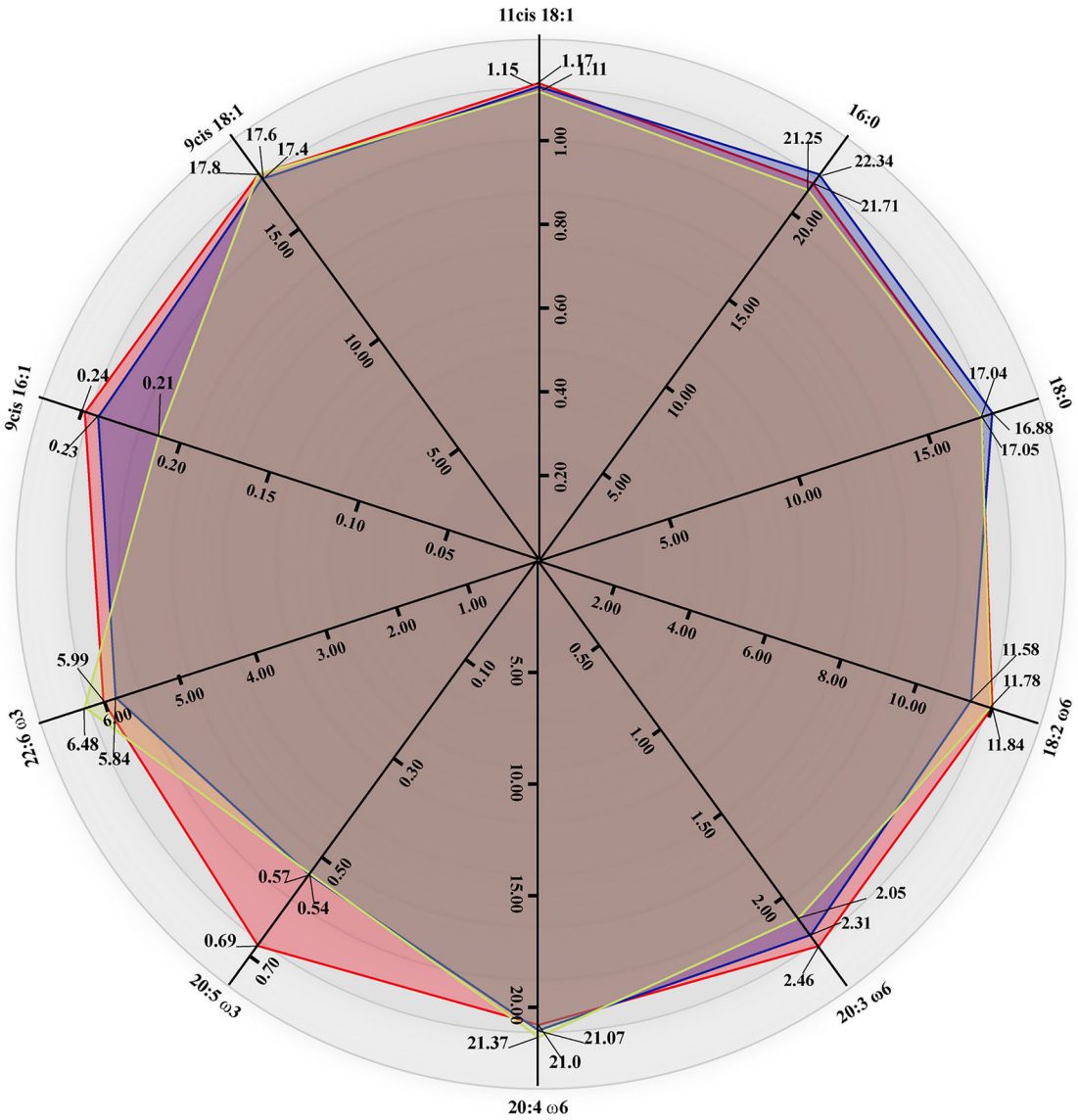

Fig. 4 | Spider maps of the membrane fatty acid profile in breast cancer patients divided by their BMI. The ten fatty acid constituents of mature RBC membrane phospholipids for the three breast cancer (BC) patients' cohorts with different body mass index (BMI) status are represented as spider maps: yellow, normal weight, NW; red, overweight, OW; blue, obese, OB. The mean value of each fatty acid interval is reported as the corner of the spider maps (full data in Supplementary Table 3).

Table 3 | Significant changes of the RBC membrane fatty acid data for normal weight breast cancer patients (BC NW, BMI = 18.5–24.9) grouped according to the Fat Mass percentages (FM%) as obtained by Bioelectrical Impedance Analysis

Fatty acid (usual name)	BC NW ($n = 12$) FAT MASS% ≤ 24.9% rel. quant. (mean ± sd)[†]	BC NW ($n = 6$) FAT MASS% 25–29.9% rel. quant. (mean ± sd)[†]	BC NW ($n = 8$) FAT MASS% ≥ 30% rel. quant. (mean ± sd)[†]
9t-C18:1 (elaidic acid)	0.09 ± 0.02	0.08 ± 0.02	0.07 ± 0.02[b]
C18:2 omega-6 (linoleic acid)	12.49 ± 1.43	10.88 ± 1.55[a]	11.99 ± 1.71
Mono-trans C20:4 (mono-trans arachidonic acid)	0.09 ± 0.02	0.06 ± 0.03[a]	0.07 ± 0.02[b]
Total Trans (elaidic acid + mono-trans arachidonic acid)	0.17 ± 0.03	0.14 ± 0.04	0.12 ± 0.03[c]

[a]Fatty acids are reported from the gas chromatographic analysis (GC) after the transformation of membrane phospholipids of mature RBC into fatty acid methyl esters (FAME); values are expressed as relative percentages (mean ± sd) of the quantitative values of each fatty acid obtained by calibration curves of the standards.
Significance (unpaired *t*-test): FM% 25–29.9 *vs* ≤ FM% 24.9 [a]$p \le 0.021$; FM% 30 *vs* ≤ FM% 24.9 [a]$p \le 0.021$; [b]$p \le 0.043$; [c]$p = 0.0018$.
Supplementary Table 6 shows the full data set.

Table 4 | Significant changes of the RBC membrane fatty acid data for normal weight breast cancer patients (BC NW, BMI = 18.5–24.9) grouped according to the Phase Angle [PA(°)] as obtained by Bioelectrical Impedance Analysis (BIA)

Fatty acid (usual name, acronym)/ Indexes	BC NW (n = 19) PA(°) ≤ 5.5% rel. quant. (mean ± sd)†	BC NW (n = 7) PA(°) ≥ 5.6% rel. quant. (mean ± sd)†
9t-C18:1 (elaidic acid)	0.07 ± 0.02	0.09 ± 0.02[a]
C22:6 (docosahexaenoic acid, DHA)	6.74 ± 1.17	5.64 ± 0.99[a]
PUFA ω3	7.42 ± 1.42	6.17 ± 1.07[a]
ω6/ω3	4.94 ± 1.04	6.05 ± 1.01[a]

†Fatty acids are reported from the gas chromatographic analysis (GC) after the transformation of membrane phospholipids of mature RBC into fatty acid methyl esters (FAME); values are expressed as relative percentages (mean ± sd) of the quantitative values of each fatty acid obtained by calibration curves of the standards.
Significance (unpaired t-test): PA (°) ≦ 5.5 vs PA (°) ≧ 5.6: [a]p ≦ 0.045.
Supplementary Table 7 shows the full data set.

causing an SFA increase can, in principle, counterbalance the PUFA fluidity contribution to the membrane, perhaps creating a membrane asset less favorable to cancer proliferative outcomes[5,56].

It must be underlined that the increase in PUFA of our BC cohort is due to the omega-6 arachidonic acid, which is known to play several roles in cancer[10,22]; however, not always the PUFA increase depend on arachidonic acid levels. The availability of the membrane lipidome profile can give an exact indication of the fatty acid status and changes of the involved PUFAs, that play very different biological consequences. In our study, we observed that: (a) the AA increase influences the increase of peroxidation index (PI) in NW and OB BC groups (Supplementary Table 3), meaning a higher propensity of lipid peroxidation, a process associated with poor prognosis parameters in breast cancer[57]; (b) the fate of AA released from membrane phospholipids is to be transformed into bioactive lipid mediators (BAL), known to increase signaling for cell proliferation and invasiveness[10,13,17,22,24]; (c) on the other hand, it is worth recalling that an overall PUFA increase—not only of AA—is associated with the ferroptosis process, studied as a control mechanism in different types of cancer[58]. From all these considerations, the membrane lipidome analysis results as a relevant marker of lipid metabolism, precisely informing on the balance of MUFA and PUFA in the fatty acid pool available for the phospholipids and the membrane formation, especially related to the fluidity and signaling contributions. It can be asked whether the increase of arachidonic acid is more connected to cancer or obesity conditions, since it is known that obesity expresses inflammatory markers[2,4,27,30].

From the point of view of patient management, our results suggest a novel approach for the follow-up of cancer patients' diets; indeed, the omega-6/omega-3 ratio from foods has an important place in epidemiology and clinical research, involving both prevention and therapeutic use of PUFA balance for avoiding inflammatory consequences in human health[13,59]. The proposed assessment panel can support a personalized calculation of the most appropriate dietary balance among omega-6 and omega-3 precursors from the diet (foods containing linoleic and alpha-linolenic acids as precursors, respectively). Moreover, this approach will open the possibility of a follow-up by the membrane lipidome analysis for the effects of dietary regimes at the personalized level, highlighting those metabolic transformations able to induce a favorable fatty acid remodeling of the membrane, important in cancer to control signals from MUFA and PUFA.

For the first time, the correlations of food categories were evaluated with the membrane fatty acid levels found in the patients and geographically matched controls. The correlations heatmaps reported in Fig. 3 indicate that the higher the BMI of BC patients, the lower the foods affecting the membrane profile. Therefore, the NW BC group is the most influenced by their dietary intakes reflected in the RBC membrane molecular asset, whereas the OB patients show only one type of food (legumes) correlated negatively with C20:4 (arachidonic acid) and positively with C16:0 (palmitic acid) (Fig. 3). In the NW cohort, it is remarkable to find the influence of fish intake on the increase of DHA (docosahexaenoic acid, C22:6) levels and total PUFA levels (p = 0.01 and p = 0.008, respectively) and the decrease of the omega-6/omega-3 ratio (p = 0.012). This result goes in the known direction of the anti-inflammatory omega-3 effects[59]. As expected from their lipid contents[21], meat/cold cuts gave a negative correlation with MUFA and a positive with PUFA and SFA/MUFA ratio, but only in the normal weight patients. Remarkably, the food correlation for the membrane lipidome profile is lost progressively going from NW to OW and OB BC patients, and this likely confirms the role of fat depots in membrane turnover[2,4,26]. No significant correlations between carbohydrate intake and fatty acid levels were found in the BC patients. The condition of an early-stage cancer disease can have influenced the observed outcome, and a larger population study is necessary to confirm these initial findings.

Moreover, statistical significance could be affected by both the variability of the foods among patients and, more importantly, by the limited number of the population.

Using the accurate chemical and analytical protocols for the RBC membrane lipidome characterization, we could have information on two geometrical trans fatty acid isomers, i.e., trans-C18:1 (elaidic acid) and mono-trans arachidonic acid in our BC cohort. Trans fatty acids (TFA) can have two origins: (a) from processed fats in foods, already known to be associated with cancer risk[60,61], and (b) from an endogenous isomerization of natural cis lipids due to an increased free radical stress[45–47]. As mentioned in the Introduction, in the erythrocyte membranes of women from the Nurses' Health Study II (NHSII) the levels of TFAs (trans fatty acids), saturated fatty acids, dairy-derived fatty acids, and omega-3 PUFAs were associated with BC risk among obese/overweight women, but not among women overall[31]. In our study, these unnatural isomers were not significantly different in BC patients of different BMIs and were further evaluated in correspondence with BIA data.

For the first time, BIA and membrane lipidome data were combined in an assessment panel of a BC cohort, thinking of the process of compartmentalization, the membrane properties, and the inflammatory status. The bioimpedance information in breast cancer patients was associated with lymphedema[6] or malnutrition, and general factors like lifestyle and quality of life, but also with the effect of chemotherapies (reduction of PA°) or survival, as described in the recent meta-analysis of 16 studies on breast cancer patients[62]. This is the first time that the FM% and PA° values in the case of normal-weight early breast cancer patients are reported for their significant correlations with the fatty acid levels. Fatty acid composition in cell membranes varied significantly in breast cancer patients with different body fat percentages (FM%) and body fat distribution (PA°), as revealed in Tables 3, 4. Notably, individuals with high body fat (FM% ≥ 30%) exhibited lower levels of all measured TFAs compared to those with lower body fat. These TFAs included 9trans C18:1, mono-trans arachidonic acid, and the total trans fatty acid content ($p \leq 0.021$, $p \leq 0.043$, and $p \leq 0.0018$, respectively) as shown in Table 3. TFAs, especially those derived from oleic and arachidonic acids, are produced within the body under conditions of oxidative stress[47]. Such stress triggers antioxidant responses involving sulfur-containing molecules, which in turn generate sulfur-centered radicals. These radicals can then convert the normal cis-configuration of unsaturated fats into the less common trans configuration found to be connected to various health conditions[46]. The analysis of membrane lipids in BC NW patients revealed evidence of higher inflammation compared to other BMI groups. Inflammation is known to increase the production of free radicals and is also linked to higher body fat levels[63]. Inflammation and early-stage breast cancer conditions might lead to a decline in the antioxidant activity of thiol compounds, and this potential impairment is evidenced in the TFA levels of BC NW patients with high body fat (FM% ≥ 30%). However, definitive conclusions cannot be drawn due to the limited number of patients in the study and the absence of data on thiol levels in our patient group. For the phase angle (PA°), PA° ≧ 5.6 correlated with a significant

decrease in the total omega-3 levels and an increase in the omega-6/omega-3 ratio (Table 4). PA° is an indicator of cellular health, integrity, and hydration[63]. For its relationship with systemic tissue inflammation, the shown correlation with anti-inflammatory PUFA levels gives clinical relevance to these body measurements[63,64].

Overall, our results are interesting for the stratification criteria of early-stage BC patients, evidencing inflammatory markers in normal-weight patients, a group normally not considered at risk for metabolic implications in cancer management, and whose body composition is rarely assessed in clinical practice.

Several molecular signatures of RBC membranes affecting cancer conditions have been previously reported in the literature and represent an important background for the advancements proposed by our study[12,35,36]. Pala and Berrino evaluated the RBC membranes in an Italian cohort of BC patients, showing an increase in MUFA biosynthesis and a decrease in the Saturation Index (stearic acid/oleic acid ratio)[41]. In a Chinese patients' cohort (322 cases, 1030 controls), RBC membrane lipidome showed a significant association between the contents of palmitic, gamma-linolenic, palmitoleic and vaccenic acids and the risk of BC, whereas the total omega-3 fatty acids, eicosapentaenoic acid (EPA) and the palmitic acid/palmitoleic acid ratio were found to be associated with a significantly lower risk of BC[42]. In a Spanish cohort of newly diagnosed BC patients, the RBC membrane lipidome reported the increase of MUFA and omega-6 PUFA, in particular AA, not connected with the levels of fat intakes, thus confirming the metabolic implications for increased lipogenesis, desaturation, and inflammation, as in our Italian cohort[37].

This research enhances our understanding of how breast cancer relates with diet and fatty acid metabolism to form cell membranes, and the connections with the anthropometric characteristics of body weight and body composition that can better stratify patients and their risk. We introduce a new way to categorize early-stage breast cancer patients based on their membrane lipidome combined with body weight and composition. This approach reveals risk conditions in early breast cancer patients with normal weight, evidencing specific nutritional and metabolic markers connected with inflammation[10,22]. The proposed simple, non-invasive protocol can be used in clinical settings, and its significance can be deepened in large-scale studies. The results can be translated into personalized dietary recommendations, with emphasis on nutritional needs of normal-weight breast cancer patients, which are often overlooked during their treatment. We recognize that dietary restrictions can cause psychological distress[65]. Therefore, personalizing the reasons for and types of foods to consume can increase patient motivation and acceptance of dietary recommendations.

Our study has some clear limitations, mainly due to the limited patient number and the lack of correlations between RBC membrane lipidome profiles and data, such as plasma cytokines, for evaluation of the inflammatory engagement of metabolism in BC patients, being very strong their association with cancer risk and outcomes[66]; the expansion of the biochemical panel to other markers such as serum homocysteine, as well as to deficiencies in folic acid, vitamin B12, and vitamin D, would be advisable for a comprehensive profile of the molecular status in these patients[67,68]. We did not include the follow-up of this BC patient group after surgery, estimating neglectable perioperative risks and complications to see differences in our small population, and also because the monitoring since enrollment was too short to draw any reasonable conclusion about the oncological outcome, due to the presence of >70% of the patients with not-so-aggressive disease (>70% of luminal breast cancer). Among the points of strength of the study, the major one is the prospective design of a homogeneous population of patients, all affected by early BC at a precise time of their cancer journey, right before the upfront surgery. This setting minimizes the risks for confounding factors, such as previous or concomitant systemic or local treatments for the same disease. In our planning, together with the larger and longitudinal study of the assessment panel, a continuous follow-up of the BC cohort is planned to record upcoming comorbidities, tumor recurrences, secondary cancers, cancer-related deaths, and overall survival.

In conclusion, the assessment panel we suggest focuses on combining non-invasive measurements of body composition (anthropometry and BIA), nutrition, and the membrane lipidome profile. This easy-to-use approach can help categorize patients during their initial visit and early stages of cancer. The results have important implications for predicting outcomes, treatment planning, and managing inflammation. Additionally, this information can quickly impact lifestyle and dietary choices, particularly for patients with a normal weight.

Methods

Study design

This observational prospective study was conducted following the ethical principles of the Declaration of Helsinki and obtained ethical clearance from the ethics committee at Fondazione Policlinico Universitario A. Gemelli IRCCS (FPG) (n.4663). Informed consent was obtained from each patient. The patients, all of Caucasian ethnicity and aged range 29–64 years, were enrolled during the pre-operatory assessments according to our integrative model, already described in a previous publication, through a preliminary psycho-oncological distress evaluation, a brief lifestyle interview, anthropometric measurements, and body composition analysis[69]. In this context, a blood sample was carried out in the morning under fasting conditions. The enrollment was carried out at FPG, Center for Integrative Oncology in Rome, according to inclusion/exclusion criteria described in the Consort diagram (Fig. 2), reaching a final cohort of 50 (fifty) patients, diagnosed with early-stage BC (I-II) undergoing upfront surgery and with no previous neoadjuvant systemic treatment.

Patients were divided, according to BMI, into three groups: NW (BMI = 18.5–24.9) $n = 26$; OW (BMI = 25–29.9) $n = 11$; OB (BMI = 30–39.9) $n = 13$.

Another cohort of 15 NW healthy women aged 22–54 (BMI = 18.5–24.9) recruited among the Hospital's employees was included in the study as geographically matched control (CTRL H). Fatty acid data were also compared with the data from an anonymized database of healthy NW age-matched Italian women ($n = 26$, CTRL DB)[12], and with interval values known for the RBC membrane fatty acids benchmark[12,14,33]. The two databases of healthy controls were merged to obtain larger control groups (CTRL) of healthy age-matched and normal-weight women ($n = 41$) for the membrane lipidome data (Fig. 3 and Supplementary Table 3).

Isolation of fatty acids from RBC membrane phospholipids and gas chromatographic analysis

Fatty acid-based membrane lipidome analyses were performed by the Lipidomic Laboratory of Lipinutragen (Bologna, Italy). Blood samples (0.5 mL) collected in vacutainer tubes with ethylenediaminetetraacetic acid (EDTA) were processed following a protocol that included the use of robotic equipment as described in previous studies[30,35–38]. Briefly, the mature cell fraction was isolated based on the higher density of the aged cells and diameter controlled by the cell counter (Scepter 2.0 with Scepter™ Software Pro, EMD Millipore, Darmstadt, Germany)[34]. The steps of phospholipid extraction and derivatization to fatty acid methyl esters (FAME) were performed to transform membrane glycerophospholipids (mainly phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidyl inositol, and plasmalogens)[39] and examine up to 90% of the RBC membrane lipidome without degrading the PUFA components. In the analytical protocol, fatty acids are evaluated quantitatively, by calibration of the gas chromatography (GC) peak areas with appropriate standards, and reported as quantitative relative percentages (% rel. quant.) of the total lipid quantity (100%)[30,35–38]. It is worth underlining that: (i) the Lipidomic Laboratory is recognized by the Italian Body of Accreditation (Lab 1836L, Accredia) to work in compliance with the ISO/EIC 17025 certification, concerning all work-up procedures made from the blood sample to the chemical analysis and the release of the final results referred to as the membrane lipidomic test; (ii) the protocol ensures reliability and repeatability of the analysis, with quality assurance (QA) checkpoints, as well as gives the inter-day and intra-day errors (<5%). In particular, PUFA

residues are not degraded during the treatment of membrane phospholipids under alkaline conditions, as ascertained for PUFA-containing plasmalogens, a lipid class that constitutes more than 20% of the total RBC lipid weight[39]; (iii) the GC conditions ensure separation of positional and geometrical isomers of MUFA and PUFA, reported as biomarkers in cancer and obesity[30,37,38].

Statistical analysis

Statistics were performed using GraphPad Prism 8.0 software (GraphPad Software, Inc., San Diego, CA, USA) applying unpaired *t*-test, one-way ANOVA test, and Spearman correlation with a 95% confidence interval. Spidermap graphs were obtained by Microsoft Power BI-Past3.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

All authors confirm that they had full access to all the data in the study and accept responsibility to submit it for publication. All authors read and approved the final version of the manuscript. C.F., R.F., R.M., and S.M. conceived and designed the study. C.F., A.F., A.S., and S.M. were responsible for data collection and curation. M.M.R., C.R., G.F., CM, A.D.M., G.B., and C.F. were responsible for laboratory procedures and made all formal analyses. C.F., S.M., and R.M. were responsible for funds used for this study. C.F. and A.S. established and supervised lipidomics methodologies of this study; A.F. and S.M. established and supervised nutritional, bioimpedance, and clinical methodologies of this study. C.F., R.M., G.F., and S.M. supervised the various phases of the study. C.F., R.F., S.M., and R.M. wrote the original draft, the revised versions, and collected the contributions from all authors.

Competing interests

C.F. is the co-founder and Scientific Consultant of the Lipinutragen company, born as a spin-off of the National Council of Research, but declares no non-financial competing interests. All other authors declare no financial or non-financial competing interests.

Additional information

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