The intake of long-chain n-3 fatty acids in typical Western diets is suboptimal. Although the Institute of Medicine has not established a recommended dietary allowance for eicosapentaenoic acid (EPA) (20:5n-3) or docosahexaenoic acid (DHA) (22:6n-3), the Dietary Guidelines for Americans recommends an intake of 8 oz seafood weekly. This is similar to the American Heart Association recommendation of fish intake twice per week for risk reduction of cardiovascular disease (CVD). Despite these recommendations, fish intake is low in the United States, with <13 g consumed daily.

A combined analysis of prospective cohort studies demonstrated that a total intake of 250 to 500 mg/day EPA and DHA is associated with a significant reduction in CVD risk. Another analysis focusing on only prospective studies in the United States suggests that approximately 500 mg combined EPA and DHA provides the most protection. The International Life Sciences Institute workshop on establishing a Dietary Reference Intake for EPA/DHA suggests 250 to 500 mg/day combined EPA and DHA as the appropriate intake for CVD risk reduction; however, greater intake may impart additional protective effects. Increasing EPA and DHA consumption at a population level will be better addressed by dietary modification than through supplementation. However, there are limited data describing the effect of various portions of high-n-3 fish intake on plasma proportions of n-3 fatty acids. Therefore, we performed a clinical trial to evaluate the effect on phospholipid fatty acid (PLFA) composition during 4-week interventions.

METHODS
Experimental Protocol
A randomized, crossover design was employed to compare the effects of different portions of high n-3 fish consumption. All participants consumed farmed Atlantic salmon in doses of...
90, 180, or 270 g twice per week in random order. Each treatment period lasted 28 days (4 weeks). Upon completion of the first and second treatment, participants returned to their usual diet for a washout period of 28 to 56 days and then crossed over to the second or third treatment in the randomization. The salmon was prepared in the metabolic kitchen and all study visits were performed at the Grand Forks Human Nutrition Research Center, Grand Forks, ND. Participants picked up their salmon portions once weekly. Compliance was monitored with the use of a weekly questionnaire administered by the research staff.

Approval for the study was obtained from the University of North Dakota Institutional Review Board. Informed consent was obtained from all study participants. The study was registered at clinicaltrial.gov (NCT01183520).

Participants
Participants were recruited using newspaper advertisements, fliers, and e-mail announcements distributed within the University of North Dakota and general community. Inclusion criteria included men and women aged 40 to 65 years, body mass index between 25 and 34.9, low intake of fish (≤1 serving monthly), and free of major medical conditions. Exclusion criteria included smoking, lipid-modifying drug or supplement use, steroid use, weight loss within the past 3 months, pregnancy or lactation, and use of fish oil or flax supplements. Potential participants were screened through an online application or telephone interview and invited to attend an informational meeting in which the study staff described the study in detail. Eligible participants were then scheduled for an examination of height, weight, blood pressure, and fasting blood glucose by fingerstick (Accu-Chek Compact Plus, Roche). Body weight (in kilograms) was measured (to 0.1 kg) using a calibrated digital scale (model 50735, Fairbanks Scales) with subjects wearing light clothing and no shoes. Stature (to 1 mm) was measured with a free-standing stadiometer (model S–214, Seca). Blood pressure was measured using a blood pressure monitor (model BPM-300, BP Medical Devices) after the patient was seated quietly for 5 minutes. Health status was determined by a medical history questionnaire. All potential participants completed a questionnaire designed to assess usual n-3 fatty acid intake.

Dietary Intervention
Filleted salmon was provided by Cooke Aquaculture. Cooked fish entrées were analyzed for fatty acids and the n-3 content is illustrated in Table 1. Complete details of the fish handling, preparation, and analysis were previously described.11 Six individual entrée recipes were developed and participants chose the entrées they were to consume. Participants were asked to maintain their habitual diet while consuming the assigned salmon portion. They were directed to replace meal entrées with the salmon and were given direction on the storage and reheating of the salmon. Dietary treatment compliance was assessed weekly by questionnaire. During each washout period, participants kept a 3-day record of their food consumption that was analyzed by a registered dietitian using the National Nutrient Database for Standard Reference, Release 22 (2009, US Dept of Agriculture, Agricultural Research Service) and a customized Grand Forks Human Nutrition Research Center nutrient analysis program.

Blood Collection
Fasting blood samples were obtained at Day 0 and Day 28 of each treatment and used to determine glucose, insulin, and PLFA proportions. Samples were obtained on Day 28 only for high-sensitivity C-reactive protein (hsCRP), and interleukin-6 (IL-6) concentrations. Whole blood samples were centrifuged to obtain serum and plasma; samples were aliquoted and stored at −80°C until analysis.

Biomarker Analysis
Serum glucose concentrations were measured by the COBAS INTEGRA 400 PLUS with Glucose HK Gen.3 kit (catalog no. 04404483, Roche Diagnostics). Serum insulin and hsCRP concentrations were determined by the IMMULITE 1000 System with the insulin kit (catalog no. LKIN1, Siemens Healthcare).

### Table 1. n-3 Fatty acid content of farmed Atlantic salmon (baked salmon) provided to participants in a study to determine influence of fish intake on plasma phospholipid fatty acid proportions

| Fatty acid | 90 g | | 180 g | | 270 g | | 90 g | | 180 g | | 270 g | | 90 g | | 180 g | | 270 g |
|-----------|------| | ------| | ------| | ------| | ------| | ------| | ------| | ------| | ------| | ------|
| 18:3n-3 (mg) | 196.2 | | 28.0 | | 392.4 | | 56.1 | | 588.6 | | 84.1 | | 1,108.8 | | 158.4 | | 2,217.6 | | 316.8 | | 3,326.4 | | 475.2 |
| 18:4n-3 (mg) | 628.2 | | 89.7 | | 1,256.4 | | 179.5 | | 1,884.6 | | 269.2 | | 1,108.8 | | 158.4 | | 1,022.4 | | 146.1 | | 1,533.6 | | 219.1 |
| 20:3n-3 (mg) | 37.8 | | 5.4 | | 75.6 | | 10.8 | | 113.4 | | 16.2 | | 511.2 | | 73.0 | | 1,022.4 | | 146.1 | | 1,533.6 | | 219.1 |
| 22:6n-3 (mg) | 1,045.8 | | 149.4 | | 2,091.6 | | 298.8 | | 3,137.4 | | 448.2 | | 4,082.4 | | 583.1 | | 8,164.8 | | 1,166.5 | | 12,247.2 | | 1,749.6 |
and the hsCRP kit (catalog no. LKCRP0, Siemens Healthcare). Serum IL-6 concentrations were measured using a Spectra MAX-190 (Molecular Device Corporation) with Quantikine HS kit (catalog no. HS600B, R&D Systems). All the procedures were conducted following the manufacturer's instructions. Insulin resistance was determined by the calculation of homeostasis model of assessment-insulin resistance. The coefficients of variance for these samples for glucose, insulin, hsCRP, and IL-6 were 1.1%, 6.4%, 6.0%, and 7.8%, respectively.

Plasma PLFA Analysis
Plasma PLFA proportion was used to evaluate salmon intake effects because they respond readily to dietary changes and provide a surrogate measure of tissue membrane fatty acid content based on plasma fatty acid exchange. PLFA analysis was performed by gas chromatography as previously described. Plasma PLFA results for baseline and 4-week results on each salmon dose are presented as mol%.

Statistics
Sample size was based on the expected mean difference of DHA using data from Calzada and colleagues. Using a repeated measures analysis of variance 17 participants give 80% power to detect a mean difference of DHA of 0.2 mol%. Data are reported as means ± standard error of the mean unless otherwise stated. SAS version 9.3 for Windows (2011, SAS Institute, Inc) was used for all statistical analyses. The mixed model procedure (Proc Mixed) in SAS was used to test for effects of treatment (90, 180, or 270 g salmon), time (Day 0 and Day 28), treatment by time interaction, feeding sequence, and time period on each outcome. Participant was treated as a random effect. Feeding sequence and time period were not significant in any of the models. When the interaction between treatment and time was statistically significant (P < 0.05), Tukey contrasts were used to perform pairwise comparisons of all group means.

RESULTS AND DISCUSSION
A total of 61 participants were screened and 22 were enrolled and allocated to treatment. Three participants withdrew from study participation and the remaining 19 (11 women, 8 men) completed all aspects of the trial. The flow of participants through the trial is demonstrated in the Figure. Mean age was 51.6 ± 1.5 years and mean body mass index was 29.2 ± 0.6. Participants reported low n-3 intake at baseline (ie, 0.04 g EPA and 0.08 g DHA) and on the 3-day dietary records kept during the washout periods between treatments (ie, 0.01 g and 0.01 g EPA, 0.03 g and 0.02 g DHA during washout periods 1 and 2, respectively). Compliance with the treatments was high with participants reporting >99% consumption of provided salmon portions. The food diaries recorded during the washout periods demonstrated that participants returned their habitual diet and baseline PLFAs at the beginning of each treatment were not different, demonstrating that the length of the washout period was adequate. There were no significant differences in body weight by time or treatment.

No changes were observed in glucose, insulin, homeostasis model of assessment-insulin resistance, hsCRP, and IL-6 in response to any level of salmon either within or between
and appeared to become saturated at the lowest level of response relationship, the proportion of DHA was elevated (P<0.0001) was dose dependent. EPA (20:5n-3) increased in a dose-responsive manner (P<0.0001), whereas DHA (22:6n-3) was enhanced by all treatments (P<0.0001), whereas DHA did not. The lack of EPA increase in the PLFA at the lowest salmon intake suggests that the EPA consumed in the 90-g portion was converted to DHA, but that at greater intake the conversion of EPA to DHA was saturated. It is possible that at the higher salmon intake, retroconversion of DHA to EPA occurred, although human data suggest that this contribution is minimal.16 Thus, the increase in EPA is likely the result of elevated consumption of EPA. Human trials with fish oil supplements indicate that plasma EPA and DHA pools are not identical with incorporation of EPA into phospholipid and cholesterol ester and DHA into phospholipid and triglyceride lipid fractions.14 Our study did not examine these other lipid pools. Nonetheless, our data indicate that PLFA pools of EPA and DHA behave differently and that this EPA pool is labile. These data have significant influence for understanding the physiologic relationship of EPA to CVD risk.

Both the absolute quantity and the bioavailability of the n-3 fatty acids affect incorporation into circulation and target tissues. Most of the n-3 fatty acids in supplemental fish oil and those in fish are in the triglyceride form with lesser quantities present in phospholipids. There appears to be much better bioavailability of n-3 in the triglyceride form compared with the ethyl ester form.17,18 The bioavailability of n-3 fatty acids from whole fish appears to be greater than that from supplements.17 Elvevoll and colleagues19 demonstrated enhanced incorporation of EPA and DHA from both smoked salmon and cooked salmon compared with almost triple the dose provided by cod liver oil. Provision of salmon twice per week resulted in dramatic increases in PLFA EPA and DHA with relatively small doses.

These data have direct relevance to the recommendations for fish consumption to meet dietary guidelines and in CVD risk reduction. Twice per week portions of 180 g and 270 g salmon during 4 weeks was effective in modifying PLFA proportions to near optimal levels of EPA and DHA for CVD risk reduction.8,18,20 Interestingly, increases in PLFA EPA, DHA,

Table 2. Plasma phospholipid n-3 and n-6 fatty acid proportions (mol %) at baseline and in response to salmon consumption for 4 weeks

<table>
<thead>
<tr>
<th>Polysaturated fatty acid</th>
<th>Salmon Portion</th>
<th>90 g (n=19)</th>
<th>180 g (n=19)</th>
<th>270 g (n=19)</th>
<th>P Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Baseline</td>
<td>4 Weeks</td>
<td>Baseline</td>
<td>4 Weeks</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>16.68±0.60</td>
<td>17.73±0.62</td>
<td>17.42±0.68</td>
<td>18.31±0.70</td>
<td>17.26±0.53</td>
</tr>
<tr>
<td>18:3n-6</td>
<td>0.79±0.02**</td>
<td>0.66±0.03*</td>
<td>0.77±0.03**</td>
<td>0.91±0.08**</td>
<td>0.72±0.03**</td>
</tr>
<tr>
<td>20:3n-6</td>
<td>4.93±0.19</td>
<td>4.02±0.15</td>
<td>4.96±0.23</td>
<td>4.21±0.18</td>
<td>4.99±0.21</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>18.75±0.90</td>
<td>17.76±0.34</td>
<td>18.22±0.91</td>
<td>16.39±0.55</td>
<td>17.63±0.79</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.85±0.04**</td>
<td>0.93±0.04**</td>
<td>0.90±0.04**</td>
<td>1.88±0.12**</td>
<td>0.88±0.03**</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>1.64±0.21</td>
<td>1.30±0.1</td>
<td>1.71±0.18</td>
<td>1.50±0.15</td>
<td>1.32±0.15</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>3.21±0.11</td>
<td>4.70±0.17</td>
<td>3.06±0.15</td>
<td>4.65±0.23</td>
<td>3.40±0.18</td>
</tr>
<tr>
<td>Σ n-3</td>
<td>5.64±0.22**</td>
<td>6.94±0.24*</td>
<td>5.53±0.22**</td>
<td>8.03±0.26**</td>
<td>5.51±0.24**</td>
</tr>
<tr>
<td>Σ n-6</td>
<td>45.23±0.45</td>
<td>43.02±0.74</td>
<td>44.24±0.50</td>
<td>42.42±0.55</td>
<td>44.64±0.75</td>
</tr>
<tr>
<td>Σ 20:5n-3, 22:6n-3</td>
<td>4.1±0.1w</td>
<td>5.6±0.2*</td>
<td>4.0±0.2w</td>
<td>6.5±0.2*</td>
<td>4.3±0.2w</td>
</tr>
<tr>
<td>20:4n-6/20:5n-3</td>
<td>22.5±1.3w</td>
<td>19.9±1.1w</td>
<td>20.7±1.2w</td>
<td>9.4±0.7*</td>
<td>20.6±1.1w</td>
</tr>
<tr>
<td>n-6/n-3</td>
<td>8.3±0.3y</td>
<td>6.4±0.3z</td>
<td>8.3±0.3z</td>
<td>5.4±0.3*</td>
<td>8.4±0.3z</td>
</tr>
</tbody>
</table>

*Based on analysis of variance. The treatment effect tests whether the response to the salmon differed depending on the amount of salmon consumed. The draw effect tests whether subjects responded to the salmon, regardless of the amount consumed. The treatment x draw effect tests for a differential response (ie, dose–response) to the amount of salmon consumed.

wwwMeans within a row not sharing a common superscript (w, x, y, z) are significantly different (P<0.05) by Tukey contrasts.
and total n-3 were enhanced in an identical manner to that of a fully controlled dietary intervention. Although the 90-g portion of salmon did not have the same magnitude of effect in increasing EPA, this portion of fish enhanced PLFA DHA and total n-3 levels. Thus, even small portions of salmon contribute significantly to n-3 status. Although more study is needed, these data suggest that incorporation of 180 g total per week of n-3–rich fish may yield optimum n-3 status.

It is unknown whether continued consumption beyond that shown here would result in continued elevation of PLFA n-3 levels. There was a dose response to intake across 4 weeks and washout PLFA proportions returned to baseline reflective of a diet with minimal n-3 intake. Whether continued intake at each of these portions would ultimately result in attainment of saturation levels of both EPA and DHA in tissue and circulation has not been evaluated. Interventions are required to determine the long-term effects of various doses of high-n-3 fish.

The primary strength of our study is the well-controlled manner in which the treatments were prepared and distributed to participants. The crossover design of the trial, the very high reported compliance, and the fact that participants returned to their usual diet between treatments and maintained their body weight throughout the trial strengthens the outcome data.

Limitations of the study include the small sample size, the fact that recruited participants were apparently healthy individuals, and the fact that no effect of salmon intake was observed on biomarkers of CVD risk. In addition, because participants’ diets were not monitored while they were receiving the treatment food, their total nutrient intake is unknown.

CONCLUSIONS

The results of this study demonstrate the effect of consuming various portions of farm Atlantic salmon on PLFA n-3 proportions in healthy men and women. Randomly assigned treatments resulted in marked changes in PLFA concentrations during the 4-week test periods in a differential manner. The proportion of DHA increased on all treatment levels, whereas EPA responded in a dose-dependent manner. Total n-3 concentration was enhanced and total n-6 and arachidonic acid concentrations were significantly reduced. The addition of farmed Atlantic salmon to the diet twice per week at portions of 180 g and 270 g modifies PLFA proportions of n-3 and n-6 fatty acids to levels associated with decreased risk for CVD.

Further work is needed to establish the optimal dose and duration of high n-3 fish intake for the modification of cardio-metabolic biomarkers in individuals who are at enhanced risk for or exhibit frank CVD. Identification of the appropriate dose of n-3–rich fish in promoting optimal n-3 status is required.

References

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STATEMENT OF POTENTIAL CONFLICT OF INTEREST
No potential conflict of interest was reported by the authors.

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