

Validation of an abbreviated FFQ for assessing DHA and EPA intake in
pregnant women.

By
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Sarah Crawford
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Chair: Susan Carlson, PhD

Heather Gibbs, PhD, RD

Beth Kerling, MS, RD

Debra Sullivan, PhD, RD

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The thesis committee for Sarah Crawford certifies that this is the
approved version of the following thesis:

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Chair: Susan Carlson, PhD

Date Approved: _____

Abstract

Background: Docosahexaenoic acid (DHA) is an omega-3 fatty acid found in variable amounts in the diet and is often accompanied by another omega-3 fatty acid, eicosapentaenoic acid (EPA) when the food chosen is seafood. While both nutrients support optimal health, DHA is of particular interest for pregnant women because of its hypothesized effect on infant visual and cognitive development. Results from randomized clinical trials (RCTs) of DHA supplementation in pregnant women, however, are mixed, and because dietary DHA intake is variable. A valid baseline measure of intake is imperative if trials are to be properly compared. The National Cancer Institute Diet History Questionnaire-II (DHQ-II) can estimate DHA and EPA dietary intake but it requires answers to 150 questions. An abbreviated 7-question food frequency questionnaire (FFQ) measures DHA and EPA intake more efficiently, however, neither the DHQ-II nor the abbreviated FFQ have been validated for DHA and EPA intake of pregnant women. Our goal was to determine the validity of each method for assessing DHA and EPA intake by comparing reported dietary intakes to red blood cell (RBC) phospholipid (PL) DHA and EPA, a standard measure of nutrient status. **Methods:** 309 pregnant women between 12 and 20 weeks gestational age who were enrolled in an on-going RCT completed both the DHQ-II and abbreviated FFQ as well as a venipuncture blood draw to assess RBC PL DHA and EPA. Pearson correlations between measures were determined. We additionally examined other potential covariables that might influence baseline DHA and EPA status. **Results:** The abbreviated FFQ was moderately correlated with both DHA and EPA status ($r=0.49$ and $r=0.56$, respectively) while the DHQ-II was poorly correlated with these two nutrients ($r=0.24$ and 0.30 , respectively). Parity and paternal education (collinear with income, non-Black race) were also significantly correlated with DHA and EPA status. Together with dietary intake estimated by the

abbreviated FFQ these three variables accounted for about 37% of the variance in DHA status and 44% of the variance in EPA status ($r^2=0.365$ and $r^2=0.44$, respectively). **Conclusion:** The abbreviated 7-question FFQ better represents DHA and EPA intake of pregnant women compared to the longer DHQ-II. Importantly this finding supports use of the abbreviated measure to increase efficiency in clinical trials and reduce participant burden. It is also important to consider variables other than dietary intake when predicting DHA and EPA status.

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Chapter 1 Introduction

Docosahexaenoic acid (DHA) is part of the omega-3 (n-3) long-chain polyunsaturated fatty acid (LCPUFA) family. Other n-3 LCPUFAs include eicosapentaenoic acid (EPA) and alpha-linolenic acid (ALA). These fatty acids have a variety of health benefits such as anti-inflammatory properties (1).

Consumption of DHA during pregnancy has a number of benefits. Some of these benefits include neurodevelopment (2, 3), reduced adiposity in body composition (4, 5), decreased risk of allergies and asthma (6), and reduction in early preterm birth (7, 8). During the third trimester of pregnancy, DHA is preferentially transferred from mother to the fetus, however, the amount of DHA transferred is dependent on the mother's dietary intake of DHA (9).

The human body can make DHA in small amounts by converting ALA to EPA and then EPA into DHA. However, this process is not very efficient and increasing consumption of ALA does not lead to an improved DHA status (10, 11). Because of this, humans must get DHA and EPA from the diet. Fatty fish are one of the richest sources of these two fatty acids (12) and supplements of fish oil or algal oil that contain DHA and/or EPA are also a significant source (13).

Studies in a number of countries have examined the various effects DHA has on health both in pregnant women and in other populations. Generally, these types of studies include some sort of dietary assessment to evaluate participants' normal dietary intake of DHA from food. In other countries, studies similar to the present one have been performed to validate different food frequency questionnaires (FFQ) for assessment of DHA and EPA intake during pregnancy, but

no such study has been done in the U.S. (14-18). Currently no validated FFQ exists for assessing DHA and EPA intake during pregnancy in the United States.

The purpose of the present study was to validate both a 7-question abbreviated FFQ and the National Cancer Institute's Diet History Questionnaire-II (DHQ-II) in a sample of pregnant women and also to establish which method is the superior indicator of DHA and EPA status. Both methods are used to assess dietary intake of DHA and EPA at baseline among individuals participating in the ADORE trial which is a phase-III, multi-center, randomized controlled trial to determine the effect of a higher-dose DHA supplement on preventing birth prior to 34 weeks (early preterm birth). Blood sample are collected at baseline and analyzed for fatty acid content of red blood cell (RBC) phospholipid (PL) DHA and EPA. Information regarding demographics, medical history, social history, and dietary intake is collected at baseline as well. (19).

The 7-question abbreviated FFQ has been validated in a small sample of healthy adults (20). However, this abbreviated FFQ has not been validated in a population of pregnant women. There are several factors that may cause diet to differ from normal during pregnancy (21). To ensure the abbreviated FFQ is appropriate for use in pregnant women, this validation study was necessary.

The DHQ-II is a validated method for assessing dietary intake over a period of time (we are using 12 months), however because it is designed to assess the whole diet, it has over 150 questions and is much more time consuming to administer than the abbreviated FFQ. The DHQ-II does include a few questions regarding supplement intake, but these are not included in the estimated DHA and EPA intake (22).

The proposed study estimated DHA and EPA intake from the abbreviated FFQ and the DHQ-II. The estimated intake from both questionnaires was compared to RBC PL DHA and EPA. These individual correlations were used to determine which method better reflected DHA and EPA status. Other factors that may influence DHA status include age, parity, socioeconomic status (SES), and race and ethnicity. We also evaluated if these variables were related to DHA and EPA status.

Primary Research Questions

1. How does the estimate of DHA and EPA obtained with the abbreviated FFQ compare to baseline RBC PL DHA and EPA?
2. How does the estimate of DHA and EPA intake obtained with the DHQ-II compare with baseline RBC PL DHA and EPA?
3. Which is a better predictor of baseline DHA and EPA status, the abbreviated FFQ or the DHQ-II?

Secondary Research Question

1. Do demographic variables influence DHA and EPA status?

Chapter 2 Literature Review

The purpose of this chapter is to establish the most feasible method of assessing n-3 LCPUFA intake in pregnant women. Currently no validated method for assessing n-3 LCPUFAs intake during pregnancy exists in the United States. This type of tool would be beneficial because two n-3 LCPUFAs, DHA and EPA, are related to positive pregnancy outcomes and are the center of ongoing research.

Most studies examining the effects of DHA and EPA during pregnancy assess dietary intake of these fatty acids and collect a blood sample to measure DHA and EPA status at baseline. Several methods exist for assessing dietary intake each with its own benefits and limitation. Choosing the most appropriate method depends on several factors such as the intended use and desired information to be gathered. This review will examine three common methods used in research for assessing dietary intake to narrow down the most appropriate method to estimate DHA and EPA intake during pregnancy for research.

DHA and EPA are n-3 LCPUFA that are important for numerous physiological processes in the human body including fetal development. It is hypothesized that during pregnancy, DHA requirements are even higher. These things will be discussed in detail later in this chapter. Humans can obtain DHA and EPA for these processes several different ways.

Sources of DHA

Endogenous DHA and EPA is one source of these n-3 LCPUFAs. The human body can make small amounts of EPA from ALA and EPA can then be converted into DHA. The evidence suggests that pregnant women cannot make enough EPA and DHA from ALA to result in

optimal pregnancy and newborn outcomes, so EPA and DHA must be obtained from dietary sources. However, increasing ALA intake alone does not increase DHA status and has little effect on EPA status (10, 11). Arterburn et al. (2006) reported that ALA supplementation resulted in a small, non-significant increase in plasma EPA and had no effect on plasma DHA (10). Harper et al. (2006) did find a significant increase in plasma EPA after ALA supplementation, but like Arterburn et al., they did not observe any change in plasma DHA after supplementation of ALA (11). The evidence shows that the most effective way to improve DHA and EPA status is by direct consumption.

The findings of Patterson et al. (2015) also support the conclusion that direct consumption is necessary to improve DHA and EPA status. Patterson et al. supplemented participants with 250 mg, 500 mg, and 1000 mg of EPA+DHA in a stepwise fashion each for four weeks. Blood samples were drawn at baseline and at the end of each supplement period to determine fatty acid composition of the blood using three biomarkers. Each biomarker showed a positive linear relationship between dose of EPA+DHA and percent EPA and DHA in the each of the three biomarkers. As the dose of EPA+DHA increased, so did the amount of those fatty acids in the blood (23). In summary, the evidence from Arterburn et al., Harper et al. and Patterson et al. indicates that to improve DHA and EPA status and get the most benefits, DHA and EPA should be consumed instead of ALA.

DHA and EPA are found in some foods and in dietary supplements. The highest concentration of DHA and EPA in foods is from fatty fish such as mackerel, bluefish, herring, and salmon. Eggs and poultry are also sources as well (12). A large portion of the population consumes DHA and EPA as a supplement. Clarke et al. (2015) found that non-vitamin, non-mineral dietary supplements were the most commonly used complementary health approach in

the United States in 2012. Of these supplements surveyed, fish oil was by far the most widely consumed supplement with over 20% of their sample reporting use of a fish oil supplement (13). Both dietary intake and supplement consumption are effective ways to consume DHA and EPA. Although the body is capable of synthesizing DHA and EPA endogenously, it is an inefficient process and in order to maximize the benefits of these fatty acids, direct consumption is necessary.

Health Benefits

DHA functions as an essential part of the phospholipid membrane of cells all throughout the body and is particularly concentrated in membranes in the nervous tissue and in retina (24). There is strong evidence that n-3 LCPUFAs are beneficial for heart health and prevention of cardiovascular disease. Other evidence supports the idea that LCPUFAs promote cognitive function in older adults, though this evidence is less sound compared to that for cardiovascular health (1). Throughout the third trimester of pregnancy, DHA is preferentially transferred to the fetus for development. The amount of DHA the fetus receives is largely dependent on the mother's diet (9). DHA intake during pregnancy has proven to be beneficial in cognitive development, childhood body composition, neonatal outcomes, and more (2-5, 7, 8).

Cognitive development is just one function of DHA during pregnancy. Hibbeln et al. (2007) observed a dose-response association between the level of seafood intake mothers consumed during pregnancy and suboptimal outcomes in the offspring. Specifically, verbal IQ, fine motor skills, prosocial behavior, and social development were measured in their offspring; and seafood intake was inversely related to suboptimal developmental outcomes; i.e. infants born to mothers with higher seafood intake had a lower risk of suboptimal developmental outcomes

(2). Consistent with the findings of Hibbeln et al., Vollet et al. (2017) reported that fish oil supplementation before and during pregnancy was associated with a lower risk of failing the problem-solving domain of the Ages and Stages Questionnaire (3). Since these two studies looked at fish oil as a whole, it cannot be ruled out that EPA and other nutrients in fish may play a role in the findings.

In addition to the positive effects on cognitive development fish oil may have, DHA, along with other n-3 LCPUFAs, has been shown to influence adiposity as a child grows and develops. Donahue et al. (2011) found that the offspring of mothers who consumed more DHA and EPA at mid-pregnancy had lower odds of childhood obesity. A similar relationship was found in cord blood. Higher levels of DHA and EPA in cord blood plasma correlated with lower adiposity (4). Pereira-de-Silva et al. (2015) also found supportive evidence that maternal DHA and n-3 LCPUFAs influence body composition of offspring. A positive association between maternal DHA intake and ponderal index was found in male offspring. In female offspring, a lower ratio of n-3:n-6 LCPUFAs was positively correlated with fat mass and percent fat mass (5). Additionally, Hidaka et al. (2018) found a positive correlation between change in maternal RBC-PL DHA and fat-free mass. They also observed positive trends between fat-free mass and both intent-to-treat (DHA supplement or placebo) and DHA status at delivery (25). Together these studies suggest beneficial effects of maternal DHA status in pregnancy on offspring body composition and the need for further research including randomized trials that look at prenatal DHA intake and body composition of the offspring.

DHA has also been shown to benefit several pregnancy and neonatal outcomes. In a randomized controlled trial, Carlson et al. (2013) found that the children of mothers in the DHA group had higher birth weight, length and head circumference compared to those in the placebo

group. Furthermore, there were fewer infants born before 34 weeks gestation in the DHA group. Of the infants in the DHA group who were born prior to 34 weeks, they had fewer complications and shorter hospital stays compared to the early preterm infants in the placebo group (7). In support of the Carlson et al. findings, Harris et al. (2015) also found that DHA intake reduced the risk of early preterm birth (8). Research is currently ongoing to further investigate DHA's influence on pregnancy outcomes such as early preterm birth (19).

Prenatal DHA and EPA are proposed to influence the development of allergies and asthma in children. In a cohort of 736 pregnant women randomly assigned to 2400 mg of fish oil or placebo, Bisgaard et al. (2016) explored the effects of maternal n-3 LCPUFA supplementation during pregnancy on the incidence of asthma and wheeze disorders in offspring. The results showed that the n-3 LCPUFA supplement reduced the risk of persistent wheeze or asthma in the offspring compared to the placebo group (6). Bisgaard et al. and the other studies mentioned have shown that DHA may function in a wide variety of developmental processes during pregnancy. It should be noted that none of the studies reported any serious adverse effects from DHA or fish oil. So, although research is still sorting out the exact role DHA plays in fetal development, expectant mothers can still consume DHA without known risk but with the possibility of achieving some or all of the possible benefits.

How much and at what time point have studies supplemented DHA?

The evidence for increasing DHA intake during pregnancy continues to grow however, these studies vary in the dose and time-period of DHA intake. Additionally, some of the studies also explore both DHA and EPA. Courville et al. (2009) performed a secondary analysis of an RCT in which pregnant women received either a DHA-rich functional food or placebo 5 days per

week starting between 20-24 weeks gestation until delivery (26). Carlson et al. (2013) randomly assigned women to receive either 600 mg DHA capsules from algae or a placebo of corn and soybean oil beginning between 8 and 20 weeks gestation and ending at delivery (7). Muhlhauser et al. (2014) randomly assigned women less than 21 weeks gestation to receive a fish oil that provided 800 mg DHA and 100 mg EPA or a placebo until delivery (27). Harris et al. (2015) randomized subject to receive either 300 mg DHA, 600 mg DHA, olive oil placebo, or nutrition education to increase DHA consumption by 300 mg per day beginning at 20 weeks gestation until delivery (8). From 24 weeks gestation until 1 week postpartum, Bisgaard et al. (2016) randomly assigned participants to receive a fish oil supplement with 2400 mg of n-3 LCPUFAs supplement that was 55% EPA and 37% DHA or placebo (6).

Both Carlson et al. (2013) and Muhlhauser et al. (2014) initiated supplementation prior to 21 weeks gestation, while Courville et al. (2009), Harris et al. (2015) and Bisgaard et al. (2016) did not begin supplementation until 20 weeks or later. Likewise, Coureville et al. (2009), Carlson et al. (2013) and Harris et al. (2015) provided only a DHA supplement, while Muhlhauser et al. (2014) and Bisgaard et al. (2016) supplemented participants with both DHA and EPA (6-8, 26, 27). The dose of supplementation also varied from 300 mg to 2400 mg among all of these studies. These are just a few examples of how different study designs can be and how difficult that makes comparing results between studies. Further research is needed to isolate the amount of DHA and/or EPA and the time period most beneficial for these outcomes. A valid and consistent way to assess dietary intake of DHA and EPA at baseline would be useful for those future studies.

Methods of Assessing Dietary Intake

There are several methods that can be used to assess dietary intake. Three methods commonly seen in research are the FFQ, the diet record, and the 24-hour dietary recall (24-hour recall).

A FFQ is typically composed of a list of foods and questions about each food. It asks about frequency of consumption for a specified time period. Sometimes respondents report portion size, while other times the questionnaire gives a portion size and asks the participant about frequency of consumption relative to the specified portion size. Estimated intake of nutrients and food groups can be obtained as well as characteristics of the diet. This type of questionnaire is typically used to estimate intake over a period of time because it can easily capture intake for the desired time period.

A FFQ can be beneficial because it has the capability to gather information regarding frequency of a wide variety of foods consumed. However, such a questionnaire is typically lengthy and time consuming to administer. To reduce burden on research participants, a FFQ can be tailored to target a specific food, nutrient, or food group of interest making it a more reasonable length. It can also be designed for certain sub-groups or populations whose dietary patterns may differ from that of the majority population. Some limitations of using a FFQ are that details of the foods are not captured and if the list of foods does not reflect the respondent's diet, then the completed questionnaire will not be representative of their dietary patterns. Furthermore, depending on the purpose of the questionnaire, a FFQ can be lengthy and somewhat burdensome to the participants. This must be considered when designing a questionnaire in order to get the best data possible while limiting participant burden.

Additionally, the FFQ has a much higher chance of measurement error compared to diet records or 24 hour recalls (28). Although it is not perfect for all situations, the FFQ has unique benefits that a diet record or 24-hour dietary recall is limited by.

A diet record is basically a diary of everything the respondent eats or drinks over a set number of days. It is typically preferred that anything consumed be recorded immediately after eating, so that portion sizes are not forgotten, and no foods or beverages get omitted. Sometimes portion sizes are measured using a scale, however household measures can be used as well. Diet records are beneficial because respondents can record what they eat throughout the day, limiting the possibility for recall bias and providing more accurate reporting of portion sizes. Diet records can be expanded to obtain detailed information regarding the foods eaten. However, it is burdensome to the respondent. Respondents require training to record their intake and after several consecutive days of keeping a diet record, they may become less careful about recording. If records are entered into an electronic system, study staff may be burdened as well. Additionally, little information is gathered regarding frequency of eating different types of foods (28). A diet record is able to obtain the most detail about food eaten but is limited by the burden placed on participants to do so.

A 24-hour dietary recall is an interview in which the respondent is asked to report everything he or she ate the day prior. A multiple-pass method ensures that the subject has not forgotten some food eaten at an unusual time and get detailed information regarding foods consumed. The interviewer first asks the respondent to recall everything eaten the day before and makes a list. Then, the interviewer goes back through the list to capture details about preparation, portion sizes, and any other relevant information. Once the interviewer feels there is enough details for all foods, he or she goes through one final pass with the respondent to review the

information and make sure all the information needed is collected. In order to be considered representative of individual intake, 24-hour recalls should be obtained on one or two occasions during a weekday and on one occasion during the weekend.

Dietary recalls place a lower burden on the participant and are able to capture general daily food intake with detailed information about the foods consumed. Literacy of the respondent is irrelevant unlike the FFQ and diet record. Since respondents are asked about the day immediately prior, it usually is not too difficult for people to remember details about what they ate. Also, because respondents are asked about what they have already consumed, they are less likely to alter dietary patterns for reporting compared to a diet record. Software exists that allows study staff to interview the respondent and enter data simultaneously, which reduces burden compared to the diet record.

The 24-hour dietary recall is limited by the large amount of training that interviewers must undergo. The interviewer must ensure enough details are reported to be reflective of the actual diet. This method may be less accurate for estimating intake of foods that are not commonly eaten, such as seafood; and if the recall is taken the day after an event or special occasion the report may not be reflective of typical dietary patterns. Importantly, for my study of EPA and DHA intake, 24-hour dietary recalls may underrepresent their intake of seafood and other food sources that contain these nutrients. Lastly, respondents report retrospectively, opening up the opportunity for recall bias and misrepresentation of portion sizes (28). All three of these methods have their own unique benefits and limitations. There are also challenges and limiting factors to consider no matter what method is used for assessment.

Challenges of Assessing Dietary Intake

Assessing dietary intake comes with several challenges. Often people will alter their eating patterns because they know it is going to be recorded. Over-reporting of healthier foods such as fruits or vegetables and under-reporting of less healthy foods such as junk foods or desserts is also common. Estimating portion size is one of the biggest concerns with almost all dietary assessment tools. Individuals differ in what they consider a “serving” and coming up with a way to get all participants on the same page can be difficult. Visual aids are often used and provide a way to ensure better patient reporting when it is important to know how much of a food is consumed. If a new method of assessing dietary intake is created, it must be validated to prove its effectiveness before professionals will want to use it. A method that has been validated in one population, may need to be validated again for a different population. For example, a FFQ validated for use in the general adult population may not be valid for use in pregnancy, when women commonly consume prenatal vitamins. Another factor to consider is that dietary intake tools can often be either self-administered or interview administered. The benefits and limitations of each must be considered when choosing a method (28). These are all challenges to assessing diet in any population, during pregnancy there are other factors to consider in addition to those general ones.

Challenges of Assessing Dietary Intakes Specific to Pregnancy

The time of assessment must be considered when assessing dietary intake during pregnancy. When a woman is trying to get pregnant or finds out that she is pregnant, she may change her dietary patterns to benefit her baby. While this is good, it is an extra factor to consider when assessing dietary intake for research purposes (21). Complications of pregnancy

must also influence dietary intake. GI distress such as nausea and vomiting are common side effects of becoming pregnant and usually resolve by the 20th week of gestation (29).

Additionally, it is not uncommon for a woman to be put on bed rest to prevent preterm labor or other serious complications. These factors could potentially play a role in a woman's eating habits. If she has an upset stomach, she is likely going to avoid the foods that trigger it. While on bed rest, a woman may not cook as much or prepare meals like usual (21). Taking these factors into consideration, the assessment should occur at the most appropriate point in time depending on what information is needed for the research question. On top of the general challenges and those specific to pregnancy, DHA and EPA are in a limited number of foods, many of which are not consumed regularly. This should also be accounted for when assessing the intake of DHA and EPA during pregnancy.

How have other DHA studies assessed DHA dietary intake in the past?

In past studies, the FFQ questionnaire is the most commonly used tool to assess dietary intake of DHA and EPA in research. Hibbeln et al. (2007) used a FFQ to assess seafood consumption at 32 weeks gestation in over 11,000 pregnant women to study the risks and benefits of seafood consumption during pregnancy (2). Donahue et. al (2011) studied how LCPUFAs impact adiposity in the offspring and also used a FFQ, at 29 weeks gestation, assessing maternal seafood consumption during pregnancy. The questionnaire was composed of over 140 foods and beverages and asked for frequency of consumption in the past 3 months (4). Like Hibbeln et al. and Donahue et al., Pereira-da-Silva et al. (2015) used a FFQ however, Pereira-da-Silva et al. assessed diet during the whole pregnancy. Women completed the questionnaire immediately after delivery and questions assessed food intake throughout the entire pregnancy. The study was primarily focused on studying how maternal LCPUFAs impact

body composition of the offspring (5). Bisgaard et al. (2016) used a FFQ that contained 360 items to assess dietary intake throughout the 4 weeks leading up to randomization in their study, which focused on the effects maternal EPA and DHA have on the development of asthma and wheeze in offspring (6). When studying length of gestation, Harris et al. (2015) uniquely used a pictorial food frequency inventory designed specifically for assessment of n-3 LCPUFAs to capture intake between 16 and 20 weeks of gestation (8).

While these studies varied among time points of assessment and whether or not the FFQ assessed whole diet or DHA and EPA only, there is a common trend in that a FFQ was used to capture dietary intake for a period of months. The food frequency inventory used by Harris et al. was developed specifically for their study and validated in a subgroup of participants against erythrocyte DHA (8). Hibbeln et al., Periera-da-Silva et al. and Bisgaard et al. all used FFQs that had been previously validated (2, 5, 6). Donahue et al. took a unique approach by modifying an already validated FFQ to be more appropriate for use in pregnant women. Their modified version was then validated against erythrocyte fatty acid concentrations in pregnant women (4). Developing a new FFQ like Donahue et al. and Harris et al. allows the investigator to tailor the questionnaire so it collects the exact information desired. However, it does require the extra steps of creating the questionnaire and validating it.

Challenges in creating a FFQ

A challenge when designing a FFQ is deciding how many questions to ask. Foods may be grouped together based on similar nutrient content. Researches must design the questionnaire in such a way that limits participant burden and still captures the necessary information for the study.

How to define portion sizes is another major factor. Some questionnaires specify a portion and the participant is asked to report frequency consumed based on that portion size. Other questionnaires have the participant report portion size based on how much they would typically eat as one serving. Both of these methods have limitations and benefits (21). Using a pre-specified portion size gathers the data in the same unit of measure for all participants, which may make analysis easier. When a visual aid is provided, it can be a very accurate method of establishing serving size for a questionnaire. A limitation is that variability will occur between individuals as to how much food they think is in that specified portion size. This could lead to under- or over- reporting. Having the participant report their serving size may make it easier for the participant to quantify how much they eat. However, this essentially adds twice as many questions to the questionnaire and many people do not truly know how much food is in an ounce or gram, etc. so they just estimate based on their opinion. This may result in inaccurate reporting as well. Again, visual aids can provide standardization for more accurate and reliable information. Both methods of establishing portion size have flaws but using visual aids can help to get accurate information.

Obtaining information regarding supplement intake is another consideration when designing a FFQ. Because there is such a wide variety of supplements available with little consistency between content, reporting of brand name and specific details is likely the easiest way to acquire the most accurate information regarding supplement use (21). Once a new FFQ has been designed, it must be validated in a sample of the target population.

Validation of Dietary Intake Methods

Validation of a FFQ typically requires another, already validated, method of dietary intake, such as diet record, obtained for the same time period as the FFQ, for comparison of intake (30). The new questionnaire is considered valid if estimated intake from both questionnaires meets a predetermined level of agreement. For a more objective and independent measurement, nutrient biomarkers can also be used if feasible (31). According to Michels (2003) biomarkers should be the standard method of validation because there is a lower chance of error. However, an easily obtainable biomarker does not exist for all nutrients making the method of comparing two dietary assessments necessary (31). Since biomarkers of DHA and other n-3 LCPUFAs are easily measured, this is a common method for validation of FFQs designed to assess n-3 LCPUFAs specifically (14-18, 20). Some studies use both a biomarker and a second dietary intake method to compare the FFQ (18, 20).

When validating a FFQ for pregnant women, the same challenges of assessing dietary intake during pregnancy would hold true for the validation process. Because pregnancy brings about those complications that may impact a woman's diet, a FFQ validated in a non-pregnant adult population may not be valid a population of pregnant women. Therefore, a validation study must be done to ensure the questionnaire is capturing accurate and precise information (21). Furthermore, while several FFQs for assessing n-3 LCPUFA intake during pregnancy have been developed and validated in other countries, there is currently no questionnaire that has been validated for use in pregnant women in the United States. Because diet is largely reflective of geographical location and culture, a questionnaire validated in another country is not necessarily valid for a similar sub-group in the United States. Validation can be done either using a separate group of subjects from the same target population or in a sub-group of participants from the

study sample. The latter is commonly done because often blood samples are already being collected for other study procedures and the participants are already completing the questionnaire. The validation study can easily be performed with minimal additional work.

Biomarkers

Several biomarkers exist for measuring DHA status in the blood and can be used in the validation of a FFQ to assess DHA intake. Plasma and RBCs appear to be the two most common methods of assessing DHA status in research (26, 32). Courville et al. (2009) concluded that both plasma phospholipids and RBC PL are suitable biomarkers to measure DHA status in pregnant women and their offspring (26). However, Sun et al. (2007) found RBCs to be a superior indicator of long-term intake compared to plasma (32). Serum fatty acid composition can also be used as a biomarker of n-3 LCPUFAs, though this method is less common in the literature. Arab et al. (2003) reports that while serum fatty acid levels are sensitive markers for LCPUFAs, there is large variability in triglyceride levels. This may be why alternative methods are more commonly used (33). These biomarkers provide an objective measurement of DHA status and are reflective of dietary intake, however some variability does exist between reported dietary intake and level of DHA or other fatty acids in the blood. This is because there are factors in addition to dietary intake that affect DHA status in the body.

An individual's DHA status can vary due to several reasons other than diet. As discussed earlier, small amounts of DHA can be synthesized but some people make DHA better than others; e.g., women synthesize DHA better than men (34). Bisgaard et al. (2016) found that participants with the minor allele FADS genotype had lower baseline levels of DHA and EPA prior to intervention (6). Muhlhauser et al. (2014) explored the effects of factors other than diet

that may impact DHA status in the cord blood of newborn infants whose mothers had similar DHA intake during pregnancy. They found that the number of DHA capsule consumed and gestational age at delivery accounted for about 35% of the variation in cord blood DHA of infants. Other factors that were significantly related include maternal BMI, parity, intake of other supplements, GDM, C-section, induction of labor, and infant sex, each accounting for less than 2% of variation. Muhlhauser et al. concluded that over 65% of variation in infant cord blood DHA is due to factors that have yet to be identified (27). Although the biomarkers and dietary intake assessments both have limitation, when used in combination the two can provide a more nuanced picture of an individual's current DHA status and typical intake.

Why is FFQ the most appropriate method?

A FFQ is the most appropriate method for studying a large population of pregnant women for several reasons, keeping in mind that the primary goal is to estimate DHA and EPA intake. First, information regarding frequency of intake is indispensable. An individual's seafood intake can be variable and inconsistent, making it almost impossible for a diet record or 24-hour dietary recall to accurately depict seafood intake for every participant. Since fish oil supplements are so common, details about supplement intake are also crucial. Second, because of the possible inconsistent intake of DHA and EPA-rich foods, dietary patterns must be assessed over a longer period of time, which is often in the past. Strengths of the other methods, such as information regarding cooking and food preparation, are less important here. A FFQ is able to gather the most important dietary information with the greatest efficiency.

This information needs to be obtained with minimal burden on the participant. Although a FFQ must be long when it tries to assess the whole diet, a questionnaire designed specifically

for assessing DHA and EPA intake can be short and simple for participants to complete. A diet record or 24-hour dietary recall would not be able to depict frequency of intake the way a FFQ can. They also make it much more difficult to capture longer-term information because several days of either diet records or 24-hour dietary recalls at different time points would have to be completed. If information is needed regarding dietary habits in the past, then the FFQ is the only feasible method as diet records and 24-hour dietary recalls assess current patterns. Even though FFQs are known to have a higher chance of error compared to diet record or 24-hour dietary recall, there are many instances in which a FFQ is the most appropriate method. They are widely used and accepted in the literature (28).

In conclusion, in the United States, a validated FFQ specific to DHA and EPA intake during pregnancy does not exist. The aim of this study is to validate a seven-question abbreviated FFQ for assessing DHA and EPA intake during pregnancy. This tool could be useful in both research and clinically. While published research points to several benefits DHA may provide during pregnancy with minimal risk as stated earlier in this review, there is still ongoing studies in many areas related to DHA intake. Establishing the validity of this abbreviated FFQ would be useful to future research in these areas. Clinically, use of a quick and easy method of assessing intake of these n-3 LCPUFAs could provide clinicians with the ability to individualize recommendations regarding intake of these nutrients.

Chapter 3 Materials and Methods

The present study aimed to validate a 7-question abbreviated FFQ by correlating estimated intake of DHA and EPA from the abbreviated FFQ to RBC PL DHA and EPA levels. We additionally aimed to examine how well the National Cancer Institute's DHQ-II estimated DHA and EPA intake compared to the abbreviated FFQ. Pregnant women between 12 and 20 weeks gestation were recruited for participation in the ADORE trial. Eligibility for participation required women to be over 18 years of age, be between 12 and 20 weeks gestation, consume the investigational supplement and 200 mg DHA supplement provided, be available by phone, and speak English or Spanish. Individuals were ineligible if they were unwilling to stop consuming another supplement with more than 200 mg of DHA, had an allergy to any components of the capsules, were expecting multiple infants, or were deemed ineligible due to clinical judgment by the research team such as women taking an anticoagulant or women who had a history of incompetent cervix (35).

Recruitment for the ADORE trial began in June 2016 and is ongoing. Participants are primarily recruited in academic hospital outpatient obstetrics clinics. At the time of analysis for the present project, there were 309 subjects enrolled in the ADORE trial with all baseline data available.

My primary questions were: (1) How do the abbreviated FFQ and the DHQ-II compare to baseline RBC PL DHA and EPA? (2) Which one is more strongly correlated with RBC PL DHA and EPA? And (3) Which is a better predictor of baseline DHA and EPA status? My secondary research question was: What variables, other than diet and supplement intake, influence baseline DHA and EPA status?

Recruitment and Enrollment of Participants

Recruitment for the ADORE trial is currently being conducted at three sites. The University of Kansas Medical Center (KUMC) in Kansas City, Kansas, The Ohio State University (OSU) in Columbus, Ohio and the University of Cincinnati Medical Center (UC) in Cincinnati, Ohio. KUMC is the central administrative and data coordinating site. At each site, eligible individuals are approached by a trial recruiter. Individuals who agree to participate are then required to attend the baseline study visit which must be completed after week 11 and before week 20 of gestation.

Ethical considerations

Prior to any study procedures taking place, participants were required to provide informed consent for the ADORE trial. The present study was covered under the existing ADORE protocol approved by the KUMC-IRB (STUDY00003455) which serves as the central IRB for the trial. The present study did not require any additional action from the participants not covered under the main trial consent.

Completion of Questionnaires

At the baseline study visit for the ADORE trial, the abbreviated FFQ, the DHQ-II, and other health and demographic questionnaires were completed and a venous blood sample was drawn. The abbreviated FFQ was interviewer-administered and focused on intake of DHA-rich foods over the past two months. A trained member of the study team completed the questionnaire with each participant. The questions specified portion size as a three ounce serving of fish/seafood and meat. A deck of cards was used as a visual aid to represent a three ounce

serving. The abbreviated FFQ included seven questions (see Appendix A). The first three questions were in regard to fish and seafood. Fish and seafood are categorized based on amount of DHA they contain: low, medium or high DHA level. Questions four and five assessed intake of liver and egg yolks, respectively. Consumption of chicken, turkey, and other poultry was evaluated in question six. The last question, number 7, asked about intake of n-3 dietary supplements and functional foods. Participants also completed the DHQ-II at the baseline study visit, which was intended to evaluate overall dietary intake for the prior 12 months. The separate abbreviated FFQ for DHA-rich foods was collected to obtain information regarding intake of those n-3 fatty acids specifically (19).

Materials required for data collection included the abbreviated FFQ, the DHQ-II, other baseline ADORE questionnaires and all supplies related to blood sample collection and fatty acid analysis. A trained interviewer administered the abbreviated FFQ and baseline questionnaires to participants using a computer. Each participant completed the electronic version of the DHQ-II after instruction from a trained researcher. The Diet-Calc software was downloaded for analysis of the DHQ-II data.

Collection and Analysis of Blood Samples

At the baseline visit, blood samples were collected for each participant by venipuncture by a trained phlebotomist. Two 4 ml potassium-EDTA tubes were obtained. Samples were placed on ice immediately and were centrifuged within 24 hours to separate plasma, buffycoat, and anticoagulated RBCs before being stored at -80 degrees C. Monthly, samples from OSU and UC participants were shipped to KUMC on dry ice for sample analysis. Further processing of samples was completed by a laboratory technician at KUMC. Gas chromatography was used to

analyze the fatty acid content of the RBCs using standard technique (35). Results were reported as RBC-DHA and EPA weight percent of total fatty acids (19).

Calculating Dietary DHA and EPA Intake from Questionnaires

Data from the abbreviated FFQ and supplement intake form was pulled by the trial administrator to calculate DHA and EPA exposure from dietary supplements and functional foods (FFQ question #7). First, participants who reported taking a DHA-containing dietary supplement during the two months that preceded enrollment were identified. A student then used a Microsoft Excel spreadsheet to calculate total exposure of DHA and EPA over the two months preceding enrollment. Briefly, each participant's study ID number, enrollment date, and supplement name were put into the spreadsheet. The dietary supplement label was searched for to find the exact amount of DHA and EPA in each supplement. The NIH Office of Dietary Supplement's Dietary Supplement Label Database (DSLD) was searched first (36). If the DSLD did not have a supplement, then the CNR's Supplement Online Wellness Library (OWL) was searched (37). If a supplement was not found at either of these sources, then the brand or a closely linked website was used as the reference. The amount of DHA and EPA in each supplement was recorded in the excel spreadsheet calculator along with start and stop dates of the supplement. The spreadsheet calculated total exposure to DHA and EPA over the two-month time period prior to enrollment.

For the remaining six questions on the abbreviated FFQ, DHA and EPA exposure were also calculated using a Microsoft Excel spreadsheet. The spreadsheet multiplied the amount of servings reported by a corresponding factor of likely DHA exposure to get intake of DHA and EPA in milligrams per day. Exposure from the first six questions was then added to exposure

from question seven to provide total DHA and EPA exposure over the two-months preceding enrollment.

Data from the DHQ-II was analyzed using Diet-Calc software (19). Diet-Calc calculated total intake servings of 176 nutrients, dietary constituents, and food groups (22). The present study only focused on estimated intake of DHA and EPA.

Statistical analysis

Microsoft Excel and SPSS were used to analyze data. General descriptive statistics include means and standard deviations to describe the population. To compare level of dietary intake to RBC PL level, Pearson's correlation coefficient was calculated. Correlations and linear regressions were also calculated to compare age, race and ethnicity, socioeconomic status (SES), number of pregnancies, and number of births to RBC PL DHA and EPA and dietary intake. Significance set at $p < 0.05$

Schedule of activities

Data collection for the ADORE trial began on June 6, 2016 and is ongoing. For the present study, analysis of data began in February 2018. Data were analyzed for all ADORE participants for whom baseline data were available by February 2018. After data analysis was completed, the write up of the thesis began. The thesis was defended on April 24, 2018.

Chapter 4 Results

Subject characteristics

Baseline data were available for 309 pregnant women enrolled in the ADORE trial. Of these, 109 women were from KUMC in Kansas City, 98 were from UC in Cincinnati, and 102 were from OSU in Columbus. Subject characteristics are described in Table 1.

Relationship of estimated intake to DHA and EPA status

The abbreviated FFQ moderately correlated with RBC DHA and EPA ($r=0.487$, $p < 0.01$ and $r=0.536$, $p < 0.01$, respectively). Correlations between the DHQ-II and RBC DHA and EPA, while still statistically significant, were much weaker ($r=0.239$, $p < 0.01$ and $r=0.297$, $p < 0.01$, respectively). When comparing the abbreviated FFQ and the DHQ-II the correlations were again significant, but weak ($r=0.318$, $p < 0.01$ for DHA and $r=0.317$, $p < 0.01$ for EPA) (Table 2).

Impact of Supplements

I investigated why the abbreviated FFQ and the DHQ-II were not strongly correlated with each other since they are both tools used to measure dietary intake. Importantly, I identified differences in how supplement intake was incorporated into the estimated DHA and EPA intake as the main cause in variation. The abbreviated FFQ asks for very specific details regarding DHA and EPA containing supplements, while the DHQ-II asks about supplement usage but does not capture supplements of DHA and EPA well and does not include supplements in the estimation of DHA and EPA.

When DHA and EPA exposure from supplements was removed from the abbreviated FFQ, the correlations with the DHQ-II improved ($r=.465$, $p < 0.01$ for DHA and $r=0.476$, $p<0.01$ for EPA) (Table 2). The strongest correlations were found between the abbreviated FFQ (which includes more detailed description of supplements) and the DHQ-II when those details of DHA and EPA supplements were added to the DHQ-II ($r=0.843$, $p < 0.01$ for DHA and $r=0.897$, $p < 0.01$ for EPA) (Table 2).

Influence of demographic variables on DHA status

Factors hypothesized to have an impact on DHA and EPA status include age, parity, SES, race, and ethnicity. We found no effect of maternal age, race, and ethnicity on DHA and EPA status, and thus each was removed from the statistical model. Several measures of SES were tested including maternal education, paternal education, income, insurance type, and marital status. Paternal education, collinear with income ($r=0.697$), was found to be the strongest predictor and was kept as the sole measure of SES. The final statistical model included RBC PL as the dependent variable, and the abbreviated FFQ, parity, and paternal education as the independent variables. A model was completed for both DHA and EPA. The abbreviated FFQ, paternal education, and parity accounted for about 37% of the variation in RBC-DHA ($r^2=0.365$) (Figure 1). and 44% of the variation in RBC-EPA ($r^2=0.44$) (Figure 2).

Table 1. Subject Characteristics

Variables	Mean \pm SD or Total (%)
RBC DHA (%)	6.17 \pm 1.52
RBC EPA (%)	0.48 \pm 0.28
FFQ DHA (mg/d)	143.97 \pm 132.26
FFQ EPA (mg/d)	67.69 \pm 103.76
DHQ-II DHA (mg/d)	56.41 \pm 68.87
DHQ-II EPA (mg/d)	27.57 \pm 45.04
Age (yrs)	30.30 \pm 5.49
Parity (no.)	1.18 \pm 1.42
Maternal Education (yrs)	14.86 \pm 2.85
Paternal Education (yrs)	14.60 \pm 2.72
Site	
KUMC	109 (35)
UC	98 (32)
OSU	102 (33)
Race	
White	181 (59)
Black or African American	93 (30)
Asian	12 (4)
American Indian or Alaskan Native	3 (1)
Other	6 (2)
2 or more races	13 (4)
Unknown	1 (0)
Taking DHA-Containing Supplement	
Yes DHA Supp.	132 (43)
No DHA Supp.	177 (57)

Table 2. Pearson's Correlation Coefficients for primary outcome variables with and without adjustment for supplement intake.

	DHA		EPA	
	Correlation (r)	Sig. (p)	Correlation (r)	Sig. (p)
Abb. FFQ vs. RBC-PL	0.487	< 0.01	0.536	< 0.01
DHQ-II vs. RBC-PL	0.239	< 0.01	0.297	< 0.01
Abb. FFQ vs. DHQ-II	0.318	< 0.01	0.317	< 0.01
Abb. FFQ No Supp. vs. DHQ-II	0.465	< 0.01	0.476	< 0.01
Abb. FFQ vs. DHQ-II+Supp.	0.843	< 0.01	0.897	< 0.01
DHQ-II+Supp. vs. RBC-PL	0.481	< 0.01	0.563	< 0.01

Abb. FFQ denotes abbreviated FFQ, RBC red blood cell phospholipid, Supp supplement.

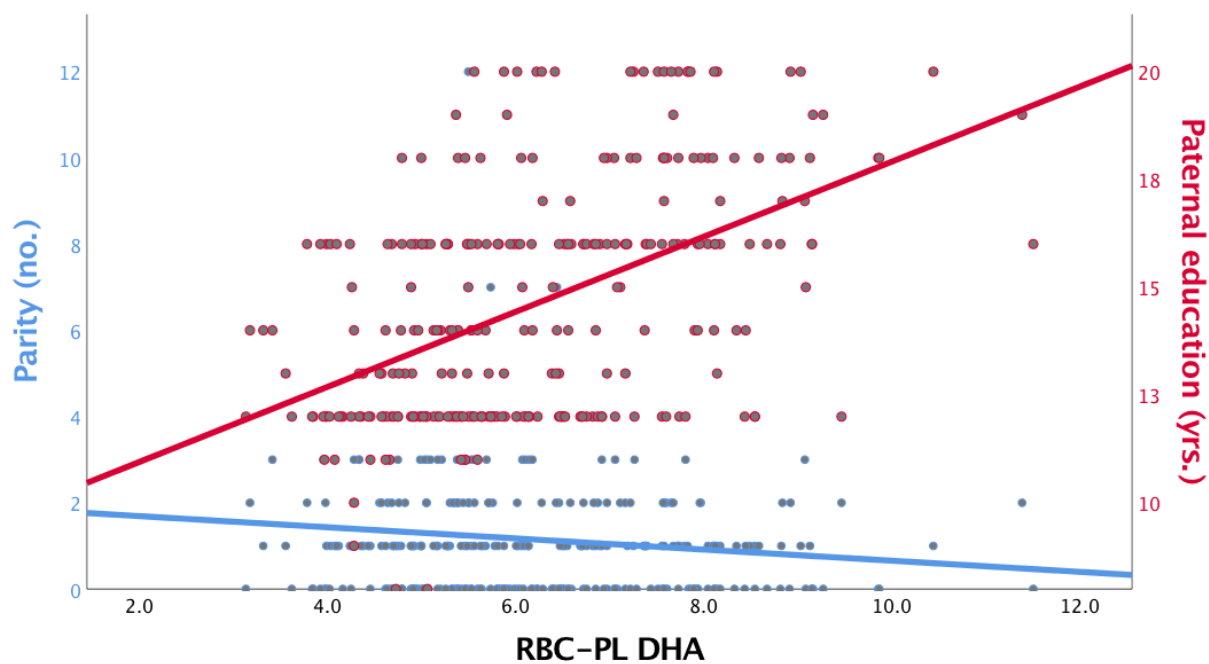


Figure 1. Secondary outcome variables for DHA.

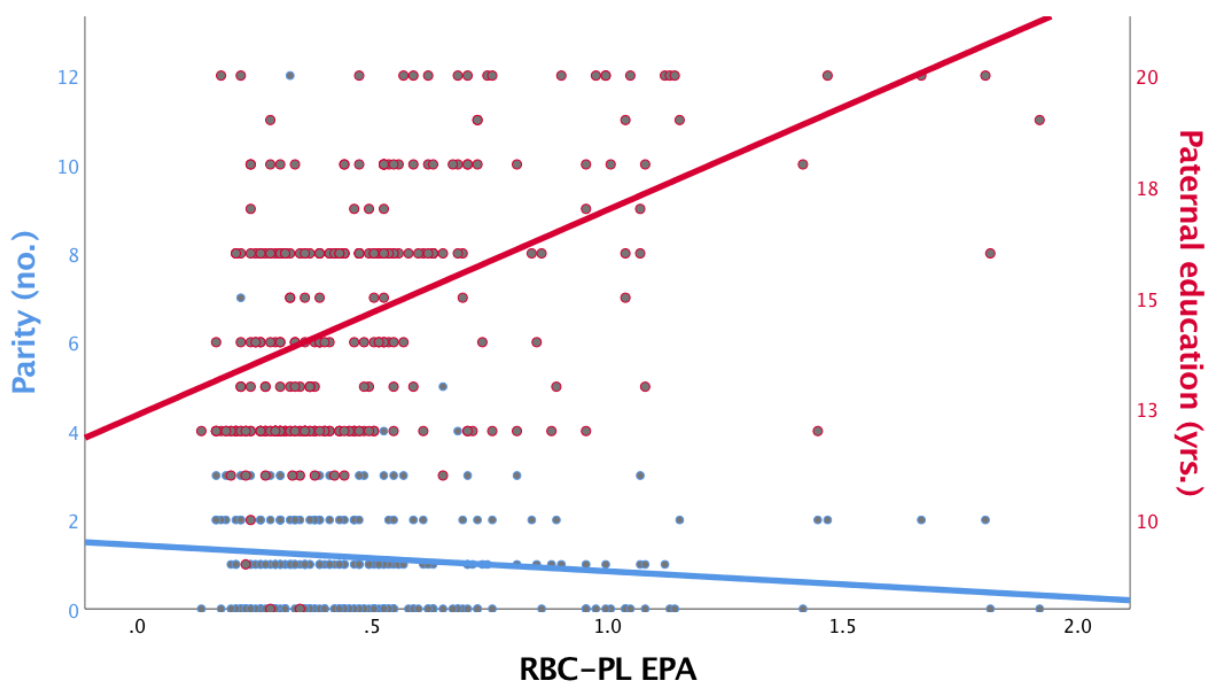


Figure 2. Secondary outcome variables for EPA.

Chapter 5 Discussion

Upon analysis the abbreviated FFQ proved to be the superior method of estimating DHA and EPA intake compared to the DHQ-II. The most likely reason is because the abbreviated FFQ obtains much more detailed information regarding supplement intake, and supplements of DHA in particular. In the United States, supplements are a very common source of these nutrients and thus it is crucial to include them when estimating intake (13). We found that almost 43% of our study sample was taking a DHA-containing supplement in the two months preceding enrollment. Questions regarding supplement intake on the DHQ-II ask about multivitamins, listing prenatal-type multivitamins as an example, and fish oil/omega-3 supplements (22). Many prenatal vitamins on the market are multivitamins that include DHA all in one capsule. There are likely inconsistencies on how women taking this particular type of supplement report it on the DHQ-II. Additionally, the amount of DHA and EPA in prenatal and fish oil supplements can vary greatly. Without knowing the exact brand supplement an individual is taking, what dose they take and the frequency at which it is taken, it is almost impossible to accurately estimate intake from that supplement. Because the DHQ-II is such a lengthy questionnaire as is, it is not feasible to add additional questions regarding detailed supplement intake. This is why an abbreviated FFQ, such as the one in this study, can be a valuable tool.

The present study is consistent with studies in other countries that have found similar results when validating FFQs for use in pregnant women. In Brazil, Lepsch et al. (2015) found weak, but significant, correlations between an 81-item FFQ and serum fatty acid composition of DHA and EPA ($r=0.209$ and $r=0.263$, respectively) in pregnant women (15). The results of Lepsch's study are comparable to correlations between the DHQ-II and RBC DHA and EPA in

the present study ($r=0.239$ and 0.297 , respectively). The FFQ used by Lepsch et al. did not include questions about omega-3 fortified foods or dietary supplements, as those things are uncommon in Brazil. Two FFQs have been validated in Japan for assessment of DHA and EPA in pregnant women. Shiraishi et al. (2015) validated a brief-type diet history questionnaire (BDHQ), in which they selected 58 items from the DHQ that they thought were most important to what they were studying. It was not reported whether supplements were included in those 58 items. They did not find strong correlations between their BDHQ and plasma DHA and EPA ($r=0.284$ and $r=0.352$, respectively, $p<0.05$) (17). Kobayashi et al. (2017) compared intake from a 167-item FFQ to serum-phospholipid DHA and EPA and also did not find strong correlations ($r=0.27$ and $r=0.37$, respectively, $p<0.05$). They reported that the FFQ underestimated DHA and EPA intake, however it was not stated if the FFQ included questions about supplement intake or omega-3 functional foods (18).

The dietary questionnaires used by Lepsch et al., Shiraishi et al., and Kobayashi et al. are all more comparable to the DHQ-II than the abbreviated FFQ in the present study. These three studies all found significant results, and each considered the FFQ that they studied to be valid. However, the weak correlations found by Lepsch et al., Shiraishi et al., Kobayashi et al., and in the present study between the DHQ-II and RBC DHA and EPA all support the need for an omega-3 specific FFQ to estimate intake of these nutrients. Because these questionnaires aim to evaluate the entire diet in the most efficient way possible, it is difficult to include detailed questions regarding the numerous omega-3 rich foods such as various species of fish. Additionally, it is challenging to obtain specifics about supplements without making such a questionnaire unfeasibly long.

In Australia, Parker et al. (2015) studied a 38-item FFQ designed to assess usual diet but included questions about omega-3 supplements and functional foods. Parker et al. found strong significant correlations between their FFQ and RBC DHA and EPA in pregnant women ($r=0.61$ and $r=0.55$, respectively) (16). In the present study, the DHQ-II with supplement intake added is comparable to the FFQ used by Parker et al. and correlations were similar, especially for EPA (DHQ-II+Supp. vs. RBC DHA and EPA correlations: $r=0.481$ and $r=0.563$, respectively). Even though the FFQ used by Parker et al. was designed to assess multiple dietary components, it is likely that correlations were strong because questions about dietary supplement intake and omega-3 fortified foods were included. The results from Parker et al. highlight the importance of capturing dietary supplement intake.

Paternal education was used as the sole indicator of SES because it is collinear with higher income and race and it was a better predictor than maternal education. A higher number of years of paternal education correlated with higher DHA and EPA RBC level. This is consistent with our hypothesis and with previous research. Rehm et al. (2016) evaluated overall diet quality using the American Heart Association (AHA) Continuous Diet Score. They found overall diet quality to be positively correlated with income. Similarly, higher income was also associated with greater intake of fish and shellfish (38). Villegas et al. (2015) found the same correlation between income and fish intake, with low income associated with low fish intake (39).

As hypothesized, parity was negatively associated with RBC DHA and EPA. Previous research has shown that DHA status decreases with subsequent pregnancies (40). Al et al. (1997) found a significant negative correlation between number of times a woman has been pregnant and plasma DHA ($r= -0.20$, $p=0.01$). Furthermore, there were also a significant negative

correlation between birth order and umbilical cord blood ($r = -0.31$, $p < 0.001$) found by Al et al. These results suggest that pregnancy depletes a woman's DHA stores and that they may not fully recover prior to the next pregnancy.

Age, race, and ethnicity were not found to have a significant effect on RBC DHA and EPA. It was hypothesized that age would have a small positive correlation with nutrient status, which is what Walker et al. (2014) found in a study of adults between the ages of 20 and 79 (41). However, the population of the present study had a much smaller age range than the population of Walker's sample. It is likely that the present study was too homogenous to show significant differences. Additionally, older women may be more likely to have had more pregnancies which we know is associated with lower DHA status and thus could counteract the effect of age.

Previous research found that African Americans had the highest level of fish intake, while being white was associated with the lowest intake. Both Rehm et al. and Villegas et al. found that African Americans consume more fish and shellfish than people who are white. The sample studied by Villegas et al. was taken from the Southeastern U.S., and their results were likely influenced by the region's culture. Rehm et al. however, used NHANES data, and should have had a sample representative of the entire United States (38, 39). Nonetheless the results of the present study were not consistent with the findings of Rehm et al. and Villegas et al. This could be due to the fact that the sample used in the present study was taken only from the Midwest U.S., a region where fish and seafood intake is typically low.

Potential limitations of the present study must be considered. The abbreviated FFQ relies on self-reported, retrospective data, which risks the possibility of recall bias. Also, the questionnaire classifies fish and seafood into three categories, low, medium, or high DHA levels.

There is some variability in DHA levels between different fish in the same category. Therefore, the calculation of DHA and EPA for each category uses an average, so only an estimate can be established. The list of fish on each question is not exhaustive and opens the risk for missing data from other fish and seafood that are not included. The questionnaire also excludes red meat intake. While DHA levels in red meat are low, red meat is a staple in the U.S. diet and could provide significant levels to some people (20). Although the abbreviated FFQ does have some flaws, our findings support it is valid in a pregnant population and is a sufficient tool for measuring dietary intake of DHA and EPA.

While biomarkers provide an objective measurement of DHA and EPA status, there are still several confounding variables other than diet that could impact RBC DHA and EPA levels. Firstly, the human body can make DHA in small amounts. Bisgaard et al. (2016) found a genetic component related to DHA status meaning some people are going to have higher levels of RBC-DHA than others simply due to genetic variations (6). Muhlhauser et al. (2014) found other possible variables in cord blood, including maternal body mass index, parity, intake of other supplements, diagnosis of gestational diabetes mellitus, delivering via induction or cesarean-section, and infant gender (27). Several of these factors also have the potential to impact maternal nutrient status as well. Because of all these confounding variables, measuring dietary intake is never going to be a perfect method for estimating DHA and EPA status.

The abbreviated FFQ is proven to be valid for use in pregnant women. It could be established as a standardized method for assessing DHA and EPA intake during pregnancy in U.S. women. This would be useful both in research and clinically. In research, using the abbreviated FFQ as a consistent method of measurement would make comparisons between

studies much easier. Clinically, the questionnaire could be used by practitioners to assess DHA and EPA status and individualize recommendations for patients.

Additionally, the present study identified that SES and parity have a significant impact on DHA and EPA status, which could further help scientists and clinicians improve recommendations and care of patients. It was shown that the more pregnancies a woman has had, the poorer her DHA status. It may be more important for clinicians to emphasize DHA intake for multiparous moms more so than for primigravida women. Lower SES is also associated with lower intake of DHA and EPA rich foods and poorer nutrient status. Individuals who fall into this category may need extra assistance obtaining sources of DHA and EPA. Sources could include a simple prenatal vitamin with DHA or supplement. Food programs such as WIC and SNAP could also incorporate ways to encourage and reward consumption of DHA and EPA rich foods.

In conclusion, we found the abbreviated FFQ to be a valid and superior method for assessing DHA and EPA intake in pregnant women compared to the DHQ-II. Stronger correlations were detected between the abbreviated FFQ and RBC DHA and EPA ($r=0.487$ and $r=0.536$, respectively) in comparison with the DHQ-II ($r=0.239$ and $r=0.297$, respectively). One potential reason why the abbreviated FFQ is a better predictor of DHA and EPA status may be because it captures detailed dietary supplement intake information. In the U.S. supplements are a common source of DHA and EPA (13). We observed that 43% of our sample was taking a DHA-containing supplement. When supplement intake DHA and EPA exposure was added to the DHQ-II correlations between the DHQ-II plus supplements and the RBC PL DHA and EPA ($r=0.481$ and $r=0.563$, respectively) were similar to correlations between the abbreviated FFQ and RBC PL DHA and EPA. Importantly, prior to supplement intake being added to the DHQ-

II, the abbreviated FFQ and the DHQ-II were not strongly correlated ($r=0.318$ for DHA and $r=0.317$ for EPA), but after addition of dietary supplements to the DHQ-II, these correlations strengthened considerably ($r=0.843$ for DHA and $r=0.897$ for EPA). These results illustrate how supplement intake overwhelmed the much smaller dietary contribution to DHA and EPA intake while the high correlation between the abbreviated FFQ DHA and EPA status support the importance of obtaining quality supplement intake information.

Though dietary and supplement intake play a key role in predicting DHA and EPA status, there are other factors that also have an influence. We found parity and SES, measured by paternal education, are also significant predictors of DHA and EPA status. Despite the numerous variables we tested, we found that diet and supplement intake, measured by the abbreviated FFQ, parity, and SES, measured by paternal education, only account for about 37% of the variation in RBC PL DHA and 44% of the variation in RBC PL EPA.

Further research needs to be done to identify other predictors of DHA and EPA status. Specifically, there needs to be more genetic studies to identify how genes influence the body's metabolism and processing of DHA and EPA. Recall from chapter 2 that Bisgaard et al. (2016) found a link between a specific genotype and whole blood levels of DHA and EPA (6). Additional research is warranted to broaden our understanding of the role genetics plays in predicting DHA and EPA status. If we can learn why some people need to consume more DHA and EPA than others, it could allow clinicians to better individualize recommendations in the future.

We found that parity has a significant effect on DHA status, which is consistent with the findings of Al et al. (1997), who found a direct relationship between the number of prior

pregnancies and DHA status in both mothers and infants (40). The question for future research is, do women who have had more pregnancies need more DHA than those with fewer? Because there are no known effects to taking high doses of DHA and EPA, it may be beneficial for women to increase their intake with each subsequent pregnancy. Further research is needed to develop specific recommendations on this subject.

Regulation of new supplements on the market does not follow the same regulation pathway as new drugs. It is the responsibility of the manufacturer to ensure marketed supplements meet all regulatory requirements. The U.S. Food and Drug Administration (FDA) will step in to remove any supplements that do not meet regulation only after they are already on the market (42). This makes it easy for new supplements to be distributed very quickly. There is a huge market for prenatal supplements, as the American College of Obstetrics and Gynecology (ACOG) recommends all pregnant women take a prenatal vitamin and mineral supplement to ensure they are getting the appropriate amounts of important nutrients such as folic acid and iron (43). According to a report by Credence Research, the prenatal vitamin supplement market is rising at a compounded annual growth rate of 8.5% (44). In our sample, we observed over 75 different over-the-counter and prescription dietary supplements being taken by participants. These supplements all vary in regard to which and how much of each nutrients(s) they contain.

ACOG considers calcium, iron, folic acid and vitamins A, D, B6, and B12 key nutrients during pregnancy (43). However, prenatal supplements typically contain many other nutrients as well. Further research is necessary to establish how much of these important nutrients pregnant women generally get through their diet. Then recommendations can be made for how much of these various nutrients should be supplemented. The ultimate goal would be to eventually

develop guidelines for the prenatal supplement market, based on scientific evidence, for the types and amounts of nutrients, including DHA, that prenatal supplements should contain.

DHA appears to have a variety of potential benefits during the prenatal period with no known adverse effects of taking high doses. However, there is still a lot we do not know in regard to the exact role it plays and what influences nutrient status. Numerous prenatal supplements are available with varying amounts of DHA and different nutrients. Research needs to continue to further investigate nutrient needs during pregnancy

Appendices

APPENDIX A. Abbreviated FFQ

FFQ to assess DHA and EPA consumption. Totals equal amount of DHA or EPA in mg/day. (20, 45)

Question	Servings (no.)	DHA per serving (mg)	EPA per serving (mg)	Total DHA (mg)	Total EPA (mg)
1) How many 3-oz servings of the following fish do you eat monthly?		×22	×14	=__	=__
Bluefish	Herring	Sardines			
Blue fin tuna	Mackerel	Salmon			
Cisco, smoked	Pollock	Whitefish			
2) How many 3-oz servings of the following fish do you eat monthly?		×10	×5	=__	=__
Bass	Mussels	Snapper			
Catfish	Perch	Sole			
Drumfish	Redfish	Swordfish			
Flounder	Rockfish	Trout			
Grouper	Shark	Tuna, canned (6-oz can)			
3) How many 3-oz servings of the following fish do you eat monthly?		×5	×6	=__	=__
Carp	Fish patties/squares	Pike			
Clams	Fish sticks	Pompano			
Cod	Haddock	Scallops			
Crab	Lobster	Shrimp (14 med)			
Crayfish Oysters	Mullet	Sturgeon			
4) How many 3-oz servings of liver (chicken, turkey or beef) do you eat monthly?		×7	×2	=__	=__
5) How many egg yolks do you eat weekly (including egg yolks used in cooking)?		×3	×0.25	=__	=__
6) How many 3-oz servings of chicken, turkey, or other poultry (not including livers) do you eat weekly?		×5	×3	=__	=__
7) Any ω-3 dietary supplements (i.e., flax-seed oil, fish oil, neuromins)?		mg/d	mg/d	=__	=__
Amount or strength _____					
Frequency _____ d/wk/mo					
Totals					

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APPENDIX B. Fatty Acid Analysis Procedures

BLOOD PREP PROCEDURE: (Dennis Hoffman procedure)

1. Centrifuge blood (4°C) at 30,000g-min [3000g x 10 minutes i.e., 3,600 RPM] in Eppendorf 5804 R refrigerated bench-top centrifuge with an A-4-44 swing-bucket rotor.
2. Pipet plasma (upper layer) to a 1.5mL, 10.8x 40.6mm plastic micro centrifuge tube (Fisher #05-408-129).
 - a. Put 0.5 mL into two plasma tubes and 0.5mL into the sRAGE tube.
 - b. Pipette remaining plasma into a fourth tube to be kept at KUMC.
3. Pipette the residual plasma, the buffy coat, and some RBCs into a tube for the buffy coat (thin white layer over RBC).
4. Pipet 0.7 mL RBC into two 1.5mL plastic micro centrifuge tubes.
5. Flush tubes with N₂ gas, cap, and store in -80°C.

Apply label and barcode to each tube.

(Used pipettes are discarded in sharps box; used gloves and other contaminated materials are discarded in the red biohazard waste bin)

Volume for each tube:

Plasma:

0.5 mL/ tube * 3 tubes (1 tube for sRAGE)
Pipette remaining plasma into a tube for KUMC

sRAGE:

0.5 mL/tube*1 tube

Buffy Coat:

Combine buffy coat from both 4mL tubes into 1 tube

RBC:

0.7 mL/tube * 2 tubes (comes to roughly 1 tube per 4ml EDTA sample)

Revised 6/1/16

Plasma and Red Blood Cell FAME Extraction

Phospholipids & Triacylglycerols

BLOOD PREP PROCEDURE (Dennis Hoffman):

1. Centrifuge blood (4°C) at 30,000g-min [3000g x 10 minutes i.e., 3,600 RPM] in Eppendorf 5804 R refrigerated bench-top centrifuge with an A-4-44 swing-bucket rotor.
2. Pipet plasma (upper layer) to a 1.5mL, 10.8x 40.6mm plastic micro centrifuge tube
3. Discard buffy coat (thin white layer over RBC).
4. Pipet RBC to a 1.5mL to a 1.5mL plastic micro centrifuge tube.
5. Flush tubes with N₂ gas, cap, and store in -80°C.

Label each tube with its contents (RBC/Plasma), Subject ID, Date, and Initials of preparer.

EXTRACTION (Smuts et. al, 2003):

1. Pipette 4mL of methanol into clean 15mL extraction tubes.
2. Add 500µL RBC or Plasma, cap, and immediately vortex for a few seconds.
3. (Optional) Add 100µL internal standard (17:0 PE) and vortex.
4. Add 8mL chloroform and vortex. Vortex 15' for RBC and 10' for Plasma.
5. Transfer contents through a funnel lined with filter paper into a clean extraction tube.
6. Add 1.6mL 0.05M KCl and vortex for 10 seconds.
7. Centrifuge for 5 minutes at 750 r.p.m. and then using a clean Pasteur pipette discard upper phase into appropriate hazardous waste bottle.
8. Evaporate the lower phase in a water bath at 35°C under N₂.
[Add 1-2mL benzene and re-evaporate if small amounts of water remain in sample].
Leave under N₂ until ready to spot onto the TLC plate.

SEPARATION OF TOTAL PHOSPHOLIPIDS (Smuts et. al, 2003):

(When plates have been heated for at least 20 minutes at 120°C, take out of oven and cool.)

9. When extract is completely dry in tube, dissolve in 150µL dichloromethane.
10. Spot the 150µL of sample, then add 50µL dichloromethane to the empty tube, swirl, and spot again (reduces volume of sample left in tube).
11. Place the spotted plate immediately in the TLC chamber containing the developing solvent 80:20:1 Hexane: Ether: Acetic Acid and that has been lined with filter paper and allowed to equilibrate.
12. Run each plate for 9 minutes, which is roughly how long it takes the solvent front to reach the top of the plate.
13. Remove from TLC chamber to dry for a few seconds before spraying with BBOT. Place on UV lamp (using cardboard frame to reduce glare).
14. Identify phospholipid line (it is the same line as the spotting line). Identify triacylglycerol line (it has an R_f of ~0.5). Scrape off the gel containing the lipid fraction of interest with a single edge razor blade onto weighing paper. Transfer the gel to clean 15mL extraction tubes (on ice) containing:
 - For PL: 1.0mL BF₃
 - For TAG: 0.5mL BF₃ + 0.4mL Benzene + 1.10mL Methanol

APPENDIX C. List of Abbreviations

ACOG	American College of Obstetrics and Gynecology
ALA	Alpha-linolenic acid
DHA	Docosahexaenoic acid
DHQ-II	Diet History Questionnaire-II
EPA	Eicosapentaenoic acid
FFQ	Food frequency questionnaire
KUMC	The University of Kansas Medical Center
LCPUFA	Long
n-3	Omega-3
OSU	Ohio State University
PL	Phospholipid
RBC	Red blood cell
SES	Socioeconomic status
UC	The University of Cincinnati

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