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Metabolic indices of polyunsaturated fatty acids: current evidence, research controversies, and clinical utility

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ABSTRACT

The n-3 and n-6 polyunsaturated fatty acids (PUFA) are among the most studied nutrients in human metabolism. In the past few decades, prospective studies and controlled trials have supported the view that the effects of these essential fatty acids are clinically relevant. PUFA profiles in different blood compartments are reflections of both diet and metabolism, and their levels may be related to disease risk. Despite widespread interest, there is no consensus regarding which biomarkers best reflect PUFA status in the body. The measurement of PUFA levels is not straightforward, and a wide variety of indices have been used in clinical studies, producing conflicting results. A major source of heterogeneity among studies is associated with research design, sampling, and laboratory analyses. To date, the n-3 index, n-6/n-3 ratio, and arachidonic acid (AA)/eicosapentaenoic acid (EPA) ratio are the most promising biomarkers associated with PUFA metabolism. Although hotly debated, these indices may be considered at least markers, if not risk factors, for several diseases, especially cardiovascular events and brain disorders. Here, we summarize the most updated evidence of n-3 and n-6 PUFA effects on human health, reviewing current controversies on the aforementioned indices and whether they can be considered valuable predictors of clinical outcomes.

KEYWORDS

Fatty acids; polyunsaturated fatty acids; n-3 index; n-6/n-3 ratio; AA/EPA ratio

Introduction

Fatty acids (FA) can be classified into three categories based on the number of double bonds present in side chains: saturated FA (SFA, no double bonds), monounsaturated FA (MUFA, a single double bond), and polyunsaturated FA (PUFA, ≥ 2 double bonds). Moreover, FA can be classified by their carbon chain length and the position of the first double bond on methyl terminal (omega; ω ; or n-FA). PUFA, mainly categorized into n-3 and n-6 FA, play key roles in regulating body homeostasis and cannot be produced endogenously. These FA are important constituents of cells where they assure the appropriate environment for membrane protein function, maintaining membrane fluidity and regulating cell signaling, gene expression and cellular function (Russo 2009; Catalá 2013). Dietary sources that are rich in PUFA include many vegetable oils, nuts, seeds and certain types of fish. The main consequence of their consumption is to be included in cellular membranes, especially of platelets, erythrocytes, neutrophils, monocytes, and neuronal cells. The impact of PUFA on health and disease has been of interest for many decades. However, the biological properties of PUFA are still the focus of considerable attention as they are thought to play an important role in several conditions, such as cardiovascular diseases (CVD), cancer, depression, insulin resistance (IR), and nonalcoholic fatty liver disease (NAFLD) (Zárate et al. 2017; Shahidi and

Ambigaipalan 2018; Fedor and Kelley 2009; Delarue and Lallès 2016). Numerous studies have reported a variety of physiological effects of PUFA, and it would appear that n-3 and n-6 PUFA may influence several pathological conditions associated with metabolic, inflammatory, and oxidative processes (Calder 2006; Patterson et al. 2012). Nevertheless, several issues are still debated, including the lack of a universally accepted biomarker that reflects PUFA status in the tissues. Although clinical studies have investigated the response of various biomarkers associated with PUFA intake, and methodological considerations have also been published (Fokkema et al. 2002; Harris, Assaad, and Poston 2006; Serra-Majem et al. 2012), we still do not have enough data in the literature to understand which biomarkers truly reflect PUFA status. The analytical determination of PUFA is challenging, mainly because of their biological diversity and their physicochemical similarity. Moreover, these compounds are produced within the same cascade and they all are part of a complex regulatory network. Therefore, this wide spectrum of compounds should be comprehensively captured to fully characterize their biological activity. Nowadays, lipidomic provides a powerful tool for the development of PUFA biomarkers to study disease states. Methods currently used include gas chromatography (GC), GC-mass spectrometry (GC-MS), GC-tandem mass spectrometry (MS/MS), liquid chromatography-mass spectrometry (LC-MS), LC-MS/MS, and LC-ultraviolet (UV)-MS/MS. LC-MS/

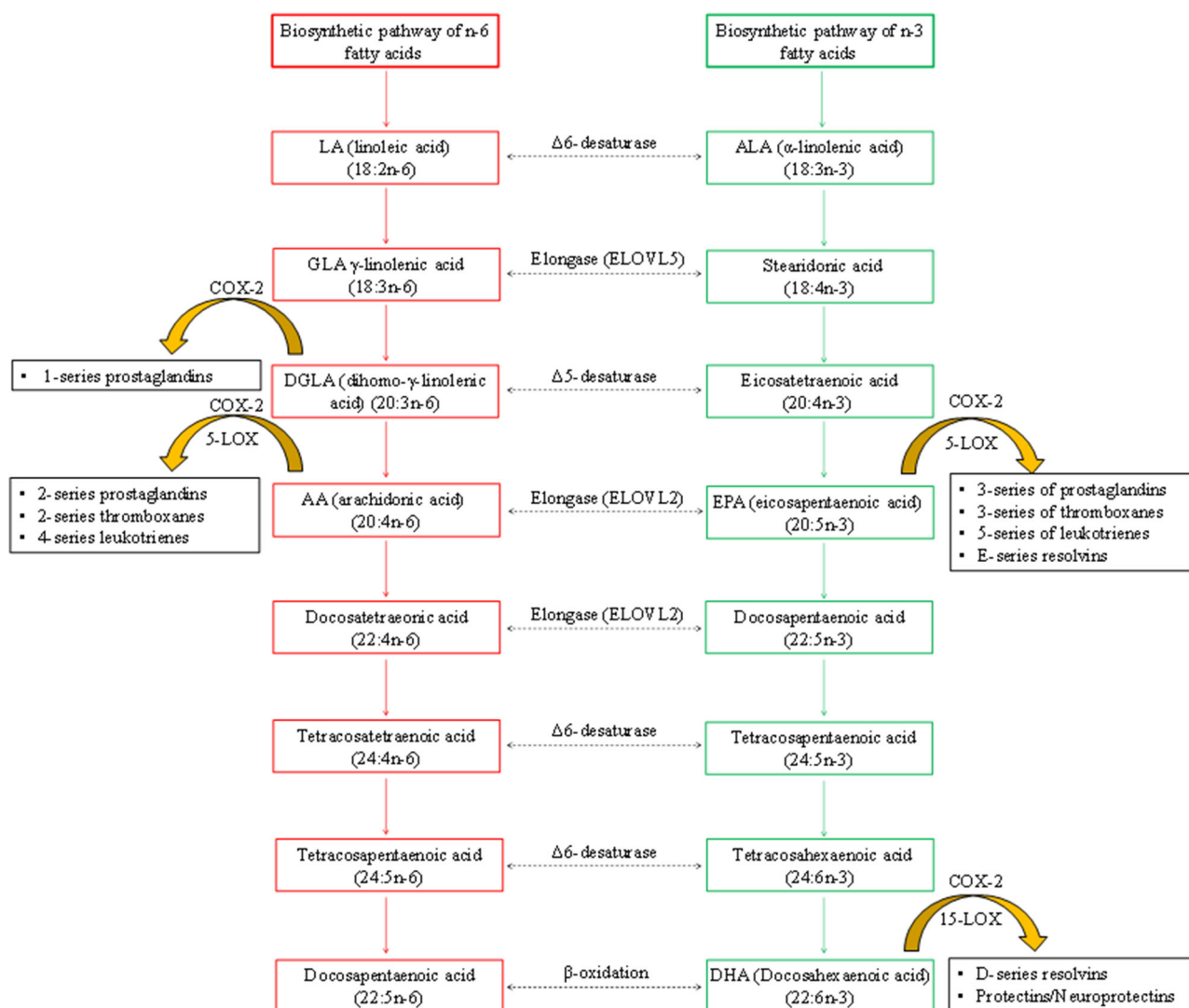


Figure 1. Biosynthesis of the principal polyunsaturated fatty acids and their metabolism. LA (18:2n-6) and ALA (18:3n-3) are PUFA obtained from the diet. Mammals convert LA and ALA to long chain PUFA using a series of desaturation and elongation reactions. Relevant intermediates to synthesize EPA (20:5n-3), DHA (22:6n-3), and AA (20:4n-6) include stearidonic acid (18:4n-3), eicosatetraenoic acid (20:4n-3), GLA (18:3n-6), and DGLA (20:3n-6). AA and EPA are substrates for the synthesis of eicosanoid products. These include the various prostaglandins, thromboxanes, and leukotrienes that mediate inflammatory processes. DHA is metabolized to resolvins and protectins that promote the resolution of inflammation.

MS is currently one of the most powerful techniques for analysis of lipid mediators due to its high analytical specificity and sensitivity (Yang and Han 2016). Additionally, it is also necessary to know the reliability of PUFA dietary intake estimations. There are several biological samples to estimate PUFA intake. Although erythrocytes and plasma are often used to assess PUFA level, dried blood spots (DBS) have become useful tools for quantifying nutrient intakes from diet as they are noninvasive and easily implemented into studies of large populations (Marangoni, Colombo, and Galli 2004). Despite this, the assessment of PUFA status using key FA indices is growing. Clinical and epidemiological studies suggest that the n-3 index, n-6/n-3 ratio, and acid (AA)/eicosapentaenoic acid (EPA) ratio may provide valuable information on nutritional needs, health outcomes, and long-term disease risk (Fokkema et al. 2002; Harris, Assaad, and Poston 2006). Therefore, the aim of this manuscript is to briefly discuss the metabolism of PUFA, describing the most updated evidence of their effects on human health and

major chronic diseases. Then, given the uncertain clinical utility of PUFA indices, we also reviewed analytical methods and the most promising biomarkers that can be used in the common clinical practice.

Metabolism and dietary sources of n-3 and n-6 PUFA

Two different pathways exist for the synthesis of the long-chain n-3 and n-6 PUFA (Figure 1). The simplest members of each family, linoleic acid (18:2n-6; LA) and α -linolenic acid (18:3n-3; ALA), cannot be synthesized by humans. The dietary intake of these essential FA promotes the synthesis of AA (20:4n-6), EPA (20:5n-3), and docosahexaenoic acid (22:6n-3; DHA) that regulate diverse homeostatic processes by acting on the synthesis of bioactive signaling lipids called eicosanoids. However, n-3 and n-6 PUFA have opposing effects on metabolic functions in the body. In general, AA synthesizes the pro-inflammatory eicosanoids, while

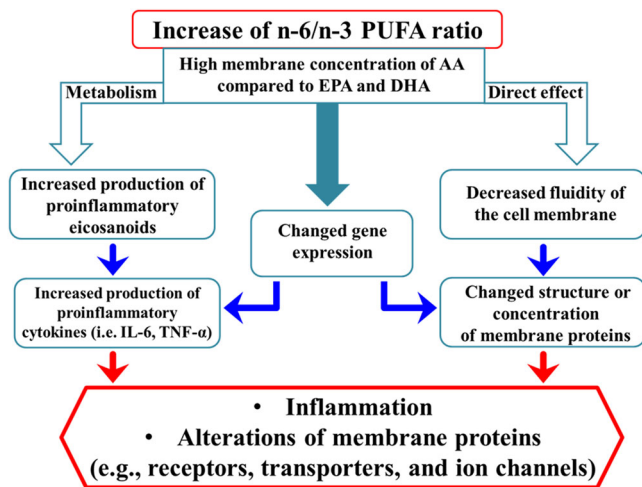


Figure 2. Biological effects of an increased n-6/n-3 ratio. The PUFA composition of the cell membranes is dependent on the dietary intake. Increased consumption of n-6 FA, especially AA (20:4n-6), replaces EPA (22:6n-3) and DHA (22:6n-3) in the membranes of probably all cells, but particularly in the membranes of platelets, erythrocytes, neutrophils, monocytes, neurons, and liver cells. The imbalance of n-6/n-3 ratio due to the high membrane concentration of AA may induce an increased production of proinflammatory eicosanoids and cytokines and lead to alterations in the lipid environment of membranes, affecting the functions of membrane-associated proteins, and thereby altering biological processes.

EPA and DHA induce the synthesis of anti-inflammatory eicosanoids (James, Gibson, and Cleland 2000; Schmitz and Ecker 2008).

Synthesis of n-3 and n-6 PUFA and production of eicosanoids

Once consumed in the diet, LA and ALA are converted to long-chain PUFA by fatty acyl-CoA synthetases, $\Delta 6$ - and $\Delta 5$ -desaturases, and their respective elongases designated as elongation of very long FA (ELOVL). In particular, LA can be converted in γ -linolenic acid (18:3n-6; GLA) by the action of the enzyme $\Delta 6$ -desaturase, and GLA is elongated to form dihomo-GLA (20:3n-6; DGLA), the precursor of prostaglandins (PG) of the 1 series. AA can be generated from DGLA by the action of the enzyme $\Delta 5$ -desaturase, and then AA synthesizes PG of the 2 series (A₂, E₂, I₂, and thromboxane A₂ [TXA₂]) by cyclooxygenases-2 (COX-2). Additionally, leukotrienes (LT) of the series 4 (B₄, C₄, and E₄) are also synthesized from AA with the action of lipoxygenases (5-LOX). These AA-derived eicosanoids are involved in several physiological actions, including pro-inflammation, pro-platelet aggregation, vasoconstriction, and immune response (Harizi, Corcuff, and Gualde 2008; Innes and Calder 2018). By an analogous set of reactions catalyzed by the same enzymes, ALA undergoes to transformation in EPA that can be metabolized by COX-2 and 5-LOX to PG of the 3 series (B₃, D₃, E₃, I₃, and TXA₃) and LT of the series 5 (B₅, C₅, and D₆), respectively. Next, EPA can be elongated again through two elongation cycles where docosapentaenoic acid (C_{22:5n-3}; DPA) is generated. The biosynthesis of DHA from DPA involves elongation and desaturation, followed by β -oxidation. DHA can be then metabolized to autacoids such as D-series resolvins (RVD1

to RVD6) and protectins (Neuroprotectin D1 [NPD1]). EPA- and DHA-derived mediators have potent anti-inflammatory activity and serve as specialized mediators that play an important role in the resolution of inflammation (Cottin, Sanders, and Hall 2011; Schwanke et al. 2016). As both n-3 and n-6 PUFA compete for the same metabolic enzymes, an imbalance in the n-6/n-3 PUFA ratio may result in an altered equilibrium of cell membrane composition, fluidity, and function, promoting an inflammatory environment (Figure 2). Therefore, the bioconversion of LA and ALA to their intermediates depends on the ratio of ingested n-3 and n-6 PUFA. N-3 and n-6 are both incorporated into membrane phospholipids when the AA/EPA ratio is between 1/1 to 5-10/1. When the ratio is higher, the incorporation of AA is preferred, giving rise to pro-inflammatory and pro-aggregation conditions (Whelan 1996; Harnack, Andersen, and Somoza 2009).

Dietary intake of PUFA

The PUFA composition of the cell membrane is influenced by several factors such as genetic variants, physical activity, and metabolic turnover. However, tissue availability of PUFA generally reflects those in the diet, affecting the capacity to synthesize long-chain PUFA from their precursors (i.e., LA and ALA) (Wood et al. 2015). Soybean, sunflower, and corn oils are all high in n-6 PUFA, and it has been estimated that LA accounts for 80–90% of total dietary PUFA (Micha et al. 2014). ALA is found in marked amounts in plant sources, including green leafy vegetables and commonly-consumed oils such as rape-seed and soybean oils. However, as the conversion of ALA to EPA and DHA is limited, it would be easier to achieve an adequate intake of these long-chain n-3 PUFA from fish (i.e. salmon, sardine, and herring oils), the richest dietary source of EPA and DHA (Williams and Burdge 2006). Many international agencies suggest that long-chain PUFA should provide approximately 7% of the total calories and the n-6/n-3 ratio should be no more than 5/1. The European Food Safety Authority (EFSA) approved several health claims related to the consumption of fish or EPA and DHA, as for the maintenance of normal level of blood triacylglycerols, normal brain function and vision, cardiac function and blood pressure. EFSA has also proposed intake values for the general population: 250 mg EPA + DHA; 2 g ALA and 10 g of LA per day (EFSA Panel on Dietetic Products 2010; EFSA 2009). Despite this, several prospective cohort studies and randomized controlled trials (RCT) provide evidence that populations in regions that consume a ratio of n-6 to n-3 PUFA closer to 1/1 have fewer chronic diseases than those in areas that consume mostly Western diets where the n-6/n-3 ratio is approximately 15/1 (Simopoulos 2006; Harris et al. 2009; Molendi-Coste, Legry, and Leclercq 2011).

Role of PUFA in health and disease

The importance of the PUFA to human health has been linked to their involvement in multiple biochemical

functions, including synthesis of inflammatory mediators, cell membrane fluidity, intracellular signaling and gene expression. Long-chain PUFA appear to play a crucial role in specialized cells and tissues such as brain, retina, heart, and liver. PUFA are particularly vulnerable to peroxidative attack and lipid peroxidation is highly deleterious, resulting in damage to cellular membranes and initiation of lipotoxicity mechanisms. Oxidized PUFA and their metabolites are implicated in a wide range of human pathologies and inflammatory conditions. With the evolution in lipidomic techniques, it has been reported that oxidized lipid mediators from long-chain PUFA (e.g., LA and AA) are correlated with diverse pathological conditions ranging from CVD, metabolic disorders, and NAFLD (Cruciani et al. 2019; Dasilva and Medina 2019). Although the interaction between n-3 and n-6 PUFA and their lipid mediators is complex and still not properly understood, the amounts of these PUFA in the body are believed to be very critical in the prevention and occurrence of chronic diseases associated with dietary patterns (Kihara 2012; Davinelli et al. 2018a, 2018b).

Brain disorders

Several mental and neurologic disorders are associated with lipid signaling, metabolism, trafficking, and homeostasis. DHA is the most abundant n-3 PUFA in mammalian central nervous system (CNS), membrane lipids of brain's gray matter (≈ 5 g in the human brain, 15–20% total FA), and visual elements of the retina (Innis 2008). Although the brain concentration of EPA is lower than that of DHA, EPA regulates several processes within the brain, such as neurotransmission, cell survival and neuroinflammation, and thereby mood and cognition. Low levels of n-3 PUFA have been associated with poor cognition, depression, anxiety disorders, and accelerated neurodegeneration in the elderly. A large number of epidemiological studies and RCT have suggested that a deficiency of n-3 PUFA may cause mood disorders, dementia, and eye diseases, increasing the production of n-6 PUFA derived pro-inflammatory eicosanoids and cytokines (SanGiovanni and Chew 2005; Innis 2008; Song et al. 2016; Deacon et al. 2017).

Nonalcoholic fatty liver disease

NAFLD is rapidly becoming a major public health concern worldwide. The top four risk factors for NAFLD are obesity, dyslipidemia, type 2 diabetes (T2DM) and metabolic syndrome (MetS) (Chalasanani et al. 2012). NAFLD affects up to 40% of the adult population, but this can exceed 85% in obese individuals (Angulo 2007). Nonalcoholic steatohepatitis (NASH) is considered the progressive form of NAFLD and is characterized by liver steatosis, inflammation, abnormal lipid composition, and different degrees of fibrosis. These features are associated with the development of IR, hyperinsulinemia, and n-3 PUFA depletion. A relationship between NASH and PUFA metabolism is supported by studies showing an increased dietary ratio of n-6/n-3 and a lower intake of PUFA among NASH patients (Zelber-Sagi

et al. 2007; Araya et al. 2004), and by lipidomic studies that demonstrate liver n-3 PUFA depletion and a higher n-6/n-3 ratio in NASH subjects (Puri et al. 2007; Musso et al. 2018). Lower hepatic n-3 FA levels have also been found to favor lipogenesis over FA oxidation (Pettinelli et al. 2009). So far, none of the interventional clinical studies with n-3 PUFA in patients with NAFLD or NASH have shown improvements in key prognostic histological features such as hepatocellular ballooning and fibrosis. However, most trials have shown a reduction in hepatic fat content (Argo et al. 2015; He et al. 2016). Interestingly, a recent RCT demonstrated that liver radiographic and histological improvements in pediatric NASH are produced during DHA-based therapy. The authors have also found that the ratio between DHA and AA and its correction by DHA-based treatment is a robust and useful indicator that needs further investigation in NASH (Torquato et al. 2019). Whether or not a high dietary ingestion of n-3 PUFA over a longer-term influences NASH progression remains uncertain but seems plausible.

Cardiovascular disease

The association between n-3 PUFA and cardiovascular health was established but the role that the n-6 PUFA play in CVD remains unclear. Recent systematic reviews and meta-analysis have yielded conflicting results, however, it is well known that n-3 PUFA can regulate cholesterol levels, adipocytes metabolism, lipogenesis, inflammation, blood pressure, thrombosis, and arterial stiffness, thus minimizing the onset of CVD (Kwak et al. 2012; Rizos et al. 2012; Colussi et al. 2017; Tortosa-Caparrós et al. 2017). Population studies still consistently show that a low n-3 PUFA status is associated with an increased risk of CVD and cardiac death (Skeaff and Miller 2009; von Schacky 2015). As mentioned, there is conflicting evidence whether increasing or decreasing n-6 intake results in beneficial effects on CVD. A recent meta-analysis of 30 prospective cohort studies in 68 659 participants found that higher in vivo circulating and tissue levels of LA and AA were associated with a lower risk of major cardiovascular events. In contrast, numerous studies have reported that high levels of n-6 PUFA have a pro-inflammatory effect by increasing the production of 2-series PG and 4-series LT. Consequently, increased intakes of n-6 PUFA may potentially worsen CVD risk (Maki et al. 2018; Marklund et al. 2019). N-3 PUFA are well-known for their hypotriglyceridemic effect and cholesterol-lowering activities. Most of the results on triglycerides are obtained from a combined effect on inhibition of lipogenesis and prompt FA oxidation in the liver. Plasma cholesterol-lowering activities are due to the suppression of cholesterol biosynthesis enzymes in the hepatic tissue (Jump et al. 2005; Sugiyama et al. 2008).

Cancer

PUFA play many key roles in all of the basic processes essential for tumor development (Abel, Riedel, and Gelderblom 2014). Several in vitro and animal studies have

established that n-3 and n-6 PUFA have effects on cancer cells, by influencing proliferation, inflammation, immune response, and angiogenesis (Berquin, Edwards, and Chen 2008; D'Eliseo and Velotti 2016). Notably, a low ratio of dietary n-6/n-3 PUFA is associated with reduced risk of several types of carcinogenesis. In race/ethnicity-specific analyses, increasing dietary ratio of n-6/n-3 FAs is correlated with higher prostate cancer risk among white men, but not among black men (Williams et al. 2011). Despite this, other epidemiological studies focusing on anticancer properties of n-3 PUFA have reported inconsistent results (Manson et al. 2019). However, the assessment of PUFA in blood and other body fluids may be useful to prevent certain types of cancer and monitor the efficacy and toxicity of anticancer treatment (Zhang et al. 2017).

PUFA indices

There is a growing interest in understanding the relationships between PUFA status and clinically important health outcomes. Concentrations of PUFA in the blood (i.e., whole blood, plasma, serum, and red blood cells [RBC]) reflect both dietary intake and biological processes (Hodson, Skeaff, and Fielding 2008; Davidson 2013). However, the measurement of PUFA levels is complex and a wide variety of biomarkers and methodological approaches have been used in experimental and clinical research. There are several reasons why PUFA analyses are challenging. First, these FA are a heterogeneous class of lipids typically organized into groups based on carbon chain length and orientation of the double bonds. Then, there are several biological materials for the measurement of PUFA, each with specific advantages and disadvantages, from whole blood to blood cells (red, white or platelet) to whole plasma or plasma lipid classes or even adipose tissue (Hodson, Skeaff, and Fielding 2008). Adipose tissue is generally considered the best source for assessing long-term PUFA intake. Erythrocytes may be a useful marker as they can provide an indication of the previous 120-day intake of long-chain PUFA. Plasma reflects the intake of FA over the past few days or more (Serra-Majem et al. 2012). PUFA are known to be involved in various physiological and pathological processes, therefore, it is difficult to define an “optimal” status or level that can be clinically used as a risk factor for a disease or as a diagnostic marker of disease. Finally, there are several analytical techniques that can be used, from enzymatic methods to lipidomic analyses. This issue has led to considerable confusion on the measurement of PUFA status, avoiding a standardization of research protocols with laboratories reporting individual results. However, over the last decade, MS-based lipidomic protocols offer a versatile, sensitive and accurate means of simultaneously assessing large numbers of PUFA-derived lipid mediators found in a single sample, and in a variety of biological samples (Zhao et al. 2015; Okada et al. 2018). These aspects are discussed in the next paragraphs, along with the main PUFA indices, which seem to be crucial to monitor clinical outcomes.

The n-3 index

The n-3 index was defined as the amount of EPA plus DHA in RBC membranes expressed as the percentage of total RBC membrane FA (Harris and Von Schacky 2004). It was originally suggested as a marker of increased risk for death from coronary heart disease (CHD), but it can also be viewed as a risk factor for several diseases. The RBC appear to be the preferred sample type in which to assess the n-3 index. Erythrocytes were chosen because numerous studies have indicated that these cells incorporate dietary EPA and DHA in a dose- and time-dependent manner (von Schacky, Fischer, and Weber 1985; Harris and Thomas 2010). It has also been shown a low biological variability of the erythrocyte FA composition (EPA + DHA), which is much lower than plasma phospholipid or whole blood FA compositions. Moreover, the content of FA in RBC is easier to measure than plasma phospholipid FA content, which requires an extra step to isolate the phospholipid fraction. It should also be highlighted that neither acute intake of n-3 PUFA, nor severe clinical events impact on the n-3 index. For all of these reasons, the n-3 index may be qualified as a “low-noise” parameter, which is suitable in epidemiologic and clinical studies (Shearer et al. 2009; Harris et al. 2013a; von Schacky 2015). Importantly, multiple observational cohort and interventional studies have shown that erythrocyte EPA + DHA is correlated to EPA + DHA in cardiac tissue and a lower n-3 index has been associated with an increased risk of CHD mortality (Harris 2008; von Schacky 2014). The n-3 index was chosen by Health Canada for the country's national health survey, also achieving a widespread use in clinical medicine in the US (Harris et al. 2013a; Langlois and Ratnayake 2015). However, before the introduction of the n-3 index in the routine clinical evaluation, reference values must be clearly established in large human studies.

The n-6/n-3 PUFA ratio

The discovery in the late 1970s of the potential health benefits of the marine n-3 PUFA by Bang and Dyerberg in Greenland Inuits opened a new era of studies on these FA (Dyerberg and Bang 1979). The PUFA/SFA (P/S) ratio, largely adopted until then, became obsolete as a biomarker of FA intake. The n-6/n-3 PUFA ratio has emerged as a new index to determine the physiological effects exerted in the body by n-6 and n-3 PUFA (Simopoulos 2002). Seminal studies demonstrated that a high proportion of n-3 FA and low n-6 FA in tissues may be beneficial to health, particularly for CVD (Gebauer et al. 2006; Harris, Poston, and Haddock 2007). This has led to the assumption that the n-6/n-3 ratio is useful to measure the balance of PUFA in the diet. Ratios between 4/1 and 7.5/1 for the n-6/n-3 ratio in the diet have been recommended, while contemporary Western diets are characterized by a ratio of around 15/1, reflecting deficient intake of n-3 PUFA and excessive intake of n-6 PUFA (Simopoulos 2002; Gebauer et al. 2006). In this context, several studies have been shown that a dietary intervention aimed at reducing the dietary n-6/n-3 ratio, through n-3 supplementation, led to a significant decrease

in the n-6/n-3 ratio (Leaf et al. 1995; Guebre-Egziabher et al. 2008). However, in recent years, the complex biochemical pathway of the eicosanoids has become clearer, and it seems that the class of n-6 PUFA can no longer be simply considered as pro-inflammatory. Indeed, the biochemical usefulness of the n-6/n-3 ratio is highly debated, and a recent individual-level pooled analysis of 30 cohort studies reported that higher in vivo circulating and tissue levels of LA and possibly AA were associated with lower risk of major cardiovascular events (Marklund et al. 2019). These new findings suggest that the use of this ratio is based on invalid assumptions and its clinical value may be not enough to correlate the overall “omega” status of the body with pathological conditions.

The AA/EPA ratio

The AA/EPA ratio is a potentially more relevant biomarker of the n-6/n-3 ratio because it focuses specifically on the two molecules that compete for the conversion to bioactive eicosanoids. Harris argues that a fundamental conceptual flaw of the n-6/n-3 ratio is its failure to distinguish between the PUFA within each class (i.e., between the n-3 PUFA, ALA, and EPA/DHA, and the n-6 PUFA, LA, and AA). Indeed, the 18-carbon species have clearly different physiologic properties than the 20- or 22-carbon species (Harris 2006). However, the interaction between AA and EPA is complex and still not properly understood. Despite this, several findings support the hypothesis that the balance between AA and EPA is important to regulate the synthesis of inflammatory mediators (Wada et al. 2007; Rizzo et al. 2010). Although less common than n-6/n-3 ratio, the EPA/AA ratio has been identified as a useful, simple, and reliable marker in a number of clinical settings. Importantly, this ratio has been shown to be a sensitive marker of cardiovascular risk as total cholesterol, low-density lipoprotein cholesterol (LDL-C), and triglycerides (Nelson and Raskin 2019; Preston Mason 2019). Additionally, a number of studies reviewed by Nelson and Raskin have found that the AA/DHA ratio is a less valuable marker of cardiovascular risk, suggesting that EPA may be superior to DHA for the prevention of CVD (Nelson and Raskin 2019). A limitation of the AA/EPA ratio is that a clinically useful threshold for identification of at-risk patients has not been clearly defined in large and prospective studies. This is complicated by the fact that different geographical regions have different AA and EPA content due to different diets, which affects not only the AA/EPA ratio, but also the cardiovascular risk. Moreover, most of the studies on this ratio have been conducted in Japan and, accordingly, there is a lack of data in Western populations (Nishizaki, Shimada, and Daida 2017; Nelson and Raskin 2019).

Measurement of PUFA

The specifics of the methods have been the subject of multiple articles and can only be summarized here (Brenna 2013; Brenna et al. 2018). Each procedure involves storage,

extraction, separation, identification and quantification stages. Recently, there has been an attempt to establish consensus and best practices for FA determinations in samples used for clinical studies. Although there is no a gold standard in PUFA measurement, it was highlighted that the type of research could vary and this needs to be considered in the analytical choices. It was established that the analytical choices need to be well justified, especially for; (1) sample collection including the type of sample and the storage conditions, (2) lipid extraction/isolation and FA derivatization (e.g., methylation to improve analytical sensitivity), (3) instrument analyses such as GS coupled to flame ionization detection (FID) or MS, and (4) data analysis and reporting, including the number of FA to report and the manner/units to express the data (Brenna et al. 2018). The measurement of PUFA indices in human tissues is one of the more demanding nutritional analytic procedures associated with disease risk and prevention. The association between dietary PUFA and health outcomes can be determined from food frequency questionnaires (FFQ). In many cases, however, the correlation coefficients between PUFA in biological samples and dietary intake of PUFA from different FFQ were not similar. Several investigators have used adipose tissue obtained by percutaneous biopsy, but this procedure is invasive and time-consuming. Currently, analysis of PUFA and their mediators can be performed using various methodologies: enzyme-linked immunosorbent assays (ELISA) and radioimmunoassays are popular but can measure only one metabolite at a time, are not always selective, can be subject to cross-reactivity, and are available only for certain lipids (Kangani, Kelley, and Delany 2008; Ostermann, Willenberg, and Schebb 2015). Recent advances in MS have underpinned the development of lipidomic, allowing the simultaneous qualitative and quantitative assessment of numerous lipid species, including PUFA. Today, LC-MS/MS, in combination with high-resolution instruments, is the most powerful tool to measure and elucidate the structures of lipid mediators derived from PUFA. In general, measurement of PUFA and their indices is preferred in RBC membrane phospholipid, as it displays less variability than measurement in plasma due to limited exchange between plasma and cells (von Schacky, Fischer, and Weber 1985; Harris et al. 2013a). In clinical research, the determination of RBC membrane phospholipid PUFA composition is a standard diagnostic procedure for evaluating PUFA indices and their association with different pathologies, particularly CVD and NAFLD. This procedure provides information about the long-term dietary intake of PUFA and their biosynthetic conversion to eicosanoids (Poppitt et al. 2005; Nishizawa et al. 2006). For example, RBC lipidomic to investigate membrane PUFA composition represents a powerful tool to diagnose lipid abnormalities in NAFLD patients and, therefore, assess liver parameters associated with lipotoxicity (Svegliati-Baroni et al. 2019). However, the determination of PUFA in RBC is a long and expensive method, and requires invasive venous blood collection, complex storage and extraction. Rather than collecting and working with venous blood, DBS technology offers a simple and convenient sample collection

option. A seminal study by Marangoni et al. described an assay for rapid profiling of the FA in whole blood using a filter paper based on DBS. These authors claimed that this method is sufficiently sensitive to detect changes in blood FA levels associated with lifestyle and dietary factors (Marangoni, Colombo, and Galli 2004; Marangoni et al. 2007).

Analytical techniques in lipidomic

Lipidomic emerged more than 15 years ago, and its progress has depended directly on the advances made in analytical technologies, particularly GC and LC. These advances allow to detect changes in lipid metabolism, pathway modulation or biomarkers and determine how these are associated with diseases. The ability of GC and LC techniques to be coupled with MS and the different ionization technologies developed including electrospray ionization (ESI), has vastly improved their sensitivity. MS measures the mass-to charge (m/z) ratio of ionized molecules. MS-based techniques for the analysis of lipids are different in the absence or presence of LC and GC prior to MS analysis. They can also be different in their analytical coverage (e.g., ‘targeted analysis’ focuses on known lipids, while ‘global analysis’ analyzes the entire lipidome, detecting every lipid species). Lipidomic has provided insights into the molecular mechanism underlying the health benefits of PUFA and their regulatory role in the inflammatory response (Yang and Han 2016). Indeed, lipidomic can provide a useful approach for profiling classes of bioactive PUFA mediators derived from AA, such as PG and LT. PUFA can be analyzed by a range of MS-based methods, and currently no method dominates the field. Lipidomic analysis of biological samples includes sample preparation (i.e., addition of internal standard; solvent extraction; and derivatization); mass spectrometry-based analysis (i.e., MS data acquisition); and data processing (i.e., spectral data analysis and quantification). Although the need to derivatize the lipids to form volatile species causes limitations such as the danger of thermal decomposition, GC–MS or GC–MS/MS has been successfully applied to PUFA and eicosanoid research in diverse biological matrices including RBC, DBS, plasma, brain, liver, cerebrospinal fluid, muscle tissues, and urine (Hewawasam et al. 2017; Dupuy et al. 2016). However, although GC–MS can be used in many cases, the required high column temperatures limit its use because many of the PUFA-derived lipid mediators are thermolabile. LC–UV–MS/MS can provide spectral information and criteria for those compounds with specific UV chromophores. In this context, many lipid mediators derived from PUFA possess conjugated double bond systems that are critical components for their bioactions; each gives a characteristic UV spectrum (Arita, Clish, and Serhan 2005). The high separation power of LC (as high-performance LC [HPLC] or ultra-performance LC [UPLC]) when coupled to MS/MS has been proven to be an excellent analytical platform for lipidomic assays with detection limits in the picogram range (Martín-Venegas et al. 2011). ESI is a low-energy ionization technique used in MS and applicable to the qualitative and

quantitative analysis of lipid species. It is emerged as one of the most popular technique for eicosanoids, since it allows the ionization of these nonvolatile compounds without the need for derivatization. These metabolites can form positive and negative ion species; however, most applications can form negative ion species in high abundance (Murphy et al. 2005). ESI is also easily coupled with LC, allowing the development of LC–MS assays that combine the separation capacity of LC (HPLC or UPLC) and sensitivity of MS. Other techniques, such as matrix-assisted laser desorption/ionization (MALDI), atmospheric pressure chemical ionization (APCI), and nuclear magnetic resonance (NMR), have also been utilized. The routine use of lipidomic for PUFA analyses is currently limited by a lack of standardization of methodological approaches, and a lack of consensus on what and how to report lipidomic data. Initiatives to standardize PUFA lipidomic approaches are ongoing and have identified pre-analytical, analytical and post-analytical challenges that need to be considered (Burla et al. 2018). Lipidomic methods and factors affecting sampling conditions, sample preprocessing and storage as well as selection of study subjects (particularly in clinical lipidomic studies), and analysis of chromatogram data have been extensively reviewed (Maskrey et al. 2013; Jurowski et al. 2017).

Effect of sampling on PUFA indices

There is a surprising amount of controversy regarding the choice of blood sample to measure PUFA status in clinical studies. Each sample has its inherent strengths and limitations, so the rationale for choosing the most appropriate sample should be based on the research design used and the specific question being asked. Plasma and serum tend to be collected as the primary blood sample for clinical studies, each with specific advantages and disadvantages (Brenna et al. 2018). Interestingly, a recent study by Giusepponi et al. provides a validated protocol on a new LC–MS/MS method and optimized sample preparation for the simultaneous detection of PUFA, tocopherols and their metabolites in human plasma and serum (Giusepponi et al. 2019). Although large human cohort studies measure PUFA levels in plasma and serum, one of the most common and reliable method of measuring PUFA indices involves RBC. This method includes several steps, such as 1) the collection of whole blood and its separation into RBC by centrifugation; 2) the washing of the RBC to remove cells and plasma contamination; 3) the extraction of the lipids from the RBC and their separation; 4) the methylation of the RBC lipids for analysis by chromatography. However, Patterson et al. demonstrated in a controlled supplement intervention study that the sum of EPA + DHA in RBC, plasma, and whole blood collected by fingertip increased linearly in these samples from 85% to 95% for every gram of EPA and DHA consumed (Patterson et al. 2015). There is a growing interest in the use of DBS sampling, usually obtained from fingertip, which allows simple and cost-effective logistics in many clinical settings. It should be highlighted that the simplicity of this method is particularly useful to measure the FA

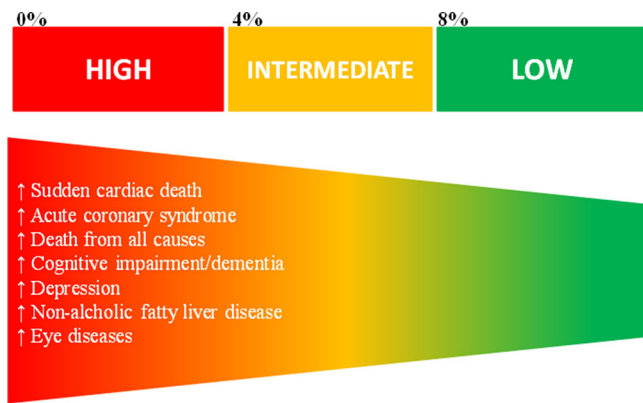


Figure 3. Omega-3 index risk zones. The protective target level for the n-3 index appeared to be about 8%, and the level associated with increased risk of disease is <4%. However, these values have yet to be prospectively tested in large human studies.

status of large cohorts. It is minimally invasive and easily understandable to clinicians and general public. For example, data from epidemiologic and intervention studies confirm the utility of DBS system for estimating PUFA indices. The n-3 index has been deduced from analysis of DBS samples in studies with U.S. soldiers, runners, adolescents, premature babies, vegans, and cardiac patients (Aarsetoy et al. 2011; Johnston et al. 2013; Sarter et al. 2015; Baack et al. 2016; van der Wurff et al. 2016; Davinelli et al. 2019). Johnston et al. calculated correlation coefficient (CC) and variation coefficient (VC) of the n-3 index in RBC against DBS from 49 participants whose samples were collected at the same visit. They found that CC and VC were 0.96 ($p < 0.0001$) and 5–6%, respectively. These values reflect a good correlation and a low variability between RBC and DBS (Johnston et al. 2013). An earlier study by Bailey-Hall compared the percentage values for AA in RBC versus DBS, which were 10.6 and 8.06, respectively. These values were similar to those recorded by Bell et al., where AA was 13.0 in RBC and 7.3 in DBS. DHA was also closer in these two studies, being 4.36 in RBC and 2.76 in DBS in the study by Bailey-Hall, and 4.84 and 2.18 in the study by Bell et al. (Bailey-Hall, Nelson, and Ryan 2008; Bell et al. 2011). Rizzo et al. determined AA/EPA in whole blood against RBC, showing that the AA/EPA ratio in whole blood was correlated with the ratio derived from RBC (Rizzo et al. 2010). In this study, the R^2 value of the AA/EPA ratio was 0.87 for RBC versus whole blood, while Bell et al. recorded a slightly higher value of 0.94 for RBC against DBS. Although capillary blood, when collected via fingertip, is often contaminated, these findings provide preliminary evidence that the DBS method may be used for large-scale studies.

Human studies and PUFA indices

Although numerous studies employ FFQ to estimate PUFA intake exposure, blood-based biomarkers of PUFA is a preferred approach to estimate circulating levels of long-chain PUFA and investigate the relationship between PUFA status and disease.

The n-3 index as marker for human diseases

The n-3 index has been shown to be a stable biomarker of dietary intake and a valid surrogate of tissue long-chain n-3 PUFA (Harris et al. 2004). As mentioned, this index was first proposed as a potential risk factor for CHD, especially sudden cardiac death, and subsequent cross-sectional and prospective studies have supported its clinical utility (Harris 2007; Jackson and Harris 2018). Blood levels of EPA + DHA from 10 studies of a meta-analysis conducted by Del Gobbo et al. were converted to the n-3 index (RBC %EPA + DHA) by Harris et al. to gain further insight into what levels of the n-3 index might be linked with higher vs. lower risk for CHD. The analysis shows that a cardioprotective target level for the n-3 index appears to be about 8%, and the level associated with the increased risk for CHD death is <4% (Figure 3) (Del Gobbo et al. 2016; Harris, Del Gobbo, and Tintle 2017). These cut-points could be used in the clinic practice to identify patients at highest risk for fatal CHD. Recently, Walker et al. developed a model to predict the effects of long-chain n-3 PUFA supplementation on the n-3 index. Data from 1422 individuals from 14 published n-3 intervention trials were included in this study. Given 850 mg/d of EPA + DHA, the model predicted that the final n-3 index, with a baseline concentration of 4.9%, would be approximately 6.5% (Walker et al. 2019). The n-3 index is also a potentially useful marker of NAFLD risk in overweight and obese adults and n-3 PUFA supplementation appears to be effective in the reduction of hepatic steatosis in adults aged >18 years (Parker et al. 2012, 2015). A recent study demonstrated an inverse association between n-3 index and NAFLD in older adults, supporting a relationship between n-3 index and NAFLD. The n-3 index was significantly lower in participants with NAFLD compared to those without NAFLD. However, the inverse association was found in female but not male participants, suggesting that sex differences may be an important consideration when evaluating the efficacy of n-3 PUFA supplementation in the prevention of NAFLD (Rose et al. 2016). The dietary intake of n-3 PUFA increased the index value improving the MetS parameters with a beneficial impact on NAFLD subjects characterized by a very low level of n-3 index. Notably, the n-3 index displayed a negative association with metabolic variables related to MetS such as homeostatic model assessment (HOMA)-IR, cholesterol concentrations of lipoprotein classes, and intima-media thickness (IMT), indicating that the n-3 index can be an appropriate predictor for MetS in NAFLD (Spahis et al. 2018). The n-3 index is also inversely correlated with major depressive disorders and inflammatory biomarkers (Baghai et al. 2011; Fontes et al. 2015). An increase in the n-3 index reduced the risk and the severity of depressive symptoms in several clinical studies. One of them found that for each 1% increase in the n-3 index, the risk of developing depression was reduced (Pottala et al. 2012). Recently, Zhang et al. found an association between n-3 PUFA supplementation and a lower risk of cognitive decline in Alzheimer disease (AD) patients (Zhang et al. 2015). This is supported by a study in healthy subjects where the n-3 index was correlated with cognitive function

Table 1. AA/EPA ratio in cardiovascular diseases, depression, nonalcoholic fatty liver disease, and cancer.

Condition	Key findings	Study authors
CAD	<ul style="list-style-type: none"> The incidence of MACE in patients who have undergone PCI is significantly associated with AA/EPA ratio. 	Domei et al. (2012)
ACS	<ul style="list-style-type: none"> The AA/EPA ratio is one of the risk indicators in patients with angina. An imbalance in the AA/EPA ratio is a common critical risk factor for ACS in middle-aged older patients as well as younger adult patients. After successful primary PCI, treatment with EPA combined with statin improves the AA/EPA ratio, reducing cardiovascular events after ACS. 	Kondo et al. (1986) Serikawa et al. (2014)
MI	<ul style="list-style-type: none"> PCI patients with higher AA/EPA ratio have a higher risk of periprocedural MI. Higher AA/EPA ratio is associated with a higher frequency of fatal arrhythmic events in the early phase of acute MI. 	Nosaka et al. (2017) Suzuki et al. (2014)
Stroke	<ul style="list-style-type: none"> The AA/EPA ratio appears a potential predictive risk factors for ischemic stroke. EPA treatment improves AA/EPA ratio reducing the risk of secondary stroke. 	Hashimoto et al. (2018) Ikeya et al (2013)
CHF	<ul style="list-style-type: none"> The AA/EPA ratio may play an important role in preventing left ventricular wall thickness in patients with diabetes. The AA/EPA ratio is an independent predictor of cardiac mortality in patients with heart failure. EPA treatment reduces the AA/EPA ratio, improving left ventricular ejection fraction. 	Tanaka et al. (2008) Okamoto et al. (2015) Watanabe et al. (2016) Kohashi et al. (2014)
PAD	<ul style="list-style-type: none"> The AA/EPA ratio is predictive of PAD diagnosis. Higher AA/EPA ratio appear to be associated with a greater risk of major adverse limb events and death from any cause after endovascular therapy in patients with PAD. 	Fujihara et al. (2013) Hishikari et al. (2015); Hiki et al (2017)
Depression	<ul style="list-style-type: none"> Higher AA/EPA ratio is associated with a greater likelihood of depressive symptoms in subjects with systemic inflammation. n-3 PUFA supplementation ameliorates symptoms in elderly depression by improving the AA/EPA ratio. 	Shibata et al. (2018) Rizzo et al. (2012)
NAFLD	<ul style="list-style-type: none"> A healthy diet and physical activity reduced the AA/EPA ratio value, improving NAFLD 	Tutino et al. (2018)
Cancer	<ul style="list-style-type: none"> An imbalance in the AA/EPA ratio is a significant risk factor for cancer death. High levels of the AA/EPA ratio is an inflammatory biomarker in tumor tissue of metastatic colorectal cancer patients. 	Nagata et al. (2017) Tutino et al. (2019)

CAD, coronary artery disease; AA, arachidonic acid; EPA, eicosapentaenoic acid; MACE, major adverse cardiac events; PCI, percutaneous coronary intervention; ACS, acute coronary syndrome; MI, myocardial infarction; CHF, chronic heart failure; PAD, peripheral artery disease; Nonalcoholic fatty liver disease, NAFLD; PUFA, polyunsaturated fatty acid.

as well as hippocampal and total brain volume (Pottala et al. 2014). Only few clinical studies used the n-3 index to monitor PUFA metabolism in cancer. However, DHA supplementation was associated with increased levels of n-3 index in breast cancer patients (Molfinio et al. 2017).

Significance of the n-6/n-3 ratio for human diseases

There are controversial data among the few studies that specifically explored the role of n-6/n-3 PUFA ratio as a biomarker of disease risk. Harris argues that the n-6/n-3 ratio lacks many of the characteristics of a useful metric, both for interpreting biomarker data and for making dietary recommendations. Additionally, the same author indicates that there is no clinical evidence that lowering n-6 PUFA intakes, which will improve the ratio, will result in reduced risk for CHD. Willet suggests that even though the n-6/n-3 ratio has been described as an important index associated with inflammatory pathways, the existing scientific evidence is inconclusive, and in humans, a high n-6 intake has not been correlated with high inflammatory marker levels (Willett 2007; Harris 2018). Similarly, a systematic review of RCT reported no significant association of the n-6 LA with a wide variety of inflammatory markers, including C-reactive protein (CRP), fibrinogen, plasminogen activator inhibitor type 1, cytokines, soluble vascular adhesion molecules, or tumor necrosis factor- α (TNF- α) (Johnson and Fritsche 2012). However, in the secondary prevention of CVD, a n-

6/n-3 ratio of 4/1 was associated with a 70% decrease in total mortality. A ratio of 2.5/1 reduced rectal cell proliferation in patients with colorectal cancer, and a ratio of 2–3/1 suppressed inflammation in patients with rheumatoid arthritis (de Lorgeril et al. 1994; Simopoulos 2002). Although measured by FFQ, a population study conducted by Goodstine et al. indicates that an improvement in the n-6/n-3 ratio may reduce the risk of breast cancer in premenopausal women (Goodstine et al. 2003). Other studies reviewed by Marventano et al. reported no association between n-6/n-3 PUFA ratio and breast, ovarian, and colon cancer (Marventano et al. 2015). Promising results have been also reported in major depression, suggesting that an increased n-6/n-3 ratio is involved in the pathogenesis of mood disorders (Husted and Bouzinova 2016; Berger et al. 2017). Lipidomic analyses coupled with enzymatic activity and gene expression profiling revealed an increased n-6/n-3 ratio in relation to NASH (Walle et al. 2016; Puri et al. 2009). More specifically, dysregulation in hepatic PUFA desaturation reactions was associated with the hepatic imbalance between n-6 and n-3 levels, thereby causing preferential synthesis of n-6-derived proinflammatory eicosanoids and accumulation of toxic lipids during NASH progression (Chiappini et al. 2017). In this context, FA elongation and desaturation processes show defects also in NASH pediatric patients. Targeted and untargeted lipidomic findings demonstrate the importance of achieving a better hepatic metabolism of DHA, and consequently of its metabolic counterpart, the n-6 AA, to treat pediatric NASH with the diet, especially

DHA (Torquato et al. 2019). Several cohort studies support the idea that the n-6/n-3 ratio may be useful to predict disease risk factors and monitor health outcomes, however, further clinical research is needed to assess the accuracy and reliability of this index.

The importance of AA/EPA ratio in human diseases

The AA/EPA ratio has been found to be more closely associated with the pathophysiology of several diseases. Epidemiological and clinical studies have shown that a lower AA/EPA ratio is associated with decreased risk of coronary artery disease (CAD) (Kondo et al. 1986; Domei et al. 2012; Preston Mason 2019), acute coronary syndrome (ACS) (Nishizaki et al. 2014; Serikawa et al. 2014; Nosaka et al. 2017), myocardial infarction (MI) (Suzuki et al. 2014; Hashimoto et al. 2018), stroke (Tanaka et al. 2008; Ikeya et al. 2013), chronic heart failure (CHF) (Kohashi et al. 2014; Okamoto et al. 2015; Watanabe et al. 2016), and peripheral artery disease (PAD) (Fujihara et al. 2013; Hishikari et al. 2015; Hiki et al. 2017) (Table 1). The potential role of EPA treatment in modulating the AA/EPA ratio and reducing cardiovascular risk was suggested in the large, prospective, randomized Japan EPA Lipid Intervention Study (JELIS), which randomized hypercholesterolemic patients to EPA 1.8 g/day for primary prevention of cardiovascular events. Treatment with EPA not only increased its level, but the risk of major coronary events was significantly reduced in patients with lower AA/EPA ratio (Yokoyama et al. 2007, Itakura et al. 2011). Recently, higher levels of AA/EPA ratio were also positively associated with depression severity, suggesting a potential role for the balance of AA/EPA in mood disorders. In support, a meta-analytic review reported that deficits in EPA and DHA are the most common lipid markers in patients with depressive symptoms (Lin, Huang, and Su 2010; Scola et al. 2018). Additionally, the association between AA/EPA ratio and depressive symptoms was also examined in a cross-sectional study involving 2,529 Japanese residents in the Hisayama Study. The authors demonstrated that a higher AA/EPA ratio was associated with an increased risk of the presence of depressive symptoms in individuals with higher CRP levels (Shibata et al. 2018). A study conducted by Rizzo et al. reported that supplementation with 2.5 g/day of n-3 PUFA for eight weeks ameliorates AA/EPA ratio and depressive symptoms in elderly subjects (Rizzo et al. 2012). Furthermore, studies in humans show that high levels of AA/EPA ratio are correlated with obesity and visceral fat accumulation (Caspar-Bauguil et al. 2012; Inoue et al. 2013). However, only one clinical study investigating the usefulness of AA/EPA ratio in NAFLD. In particular, the combined effect of diet and physical activity reduced the AA/EPA ratio value, improving the steatosis of NAFLD patients (Tutino et al. 2018). Finally, a large-scale prospective cohort study involving 3098 subjects has demonstrated that increased level of the AA/EPA ratio is a significant risk factor for cancer death in the general population (Nagata et al. 2017). It was also demonstrated that high levels of AA/EPA ratio in metastatic

patients induce an inflammatory microenvironment more susceptible to tumor progression (Tutino et al. 2019). Therefore, EPA-rich foods may be effective for reducing the risk and the progression of cancer.

Conclusions

The essential long-chain PUFA play crucial roles in maintaining normal physiological conditions. Recently, there has been much interest to measure PUFA indices that are assumed to reflect whole-body activities of enzymes in PUFA biosynthetic pathways. The indices reviewed here may be useful biomarkers to investigate the associations between intake of PUFA and various health outcomes, especially in large observational studies and clinical intervention trials. Although these metrics have been significantly associated with the onset of several diseases, there are concerns regarding their use. At present, there is no consensus regarding which indices best reflect PUFA status in the body. Undoubtedly lipidomic has allowed tremendous advances in understanding and determining the true importance of PUFA in many physiological and molecular mechanisms implicated in the establishment of healthy or diseased status. However, the routine use of PUFA indices in the common clinical practice is currently limited by a lack of standardization of methodological approaches. There is also a myriad of pre- and post-analytic variables that can affect the final outcomes. To date, n-3 index and AA/EPA ratio appear more robust biomarkers in a number of clinical settings and human studies. Conversely, the n-6/n-3 ratio is still widely debated and conflicting results were reported. However, the PUFA indices described in this review may be clinically useful because they meet two important criteria: 1) they are related to clinical outcomes; and 2) they are generally modifiable by lifestyle habits such as dietary changes and physical exercise. Further epidemiological and clinical studies will help to better define the prognostic value of these biomarkers and their definitive thresholds for treatment targets.

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