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Review Article

Dietary lipids from an evolutionary perspective: sources, structures and functions

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Abstract

Lipids are a complex group of biomolecules whose precise functions remain poorly understood. As a result of this poor understanding, it is difficult to make mechanistically based recommendations for appropriate dietary intakes. It is equally difficult to develop methods that are capable of diagnosing functional impairments because of insufficiencies or excesses in particular fatty acids. Lipids are abundant building blocks of cellular membranes, supply components for lipid particle assembly and substrates for metabolic fuel, and provide a precursor pool for an astonishingly diverse range of signalling molecules. In each of these broad functions, the functional consequences of different structures of fatty acids are not fully understood. According to research on membrane functions through early evolution, docosahexaenoic acid provides two biophysical properties to membranes – accelerating the lateral motion of lipids and proteins within the plane of the membrane and simultaneously slowing the rate of diffusion/leakage of charged species across the plane of the membrane. The range of fatty acid structures used as substrates for assembly of either lipoproteins or milk fat globules is broad, yet the functional consequences of differences are not known. Different lipids signal into a remarkable range of biological processes. Saturated and monounsaturated fatty acids are becoming recognized as signal molecules in their own right. The complex composition of human milk lipids implies that diets with a diversity of fatty acids in complex lipid forms and structures is more beneficial than a narrow range of any particular group of fatty acids.

Keywords: evolution, docosahexaenoic acid, membrane structure and function, lipid signals, milk fat.

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Introduction

Lipids are the least understood of the cellular biomolecules. Compared with polynucleotides (DNA, RNA), proteins, organic acids, or sugars, lipids remain a poorly described and perplexing black box. Even in the era of genomics, in which the entire genomes of the major classes of life are being assembled – and with this sequence information propelling a new wave of discovery in the life sciences – lipids remain a vague and under-appreciated subset of the 'metabolome'. Lipids and other metabolites that are not direct products of gene sequences are not as easily addressed with the modern tools of genomics and bioinformatics. Similarly, lipid ensembles that are found in biological cells and fluids, even in isolated chemical studies, have not been as amenable to detailed analysis of their structures as proteins and polynucleotides. There is a critical need for mechanistic research to understand the composition, structures and functions of lipids as the basic understanding against which dietary delivery of lipids can be pursued. This research will need to create new tools for examining cellular structures and combined

together with the tools that are emerging from the genomics era to illuminate lipid functions in molecular detail. From the origins of life to the composition of mammalian milks, lipids evolved as a diverse group of highly functional biomolecules. An evolutionary perspective provides insights into possible roles of lipids, and considerations for research priorities and conjectures as to how dietary fats should be formulated for diverse individuals at different ages with various health conditions.

Typically, the first-generation scientific process of disassembling biology into discrete variables is a simple compositional analysis. Lipids have been addressed from the perspective of the composition of different organisms, cell types, tissues and biofluids. Lipid metabolites have been measured for decades, and the knowledge of the biochemical pathways that use and produce metabolites is extensive. There is a long history of successful application of metabolites as measured biomarkers (blood cholesterol, triglycerides, etc.), yet there is still a considerable lack of basic compositional information.

The same analytical principle of measuring soluble metabolites (high resolution chromatography, mass spectrometry, nuclear magnetic resonance) has been modified for lipids. These tools are being used to build the complete compositional databases of cellular lipids (Quehenberger *et al*. 2010). However, annotating these databases into a comprehensive, predictive model of how the structures and compositions of biological membranes relate to their functions will be a major challenge to future research across all of life science. This knowledge will be critical in determining the role of diet in altering lipid compositions.

Lipids are a distinctive class of biological molecules and their uniqueness is part of the basis of their scientific challenge. Lipids as biological molecules are responsible for: (1) a unique class of cellular structures, particles and organizations; (2) providing life's most dynamic and efficient fuelling and energetic schemes; and (3) complex signalling systems within and between cells. Why are we still so ignorant of the structural basis of their functions? Lipids have provided remarkable capabilities to the processes of cellular life, but the properties that make them so vital to life's processes render them difficult for scientists to study, much less understand. It is instructive to consider why science has such difficulty with lipid research.

Membrane structures

The property of complex lipids to self-assemble in water to form a highly organized bilayer membrane seems almost magical. Apparently, early life found it magical and useful. This self-assembly process of complex lipid bilayers provides essential properties to the barrier between cells and the outside world in virtually all life forms. Furthermore, this same bilayer structure enables the lipid membrane to simultaneously separate charged molecular species and 'house' the wide range of proteins that direct the energy associated with the collapse of charge separation into biological processes.

Ever since the seminal events of early molecular evolution that produced the lipid bilayer, living organisms have expanded the permutations of its composition, structure and utility. Life 'found' that increasing diversity of lipids yielded remarkable diversity of structures available for disparate membrane configurations. Throughout evolution, lipids have been an apparently irresistible molecular scaffold for cellular structures, intracellular and extracellular surfaces, vesicular and clustered transporters, and *trans*-membrane channels. Why did science take so long to recognize this biological dimension? The very nature of the forces underlying the spontaneous assembly of lipids into ephemeral, diaphanous structures is the reason the traditional experimental strategy of biochemistry (smash and spin), so successful

Key messages

- There are multiple actions of dietary lipids beyond the provision of essential fatty acids.
- Human milk contains a broad range of fatty acids and complex lipids.

with soluble proteins and polynucleotides, is so disastrous with lipids. The tools that worked so well on the 'rugged' molecules of biology – proteins, polysaccharides and polynucleotides – tear the structures of lipids apart. As a result, we still do not know how the structures of lipids form and change, how they function, nor even why the striking diversity of lipid composition is necessary at all.

Energy and fuelling

Although their roles as fuel molecules and emergence in higher life forms as a favourite storage form of energy are the best understood of lipids' functions, they still pose difficulties for study. When is a lipid intermediate a precursor for complex lipids, for signals and for simple fuel? The promiscuous nature and interconnectedness of lipid biochemistry frustrates attempts to understand the regulation of lipid biosynthetic pathways and their effective modelling to this day. The simple property of insolubility of neutral lipids that makes them such a useful, inert fuel storage also means that the surface properties of their globule boundaries and the transport systems to move these insoluble lipids around as aggregates (e.g. lipoproteins) are as critical to their functions as are the structures and reactions of individual molecules. Yet again, the standard biochemistry experimentation that unravelled the myriad enzymes and pathways of cell metabolism by the chemical reactions that they catalyse in isolation provides no means to study and understand protein components that simply bind and transport lipids from one cellular compartment to another. This problem was especially true when the first step of a traditional biochemical experiment was to disassemble (homogenize) the cellular compartments.

Signalling actions

The signalling functions of lipids have proven almost as difficult to study as the membrane structures. Nonetheless, organisms were apparently at considerable Darwinian advantage when they used lipids as a precursor pool for ingenious signalling systems within and between cells. Though proteins are typically the transducers of signal actions, lipids form an astonishing diversity of the pool of cellular signal molecules. The cellular membrane is ubiquitous and fragile. It is thus logical that these molecules are the basis of much of biology's alarm communication system triggered by various forms of 'stress' and mediation of appropriate cellular responses. Lipid structures are fragile in many chemical aspects; they can readily breakdown, via simple, thermodynamically favourable chemical reactions (hydrolysis, oxidation), into products with solubility and structural features distinctly different from their intact precursors. This means that virtually every type of stress to a cell is likely to liberate lipid fragments, the presence of which could easily be converted into a signalling system appropriate to the recognition and management of those same stressors. Nonetheless, this same tendency of complex lipids to form 'signal molecules' so readily and rapidly in cells makes them difficult to study. Signal molecules form so easily under the conditions of experimental study that they form and disappear purely because of artefact in spite of the best laid plans to carefully study them. The problems of false positive and false negative errors have bedevilled the field of lipid signalling.

With the challenges to studying lipids so apparent, it is impossible to make confident predictions of dietary requirements and consequences based on simple compositional abundance. Nutrition research will be frustrated for decades as the mechanisms are established for underlying the compositions, structures and functions of lipids against which dietary intakes can then be interpreted. Nonetheless, even before detailed mechanisms are known, it is possible to assemble principles on which to make educated recommendations.

Membrane structures and functions

The biological membrane is a highly complex ensemble of self-associating lipids into which are embedded a diverse array of proteins and other biomolecules. It is estimated that the average cell membrane contains thousands of different molecular species of lipids (Quehenberger *et al*. 2010). This

remarkable diversity of composition of different cellular and subcellular membranes implies considerable functions associated with the molecular details of the membrane composition. For example, the inner mitochondrial membrane contains lipids – most notably cardiolipin – not found in any other membrane in the cell. At the level of cells, neurons are abundant in sphingomyelin, cells of the outer rod segment are enriched in phosphatidyl ethanolamine and epithelial plasma membranes are rich in glycolipids. The fatty acid composition also varies widely, but nonetheless, the pattern of fatty acids tends to be conserved within each complex lipid class. There is substantial evidence that this conservation of composition is tightly controlled within cells against a variety of external influences, including diet. Yet, it is not known how different fatty acids are sensed and their levels appropriately adjusted.

In certain tissues, there is a functional role to the secretion of extracellular lipids. Epithelial cells secrete lipids in various vesicular or globular particles that sequentially spontaneously self-assemble and then, in some cases, are further acted upon by extracellular enzymes to form macroscopic extracellular lipid structures. Both the lipid classes and fatty acid compositions of these secreted lipid particles are distinctive. For example, lung epithelial cells secrete lung surfactant that is conspicuously enriched in the single molecular species dipalmitoylphosphatidylcholine. Skin epithelia secretions are enriched in glycolipids, cholesterol, diacylglycerides and free fatty acids. The demands on the precise composition–structure-function–demands of these lipids appear to be critical. In genetic or developmental conditions where individuals fail to produce them, the results are catastrophic. Premature infants who cannot assemble dipalmitoylphosphatidylcholine are unable to breathe normally (Engle 2008), and those who cannot assemble linoleate-containing glycolipids do not form a functional water barrier in their skin (Jackson *et al*. 1993). Neither condition is survivable, but each can be diagnosed by simple compositional analysis. Much of scientific research into cellular lipids has pursued a compositional description strategy as a logical first step to understanding the mechanisms by which lipids act.

Composition description of specific samples, cell types and subcellular organelles

The first step in understanding the structures and functions of biomolecules has been to describe their distribution within functionally or structurally distinct compartments. Such a strategy has been a hallmark in biochemistry research of mitochondrial and nuclear proteins, ribosomal RNAs, etc. This basic logic has been critical to gaining the understanding we now have of the importance of diet in providing adequate quantities of particular fatty acids to supply the composition of normal membranes. Unfortunately, the complexity of the membrane does not allow for simple fatty acid composition of a membrane to infer function or its failure. Lipidomics is an application of analytical chemistry and compositional biology that are taking a similar approach to 'the study of the composition, metabolism, and biological role of lipids in cells at the levels of molecular species' (Hunt 2006). The combination of more comprehensive analytical techniques with specific sample collection, cell isolation and subcellular fractionation methods is capable of gaining insights into possible associations between composition and location, though again, not yet function. The inability to interpret compositional changes in lipids in terms of function also extends to biochemical defects in synthesis, in spite of altered compositions having devastating effects on health. A case study of a young patient with very distinctive defects in lipid metabolism and health was instructive of just how critical lipid metabolism can be to overall health. Williard *et al*. (2001) identified a highly unusual genetic deficiency of Δ6-desaturase activity in a young patient. This genetic deficiency was responsible for a wide range of phenotypic problems, from linear growth to skin function, and even behavioural alterations. Skin fibroblasts taken from the patient were cultured and challenged with a series of labelled fatty acids which comprised both precursors and products of the major lipid metabolism enzymes. The results indicated a defect in the Δ6-desaturase activity of the patient. Based on these results, simple supplementation was pursued to increase the patient's lipid intake of fatty acid products of the Δ6-desaturase activity. Interestingly, supplementation mitigated much but

not all of the phenotypic consequences of the condition. Perhaps the most informative aspect of this unusual case history is the length of time it took to even begin to address lipids as a target for diagnostics and intervention. The young girl had been exhibiting increasingly debilitating symptoms soon after birth, yet she was 6 years old before clinicians began to examine her blood lipid composition as a possible underlying basis of her diverse problems. Quantitative lipid metabolome data as the basis of routine health measures will yield a breadth of information for individual assessment once lipid composition is finally understood in functional detail. The one clarifying conclusion that can be made from a wealth of diet and its effects on simple lipid composition studies is that the unifying assumption that all members of a lipid family are equivalent is demonstrably incorrect. Fatty acids are distinct metabolic entities; they interact metabolically and should be addressed as such (Friesen & Innis 2010).

Lipid composition and membrane function

The experimental practice of separating biomolecules in order to characterize their functionality is relatively easy with proteins as enzymes because their function, i.e. catalytic activity of a particular reaction, typically persists even in an isolated protein. However, the diversity of complex lipids in eukaryotic cells, and the fact that they apparently function as aggregates or ensembles, have made it very difficult to assign specific functions to specific lipids. We remain discouragingly ignorant of the basic functioning of the biological membrane and of the role of specific lipid components in it. This is, in part, because of the lack of methods to identify lipids precisely and in part to the lack of methods to separate functional subunits of cells, and especially to separate functional units of biological membranes. The case of cholesterol illustrates this point well. For over 100 years since the realization that cholesterol is abundant in the arteries of heart attack victims (recognized literally prior to the 20th century), cholesterol has been the subject of intensive scientific investigation. More Nobel prizes were awarded for research on cholesterol than for on any other molecule in the entire 20th century. Thousands of studies characterized how it was made, how it was transported, even how it was metabolized and, of course, how it accumulated in the arteries of those developing heart diseases. However, cholesterol's actual biological functions remained elusive. Textbooks routinely referred to cholesterol's function in the membrane as providing 'fluidity'. The fact that one of the most ubiquitous and vital molecules in cellular life was so crudely defined speaks volumes to science's collective ignorance as to how lipid membranes actually work in living cells. Only with the seminal studies of Simons, Brown and various colleagues did the true function of cholesterol and its close structural relatives in the biological membrane begin to emerge (Brown & London 1998; Simons & Gerl 2010). The simple fluid mosaic model of synthetic bilayers, elegant in concept, is now considered naïve in terms of actual biomembrane structures and functions. The cellular membrane is highly structured in the bilayer domain, and this structural dimension is responsible for a substantial portion of membrane activities. Cholesterol provides the structural nucleus for the two-dimensional organization of the biological membrane. As such, cholesterol is the central player in a unique physical property of all biological cells that is still not understood (Lingwood & Simons 2010). Given the discouraging reality that only in the past decade have hints appeared as to what cholesterol – the most studied of lipids – actually does in membranes, it is not surprising that for none of the other complex lipids has a detailed structure function relationship appeared. Scientists lack the basic tools to study soft matter; hence, we are still perhaps decades from being able to say with confidence what, for example, docosahexaenoic acid (DHA) functions in neurological, optical or energetic membranes. Given the lack of basic understanding of membrane structure and function, it is virtually impossible to place variations in membrane composition because of diet into a coherent predictive framework. Nonetheless, scientists are making do with the tools available and some important progress has been made, thus the walls of the black box are beginning to dissolve.

One of the most perplexing questions of lipid biology is 'What is the original role of polyunsatu-

rated fatty acids?' What was the primordial value to cells of the fatty acids containing multiple double bonds? Even the first discernible life forms – unicellular algae – contain DHA in their membranes. The presence of a single double bond is easily understood. Fatty acids that are fully saturated readily crystallize and do not become liquid until temperatures are above 50°C. Because the biological membrane functions as soft matter, i.e. a two-dimensional liquid, membranes that are composed of solely saturated fatty acids are crystalline and would be incompatible with normal life. However, a single double bond in the middle of a fatty acid chain confers a dramatic change in melting point and brings it close to that of water. Hence, from a simple biophysical perspective, only single double bonds, i.e. monounsaturated fatty acids, are necessary to sustain the cellular life forms utilizing soft matter bilayer membranes. Why then did these early cells go to the trouble to synthesize and accumulate fatty acids with up to six double bonds?

A compelling theory has begun to emerge from the intensive investigations of marine bacteria and their membrane functions (Valentine & Valentine 2004). This theory formulates a role for long-chain, highly unsaturated fatty acids. The singular property that made membranes so valuable to the emergence of life was the ability to separate charge. By holding charges separate across a non-polar bilayer, the membrane stored energy, i.e. formed a nanobattery. The energy, stored as separated charges, could be accessed by allowing the charge to flow back across the membrane in controlled, coupled reactions. Even more intriguingly, the membrane dimensions were ostensibly the same as those of a folding domain of proteins. Thus, the membrane formed an energy-rich folding scaffold ideal for protein evolution. This ingenious nanobattery membrane unit became the central coin of energy for much of early cellular life, and remains today as a key basis for energy transduction from bacteria to humans. Yet, the model has a flaw. Separating small charged ions, particularly protons ($^{+}$H), can be accomplished by many chemical and physical processes, but once separated across a bilayer, holding such small charges apart by soft matter phospholipid membranes is problematic, they leak. Fluid bilayers of fatty acid-based lipid membranes tend to form water wires within the membrane, down which protons and similar charged species leak, dissipating the hard-won energy (Gutknecht 1987). Ion leaks remain the major energy cost for bacteria (Oren 2010). This energy deficit became a major Darwinian pressure on early cellular photosynthetic life. What emerged in early evolution to manage this problem? – polyunsaturation. The presence of double bonds within the fatty acids of the membrane attenuates the formation of water wires and the leaking of small ions (Valentine & Valentine 2004). In the early forms of unicellular photosynthetic algae, the basic energy dissipation theory holds that energy rapidly became limiting as cells moved below the water surface, away from the light. Adding double bonds to their fatty acids slowed the leaking, allowing these cells to survive lower in the water, thus extending the ecological window capable of supporting life. As the cells went deeper, the double bonds increased up to six. With such a strategy for membrane leak protection in place, unicellular marine algae became highly enriched in polyunsaturated fatty acids, particularly DHA, 22:6n3. The biosynthetic enzymes necessary in producing *cis* double bonds within long-chain fatty acids became a central pathway in lipid metabolism. Once in place, this dimension of fatty acid structure was fully available for subsequent membrane roles as evolution proceeded.

A second proposed role for polyunsaturated fatty acids in complex phosholipid membranes relates to their physical properties within the plane of the membrane. Fully saturated fatty acids are highly flexible molecules, perhaps the most unconstrained structure in the biomolecule repertoire. An easily perceived consequence of this intramolecular flexibility is the tendency to readily associate with similar biomolecules into ensembles, including gels and crystals. Saturated fatty acids, either free or as various esters, readily crystallize far in excess of room/body temperature (60–70°C melting points, depending on chain length). The presence of a single double bond disrupts the ease of association and dramatically lowers the melting points of monounsaturated fatty acids (2–4°C). Nonetheless, the relatively high content of saturated fatty acids and the ease of association between these fatty acids within membranes confer a viscous property to the lateral mobility of molecules within the membrane,

including both lipids and proteins. Lateral diffusion measurements of artificial bilayers and biological membranes indicate a viscosity similar to that of olive oil (Lindblom & Orädd 2009).

Evolution has apparently favoured another property of highly unsaturated fatty acids in altering these viscous properties for specific membranes and functions. The fatty acid DHA, when present in a phospholipid, has recently been shown to provide another property to the membrane: ultra-rapid lateral motion (Gawrisch *et al*. 2003). Dynamic simulations of the rotational velocity of phospholipids containing DHA illustrate that the regular array of double bonds confers a significant increase in rotational motion to the phospholipid. According to this model, the consequence of such motion in a membrane is that DHA-containing phospholipids act ostensibly like two-dimensional bearings. Importantly, in the vicinity of DHA-containing phospholipids, the mobility of membrane-bound proteins is accelerated as well (Valentine & Valentine 2004).

These recent biophysical models of DHA's properties within early membranes imply that the advantages of polyunsaturated fatty acids in the evolution of simple life forms would be of similar value to complex life forms. Through evolution, cells that accumulated DHA-containing phospholipids, and thus contained membranes with unique properties, were at selective advantage when these properties conferred value. The composition of membranes that selectively accumulate DHA appears to support these models; notably, neural cells that must conduct signals rapidly at long distances (Jutras & Buffalo 2010), retinal membranes that must permit rapid rotational motion of rhodopsin (Feller 2008) and mitochondrial membranes that must shuttle electrons rapidly between proteins while separating charge across the same membrane (Watkins *et al*. 1998). The biophysical properties of DHA in membranes need not be the sole value in higher organisms as all biomolecule classes acquired multiple higher functions through evolution. Recent research has demonstrated, for example, that DHA-containing phospholipids tend to disrupt cholesterol – sphingomyelin-based clusters (rafts) – and shift the membrane structure topology towards greater non-clustered regions (Shaikh *et al*. 2009).

If an important aspect of DHA's role in neuronal membranes is to alter the local physical properties consistent with higher lateral mobility and lower ion permeability, nutrition scientists have not been measuring these properties as a diagnostic of DHA status. In fact, the tools to conduct such studies have not yet been assembled by the basic scientists who interrogate membrane biology. If nutritional evaluation of DHA status is to move to a more mechanistic basis, it will be critical to develop methods capable of assessing cellular and tissue properties related to membrane structure with sufficient sensitivity to detect the consequences of varying DHA concentration. With these in place, accurate measures of DHA composition and function can guide dietary recommendations.

Lipids as sources of energy and fuelling

Lipids as fuel

Evolutionary pressure has favoured organisms in which fat plays diverse roles as a fuel. The coemergence of insoluble complexes of fatty acids, principally triacylglycerides, as a molecular form of energy storage and the oxidation of fatty acids for immediate energy is one of the enabling metabolic systems of higher eukaryotes (Turkish & Sturley 2009). The complex enzymatic machinery necessary to synthesize fatty acids and assemble triacylglycerides on the endoplasmic reticulum attests to the substantial genetic investment in this biochemistry (Athenstaedt & Daum 2006). The investment in lipid oxidation itself must be said to be even more prodigious from the perspective of evolution, as it is clear that entire prokaryotic organisms with their genomes intact moved into eukaryotes and acquired an adopted intracellular role as mitochondria (Zimmer 2009). The complexities of controlling this fuelling system extend well beyond the simple management of substrates to including intermediate transport, mitochondrial biogenesis and fuel partitioning (Gohil & Greenberg 2009, Hock & Kralli 2009). Not surprisingly, a host of human diseases is associated with subtle failures or dysfunctions of mitochondrial lipid oxidation (Wallace 2009), intracellular lipid

storage (Simha & Garg 2006; Schaefer & Asztalos 2007; Meex *et al*. 2009) and lipid transport (Salter & Mangiapane 1999).

From the perspective of evolution and early diet of infants and children, is there evidence that diet influences fuel metabolism acutely and or chronically, most importantly as a form of imprinting? There is no direct mechanistic studies documenting how milk is imprinting infant metabolism; however, considerable evidence in a variety of mammalian models implies that early diets, notably milk, have a persistent effect on whole body metabolism.

Lipid transport

Lipoproteins, the colloidal particles that transport insoluble lipids within blood, lymph and cerebral spinal fluid, are among the most studied structures in biology. There are excellent reasons for this intense interest. Dysregulations of lipoprotein metabolism, even when expressed as crudely as inordinately high or low concentrations of individual components of these complex particles in human blood (e.g. cholesterol), are responsible for the leading cause of mortality within human populations in the Western world (Salter & Mangiapane 1999). Literally, thousands of studies have followed the raising and lowering of the lipid constituents of lipoprotein particles in humans and surrogate animal models as a function of experimental variables, including genetics, diet, drugs, lifestyle, toxins and exercise. Despite this massive effort, the precise structures and functions of lipoprotein particles in the nanometer length scale at which they function remains poorly understood (German *et al*. 2006; Schaefer & Asztalos 2007; Vuorela *et al*. 2010). For example, it is not yet known how to treat individuals with drugs that raise functional high-density lipoprotein (HDL), nor construct artificial lipoproteins for therapeutic purposes, nor if it were, what compositions and structures would be optimal for particular conditions. Perhaps the most discouraging omission is the lack of knowledge of how human breast milk guides the development of lipoprotein biology in the neonate. Little research has pursued this obvious target, and most of the research that has been performed to date on infant lipoproteins and diet has taken the perspective of long-term cardiovascular risk (Kallio *et al*. 1992). Thus, human milk remains poorly understood in terms of its ability to guide or even influence infant lipoprotein metabolism. It is also not known if these effects are of net benefit to the infant. Nonetheless, human milk composition is the most appropriate perspective to begin this research.

Human milk

The composition of fatty acids in human milk is interestingly variable depending on stage of lactation, health of the mother, birth order of the infant and the diet consumed by lactating women, although there are insufficient data showing the ranges of the biologically important fatty acids (Jensen 1999). Smit *et al*. (2002) reported the large biological variation of 28 fatty acids in 465 mature human milk samples from five regions of the world, documenting that long-term maternal diet has a strong effect on fatty acid composition of milk. Lipid composition also changes as mammary secretions move from colostrums to mature milk. Bitman *et al*. (1986) reported that the total fat content was 1 g dL^{-1} in the pre-partum secretions and remained at 3–4 g L^{-1} in colostrums at 3 days, through transitional milk at 7.2 days and mature milk at 56.2 days of lactation. Most lipid (93–97%) was triacylglyceride, except that prepartum secretions contained higher amounts of the membrane components, phospholipid (3.2 g dL^{-1}), cholesterol (2.3 g dL^{-1}) and cholesteryl ester (1.1 g dL^{-1}), which declined post-partum to 0.65 g dL^{-1} at 3 days, 0.37 g dL^{-1} at 7.2 days and 0.09 g dL^{-1} at 56.2 days. Synthesis and secretion of fatty acids pre-partum were similar to those occurring post-partum. The fat content variation in human milk is clearly the result of different dietary, metabolic and physiologic controls. Agostoni *et al*. (2001) documented fatty acid concentrations in colostrum and breast milk throughout a 12-month nursing period in a group of mothers after delivery of full-term infants. Among long-chain polyunsaturated acids, the concentrations of arachidonic acid (20:4n-6) and DHA (22:6n-3) remained stable from colostrums up to the 12th month of nursing, and their percentage concentrations were highest in colostrums. The data indicated that secretion of arachi-

donic acid and DHA remained constant during lactation, although there were changes in the total fat and the linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) contents of milk.

Milk fat globule

The lipids are present in milk not as soluble molecules but as dispersed multimolecular aggregates with a distinctly varied array of complex lipids on their surfaces. The particles themselves are highly disperse and exhibit an average diameter, depending on stage of lactation, health of the mother, etc., between 1 and 10 μm (Hamosh *et al*. 1999). The fat globule is primarily a core of triacylglycerides synthesized within the endoplasmic reticulum of the mammary epithelial cell. This core of triacylglyceride is bounded by a single monolayer of polar phospholipids derived from the endoplasmic reticulum membrane (Bauman *et al*. 2006). The entire globule, in turn, is bounded by a bilayer membrane, which enrobes the globule as it exits the epithelial cell. This membrane is composed of the lipids and proteins of the epithelial cell plasma membrane, including significant quantities of cholesterol, phosphatidylcholine and sphingomyelin (reviewed by German & Dillard (2006). Further complexity can be seen in components unique to the external surface of the native fat globule, including glycolipids, gangliosides and significant quantities of membrane glycoproteins and mucins. Research has documented that these complex lipids have effects on composition and function of tissues literally from intestine to brain (Carlson 2009; McJarrow *et al*. 2009; Schnabl *et al*. 2009). Nonetheless, no dietary recommendations for infants have been made for these components in spite of explicit pathways of synthesis within the mammary gland.

The role of diet in bovine milk fatty acid synthesis has been reviewed, and the processes by which the mammary gland assembles lipids for secretion into milk combine dietary fatty acids, tissue fatty acids and mammary gland *de novo* synthesis (Bauman & Griinari 2003). The basic mechanisms appear to be similar in humans, though a more significant variation has been seen in human milk lipid compositions in response to dietary changes, even changes in saturated fatty acids as diet varies over a wider range (Nasser *et al*. 2010). The transfer of dietary fatty acids directly to milk is covered in various reviews, and all attest to the role of maternal diet in the composition of milk. Such variation makes it difficult to determine an optimum composition from a purely evolutionary perspective. Studies documenting the role of diet in the composition of complex lipids of milk have not been published to date.

To date, little research has addressed the nutritional implications of the structural dimensions of human milk fat globules. Genomic analysis of lactation has demonstrated that the most assiduously retained lactation genes through mammalian evolution are those associated with fat globule production (Lemay *et al*. 2009). The retention of milk's lipid production through the evolution of lactation in mammals implies that this remarkable structure is of considerable functional value to the mother–infant pair. However, the techniques necessary to describe the structures of lipids in detail are not yet developed, hence their nutritional values to infants are still being studied (Argov-Argaman *et al*. 2010). Nonetheless, in mammalian milks, including those of humans, the fatty acids are not simply randomly distributed on the glycerol of triacylglycerides. They are positioned on specific complex lipids arrayed within distinct structures that are in turn likely to have effects on the structures, digestion, absorption and ultimate disposition of fatty acids consumed by infants.

Lipids that function as signals

The field of lipid signalling continues to expand rapidly as entire classes of signalling molecules are still being discovered (Bankaitis *et al*. 2010; Ringseis & Eder 2010). The diversity of lipid molecules that have now been documented to perform a signalling function, i.e. to influence cellular processes beyond their simple structural and metabolic functions, is astonishing. Cell biology has elaborated a remarkable diversity of protein receptors that bind to different lipids as a first step to a cascade of processes (Lin *et al*. 2010; Singh *et al*. 2010; Zeidan & Hannun 2010). This aspect of cellular function has been unusually frustrating for cellular biologists, in part because of the

spatial, temporal and chemical nature of the molecular events. Most lipid signals can be traced to the release of membrane-bound constituent lipids due either to a hydrolytic or oxidative event, both of which are spontaneous chemical reactions of complex lipids. The chemistry and biology of the lipid signalling pathways studied intensively to date thence form a relatively well-established pattern. Once liberated from the membrane, constituents that are normally insoluble and confined to the membrane compartment are *de facto* signals of the events that caused their release. The field of lipid signalling has been dominated for the past 50 years by the eicosanoids – oxidized arachidonic acid derivatives, including the prostaglandins, leukotrienes, etc. (Harizi *et al*. 2008). These are relatively well understood as signals of local stress, and are in keeping with the principle of lipids being unstable membrane components. The essentiality of n6-polyunsaturated fatty acids is, in part, a result of the role of arachidonic acid for these signalling functions (Horrobin 1983).

It is now becoming clear that a great many more lipids play a much more elaborate array of signalling roles than solely indicating stress and, in fact, lipids are key signals in a host of biological processes. After five decades of research, it can now confidently be stated that virtually every class of lipid in the membrane becomes a signalling system once released. Considering the diversity of complex lipids in human milk, the digestion of milk produces a cornucopia of potential signals. It is certainly beyond the scope of this review to explore all aspects of lipid signalling of potential relevance to infant health; however, some examples are illustrative of an underlying principle – lipids are intensively and comprehensively involved in guiding biological processes via cellular signalling functions. Three new classes of lipid-based signals – palmitic acid, palmitoleic acid and arachidonoylethanolamide – illustrate the complexity and biological implications of milk's composition.

Can palmitic acid be a signal? Palmitic acid is the end product of fatty acid synthesis in virtually all organisms. It is difficult to imagine a more ubiquitous biomolecule. Palmitic acid serves as a constituent fatty acid in virtually all cellular membranes; it is a fuel, producing 106 moles of adenosine triphosphate energy via beta oxidation; it is acylated to various membrane proteins; and it is a major component of storage lipids. With these multiple and ubiquitous substrate roles for palmitic acid, it was not suspected to be a signalling fatty acid. Although it was long recognized that saturated fatty acids, including palmitic acid, when consumed in the diet in high quantities, tended to elevate levels of circulating cholesterol, it was not known how. In 2003, Puigserver & Spiegelman (2003) found that the peroxisome proliferator activating receptor (PPAR) is controlled by a higher-order protein complex that they termed the PPAR gene transcription coactivator (PGC-1α). This transcription factor coactivator assembles multiple protein complexes into transcriptional regulatory units in the nucleus to control the transcription of higher-order subcellular systems such as mitochondrial biogenesis. Such subcellular systems had been known for some time to be controlled in a coordinated fashion (Wu *et al*. 1999). With this new understanding of a higher level of gene expression control, transcriptional coactivation, scientists began to examine the proteins responsible. A major breakthrough came when Lin *et al*. (2005) recognized that, when exposed to high levels of saturated fatty acids, liver cells both *in vivo* and *in vitro* actively turned on a closely related protein, PGC-1β. The actions of this newly described transcription coactivator homologue to PGC-1α were remarkable. Turning on PGC-1β simultaneously switched on fatty acid synthesis, triglyceride assembly, phospholipid synthesis and cholesterol biosynthesis. Interestingly, this same coactivation switched off the low-density lipoproteins (LDL) receptor. These results thus explained the perplexing link between dietary saturated fat and cholesterol. The basic molecular switch linking dietary saturated fat and serum cholesterol is PGC-1β and its most potent ligand is palmitic acid. Hence, palmitic acid is the signal to activate PGC-1β and all of its cellular functions. This mechanism within the liver stimulates the net production of very low density lipoproteins (VLDL) and their molecular constituents, and simultaneously slows the uptake of their products, intermediate density lipoproteins and LDL.

What would explain why such a control mechanism would evolve? In essence, when saturated fatty acids

arrive in the liver, VLDL synthesis is activated. When would this situation occur?

1. Lactation – During lactation, fats are mobilized from adipose and circulate to the liver, are packaged into VLDL, and delivered to the mammary gland (Neville 1999).
2. Exercise – In response to intensive physical activity, fat is mobilized from adipose, circulates to the liver, packaged into VLDL and delivered to muscle (Zimmermann *et al*. 2004).
3. Acute infection or injury – During an acute phase of infection, fats are mobilized from the adipose, circulate to the liver, produce VLDL and are delivered to immune cells (Khovidhunkit *et al*. 2004).
4. And of course, during infancy, fats are consumed in milk, circulate to liver via the intestine, and delivered to adipose and growing tissues. All of these processes would benefit from the PGC-1β coactivation of VLDL synthesis by palmitic acid. In this context, the simple fatty acid palmitic acid acts as a hepatic signal to stimulate VLDL synthesis and assembly. This specific signalling action in the liver and the consequences to VLDL production may be quite physiologically and metabolically important both in infancy and throughout life.

Can palmitoleic acid be a signal? Palmitoleic acid (16:1n7) is a minor fatty acid formed by the desaturation of palmitic acid by the stearoyl Coenzyme A (CoA) desaturase enzyme. Normally, palmitic acid is elongated to stearic acid prior to the desaturation step taking it to oleic acid, the most abundant monounsaturated fatty acid in all life forms. In essence, palmitoleic acid is produced when the enzymes of fatty acid synthesis and stearoyl CoA desaturase are more active than the elongase. This turns out to be the case during active lipogenesis in humans and other animals. In an elegant study in mice, Cao *et al*. (2008) identified and characterized palmitoleic acid as a novel lipokine, or lipid hormone. Palmitoleate has been recognized for some time to be a unique marker of *de novo* lipogenesis because of the simultaneous activation of fatty acid synthesis and stearoyl CoA desaturase in fat synthesizing tissues, including liver and adipose. However, the researchers made a bold hypothesis that 16:1n7 is involved in a regulatory role that mediates the insulin-sensitizing effects of diet. The researchers developed a series of assays demonstrating that palmitoleate itself was the metabolic regulator. Plasma lipids from mice enriched in 16:1n7 suppressed stearoyl-CoA desaturase 1 expression in adipose tissue and liver. Thus, increased lipogenesis in the adipose tissue resulted in both higher adipose tissue levels and higher circulating levels of 16:1n7, which was directly linked to the suppression of lipogenesis in the liver – with a resultant resistance to steatosis – and increased muscle insulin sensitivity and glucose uptake. These studies are a stunning demonstration not only of a role of palmitoleate as a lipid signal, but the entire concept that metabolic products are capable of acting to modify metabolism far beyond their simple biochemical pathways of origin.

Arachidonoylethanolamide constitutes one of a newly described class of lipid signalling molecules, anandamides, for which seemingly all of the principles underlying the basic roles of lipid signals are changed. First, the molecule is not a product of membrane and fatty acid deterioration. In fact, arachidonic acid is esterified intact as a conjugated complex with ethanolamide as an immobilized precursor on a phospholipid in the membrane. It is not consumed by the signalling pathway and can be reinserted in the membrane after its activity. Second, the molecule does not signal stress. In fact, the anandamide pathway appears to signal the opposite of stress. The pharmacology of anandamides was worked out long before arachidonic acid was implicated as the natural ligand. Scientists had discovered a class of receptors (termed cannabinoid receptors) as the target for the cannabis recreational drugs (Munro *et al*. 1993). Antagonists to the receptor class had already been developed as anti-abuse drugs before the natural ligand for the receptors was discovered to be arachidonoylethanolamide (Devane & Axelrod 1994). A substantial literature has now documented that the cannabinoid receptors are expressed on a wide variety of cells and mediate food intake, metabolism, immune functions and mood (Di Marzo & Matias 2005). In general, anandamides are considered to be strong anabolic signals that promote food intake and growth. The presence of arachidonoylethanolamide in membranes could be

considered both a storage form and a sensor for arachidonic acid status. Furthermore, release of arachidonoylethanolamide from the membrane and the activation of the cannabinoid receptors would be consistent with a logical biological response to sufficient stores of an essential nutrient class. Long-term dysregulation of the anandamide system has been linked to inappropriate weight gain, hyperphagia and metabolic disorders.

These three examples of novel signalling systems involving fatty acids illustrate multiple roles of lipids in regulating cellular, tissue and whole body functions. Each of these lipid signals is present not only in the diet, and conspicuously in human milk, but also exerts differential effects based on their quantitative abundance. In view of the potentially immediate benefits of dietary lipids in acute health such as inhibiting pathogens (Hernell *et al*. 1986), increasing HDL production (Wang *et al*. 2001), providing innate immune protection from infection (Feingold *et al*. 1995; van Leeuwen *et al*. 2001) and in long-term health from providing substrates to key structures (Massiera *et al*. 2003; Langelier *et al*. 2010) to programming of metabolism (Sawaya *et al*. 1998), it seems prudent to consider the complexity of lipids within human breast milk and in mammalian milks in general.

Conclusions

The diverse roles of lipids throughout evolution are a compelling story of biological innovation and success, and science is making progress to understand this critical dimension of biology. Though the research is by no means complete, certain principles can be taken forward in considering optimal dietary lipid compositions and structures for various age groups, including infancy. The dietary polyunsaturated fatty acids are unquestionably a focus for attention. Insufficient intake of essential fatty acids has far-ranging implications for development, maturation and ultimately the lifelong health of humans. However, the case of the omega-3 fatty acids and particularly DHA is unusually complex. This fatty acid is clearly a unique and functional biomolecule that emerged in early evolution and has proliferated its functional roles as evolution progressed. Its remarkable accumulation in specific tissues and compartments implies that its insufficiency would be potentially devastating. Why then, is the evidence of profound dysfunction not abundant? For the balance of essential nutrients, deficiency is devastating to the organism and if severe is lethal. Insufficiencies of DHA in the diet do not appear to produce deficiency-like symptoms. If this fatty acid is so critical in a variety of key functions, why are there not easily measurable indications of dysfunction in the case of dietary insufficiency? Two possible explanations for compensation emerge from examination of existing results. One, the ability of multicellular organisms to increase the metabolic conversion of structurally similar isomers, omega-6 and omega-9 (22:5n6 and 22:4n9, respectively) may partially compensate for a lack of DHA in key membrane functions. Second, the dynamic range of construction of DHA-rich tissues may be altered. If tissues are capable of sensing DHA, it is possible that the process of neurogenesis is limited by available DHA (Wurtman *et al*. 2009). If this mechanism is true, then the consequence of insufficient DHA is not to produce the same quantity yet structurally compromised neurological tissues, but instead to produce less neurological tissue. If this mechanism is operating in humans, then attempts to identify signs of deficiency as neurological dysfunction are unlikely to be successful. Instead, it will be necessary to interrogate subtle variations in neurological performance (McNamara *et al*. 2010).

The roles of lipid molecules in signalling are proving to be astonishingly diverse. Whereas lipid signalling was once thought to be a simple response to stress via the oxygenation of polyunsaturated fatty acids, a host of lipid molecules are now implicated in normal metabolism, physiology, immunology, and even cognition and behaviour. Importantly, the fatty acids that exhibit signalling properties are not just long-chain polyunsaturated fatty acids. Saturated and monounsaturated fatty acids, short-chain fatty acids and a dizzying host of lipid conjugates are now clearly involved in controlling cellular processes. With such new knowledge in place, it becomes more and more compelling that foods should provide a diversity of fatty acids in a wide variety of complex lipid forms. If one examines human milk, the most notable property of the lipid composition is indeed

its striking complexity. Although it is technologically easier to produce homogeneous supplements of conspicuously few lipid species, the available evidence does not support such a strategy for optimal lipid intakes for health.

Finally, lipids are considered by biologists to be a unique class of biomolecules principally because of the structures that they make within cells. From the self-assembling bilayer membrane to the diverse globules – vesicles and lipoproteins present as colloidal particles in biofluids – the structural dimension of biological lipids is defining. The evolution of mammalian milk has retained the unique fat globule structure across marsupials and all mammals. Although there are few data to begin to assign specific functions to the structural dimension of dietary lipids, and this is a critical area for future research, the available evidence to date would argue that lipid formulations that include a diverse array of colloidal lipid particles, including complex phospholipids and glycolipids on their surface, would provide benefits to health of infants and children.

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Conflicts of interest

There were no potential conflicts of interest for this paper's subject.

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