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Short-chain fatty acids are key mediators of the favorable effects of the Mediterranean diet on intestinal barrier integrity: data from the randomized controlled LIBRE trial

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ABSTRACT

Background: The Mediterranean diet is associated with the prevention of diabetes, cardiovascular disease, and cancer, all of which are linked to intestinal barrier impairment.

Objectives: Here, we hypothesize that the Mediterranean diet, possibly via the induction of short-chain fatty acids (SCFAs), improves intestinal barrier integrity. Furthermore, we aim to establish novel personalized nutrition advice based on machine learning algorithms.

Methods: We studied 260 women with intestinal barrier impairment. The women were allocated to follow either a Mediterranean diet or a control diet for 3 mo. Intestinal permeability was assessed by measuring lipopolysaccharide binding protein (LBP) in plasma and zonulin in feces. SCFA concentrations were analyzed in feces. Bi- and multivariate analyses and machine learning algorithms (random forest classification) were conducted.

Results: Particularly in the intervention group, adherence to the Mediterranean diet increased, whereas plasma LBP and fecal zonulin concentrations decreased (all $q < 0.001$ for the intervention group, all $q < 0.1$ for control group). In the intervention group, fecal SCFA concentrations increased (propionate + 19%; butyrate + 44%; both $q < 0.001$). Multivariate analyses showed that adherence to the Mediterranean diet was associated with SCFA concentrations (all $q < 0.001$) and inversely associated with LBP and zonulin concentrations (all $q < 0.02$). Mediation analyses identified propionate and butyrate as the key mechanistic link between diet and intestinal permeability integrity. Accordingly, using baseline SCFA data, we could predict the effect of the Mediterranean diet on intestinal permeability using a machine learning algorithm (receiver operating characteristic AUC: 0.78–0.96).

Conclusions: Our data suggest that SCFAs are key mediators for the relation between diet and gut health. Assessment of SCFAs may form a basis for personalized nutrition in future clinical care. These results need to be verified in larger studies powered for this purpose, comprising different study populations. The trial was registered at clinicaltrials.gov as NCT02087592 and NCT02516540. *Am J Clin Nutr* 2022;116:928–942.

Keywords: gut barrier, short-chain fatty acids, Mediterranean diet, dietary fibers, mediation analyses, machine learning, precision nutrition, personalized nutrition, breast cancer

Introduction

The Mediterranean diet has been shown to be beneficial in gastrointestinal, cardiometabolic, and malignant diseases (1–6). This dietary pattern is well defined and characterized by a high intake of fiber-rich plant foods such as fruit, vegetables, legumes, and nuts and a high intake of olive oil and seafood, while the consumption of red meat and confectionery is reduced (7). So far, the underlying mechanisms are not fully understood, yet the diet's fatty acid pattern and its large amounts of polyphenols and fibers have been proposed as the most relevant dietary factors for the beneficial effects of the Mediterranean diet on health.

The intestinal barrier is a key feature of intestinal and overall health. Thus, intestinal barrier impairment has been associated with those diseases that profit from the Mediterranean diet (8, 9). Therefore, we hypothesized that adherence to the Mediterranean diet could improve intestinal barrier integrity. Apart from diseases, permeability disorders have been linked to aging (10), overweight (11), and low or extreme physical activity (12). Such findings clearly establish the central role of the gut barrier in health and disease, yet the mechanisms by which barrier function is regulated are not well known.

We recently showed that plasma lipopolysaccharide binding protein (LBP) and fecal zonulin concentrations are suitable biomarkers for intestinal permeability in humans (13), whereas other markers, such as fecal albumin and calprotectin, are less valid in this respect (13–15).

In the present study, we investigated the effect of a change to the Mediterranean diet on intestinal barrier function in a cohort of 260 women with breast cancer gene (BRCA) 1/2 mutations and impaired barrier function (16). The women participated in the Lifestyle Intervention Study in Women with Hereditary Breast and Ovarian Cancer (LIBRE) and were studied at baseline and 3 mo after the start of intervention. Here, we hypothesize that the Mediterranean diet improves intestinal barrier integrity via the induction of short-chain fatty acids (SCFAs). Furthermore, we aimed to deduct personalized nutrition advice from the data.

Methods

LIBRE study

From 2014 until 2018, 432 women were recruited to participate in the LIBRE study (17), 260 of whom provided all dietary questionnaires as well as fecal and blood samples at baseline and after the 3-mo intervention and were therefore eligible for our current study. A total of 124 of the individuals were allocated to the intervention group (trained for the Mediterranean diet and exercise), and 136 were allocated to the control group (lectured once on a healthy diet) (17), as shown in **Figure 1** and **Supplemental Figure 1**. Randomization was performed with a 1:1 ratio using an Internet-based central randomization system, stratifying for center, breast cancer disease status, age, physical activity, and BMI (in kg/m^2). The LIBRE study is a 2-armed, randomized, controlled, and openly designed clinical multicenter trial conducted in Germany. It examines the effect of the Mediterranean diet combined with physical activity in a cohort of women at genetic risk for breast and ovarian cancer. Primary outcomes comprise BMI, adherence to the Mediterranean diet, and physical fitness (expressed as the ventilatory threshold 1, VT1). VT1 is an objective marker of physical fitness and represents the level of physical activity at which blood lactate accumulates faster than it can be cleared in spiroergometry. Further outcomes comprise, among others, gastrointestinal metabolites and circulating markers of inflammation and endotoxemia, as shown in the present analysis. Here, we included individuals from the LIBRE-1 feasibility trial (18), started in 2014, and the LIBRE-2 main study, started in 2015 (17). In both studies, the inclusion criteria comprised female sex, age between 18 and 69 y, a pathogenic BRCA1 or BRCA2 mutation, and written informed consent. The exclusion criteria comprised a BMI <15, pregnancy, veganism, or food allergies not allowing adherence to a Mediterranean diet. In the LIBRE study, both women with a history of breast cancer and women without previous breast cancer who were at high risk were included. These 2 subgroups showed no difference in age, BMI, diet, SCFAs, intestinal permeability biomarkers, or physical fitness (data not shown).

The intervention group received a 3-mo intervention program consisting of biweekly courses on the Mediterranean diet and endurance-oriented training. The control group received 1 initial lecture on the dietary recommendations of the German Nutrition Society and 1 lecture about the benefits of physical activity on breast cancer incidence, prognosis, and recurrence. The ethics review board of the Technical University of Munich approved the study protocols [REFs: 5686/13 (18) and 5686/13 s (17)]. The trial has been registered at clinicaltrials.gov as NCT02087592 and NCT02516540.

Dietary measurements

Two questionnaires were used to assess dietary behavior and intake. The Mediterranean Diet Adherence Screener (MEDAS) was developed and validated in the Prevención con Dieta Mediterránea studies (19). We translated the English version into German and validated the German version again (20). The MEDAS comprises 14 dichotomous questions. Each question is scored with either 0 or 1, with 1 representing the answers that are related to the Mediterranean diet.

Two MEDAS questions (Q5: How many servings of red meat, hamburger, or meat products do you consume per day? <1 or ≥1; Q13: Do you prefer to eat chicken, turkey, or rabbit instead of beef, pork, hamburgers, or sausages? Yes or no) implied meat consumption and were occasionally left unanswered by vegetarians, which represented 8% of the cohort. If Q5 was unanswered by vegetarians (applicable to 5% of all 260 individuals), we set the answer to 0 points, corresponding to <1 meat servings. Q13 was not applicable to vegetarians. Because many vegetarians left it unanswered, we decided to omit this question for all vegetarians, who, by doing so, achieved a maximum score of 13 instead of 14. We therefore present the results from the MEDAS as the MEDAS-Score, with the percentage of the achieved score related to the achievable score.

This study is part of the FiberTAG project (www.fibertag.eu), which was initiated from the European Joint Programming Initiative, "A Healthy Diet for a Healthy Life" (www.healthydietforhealthylife.eu). Funding for the team from the University of Hohenheim for the FiberTAG project derived from the German Federal Ministry for Education and Research (BMBF; grant no. 01EA1701). Other funding sources related to the project: The LIBRE study was funded by the German Cancer Aid (Deutsche Krebshilfe; http://www.krebshilfe.de; grant no. 110013 and 111888), Sphingotec GmbH, Senator Rösner Foundation, and Waltraut Bergmann Foundation. JW acknowledges support by the Science Foundation Ireland through a Centre award (APC/SFI/12/RC/2273_P2) to APC Microbiome Ireland and an SFI Professorship (19/RP/6853). The funding sources had no contribution in the design, implementation, analysis, and interpretation of the data of the LIBRE study and the present analysis.

BS and NKN contributed equally to the article.

Data described in the manuscript, code book, and analytic code will be made available upon request pending approval by the corresponding author.

Supplemental Figures 1–4 and Supplemental Tables 1–3 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn/.

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Abbreviations used: BRCA, breast cancer genes; LBP, lipopolysaccharide binding protein; LIBRE, Lifestyle Intervention Study in Women with Hereditary Breast and Ovarian Cancer; MEDAS, Mediterranean Diet Adherence Screener; MedD-Score, Mediterranean Diet Score; MLR, multiple linear regression; PERMANOVA, permutational analysis of variance; SCFA, short-chain fatty acid.

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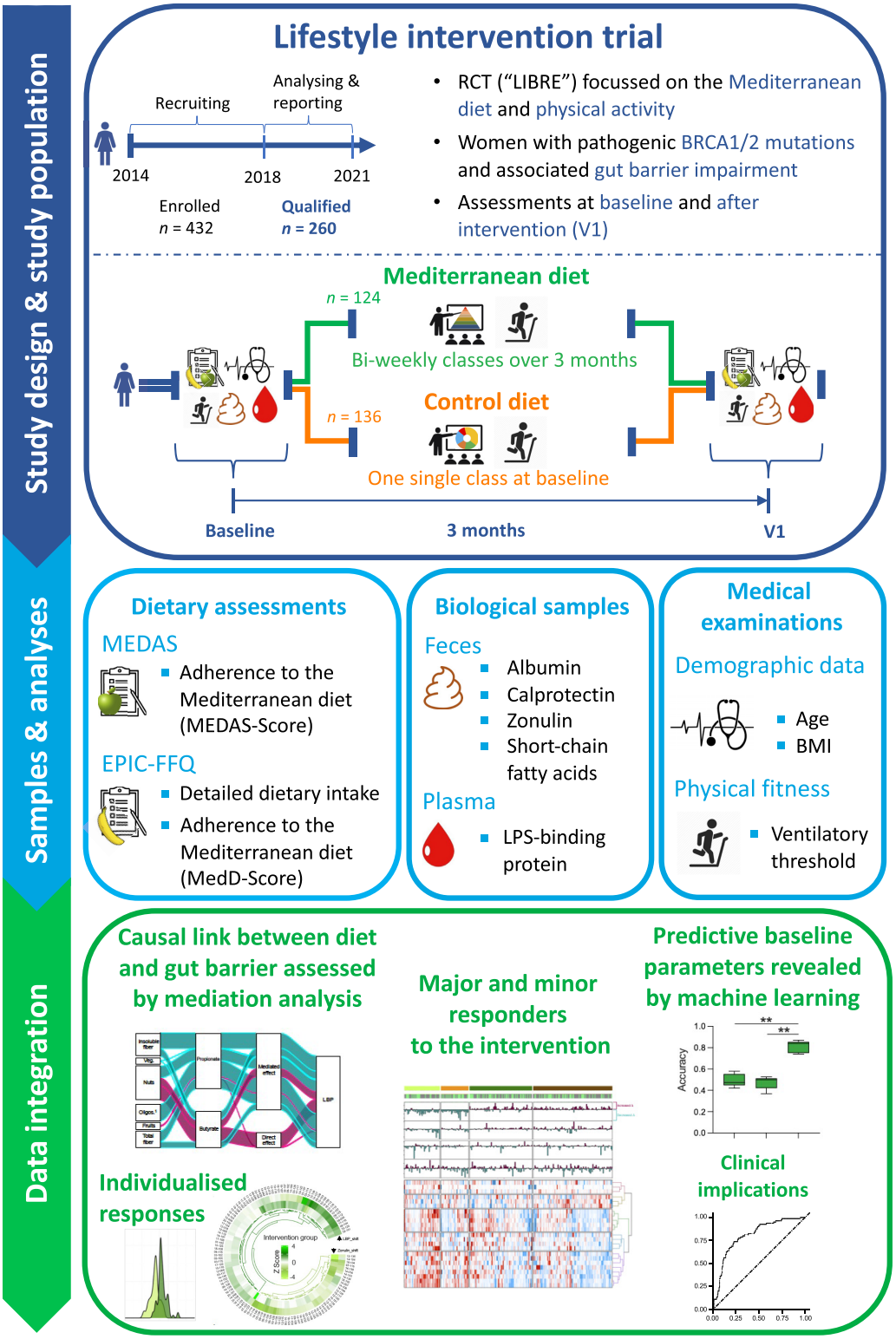


FIGURE 1 Experimental approach to assess the effect of a 3-mo lifestyle intervention on intestinal permeability. From the randomized controlled trial (RCT) Lifestyle Intervention Study in Women with Hereditary Breast and Ovarian Cancer (LIBRE), 260 women with pathogenic breast cancer (BRCA) gene mutations were included in the present evaluation comprising data assessed at baseline as well as after intervention (V1). Dietary habits were assessed by 2 independent questionnaires, the Mediterranean Diet Adherence Screener (MEDAS) and the European Prospective Investigation into Cancer and Nutrition FFQ, which constitutes the basis for the Mediterranean Diet Score (MedD-Score). Intestinal permeability was assessed by 4 biomarkers: the fecal biomarkers albumin, calprotectin, and zonulin, as well as plasma lipopolysaccharide (LPS) binding protein (LBP). Fecal concentrations of the short-chain fatty acids acetate, propionate, and butyrate were assessed by gas chromatography. Comprehensive medical examinations at the study visits provided demographic data, as well as age, BMI, and physical fitness, assessed by bicycle spiroergometry. Multivariate analyses and machine learning algorithms were conducted to assess individualized responses to the lifestyle intervention and to obtain novel insights into the mechanistic framework of how diet affects gut health.

The FFQ was established and validated by the European Prospective Investigation into Cancer and Nutrition consortium (21). It contains various questions about dietary behavior to assess it in a qualitative and quantitative manner, covering the previous 12 mo (baseline) or the 3-mo intervention phase, respectively. As an outcome, the FFQ provides the daily intake of food groups (such as fruit and vegetables) and nutrients (such as carbohydrates and fibers). We adjusted the FFQ data for energy intake according to Willett et al. (22). Because the FFQ does not provide information about adherence to the Mediterranean diet per se, we calculated the adapted Mediterranean Diet Score (MedD-Score) according to Trichopoulou et al. (23). This score ranges from 0 to 9, with higher scores indicating greater adherence to the Mediterranean diet.

Blood and fecal sample collection

Fasting plasma samples and fecal samples were collected between 2014 and 2018 and analyzed between 2017 and 2018. Prior to and after processing, the samples were stored at –80°C.

Intestinal permeability biomarkers

All biomarkers were analyzed using enzyme-linked immunosorbent assays following the manufacturer's protocols. Fecal biomarkers comprised albumin, calprotectin, and zonulin (REFs: K5600, K6330, K6750, K6927; Immundiagnostik AG). LBP (REFs: DY870-05 and DY008; Bio-Techne GmbH) was measured in plasma.

Fecal SCFA quantification

Fecal SCFA and dry mass methodology has been described in detail elsewhere (24). In brief, fecal samples were homogenized, diluted 1:4 in water, derivatized, and analyzed by gas chromatography. Fecal dry mass was determined by weighing oven-dried fecal samples. The SCFA data are expressed in relation to dry mass to overcome bias due to differing fecal water contents.

Statistical analyses

All univariate analyses were performed using Prism version 9.0.1 (GraphPad Software), whereas multivariate analyses, mediation, clustering, and machine learning were performed using R version 3.6.2 (www.r-project.org). A $P < 0.05$ and a false discovery rate (FDR)–adjusted P value (q) < 0.05 were considered statistically significant. All authors had access to the study data and have reviewed and approved the final version of the manuscript.

Differences between the intervention group and the control group were tested using the 2-sided Mann–Whitney U test (numerical data) or the χ^2 test (categorical variables); differences between time points were evaluated using the 2-sided Wilcoxon signed-rank test; differences between tertiles were tested using the Kruskal–Wallis 1-factor ANOVA on ranks. We calculated the shift (baseline concentrations subtracted from the respective values after the 3-mo intervention, Δ) to determine the change over time. Correlation analyses were performed using Spearman rank correlation coefficient. The receiver operating characteristic (ROC) curves are based on the Wilson–Brown method (25). Before performing multiple linear regression (MLR) models, the variables were tested for intercorrelations, and variables with a Spearman correlation coefficient >0.8 were excluded from the model.

In multivariate analyses, differences between groups were assessed by permutational analysis of variance (PERMANOVA) with 999 permutations using the "Adonis" function (26). Principal component analysis was used for visualization using "Factoextra" and "FactoMineR" (27). For missing values, the median value of all other values of the corresponding group and time point was used (<5% data for each parameter included was missing). Multiple testing was adjusted with an FDR of 5% according to Benjamini and Hochberg (28). A $P < 0.05$ and an FDR-adjusted P value (q) < 0.05 were considered statistically significant.

Mediation analysis

To explore the contribution of SCFAs in the association between dietary components and intestinal permeability biomarkers, we performed a model-based causal mediation analysis (29). Here, only the shift value was used (baseline concentrations subtracted from the respective values after intervention). Eighteen dietary factors significantly changed in the course of the intervention in either the intervention group and/or the control group and were included in the mediation analysis.

Model-based causal mediation analysis comprises a set of different linear regressions, as described in detail by Tingley et al. (29) and described briefly as follows. First, 3 screening linear regression models were performed. Main components of each of the 3 models were *1*) 1 *health outcome* (either zonulin or LBP) as a dependent variable and 1 predictive *dietary factor* (e.g., total fiber), *2*) 1 *potential mediator* (either propionate or butyrate) as a dependent variable and the same *dietary factor* from model 1, and *3*) the same dependent variable from model 1 and the *potential mediator* from model 2 as the first predictive factor and the *dietary factor* from model 1 as the second predictive factor. Second, the fitted objects from the screening linear models 2 and 3 were used as the main inputs to the "mediate" function in R using a nonparametric bootstrap option with 1000 simulations. All diagnostic plots and data transformation for the assessment of the assumptions were performed as follows. Linearity was checked by plotting residuals compared with fitted values; normal Q–Q plots were used to examine normality; homogeneity was checked by using scale-location plots, as suggested by Tingley et al. (29). An estimated coefficient of the *dietary factor* from model 1 was named a "single effect." Similarly, estimated coefficients of the *potential mediator* and the *dietary factor* from model 3 were named "mediated effect" or "direct effect," respectively.

A P value for each mediated effect, direct effect, and single effect was obtained from each set of linear regression models and adjusted within its same type of effect using the FDR method. An FDR-adjusted P value (q) <0.05 was considered statistically significant. Based on the adjusted P value, relations between *health outcome*, *potential mediator*, and *dietary factor* were classified as "complete mediation," "incomplete mediation," "no mediation," or "no effect". Furthermore, the direction of the effect (positive or negative value of the estimated β-coefficient of the predictor in the linear regression model) was determined whether it is

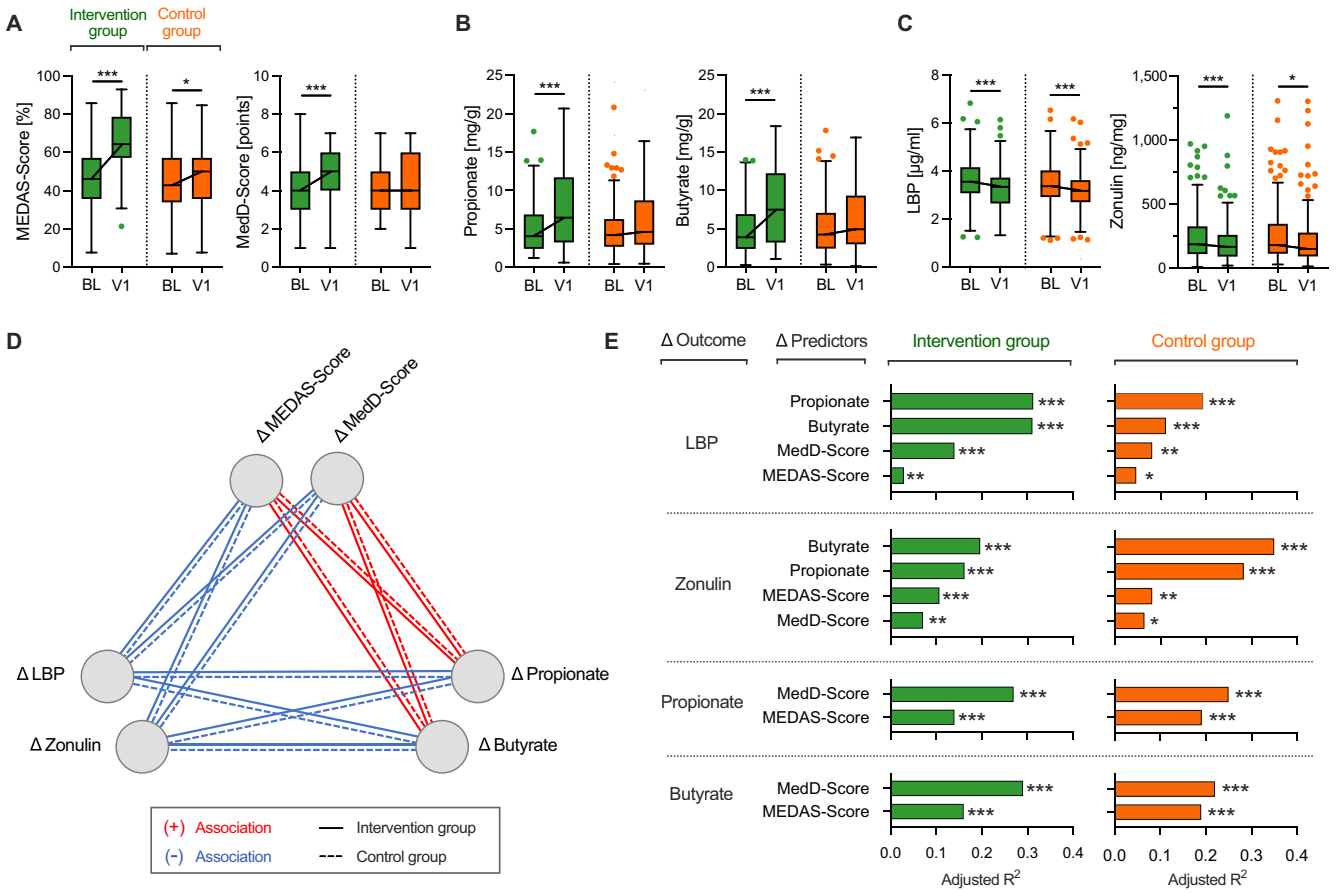


FIGURE 2 Causal relation between changes in diet, fecal short-chain fatty acids (SCFAs), and intestinal permeability. (A–C) Change of study-specific parameters from baseline (BL) to the end of intervention (V1) for (A) the Mediterranean Diet Adherence Screener (MEDAS)–Score and the Mediterranean Diet Score (MedD-Score); (B) fecal amounts of the SCFAs propionate and butyrate; (C) plasma lipopolysaccharide binding protein (LBP) and fecal zonulin. Tukey boxplots with medians, whiskers (1.5× IQRs), and outliers are shown in green (intervention group; $n = 124$) and orange (control group; $n = 136$). Changes in the course of the study are indicated by asterisks ($^{*}q < 0.05$; $^{***}q < 0.001$; Wilcoxon signed-rank test with a false discovery rate of 5%). (D) Network diagram showing the results of 24 multiple linear regressions using the shift (BL values subtracted from the respective values at V1, shown as Δ). (E) Overview of the results from the 24 multiple linear regressions, showing the adjusted R^2 as well as the 5% false discovery rate–adjusted level of significance ($^{*}q < 0.05$; $^{**}q < 0.01$; $^{***}q < 0.001$; $n = 260$). The adjusted level of significance and the adjusted R^2 shown are calculated by also including age, BMI, physical fitness, and previous disease state as potential confounders; however, here, we only show the shift in the outcomes and the main predictors (data shown in detail in Supplemental Table 2).

"changed" or "unchanged." "Changed" shows that the total effect has a different direction than the direct effect or the mediated effect. "Unchanged" shows that the total effect has the same direction as the direct effect and the mediated effect. Only dietary factors that are mediated (either completely or incompletely) with unchanged direction were visualized in alluvial plotsusing the "alluvial" (30) and the "ggalluvial" (31) packages.

Clustering

In order to explore the complex individualized response of plasma LBP and fecal zonulin to dietary intervention, we classified participants using a hierarchical clustering method based on the Euclidean distance of the individual shift (baseline concentrations subtracted from the respective value at study end) in LBP and zonulin. Volunteers who responded similarly were grouped together, forming 2 separated clusters of major and minor responders based on the "Ward.D2 criterion" (32). Different patterns in the response of the 2 barrier biomarkers are shown by different colors assigned in a dendrogram using the "dendextend" package (33), summarized as a circle form of a heatmap, showing the subject ID on the outer layer ("circlize" package) (34). The 2 detected clusters (low and high responders) were characterized by plotting their kernel density [estimated by the "geom_density" function in the "ggplot2" package (35)] on its z-score. Difference between the 2 clusters was confirmed by a PERMANOVA test with 999 permutations on Euclidean distance between the shift value ("vegan" package) (26).

Random forest classification

To identify the intrinsic factors that might contribute to the individualized responses of the intestinal permeability biomarkers that resulted in high and low responders, we applied a random forest classification (RFC) using different baseline features to distinguish 2 subgroups of response in each arm. Three different data sets containing baseline data were used as in input information for the machine learning algorithm: *food groups*, *nutrients* (the respective food components are listed in **Table 1**), and the SCFA *butyrate*. For each intervention arm,

TABLE 1 Baseline characteristics[1]

Characteristic	Intervention group	Control group
N	124	136
Diseased,[2] *n*	71	77
Age, y	43.9 (42, 46)	44.8 (43, 46)
BMI, kg/m^2	25.0 (24, 26)	25.0 (24, 26)

[1]Means (95% CIs) are shown unless otherwise indicated.
[2]Previously diagnosed with breast cancer.

individuals were randomly stratified into a training set and a validating set in a 7:3 ratio. Due to an unbalanced number between low and high responders in each arm (intervention group: $n = 45/79$, low responders/high responders; control group: $n = 35/100$ low responders/high responders), we performed 5 different machine learning models to counter possible bias. A gradient boosting machine method was used to handle potential interactions and nonlinearities that might appear in the input data. The models were tuned using cross-validation on the training set, repeating 3 times with 10 folds used in each cycle. The 5 models were "original" model (model 1), which does not consider the imbalanced data, whereas the "weighted" model (model 2) puts more weight on the minority class. On the other hand, the "up" (model 3) and "down" (model 4) models correct for the imbalanced data by randomly subsampling all low/high responders in the training set, implemented in the caret package (36). The last model, "ROSE" (Random Over-Sampling Examples, model 5), uses a hybrid method that downsampled in the high responders and synthesized new data points in the low responders, performed by the "ROSE" package (37). Each of the 3 baseline data sets (food groups, nutrients, butyrate) was evaluated by the 5 different models to obtain an accuracy value and AUC of the ROC for each model derived from the data from the validating set.

Results

Study population at baseline

At baseline, there was no substantial difference between the groups regarding clinical parameters (Table 1), diet, fecal concentrations of SCFAs, and intestinal permeability biomarkers (**Table 2**).

BRCA mutations are associated with gut barrier impairment

The study participants included showed a higher median concentration of fecal zonulin at baseline (178 ng/mg) compared with a healthy cohort of 36 women with a similar range of age and BMI (110 ng/mg, $P = 0.001$, Mann–Whitney *U* test) (13). In contrast, other barrier-related parameters, including plasma LBP, did not differ from those of healthy individuals (13, 38).

Baseline diet is associated with intestinal permeability

We allocated all included individuals into tertiles according to their baseline adherence to the Mediterranean diet, determined by 2 scores, the MEDAS-Score and the MedD-Score, derived from 2 independent questionnaires. Compared with individuals with low baseline adherence, individuals with high baseline adherence to the Mediterranean diet had lower concentrations of plasma LBP and fecal zonulin but not fecal albumin and calprotectin (**Supplemental Figure 2**).

Using MLR models, we examined whether these associations still existed when considering possible confounders such as age, BMI, physical fitness, or previous cancer disease state. The MLR models confirmed the inverse relation between adherence to the Mediterranean diet and the 2 biomarkers, plasma LBP (adjusted $R^2 = 0.14/0.08$; MEDAS-Score/MedD-Score) and fecal zonulin (adjusted $R^2 = 0.04/0.07$; MEDAS-Score/MedD-Score; **Supplemental Table 1**). Although the relation between the Mediterranean diet and zonulin occurred independently of the confounders, a higher BMI was associated with higher LBP concentrations. Again, these findings were consistent for both Mediterranean diet scores.

Effects of the intervention on SCFA concentrations and barrier biomarkers

Training of the study participants for adherence to the Mediterranean diet resulted in a clear increase in both the MEDAS-Score and the MedD-Score in the intervention group. In the control group, we observed a minor increase in the MEDAS-Score but not in the MedD-Score (**Figure 2**A), an effect also seen in previous analyses of the LIBRE study (39). In the intervention group but not in the control group, the mean intake of vegetables, legumes, fruit, and nuts increased. As expected, the mean intake of total, water-insoluble, and water-soluble fiber, as well as resorbable and nonresorbable oligosaccharides, increased in the intervention group but not in the control group (Table 2). The most pronounced difference between the intervention and the control groups was the change in the amounts of the fecal SCFAs propionate (+19% in the intervention group) and butyrate (+44%), whereas these parameters remained unchanged in the control group (Figure 2B). Fecal acetate concentrations did not change in any of the groups. Plasma LBP decreased in both groups similarly after the 3-mo intervention (–6% in both groups), and fecal zonulin also decreased in the intervention group (–30%) and by trend in the control group (–21%; Figure 2C), but the responses of the 2 groups did not differ significantly. There was no change in fecal albumin or fecal calprotectin in either of the groups. Furthermore, fecal dry mass did not change over time (data not shown). A detailed comparison of the intervention and control groups regarding data on diet, SCFAs, and the biomarkers is presented in Table 2. The mean BMI (–0.4, $P = 0.028$, $q = 0.090$) as well as the waist-to-hip ratio (–0.02, $P = 0.001$, $q = 0.005$) decreased in the intervention group but not in the control group (Table 2), whereas physical fitness did not change in either of the groups (data not shown).

Functional relation between diet, fecal SCFA concentrations, and intestinal permeability

To further investigate whether diet changes, fecal SCFA concentrations, and permeability biomarker concentrations might have a functional relation, we ran MLR models, including age, BMI, physical fitness, and previous cancer disease state as possible confounders. For this analysis, we did not include the SCFA acetate and the biomarkers albumin and calprotectin because

TABLE 2 Overview of the study-specific parameters at baseline and V1, 3 mo after baseline[1]

	Intervention group ($n = 124$)				Control group ($n = 136$)				
Characteristic	Baseline	V1	Δ (V1 – Baseline)	Within group, P (adj.)	Baseline	V1	Δ (V1 – Baseline)	Within group, P (adj.)	Between group (Δ), P (adj.)
MedD scores									
MEDAS-Score, %	48 (45, 50)	65 (62, 67)	17 (14, 21)	0.0001 (0.001)	44 (41, 47)	48 (45, 51)	4.1 (0.9, 7.2)	0.012 (0.047)	0.0001 (0.001)
MedD-Score, points	4.0 (3.7, 4.2)	4.8 (4.5, 5)	0.8 (0.4, 1.2)	0.0001 (0.001)	4.1 (4, 4.5)	4.4 (4, 4.6)	0.3 (–0.3, 0.4)	0.965 (0.986)	0.005 (0.021)
Food groups, g/d									
Potatoes	49 (42, 55)	43 (39, 48)	–5.3 (–11, 0.4)	0.109 (0.243)	45 (40, 50)	44 (40, 49)	–0.5 (–4.1, 3.2)	0.756 (0.900)	0.113 (0.245)
Vegetables	157 (143, 171)	182 (167, 198)	25 (6, 44)	0.001 (0.005)	147 (135, 159)	158 (144, 172)	11 (–2.9, 25)	0.21 (0.373)	0.104 (0.240)
Legumes	4.7 (3.9, 5.5)	7.6 (6.6, 8.7)	2.9 (1.8, 4.1)	0.0001 (0.001)	4.1 (3.4, 4.8)	4.4 (3.8, 5)	0.3 (–0.5, 1)	0.294 (0.484)	0.0003 (0.002)
Fruit	214 (191, 237)	265 (244, 287)	51 (27, 75)	0.0001 (0.001)	206 (184, 228)	216 (194, 237)	9.5 (–12, 31)	0.206 (0.370)	0.002 (0.009)
Nuts	4.7 (3.8, 5.5)	6.7 (5.9, 7.5)	2.0 (1.3, 2.8)	0.0001 (0.001)	3.8 (3.1, 4.5)	3.9 (3.2, 4.5)	0.1 (–0.5, 0.7)	0.276 (0.463)	0.0001 (0.001)
Olives	0.4 (0.3, 0.6)	0.9 (0.6, 1.1)	0.5 (0.2, 0.6)	0.0001 (0.001)	0.4 (0.3, 0.5)	0.4 (0.3, 0.5)	0.03 (–0.1, 0.1)	0.152 (0.304)	0.002 (0.009)
Milk	138 (117, 160)	139 (119, 159)	0.4 (–14, 15)	0.946 (0.986)	151 (130, 172)	139 (120, 158)	–12 (–29, 4.4)	0.197 (0.361)	0.433 (0.635)
Yogurt	57 (48, 67)	57 (47, 67)	–0.2 (–11, 11)	0.687 (0.858)	51 (42, 60)	43 (36, 50)	–8.2 (–15, –0.9)	0.068 (0.187)	0.428 (0.633)
Cereals	186 (173, 200)	204 (187, 221)	18 (1.3, 34)	0.040 (0.119)	191 (178, 204)	184 (170, 198)	–6.4 (–21, 7.8)	0.420 (0.626)	0.040 (0.119)
Red meat	26 (21, 31)	18 (15, 22)	–7.9 (–12, –3.5)	0.001 (0.005)	27 (22, 32)	25 (21, 30)	–2.2 (–7.1, 2.7)	0.464 (0.675)	0.077 (0.199)
Poultry	15 (13, 18)	16 (14, 18)	0.9 (–1.9, 3.6)	0.113 (0.245)	14 (12, 17)	14 (12, 16)	–0.8 (–3.3, 1.8)	0.713 (0.884)	0.146 (0.299)
Processed meat	33 (29, 38)	23 (20, 25)	–10 (–14, –7.2)	0.0001 (0.001)	31 (28, 35)	32 (28, 36)	1.2 (–2.1, 4.4)	0.833 (0.929)	0.0001 (0.001)
Fish	14 (12, 16)	22 (20, 25)	8.6 (6.4, 11)	0.0001 (0.001)	14 (12, 16)	13 (12, 15)	–0.9 (–2.8, 1)	0.623 (0.824)	0.980 (0.986)
Egg	13 (11, 15)	14 (12, 15)	0.9 (–0.4, 2.2)	0.167 (0.322)	12 (11, 13)	12 (11, 13)	–0.2 (–1.4, 1.1)	0.470 (0.678)	0.105 (0.240)
Vegetable oils	16 (13, 19)	20 (17, 23)	3.8 (–0.2, 7.8)	0.002 (0.009)	14 (12, 15)	15 (12, 18)	1.7 (–1.3, 4.6)	0.609 (0.812)	0.028 (0.090)
Confectionary	40 (35, 45)	30 (26, 34)	–9.7 (–13, –6.3)	0.0001 (0.001)	40 (36, 44)	35 (31, 39)	–4.7 (–8.1, –1.2)	0.007 (0.029)	0.069 (0.187)
Wine	68 (51, 85)	85 (68, 103)	17 (2.8, 32)	0.002 (0.009)	66 (49, 83)	64 (48, 81)	–1.6 (–11, 7.5)	0.866 (0.929)	0.034 (0.105)
Nutrients, g/d									
Plant protein	26 (24, 28)	27 (25, 29)	1.2 (–0.3, 2.8)	0.027 (0.095)	26 (24, 27)	25 (23, 26)	–1.2 (–2.5, 0.2)	0.258 (0.441)	0.009 (0.036)
Animal protein	43 (40, 46)	41 (39, 43)	–1.7 (–4.2, 0.7)	0.356 (0.555)	42 (40, 45)	40 (38, 43)	–1.9 (–4.4, 0.6)	0.132 (0.273)	0.764 (0.900)
Carbohydrates	209 (196, 222)	211 (198, 223)	1.5 (–8.9, 12)	0.585 (0.792)	207 (196, 217)	199 (187, 210)	–8.3 (–17, 0.3)	0.02 (0.072)	0.097 (0.239)
Cellulose	4.7 (4.4, 5)	5.2 (4.9, 5.6)	0.5 (0.2, 0.9)	0.0001 (0.001)	4.6 (4.3, 4.9)	4.6 (4.3, 4.9)	0.0 (–0.2, 0.2)	0.594 (0.798)	0.002 (0.009)
Lignin	1.4 (1.3, 1.5)	1.4 (1.3, 1.5)	0.0 (0.0, 0.1)	0.196 (0.361)	1.3 (1.2, 1.4)	1.3 (1.2, 1.4)	0.0 (–0.1, 0)	0.330 (0.519)	0.098 (0.239)
Water–ins. fibers	14 (13, 15)	17 (16, 18)	3.0 (1.8, 3.4)	0.0001 (0.001)	14 (13, 15)	14 (13, 15)	–0.1 (–0.7, 0.6)	0.775 (0.900)	0.0001 (0.001)
Water–sol. fibers	6.9 (6.5, 7.4)	8 (7.5, 8.5)	1.1 (0.6, 1.5)	0.0001 (0.001)	7 (6.6, 7.4)	6.9 (6.5, 7.3)	–0.1 (–0.5, 0.3)	0.931 (0.981)	0.0001 (0.001)
Polysaccharides	101 (95, 108)	106 (98, 113)	4.2 (–2.6, 11)	0.215 (0.378)	104 (98, 110)	100 (94, 107)	–3.3 (–9.3, 2.7)	0.316 (0.506)	0.104 (0.240)
Non–res. oligos	0.7 (0.4, 1)	1.8 (1.5, 2.1)	1.1 (0.8, 1.4)	0.0001 (0.001)	0.7 (0.4, 1)	0.5 (0.3, 0.7)	–0.1 (–0.3, 0.1)	0.977 (0.986)	0.0001 (0.001)
Res. oligos	2.4 (1.8, 3.1)	2.7 (2.3, 3)	0.3 (–0.4, 0.8)	0.0001 (0.001)	1.9 (1.5, 2.4)	1.8 (1.4, 2.1)	–0.1 (–0.4, 0.2)	0.737 (0.900)	0.0001 (0.001)
Total fibers	21 (19, 22)	26 (24, 27)	4.8 (3.5, 6.1)	0.0001 (0.001)	21 (20, 22)	21 (20, 22)	–0.2 (–1.2, 0.8)	0.858 (0.929)	0.0001 (0.001)
Fat	93 (87, 98)	91 (86, 97)	–1.4 (–6.9, 4.2)	0.777 (0.900)	89 (85, 93)	87 (82, 92)	–1.7 (–6.3, 2.9)	0.076 (0.199)	0.306 (0.496)
Barrier markers									
Albumin, ng/mg	2.8 (1.7, 3.8)	2.9 (2, 3.9)	0.2 (–1.1, 1.4)	0.652 (0.838)	2.2 (1.7, 2.7)	4.2 (1.9, 6.6)	2.0 (–0.4, 4.3)	0.965 (0.986)	0.777 (0.900)
Calprotectin, ng/mg	49 (41, 58)	46 (38, 55)	–2.9 (–12, 5.7)	0.417 (0.626)	55 (44, 67)	69 (50, 88)	13 (–7, 34)	0.128 (0.271)	0.103 (0.240)
LBP, μg/mL	3.6 (3.4, 3.8)	3.3 (3.1, 3.4)	–0.3 (–0.5, –0.2)	0.0001 (0.001)	3.4 (3.3, 3.6)	3.2 (3, 3.4)	–0.2 (–0.4, –0.1)	0.001 (0.005)	0.066 (0.184)
Zonulin, ng/mg	282 (227, 337)	203 (171, 234)	–79 (–129, –30)	0.0001 (0.001)	262 (221, 303)	247 (193, 300)	–15 (–66, 36)	0.022 (0.077)	0.320 (0.507)
SCFAs, mg/g									
Acetate	12.2 (11, 13)	14.3 (13, 16)	2.1 (1, 3)	0.015 (0.057)	13.6 (13, 15)	14.4 (13, 16)	0.8 (–1, 2)	0.563 (0.768)	0.219 (0.378)
Propionate	5.1 (4, 6)	7.9 (7, 9)	2.8 (1, 4)	0.0001 (0.001)	5.2 (5, 6)	6.3 (5, 7)	1.1 (0.1, 2)	0.099 (0.239)	0.029 (0.091)
Butyrate	5.1 (4, 6)	8.0 (7, 9)	2.9 (2, 4)	0.0001 (0.001)	5.1 (5, 6)	6.4 (6, 7)	1.3 (0.2, 2)	0.061 (0.173)	0.017 (0.064)

(*Continued*)

TABLE 2 (*Continued*)

Characteristic	Intervention group ($n = 124$)				Control group ($n = 136$)				Between group (Δ), *P* (adj.)
	Baseline	V1	Δ (V1 – Baseline)	Within group, *P* (adj.)	Baseline	V1	Δ (V1 – Baseline)	Within group, *P* (adj.)	
Anthropometry									
Body weight, kg	71 (68, 73)	70 (67, 72)	−1.5 (−1.9, −0.6)	0.0001 (0.001)	70 (67, 72)	69 (66, 71)	−1.2 (−2.1, −0.2)	0.026 (0.090)	0.070 (0.187)
BMI, kg/m^2	25 (24, 26)	24 (24, 25)	−0.4 (−0.8, −0.1)	0.028 (0.090)	25 (24, 26)	25 (24, 26)	−0.3 (−0.6, 0.1)	0.080 (0.204)	0.647 (0.838)
WC, cm	82 (80, 84)	79 (77, 81)	−2.9 (−4.4, −1.3)	0.0001 (0.001)	82 (80, 84)	81 (79, 83)	−0.8 (−2.2, 0.6)	0.801 (0.915)	0.001 (0.005)
HC, cm	101 (99, 103)	99 (97, 101)	−1.2 (−2.2, −0.3)	0.004 (0.019)	101 (99, 103)	99 (97, 102)	−1.4 (−2.8, 0)	0.028 (0.090)	0.517 (0.722)
WHR, WC/HC	0.81 (0.8, 0.8)	0.78 (0.7, 0.8)	−0.02 (−0.03, 0)	0.001 (0.005)	0.81 (0.8, 0.8)	0.83 (0.8, 0.9)	0.02 (−0.02, 0.1)	0.291 (0.483)	0.019 (0.070)

[1]Shown are the adherence to the Mediterranean diet (MedD), the intake of food groups and nutrients according to the FFQ, the concentrations of intestinal barrier biomarkers, and the amount of fecal short-chain fatty acids (SCFAs) related to fecal dry weight. Means (95% CIs) for baseline, V1, and shift (baseline subtracted from V1, Δ) are shown. Difference between the study groups at baseline and difference between the group's shifts was tested using the Mann–Whitney *U* test. There was no difference between the groups at baseline for all parameters shown. Within-group difference between baseline and V1 was tested using the Wilcoxon signed-rank test. The *P* values were adjusted with a false discovery rate of 5%. HC, hip circumference; ins., insoluble; LBP, lipopolysaccharide binding protein; MEDAS-Score, Mediterranean Diet Adherence Screener–Score; MedD-Score, Mediterranean Diet Score; oligos., oligosaccharides; res., resorbable; sol., soluble; WC, waist circumference; WHR, waist-to-hip ratio.

they were not found to be associated with the intervention in any of the groups. For the MLR models, we used the shift values (baseline concentrations subtracted from the respective values after intervention). The MLR analyses revealed that the increased adherence to the Mediterranean diet was indeed linked to an increase in fecal SCFA concentrations and a decrease in permeability biomarkers (Figure 2D). Specifically, adherence to the Mediterranean diet explained the reduction in both plasma LPB and fecal zonulin as well as the increase in the SCFAs propionate and butyrate (Figure 2E; **Supplemental Table 2**). These results were consistent for both study groups and for both Mediterranean diet scores used. Age, BMI, physical fitness, and previous disease were not associated with intestinal permeability biomarkers or with SCFAs.

Next, we hypothesized that the changes in plasma LBP and fecal zonulin resulted from increased SCFA concentrations induced by the Mediterranean diet. The increase in propionate and butyrate resulted in a decrease in both biomarkers in both study groups, independent of the Mediterranean diet score used (Figure 2E; Supplemental Table 2). These results were most dominant for LBP and the SCFAs in the intervention group (propionate/butyrate: adjusted R^2 = 31%/32%) and for zonulin and the SCFAs in the control group (propionate/butyrate: adjusted R^2 = 28%/35%). Again, there was no effect of age, BMI, physical fitness, or previous cancer disease on the permeability biomarkers in either of the groups. These data show that adherence to the Mediterranean diet increased the amount of the fecal SCFAs propionate and butyrate, resulting in a decrease in the permeability biomarkers plasma LBP and fecal zonulin.

SCFAs mediate favorable effects of typical Mediterranean diet components on intestinal permeability

To further identify a possible causal link between the changes in the Mediterranean diet, the SCFAs propionate and butyrate, and intestinal permeability biomarkers, we were interested in *1*) dietary components of the Mediterranean diet most likely responsible for the effect seen on SCFAs and intestinal permeability and *2*) SCFAs causing the effect of diet on intestinal permeability.

First, we created a correlation matrix that included all dietary components, SCFAs, and biomarkers that were statistically significantly altered during the study. These criteria were fulfilled by 18 typical Mediterranean food groups. Nine of them (vegetables, fruit, legumes, nuts, olives, wine, total fiber, water-insoluble fiber, and nonresorbable oligosaccharides) were correlated with the shift in propionate and butyrate and inversely correlated with the shift in LBP and zonulin (**Supplemental Figure 3**). These results were consistent for both study groups at different significance levels, showing that selected Mediterranean food items, especially fiber-rich food groups, are associated with an increase in SCFAs and a decrease in permeability biomarkers. We further assessed whether changes in the adherence to the Mediterranean diet correlated with changes in body weight, BMI, waist and hip circumference, and the waist-to-hip-ratio, showing no results (data not shown).

To further investigate the causal role of SCFAs in the interplay between diet and changes in intestinal permeability biomarkers, we performed a model-based causal mediation analysis. These analyses allow us to determine whether a variable (e.g., SCFAs)

acts as the mediator between a predictor (e.g., a typically Mediterranean food component) and an outcome (e.g., intestinal barrier biomarkers) and enables us to distinguish between SCFA-mediated effects and non-SCFA-mediated effects marked as direct effects (**Figure 3**A–D). We performed these analyses separately for LBP and zonulin in the intervention and control groups, resulting in a total of 4 alluvial plots, as presented in detail in **Supplemental Table 3**.

According to the results of the mediation analysis, the effect of the dietary factors on plasma LBP concentrations was robustly mediated by the SCFAs propionate and butyrate (Figure 3A, B). On the other hand, the effect of the dietary factors on fecal zonulin concentrations was both mediated by SCFAs and unmediated by SCFAs (so-called direct effects, which could be unmediated by SCFAs or mediated by unknown factors) (Figure 3C, D). Looking at single food groups, we observed that legumes and different fibers (especially insoluble fibers) induced exclusively mediated effects on LBP. There were only minor direct effects of fruit and nuts on plasma LBP concentrations. Interestingly, dietary fibers (again especially insoluble fibers) and olives both showed mediated as well as distinct direct effects on fecal zonulin concentrations. This effect occurred most prominently in the intervention group, where the intake of dietary fibers increased during intervention.

Our clinical data propose a further mechanistic understanding of the relation between dietary components, SCFAs, and intestinal barrier integrity. Although SCFAs drove most of the dietary impact on LBP and zonulin, there are still considerable SCFA-independent effects, indicating possible "direct effects" of dietary fibers or indicating other mechanisms related to nonfiber food components. Such components may comprise polyphenols, polyunsaturated fatty acids, and vitamins, also thought to have an effect on intestinal barrier integrity (40).

Analysis of individualized responses to the Mediterranean diet

Even though we detected a significant decrease in 2 intestinal permeability biomarkers in response to the Mediterranean diet, we observed large interindividual variability. This suggests that not all participants benefited to the same extent from adhering to the Mediterranean diet. To gain more insight into the drivers of this variability, we classified participants in each study arm based on their individual shift in both plasma LBP and fecal zonulin into major and minor/no responders using an unsupervised clustering technique. Classification is based on the combination of the 2 validated biomarkers to reflect the complex intestinal barrier structure and function more robustly than analyzing single markers. This approach identified 45 major responders in the intervention group (36%) and 35 in the control group (26%; **Figure 4**A). In the control group, 1 subject could not be classified, resulting in 259 individuals being included for further analyses. Characterizing major or minor responders in each treatment arm by density plots and multivariate techniques (PERMANOVA tests), we showed a significant difference in the shift of the gut barrier biomarkers between major and minor responders, confirming a valid allocation into either the major or minor responder groups (**Supplemental Figure 4**).

Figure 4B–D characterizes major and minor responders on an individual level, showing a clear decrease in both LBP and zonulin in the major responders (LBP: –25%/–26%; zonulin: –44%/–40%, intervention/control group; all $q < 0.001$; Wilcoxon test), whereas there was no significant overall decrease in the biomarkers in the minor responders (LBP: –3%/–1%; zonulin: –8%/–13%). There was no significant change in physical fitness or BMI during the intervention, neither among major responders nor among minor responders (Figure 4C). Major responders in both the intervention and control groups were characterized by an increased adherence to the Mediterranean diet; an increased intake of vegetables, fruit, and nuts; and an increase in the fecal amounts of the SCFAs propionate and butyrate (all $q < 0.05$; Wilcoxon test), whereas there was no consistent change in diet or SCFAs in the minor responders in the intervention and control groups (Figure 4D). There was no difference between major and minor responders in baseline age, BMI, or physical fitness (data not shown).

Individualized responses to the intervention depend on baseline SCFA concentrations

Based on our findings that major and minor responders were found in both arms, we hypothesized that preintervention dietary habits, as well as fecal concentrations of the SCFA butyrate, determine an individual's response in intestinal permeability induced by dietary interventions. To test our hypothesis, we applied RFC, assigning the individuals in each arm into a training and a validation set with a 7:3 ratio. Because the ratio between major and minor responders was unbalanced in the intervention group ($n = 45/79$, major/minor responders) and markedly unbalanced in the control group ($n = 35/100$), we used 5 different RFC models to account for these unbalanced groups and to guarantee that possible statistical effects do not derive from choosing the most suitable model. As shown in **Figure 5**A, the algorithm accurately predicted the effect of the 3-mo intervention on the permeability biomarkers using baseline fecal SCFA data (butyrate) but not using dietary data. Due to intercorrelations, we did not include the other SCFAs (acetate and propionate) in the model. Furthermore, we did not include baseline data of the biomarkers in the machine learning algorithm to guarantee independence of the predictors and outcomes. As shown in Figure 5B, C, the AUC for the ROC curve was 0.87–0.96 in the intervention group and 0.78–0.86 in the control group for the predictive effect of baseline butyrate. As shown in Figure 5D, there were substantial differences in the baseline fecal amounts of butyrate between major and minor responders. Results from our machine learning algorithm showed that the baseline concentration of the SCFA butyrate accurately predicted the effect of the 3-mo intervention program on intestinal permeability.

Baseline concentrations of SCFAs and baseline permeability biomarkers predict the success of the 3-mo intervention program on intestinal permeability

Finally, we investigated whether women with impaired intestinal barrier integrity would benefit more from the intervention program than women without barrier impairment. Here, we stratified all 259 women with known allocation as major or minor responders, independent of their allocation to the intervention or control group, into a low or high baseline group based on

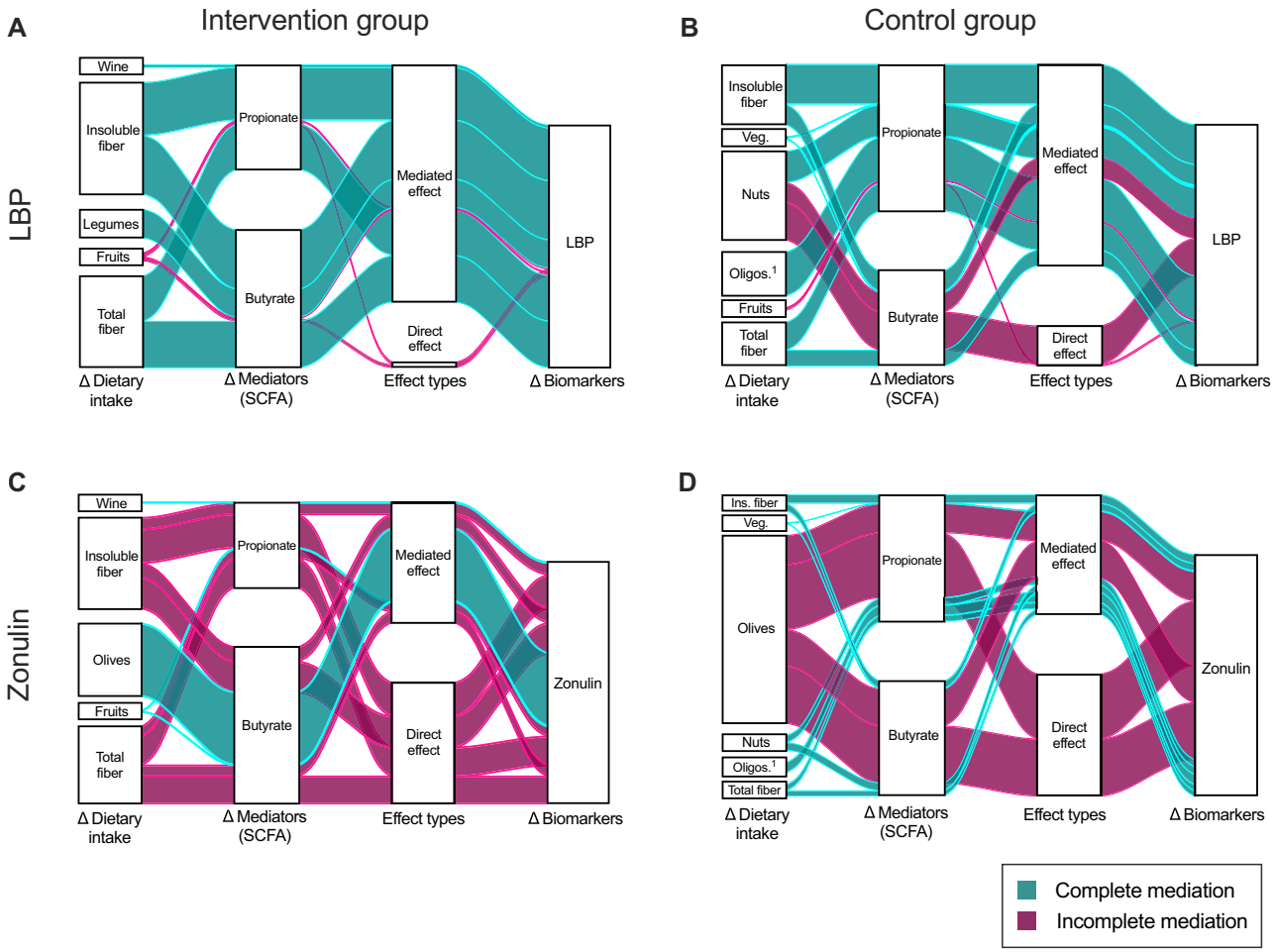


FIGURE 3 Short-chain fatty acids (SCFAs) mediate the effect of diet on intestinal permeability. (A–D) Estimated effects between the intake of different dietary components on plasma lipopolysaccharide binding protein (LBP) and fecal zonulin. The width of each flow line represents the absolute value of the β- coefficient indicating the effect size. For all models, the shifts (baseline values subtracted from the respective values after intervention, shown as Δ) were included for the predictors (Δ dietary intake), mediators (Δ SCFAs), and outcomes (Δ biomarkers). The panels show whether the effect of a given dietary component on the biomarkers was mediated by SCFAs (mediated effect) or not (direct effect). Dietary effects that were completely mediated by either propionate or butyrate are shown in cyan (complete mediation); dietary components that have both mediated and direct effects on the biomarkers are shown in dark pink (incomplete mediation). The alluvial plots summarize the significant results of 4 mediation analyses (lines are only shown for variables with negative β-coefficients for mediated and direct effects), showing the association between diet and plasma LBP (top row) or fecal zonulin (bottom row) in the intervention group (left column; $n = 124$) and the control group (right column; $n = 136$), respectively (details are shown in Supplemental Table 3). Ins. fiber, water-insoluble fiber; Oligos.[1], non-resorbable oligosaccharides; Oligos.[2], resorbable oligosaccharides; Veg., vegetables.

their biomarkers being below or above the median (medians: LBP, 3.5 μg/mL; zonulin, 178 ng/mg; for both biomarkers: $n = 129/130$ above/below median). We found that women with a high baseline concentration of LPB were major responders in 43% of the sample ($n = 55$), whereas women with low baseline concentrations of LBP were major responders in only 19% of the sample ($n = 25$; χ^2, $P < 0.001$). A similar observation was made for zonulin [40% responders in the high baseline zonulin group ($n = 51$), 22% in the low baseline zonulin group ($n = 29$); χ^2, $P = 0.003$]. Using stratification into major and minor responders, we generated ROC curves to assess whether the baseline concentrations of the permeability biomarkers and SCFAs can predict individual responsiveness to the intervention. As shown in Figure 5E, there was some predictive effect for baseline LBP and zonulin and a strong predictive effect for baseline propionate and butyrate.

Discussion

Loss of intestinal barrier integrity is thought to be a major cause that drives low-grade inflammation found in numerous chronic diseases, such as cardiometabolic diseases, type 2 diabetes, and cancer (8, 41). The Mediterranean diet has been suggested to be beneficial in all these chronic diseases, and we provide evidence that this beneficial effect is triggered to a large extent by the fiber content and mediated by SCFAs.

The causes of intestinal barrier impairment in such chronic diseases are not always clear and might differ depending on the type of disease. Strategies to restore barrier functions are scarce, but dietetic approaches might be of particular interest because they could act independent of the type of disease. Indeed, there is evidence that dietary fibers can improve intestinal integrity (42), whereas consumption of sugars, especially fructose, is associated with loss of barrier function (43). Here, we show that a high-fiber,

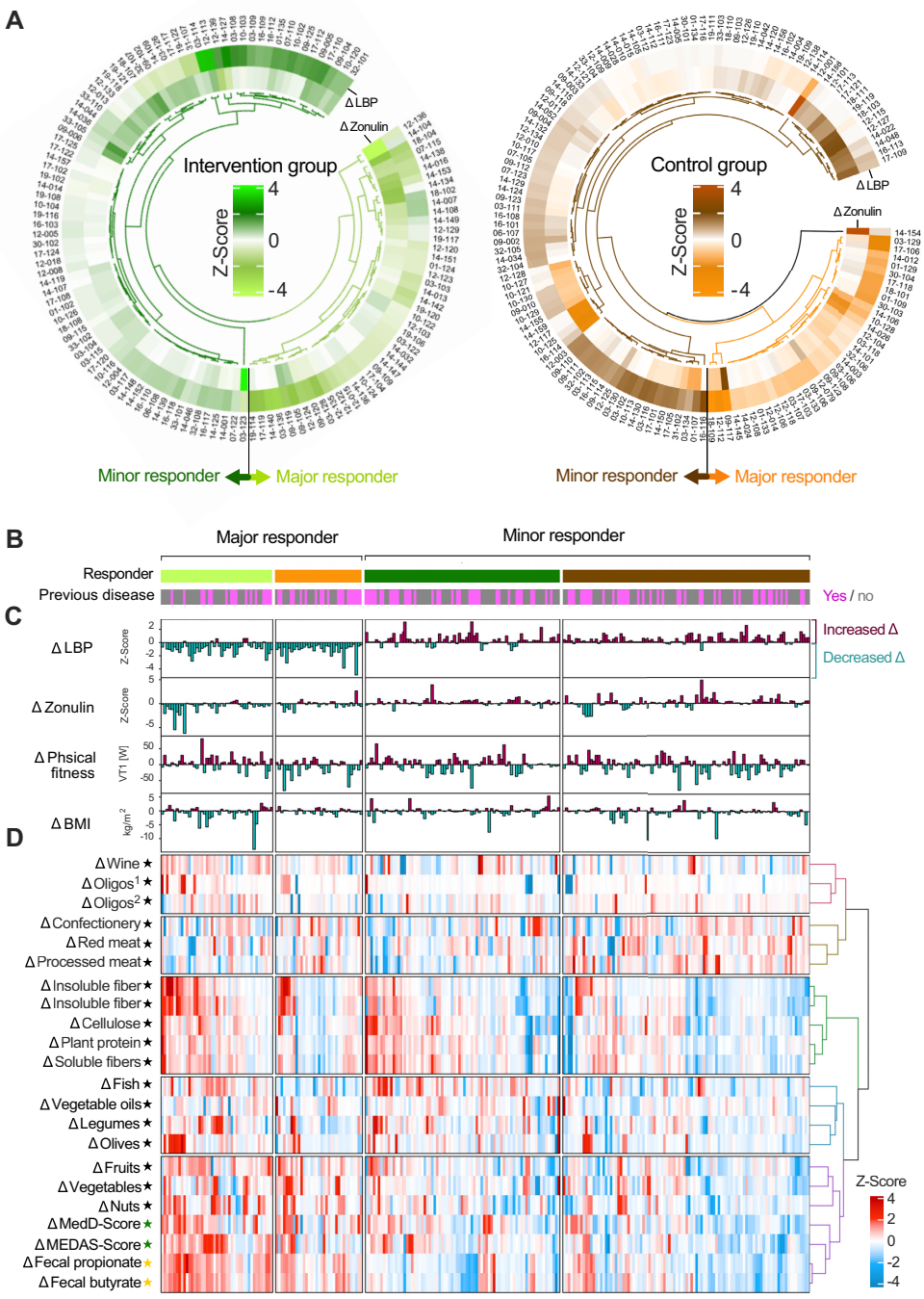
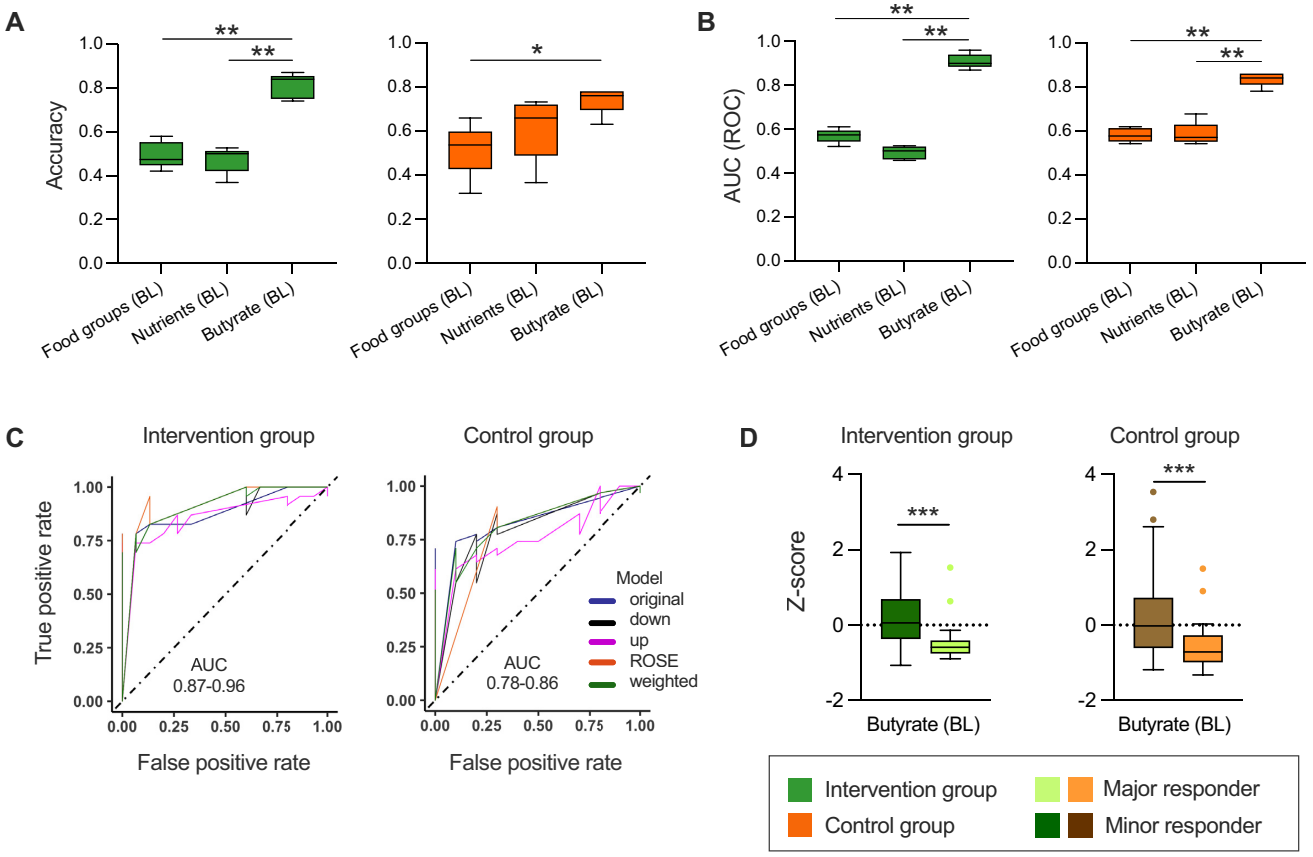
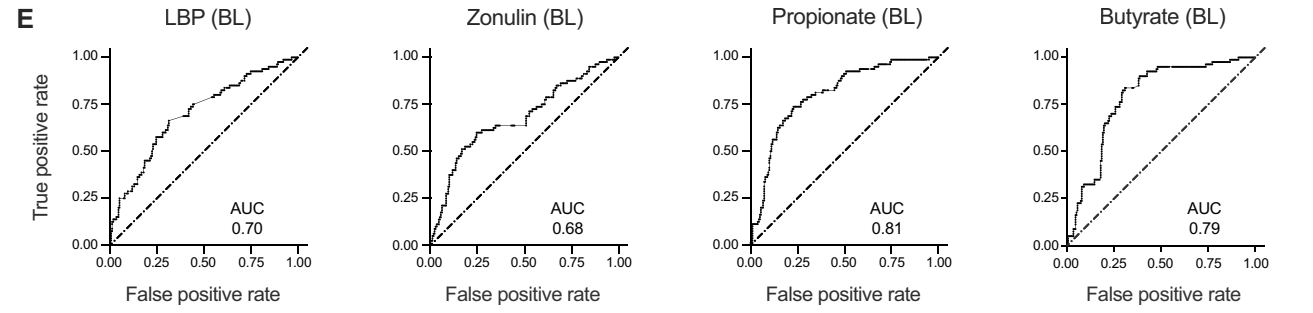


FIGURE 4 Individualized responses to the Mediterranean diet. (A) Circle heatmaps showing the clustering of the 260 individuals into major and minor responders based on the *z*-scores for the shift (baseline values subtracted from the respective values after intervention; Δ) in plasma lipopolysaccharide binding protein (LBP; outer circle) and fecal zonulin (inner circle) in individuals from the intervention group (left circle; light and dark green depending on responsiveness to the intervention) and the control group (right circle; light and dark orange). In the control group, 1 subject was not classified as either a major or minor responder and was excluded from further analyses (shown in black in the dendrogram), resulting in 124 individuals in the intervention group (45/79, major/minor responders) and 135 individuals in the control group (35/100, major/minor responders). (B–D) Individual changes in diet, short-chain fatty acids (SCFAs), intestinal permeability biomarkers, and the potential confounders age, BMI, and physical fitness. (B) The 2 top rows show the allocation into either major or minor responders and show previous breast cancer disease state. (C) Shifts in the amounts of plasma LBP, fecal zonulin, physical fitness, and BMI. (D) The heatmap shows the shift of dietary components (black stars), fecal concentrations of SCFAs (yellow stars), and adherence to the Mediterranean diet, assessed by the Mediterranean diet Adherence Screener (MEDAS)–Score and the Mediterranean Diet Score (MedD-Score). Individuals (shown as columns) and the study-specific parameters (shown as rows) with similar responses were grouped together based on Spearman correlation distance. Oligos.[1], non-resorbable oligosaccharides; Oligos.[2], resorbable oligosaccharides.





Condition		LBP
Se = Sp	Cut-off	3.6 μg/ml
	Se / Sp	66 / 69
Sp = 75%	Cut-off	3.8 μg/ml
	Se / Sp	58 / 75

	Zonulin
Cut-off	184.4 ng/mg
Se / Sp	66 / 64
Cut-off	239.0 ng/ml
Se / Sp	60 / 75

	Propionate
Cut-off	3.3 mg/g
Se / Sp	74 / 75
Cut-off	3.3 mg/g
Se / Sp	74 / 75

	Butyrate
Cut-off	3.4 mg/g
Se / Sp	74 / 74
Cut-off	3.2 mg/g
Se / Sp	70/75

FIGURE 5 Baseline characteristics predict the individual response in intestinal permeability to a 3-mo lifestyle intervention using a machine learning algorithm. The individuals were clustered into major and minor responders [124 individuals in the intervention group (45/79, major/minor responders), 135 individuals in the control group (35/100, major/minor responders); see Figure 4] and then randomly assigned into a training and validation set in a 7:3 ratio. Three different data sets of baseline (BL) characteristics were used as input information for the random forest classification models: (i) food groups, (ii) nutrients [the components of (i) and (ii) are listed in Table 2], and the short-chain fatty acid butyrate. (A) Accuracy of the prediction models for the intervention group (left, green boxes) and control group (right, orange boxes). (B) AUC for the receiver operating characteristic (ROC) curve for the intervention and control groups. (C) ROC curves for the 5 models "down," "original," "ROSE" (Random Over-Sampling Examples), "up," and "weighted" for the baseline butyrate data set. (D) Tukey boxplots with medians and whiskers (1.5× IQRs) showing the difference in baseline butyrate between major and minor responders. (E) ROC curves showing the predictive effect of selected BL data for allocation into major or minor responders (top) and proposed cutoffs (bottom; Se, sensitivity; Sp, specificity). Other baseline data (see Tables 1 and 2) yielded no or only minor predictive value (AUC < 0.6). Statistics: (A, B) Kruskal–Wallis tests with a false discovery rate of 5% ($q < 0.06$; $^{*}q < 0.05$; $^{**}q < 0.01$; $^{***}q < 0.001$, $n = 259$); (D) Wilcoxon signed-rank test ($^{***}P < 0.001$); (A, B, D) Tukey boxplots with outliers are shown; (F) Wilson–Brown hybrid method, all $P < 0.001$.

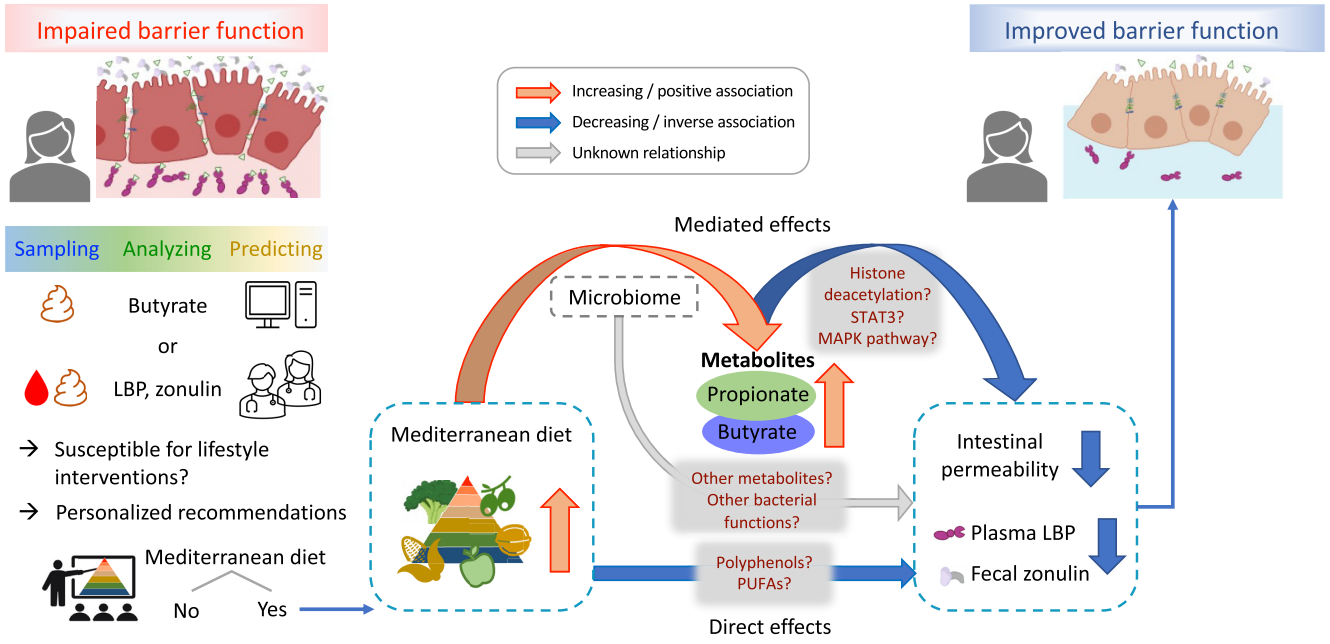


FIGURE 6 Clinical implications and proposed mechanism. (Left side) Women with impaired intestinal barrier function underwent an analysis for eligibility for lifestyle intervention. The analysis consists of a quantification of the short-chain fatty acid (SCFA) butyrate in feces, or—if this analysis is not available—measurement of plasma lipopolysaccharide binding protein (LBP) and fecal zonulin with less predictive power compared to the SCFA. Based on these analyses, individual recommendations for the Mediterranean diet can be given. (Right side) The diet leads to an increased generation of SCFAs and possibly other bacterial metabolites, improving barrier functions by histone deacetylation and STAT3 and MAPK signaling. The diet might have other effects not mediated by the microbiota, such as an increase in polyphenols and PUFAs in blood. According to our data, the majority of effects are mediated by propionate and butyrate, which are key mediators of improved intestinal barrier function.

low-sugar Mediterranean diet has favorable effects on intestinal barrier integrity in a population at risk for intestinal barrier impairment (16), as confirmed by our present data.

After 3 mo of intervention, LBP and zonulin decreased, strongly suggesting a barrier-stabilizing effect of the Mediterranean diet. Previous data from the literature on the possible effects of the Mediterranean diet on intestinal barrier function are rare and inconsistent (44, 45). These uncertainties might be related to small study sample numbers, different underlying diseases, unidentified covariables such as metabolic disorders (46), or different methods used for intestinal barrier assessment. Our study included a control group of individuals who were not counseled for the Mediterranean diet. However, since all study participants were informed about the study purpose to test the effect of a healthy diet on breast cancer, some people in the control group also changed their food habits toward a healthier diet, resulting in some dietary changes (e.g., a mild increase in the MEDAS-Score, in the control group, not only in the intervention group) (39).

The value of zonulin measurements has been questioned in the past because antibody-based assays detect several proteins structurally and functionally related to zonulin rather than a single protein (47). However, from a clinical standpoint, cross-reactivity is not a limitation as long as the biomarker concentrations correlate well with the gold standards of permeability measurements (13, 48).

Thus far, it has been largely unclear how a change to the Mediterranean diet exerts its beneficial effects in chronic diseases. Here, we show that a major mechanism is the increased induction of SCFAs, namely, propionate and butyrate, which are produced by commensal bacteria from dietary fibers. Indeed, we could show by multivariate mediation analysis that most nutritional effects are mediated by propionate and butyrate. According to our data, nutrients containing fibers, especially water-insoluble fibers, such as cellulose, as well as insoluble oligosaccharides, including fermentable oligo-, di-, monosaccharides and polyols, found in legumes, fruit, and nuts, play a particular role in the SCFA-mediated dietary effect on intestinal barrier function. We assume that we found only a few significant results for water-soluble fibers, such as inulin and other fructooligosaccharides, found in fruit and vegetables because SCFA measurements in feces might underestimate the effect of these fibers, as they are thought to be metabolized more proximally in the colon. In particular, arabinoxylan and β-glucan, fibers that comprise water-soluble and water-insoluble components found in nuts and cereals, might have an effect on SCFA production and permeability biomarkers.

Depending on which barrier marker is used, differences in the weighting of individual food components were observed. For example, olives affected mostly zonulin via butyrate, whereas legumes affected mostly LBP. Moreover, LBP changes were mediated almost exclusively by SCFAs, whereas zonulin changes were mediated only in part by SCFAs. The reasons for this are unclear at present and suggest that LBP and zonulin assess different components of barrier functions. Moreover, our data show that the Mediterranean diet also exerts its effects by SCFA-independent mechanisms, including polyphenols, polyunsaturated fatty acids, and vitamins, known to affect intestinal barrier integrity (40).

An increase in SCFAs and/or SCFA-producing bacteria induced by the Mediterranean diet was also found in randomized controlled trials from Spain (49, 50), Italy (51), and a European multicenter study (52), but the link to the gastrointestinal barrier was missing. In vitro studies and preclinical studies revealed that butyrate (53, 54) and propionate (55) can improve intestinal integrity. For example, we showed before that butyrate decreases zonulin concentrations (56) in mice. In the present study, we can confirm this in humans and provide a causal relation between dietic fibers, SCFAs, and barrier function. The molecular mechanisms by which SCFAs affect intestinal permeability are not fully understood, but the transcription factors STAT3 and SP1 (57), the hypoxia-inducible factor (58), and histone deacetylation and modifications in the MAPK pathway have been discussed lately (own unpublished data).

Our findings can be used for novel personalized nutritional advice because we can predict who will most likely benefit from a Mediterranean diet (**Figure 6**). If available, such individualized diet counseling should be based on fecal butyrate measurement. If not available, prediction can also be based on baseline LBP and zonulin concentrations reflecting baseline barrier function. The possibility to select those individuals who likely will profit from the dietetic approach will make the intervention aiming to improve intestinal barrier function most efficient in populations with impaired barrier function.

Microbiome data in the clinical setting show associations but sometimes suffer from a lack of mechanisms and causal links (59). A strength of our study is that it provides evidence for a novel mechanistic framework based on the causal relation between diet and intestinal permeability with gut bacteria-derived SCFAs as key mediators, as summarized in Figure 6. Whether the observed increase in SCFAs derived from changes in the gut microbiota composition or derived from an increased bacterial metabolic activity from an unchanged gut microbiota composition will be assessed in a subsequent analysis of the LIBRE study. Also, the effect of the Mediterranean diet on further gastrointestinal biomarkers such as fecal bile acids and possible interactions between fecal bile acids and gut barrier integrity will be assessed subsequently. A limitation of our study is that we included only women with BRCA mutations and associated intestinal barrier dysfunction. To what extent our finding will also hold true for other populations with intestinal barrier dysfunction needs to be explored in future studies. Our results should be verified in larger studies powered for this purpose and include further clinical endpoints or markers for chronic diseases.

In conclusion, our study showed that a fiber-rich Mediterranean diet improves intestinal barrier function within 3 mo of intervention by increasing SCFA production. Machine learning algorithms including SCFA data may allow novel personalized nutrition advice for improving chronic diseases associated with barrier dysfunction and low-grade inflammation.

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The authors' responsibilities were as follows—NMD, SCB, and JW: designed research (FiberTAG); MK and SCB: designed research (LIBRE); BS, MB, and SCB: conducted research; NKN and BS: analyzed data, performed statistical analyses, and created figures; BS, NKN, and SCB: wrote paper; SCB: had primary responsibility for the final content; and all authors: have read and approved the final manuscript.

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