Applications of Innovative Lipidomic Methods for Blood Lipid Biomarkers

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Abstract: Assessing dietary intake is critical for understanding the relationship between diet and health. Fatty acid blood biomarkers have been particularly useful in determining dietary intakes and assessing the risk of chronic disease. However, fatty acid analysis involves the removal of fatty acids from their complex lipid structures resulting in a loss of potentially useful biological information. “Lipidomics” involves the use of mass spectrometry to identify lipids in their native form. Lipidomics approaches present challenges as an alternative to fatty acid analysis. This includes different types of lipidomic approaches and a lack of consensus on the lipids reported in different studies. Distinguishing between macro- and microlipidomic approaches to characterize highly abundant lipids and microlipidomic approaches examining low abundant bioactive lipids and the use of brutto, medio, genio, and infinio to describe the level of information of lipidomic data can provide clarity to the field. Using lipidomic measurements for understanding docosahexaenoic acid metabolism during pregnancy will also be examined.

Key words: fatty acids, lipidomics, biomarkers, dietary intake, omega-3 polyunsaturated fatty acids

1 Introduction

The understanding of the biological role of lipids and fat in human health has shifted dramatically over time. Initially, dietary fat was viewed as a nonessential source of energy. While George and Mildred Burr would demonstrate the essentiality of linoleic and α-linolenic acid in the 1930s, acceptance of the importance of these essential fats and their metabolites would take much longer. In between, dietary fat would be linked to elevated serum cholesterol and heart disease risk in the 1940s and 1950s with recommendations to replace saturated fat with unsaturated fats emerging in the 1960s that would then get transformed into a oversimplified consume low-fat message in the 1980s and 1990s. During this vilification of dietary fat, there would be an accelerated accumulation of evidence suggesting health benefits from increased dietary intakes of omega-3 polyunsaturated fatty acids (PUFA). Suboptimal nutrition is a considered a leading cause of poor health with fatty acid intake identified as a key component of proper nutrition. Recently, lipids and fatty acids have been redefined as critical components in biological processes as components of cell membranes and signalling molecules or lipid mediators which has resulted in an increasing appreciation of the bioactivities of individual lipid and fatty acid molecules which is starting to be reflected in dietary recommendations.

There is considerable dietary fatty acid intake data in the literature. A recent global summary of national nutrition surveys identified considerable variation in the intakes of the classes of fatty acids and dietary cholesterol, and identified concerns about low intakes of polyunsaturated fatty acids (PUFA), particularly omega-3 PUFA derived from seafood in across the globe. Unfortunately, information on the intake of individual fatty acids was not examined in this review. When the intake of individual fatty acid are examined, palmitate (16:0), stearate (18:0), oleate (18:1n-9) and linoleate (18:2n-6) comprise approximately 90% of the total fatty acids consumed across various industrialized countries (Table 1). However, the contribution of these four main dietary fatty acids can fall below 50% of the total fatty acid intake in populations consuming a hunter/gatherer based diet. Interestingly, the intake of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) can be quite variable, with the percentage EPA + DHA intake ranging from as low as 0.35% in North America to over 2.0% in Japan and over 10% of total

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Accepted March 13, 2019 (received for review February 7, 2019)
Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
http://www.jstage.jst.go.jp/browse/jos/ http://mc.manuscriptcentral.com/jjocs
fatty acids in the traditional Greenland Inuit diet\(^{17}\). These dietary fatty acids are mainly believed to be in the form of triacylglycerols\(^{11}\). It has been estimated that triacylglycerols make up 95% of the total fat consumed with phospholipids contributing 4.5% and sterols and other lipids making up the remaining 0.5%\(^{14}\). Precise quantitative data on the type of complex lipids consumed is limited as nutrition databases contain the fatty acid and cholesterol content of food items rather than the naturally occurring chemical form of lipids. Lipidomic analytical approaches has the potential to characterize and quantitate the intake of complex lipid species.

Lipidomics has emerged as a field of study due to the advancements of analytical tools, particularly increased usability of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Lipidomic initiatives have led to numerous advances including the development of a nomenclature and classification system for lipids\(^{15}\) but methodologies continue to evolve and truly comprehensive quantitation of complex lipids remain elusive due to the structural complexity and the diversity in abundancies of the various lipids in biological samples\(^{14}\). The aim of this review is to revisit the role of fatty acid biomarkers in understanding diet and health with the emergence of lipidomic analyses. This will include an overview of biomarkers of fat intake and challenges in establishing the biomarkers as indicators of health status and disease risk. The role of fatty acids within metabolism and the challenges they present within the field of lipidomics will be examined. This will include an example of how lipidomic based approaches can provide additional insight into lipid metabolism that are not available with fatty acid determinations.

### Table 1

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Japan(^{10})</th>
<th>Canada(^{8})</th>
<th>United States of America(^{9})</th>
<th>Greenland(^{3})</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>20</td>
<td>22</td>
<td>22</td>
<td>14</td>
</tr>
<tr>
<td>18:0</td>
<td>8</td>
<td>10</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>18:1n-9</td>
<td>37</td>
<td>41</td>
<td>39</td>
<td>25</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>24</td>
<td>17</td>
<td>17</td>
<td>5</td>
</tr>
<tr>
<td>Sum</td>
<td>89</td>
<td>90</td>
<td>87</td>
<td>47</td>
</tr>
<tr>
<td>20:5n-3+22:6n-3</td>
<td>2.15</td>
<td>0.29</td>
<td>0.3</td>
<td>13.1</td>
</tr>
</tbody>
</table>

2.1 Biomarkers of omega-3 PUFA

Omega-3 PUFA intakes have been associated with various health benefits, including a decreased risk of cardiovascular mortality\(^{22}\), support of neural development\(^{19}\) and a reduced risk of inflammatory based diseases such as asthma\(^{44}\). However, the strength of these health benefits are not reported across studies consistently which has been examined and discussed\(^{26}\). One of the major concerns is the lack of accurate determinations of background diet and poor measures of compliance/adherence to omega-3 PUFA intervention\(^{26}\). Most clinical trials claim high adherence to omega-3 PUFA intervention despite the literature indicating that changing dietary behaviour is very difficult\(^{27}\). These claims of high adherence are usually based on simple surveys and/or capsule count to determine intake which are unreliable due to response bias\(^{28}\). Examining adherence to omega-3 PUFA intervention by measuring blood fatty acid levels can be more informative\(^{17,29}\).

Various blood measures reflecting omega-3 PUFA blood status have been proposed\(^{90}\), and large regions of the world have been shown to have low blood levels of omega-3 PUFA\(^{31}\). The most commonly used omega-3 PUFA biomarker is the “omega-3 index” which was first proposed as a potential risk factor for coronary heart disease\(^{27}\). Despite the success of the “omega-3 index”, the acceptance and

### 2 Fatty Acids as Biomarkers

Blood biomarkers can improve the assessment of dietary fat intakes beyond tools such as dietary records and food frequency questionnaires\(^{15–17}\). The relationship between dietary intakes of specific fatty acids and their corresponding blood levels was examined in the early 1990s\(^{18}\) which demonstrated high diagnostic values for blood levels of n-3 and n-6 PUFA in comparison to saturated and monounsaturated fatty acids. This is largely dictated on the dependency of diet as a source of PUFA whereas saturates and monounsaturates can be generated de novo from the carbon backbones of carbohydrates and amino acids. Biomarkers of dietary fat consumption can be based on fatty acid analyses of various tissues and fluids\(^{19}\), and although adipose tissue is often preferred for the assessment of long term dietary intake, the use blood based biomarkers are more feasible for routine sample collection\(^{20,21}\).
application of omega-3 PUFA biomarkers in clinical health assessment has limited by a lack of standardization of analytical methods\(^\text{30, 31, 33}\). Recently, there has been an attempt to establish consensus and best practices for fatty acid determinations in samples used for clinical studies\(^\text{33}\). These best practices were not prescriptive towards a single "gold standard" as it was recognized that the type of research and the research questions could vary and these need to be considered in the analytical choices. However, it was established that the analytical choices need to be well documented and justified, especially for: 1) sample collection including the type of sample and the storage conditions, 2) chemical preparation of the sample such as lipid extraction/isolation and fatty acid derivitization, 3) instrument analyses such as gas chromatography coupled to flame ionization detection or mass spectrometry, and 4) data analysis and reporting including the number of fatty acids to report and the manner/units to express the data. Ultimately, standardization of fatty acid measurements will improve reproducibility and consistency in study outcomes.

3 Fatty Acids within a Lipidomic Approach

Fatty acids as "free" or "non-esterified" fatty acids are relatively limited in human biology. Most of the dietary fat consumed is in the chemical form of triacylglycerols, with three fatty acyls esterified to a glycerol backbone. Digestion and absorption initiates their chemical rearrangement as lipases hydrolize fatty acids from their dietary precursors but they are quickly repackaged as fatty acyls within the complex lipids of chylomicrons before entering the circulation. The chylomicrons then release fatty acids and triacylglycerols to tissues, with hepatic lipid metabolism playing a central role in the fate of fatty acids through uptake, biosynthesis, elongation, desaturation, and lipoprotein assembly. Adipose sites serve as reservoirs of triacylglycerols, than can release "free" fatty acids to the circulation to support energy demand. These are free or non-esterified fatty acids complex with albumin for transport in the plasma to cardiac, skeletal muscle and hepatic tissues. At the tissue, fatty acids dissociate from albumin and translocate across the cell membrane and into the cell\(^\text{34, 35}\). Within the cell, various enzymes are involved in fatty acid release from and incorporation into complex lipids such as lipases, acyl-CoA synthetases and acyltransferases that can reshape the acyl species of complex lipids\(^\text{36–38}\). There is evidence that certain fatty acids are preferentially placed within certain complex lipids. For example, highly unsaturated fatty acids (HUFA, ≥ 20 carbons and ≥ 3 carbon-carbon double bonds) are preferentially incorporated into the sn-2 position of glycerophospholipids\(^\text{39}\), while the fatty acids of triacylglycerols are comprised mainly of 16-18 carbon fatty acids, specifically 16:0, 18:0, 18:1n-9 and 18:2n-6\(^\text{40}\). Information about these distinct fatty acyl lipid species are lost with traditional fatty acid based approaches as the fatty acids are removed from the native complex lipid prior to analysis. A lipidomic approach allows for the determination of the lipids in their native molecular form and these acyl-specific lipid species can have unique characteristics in regards to location in blood pools and in synthesis and metabolic turnover\(^\text{40}\).

3.1 The Blood Lipidome

Lipidomic analytical developments are very promising, but progress is tempered by challenges facing the field\(^\text{28, 41, 42}\). Similar to fatty acid analyses, the choice of the type of sample can greatly influence the results\(^\text{31}\). Plasma lipidomics are largely influenced by circulating lipoproteins and as such can have considerable amounts of triacylglycerols and cholesteryl esters, and a phospholipid profile that is dominated by phosphatidylcholines. These three lipid classes have been estimated to contain approximately 95% of the fatty acids in plasma, with most of the remainder as nonesterified fatty acids bound to albumin\(^\text{43}\). In contrast, erythrocytes consist of a more complex mixture of membrane polar lipids and very little of the nonpolar triacylglycerol and cholesteryl ester fractions. Whole blood is obviously a mixture of these lipidomic profiles, and while not analyzed as frequently as plasma and erythrocytes, lipidomic analysis of dried blood spots is possible\(^\text{44}\).

The plasma lipidome has received the most attention in regards to standardization of lipidomic measurements of a blood fraction based on the clinical availability and the availability of plasma based standard reference materials\(^\text{45}\). However, the plasma lipidome is not as well defined as it should be. A recent interlaboratory exercise revealed considerable variation in what was measured and reported, with over 1500 unique lipids being measured but consensus location estimates and associated uncertainties were determined for only 339 lipids\(^\text{41}\). This variation indicates a lack of standardized protocols, but also highlights that the lipidomic field is a mixture of researchers with interests and analytical procedures for specific types of lipids to answer individual research questions. Full characterization of the plasma lipidome appears to require several quantitative analytical procedures\(^\text{45}\), but even then not all lipid classes are examined at the same level of detail. In general, there is a tendency for "lipidomics" to focus on the detailed analysis of low abundant and highly bioactive lipid mediators, rather than full characterization of acyl species of complex lipids\(^\text{41}\).

3.2 Analytical Approaches

Initiatives to standardize blood lipidomic approaches are ongoing and have identified pre-analytical, analytical and post-analytical challenges that need to be considered and are presented in detail elsewhere\(^\text{42}\). The variety of analyti-
tical platforms and techniques available to the mass spectrometrist is a boon in the pursuit of identifying different types of lipids, but makes the acceptance of standardized protocols challenging. "Shotgun" approaches in lipidomics with minimized sample preparation and direct infusion can increase sample throughput, but proper identifications in very complex samples and quantitative results can be difficult. Front-end separation of analytes prior to mass spectrometry such as liquid chromatography can improve identifications in global or "untargeted" approaches but quantitation remains a challenge and throughput decreases due to the additional time for separation. The use of "targeted" approaches provide the best quantitative results, but by definition, the analytes are predetermined, which one could argue is no longer a true "omic" endeavour. Previously, we have proposed categorizing types or the focus of a lipidomic approach on the abundance of the analytes being measured\(^{46}\) to shift the focus on the lipidology rather than the analytical conditions. Examining high abundant lipids is "macrolipidomics" and examining low abundant lipids is "microlipidomics". Macrolipidomics examines common structural and energy storage lipids and methods could potentially be standardized as common sample preparation techniques could be established relatively easily. Microlipidomics examines the highly bioactive and transient lipid signalling molecules that require "targeted" approaches for proper determinations as how the sample is collected, stored and processed can impact the type and concentration of the analyte measured.

3.3 Levels of Information in Macrolipidomics

Macrolipidomics has a considerable focus on highly abundant complex lipids that contain fatty acyl chains. The level of information reported about the fatty acyl containing lipids varies considerably in the literature\(^{47}\). Unfortunately, the practice of reporting the lowest level of information about fatty acyl containing complex lipids is common\(^{41}\). This lowest level is known as the sum compositional level or "brutto" level where the lipid class followed by the total number of carbons and double bonds across all the constituent fatty acyl chains\(^{46}\). For nutritional research, brutto information can severely limit the ability to determine the acyl species of complex lipids as various combinations of fatty acids can make up a singular brutto definition as a phosphatidylcholine (PC) with a sum composition of 38:6 could be PC 16:0/22:6, PC 16:1/22:5, PC 18:1/20:5, PC 18:2/20:4, or PC 18:3/20:3. For macrolipidomic data to be informative to the nutritional scientist, information about the distinct acyl chains or the specific isomer of the complex is required, which we have defined as "medio" level of information\(^{40}\). This medio level of information is defined by an underscore between the acyl chains (i.e. PC 16:0_22:6) to denote that the sn location of each acyl chain within the lipid is unknown. Tandem mass spectrometry is typically required to confirm the acyl chain fragments of the parent lipid. The acyl MS/MS fragmentation patterns can also inform the analyst of the location of the acyl chains, as the acyl in the sn-2 of a phospholipid will be more abundant than that in the sn-1 position. Knowing distinct regio-isomers has been termed as the "genio" level of information\(^{40}\) and are denoted by a forward slash between the ordered fatty acyls such as PC 16:0/22:6 versus PC 22:6/16:0. Finally, the "infinio" level of information is reserved for cases when geometric (cis vs trans) and positional (carbon-carbon double bond location) isomers of the fatty acyls within the complex lipid are known\(^{40}\). While it is possible to determine infinio levels of information with tandem mass spectrometry, it requires specialized analytical procedures\(^{40}\). In nutritional research, various dietary databases contain information the individual fatty acid content of foods\(^{5}\). Therefore, the minimum standard level of information for macrolipidomic analyses of complex lipids needs to be at the medio level to provide useful nutritional insights. While genio and infinio levels would provide additional information, the increased analytical effort at this time to acquire such information is a considerable burden.

3.4 Using Macrolipidomics to Examine Maternal Docosahexaenoic Acid Status during Pregnancy

Recently, we demonstrated the value of using a lipidomic approach in a nutritional research study examining fatty acid metabolism during pregnancy\(^{46}\). Maternal DHA levels are important for infant health\(^{24,40}\) and there is evidence of metabolic adaptions during pregnancy to increase blood levels of DHA\(^{50}\). From fatty acid metabolism studies, the increased blood levels of maternal DHA during pregnancy appear to be driven by increased biosynthesis from other omega-3 PUFA precursors through upregulated Δ6 desaturase or FADS2 because of increased circulating estradiol\(^{51,52}\). Using traditional fatty acid techniques in a rodent model of pregnancy, we were able to determine that the increase in plasma DHA that is associated with pregnancy was mainly in the PC fraction\(^{40}\). PC is a major component of lipoproteins. During pregnancy, hepatic lipoprotein assembly and secretion into the plasma is upregulated\(^{51}\. PC in lipoproteins have the potential to serve as a source of fetal fatty acids as the placenta has considerable phospholipase activity\(^{44}\). Using lipidomic analyses, we identified that the increase in plasma DHA was largely due to increases in PC 16:0/22:6. With this insight, the metabolic pathways that could be involved with the specific hepatic synthesis of PC 16:0/22:6 were examined. PC is synthesized de novo through the Kennedy pathway or by methylation of phosphatidylethanolamine (PE) by PE methyltransferase (PEMT)\(^{50}\). With de novo synthesis, the fatty acyl composition can be "immature" in the initial phospholipid, which can then be remodelled through the Land's cycle\(^{46}\). The
incorporation of DHA into phospholipids occurs during this remodelling through an acyl transferase, or in the case of PC remodelling, a lysophosphatidylcholine acyltransferase (LPCAT). A LPCAT with a high specificity for DHA has not been identified suggesting that other mechanisms are involved in the hepatic synthesis of PC 16:0/DHA during pregnancy. PEMT is upregulated during pregnancy as determined by studies examining choline metabolism, which we confirmed in our rodent model. The DHA content of PE is typically higher than the DHA content of PC, therefore increased hepatic methylation of PE by PEMT during pregnancy could be a mechanism of increased DHA in maternal PC. In primary cultures of rat hepatocytes, Ridgway and Vance demonstrated that newly formed PE from PE is highly enriched in PC 16:0_DHA as compared with other lipid species. The mechanistic insights gained from a lipidomic approach in this study highlight the potential mechanistic insight that can be gained from the use of lipidomics in nutritional fatty acid and lipid research.

4 Conclusions
Fatty acid levels in blood are useful biomarkers of dietary intake and can be used to determine associations between specific fatty acids and various health outcomes in large observational studies. In clinical intervention trials, fatty acid blood biomarkers can confirm background dietary intakes and assess adherence to intervention protocols. This information is important to understand the effectiveness of interventions and to determine target intake levels for dietary recommendations. Lipidomic blood biomarkers have the potential to be even more informative than fatty acid biomarkers as the location of the fatty acyls within the complex lipids are not lost during analysis. In particular, lipidomic analyses will raise the mechanistic understanding of the role of fatty acids and lipids in health. The routine use of lipidomics is currently limited by a lack of standardization of methodological approaches, a lack of consensus on what and how to report lipidomic data, and challenges in quantifying the diverse types of biological lipids. These challenges become less daunting when lipidomics is categorized into macrolipidomics and microlipidomics according to the abundance of lipids in a biological sample. In addition, for lipidomics to be fully embraced in nutritional research, the reporting of sum compositional data or brutto species of lipids needs to be discontinued in favour of reporting medio, genio or infinitio species.

Acknowledgements
Ken D. Stark is supported through a Canada Research Chair in Nutritional Lipidomics and received travel support from the American Oil Chemists’ Society to present this review at the JOCS-AOCS Joint Symposium in Kobe, Japan on September 6, 2018. The author would also like to thank Juan J. Aristizabal Henao for lipidomic contributions to key publications related to this review.

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