HUMAN BRAIN LIPID FATTY ACID COMPOSITION

IN RELATION TO INFANT DIET

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Abstract

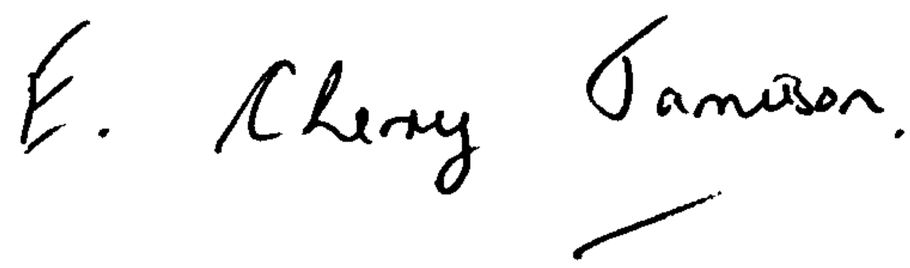
Brain tissue, both grey and white matter from the cerebral parietal region and the cerebellum, was obtained from 66 infants dying of sudden infant death syndrome. The fatty acid composition was analysed in these tissues by gas liquid chromatography after extraction and derivatisation. The subjects were divided according to their dietary history, either breast or formula feeding. Formula-fed infants were further subdivided according to the content of α-linolenic acid in the formula milk. At the time of this study no formula milks analysed contained long chain polyunsaturated fatty acids.

Dietary related differences were found in the accretion of polyunsaturated fatty acids into neural membranes. Docosahexaenoic acid concentrations were higher and conversely n-6 series fatty acids lower in breast-fed than formula-fed infants. In cerebral white matter, nervonic acid, the long-chain fatty acid associated with myelination, appeared in breast-fed in advance of formula-fed infants. Similar dietary related differences in polyunsaturated fatty acid compositions were found in the cerebellar cortex and the cerebellar white matter was associated with an earlier accretion of nervonic and lignoceric acids when compared to the cerebrum. Analysis of the phospholipid and glycolipid composition of the cerebral and cerebellar white matter tissues was achieved by means of separation by high performance thin layer chromatography followed by scanning densitometry.

The results of this study support the need for breast feeding for a minimum of four months. Formulation of manufactured milks should include long chain polyunsaturated fatty acids and nervonic acid at concentrations similar to those of breast milk.

DECLARATION

I certify that this thesis does not contain material previously published by any other person, except where referred to in the text, and that the results in this thesis have not been submitted for any other degree or diploma.



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Abbreviations

AA	arachidonic acid
ANCOVA	analysis of covariance
BHT	butylated hydroxytoluene
C	carbon
°C	degrees centigrade
CGOST	composite feeding group comprising Cow & Gate and Farley's Ostermilk
CoA	coenzyme A
CV	coefficient of variation
DGLA	dihomo-γ-linolenic acid
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
DTA	docosatetraenoic acid
EFA	essential fatty acid
EPA	eicosapentaenoic acid
EQ	educational quotient
ERG	electroretinogram
ESPGAN	European Society of Pediatric Gastroenterology and Nutrition
FAME	fatty acid methyl ester
g	grams
GalCer	galactocerebrosides
GABA	γ-amino butyric acid
GCMS	gas chromatography mass spectrometry
GLC	gas liquid chromatography
HPLC	high performance liquid chromatography
HPTLC	high performance thin layer chromatography
IQ	intelligence quotient
kg	kilograms
L-AP4	L-2-amino-4-phosphonobutanoic acid

LC	language comprehension
LCPUFA	long chain polyunsaturated fatty acids
MDI	mental development index
mg	milligrams
ml	millilitres
mm	millimetres
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide (reduced form)
NADPH	nicotinamide adenine dinucleotide 2' 3' cyclic monophosphate (reduced form)
nm	nanometres
PC	phosphatidylcholine
PDI	psychomotor development index
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PQ	performance quotient
PS	phosphatidylserine
PUFA	polyunsaturated fatty acids
r.p.m.	revolutions per minute
SEM	standard error of the mean
SIDS	sudden infant death syndrome
SPH	sphingomyelin
SU	sulphatides
TLC	thin layer chromatography
ul	microlitres
umol	micromoles
UV	ultraviolet
VLCFA	very long chain fatty acids
WISC-R	Weschler intelligence scale for children

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Summary

This work examines the hypothesis that the fatty acid composition of milks fed to infants during the first months of life significantly affects the fatty acid composition of the human infant brain.

Breast milk is the human infant's natural food and for the first four to six months of life meets his or her total nutrient requirements. In Britain although more than 80% of mothers breast feed their babies for the first week of life fewer than 35% continue for four months. In the Greater Glasgow Health Board Area, which is renowned for its bad health record, fewer than one third of mothers breast feed their infant for even one week. In the last one hundred years mothers have increasingly relied on manufacturers' milks to feed both term and preterm infants.

This thesis examines the fatty acid components of human milk and manufactured formula milks in relation to the phospholipid and glycosphingolipid fatty acid components in the brains of infants fed different milks.

During the first year of life the infant brain weight increases threefold; 50 - 60 % of this increase is due to structural membrane lipid deposition required for the increase in neuronal cell size, the formation of synaptic connections and the deposition of white matter (myelination). It is known that linoleic acid and α-linolenic acid are essential fatty acids (EFA) and these two fatty acids are present in most formula milks although not generally in the proportions present in breast milk. There is increasing evidence that the longer chain polyunsaturated fatty acids (LCPUFA), docosahexaenoic acid and arachidonic acid, which are both minor constituents of breast milk are also essential fatty acids for infants.

Cerebral cortex grey, cerebral parietal white, cerebellar grey and cerebellar white matter from brains of both term (57) and preterm (9) infants dying from sudden infant death syndrome (SIDS) were studied. Once other causes of death had been eliminated after post-mortem by an experienced paediatric pathologist, a full dietary history and birth details were obtained for the infants.

Total tissue lipid was extracted by the method of Folch. Grey and white matter fatty acids were transesterified with boron trifluoride in methanol or methanolic HCl respectively. Selected cerebral cortex lipid samples were further separated into individual phospholipids by two-dimensional chromatography followed by esterification. Fatty acids were separated by gas liquid chromatography (GLC) or gas chromatography-mass spectrometry (GCMS), identified by retention times with respect to known standards and the weight percentage of each fatty acid calculated.

Infants were assigned to feeding groups as described. The breast fed group received exclusively their mothers' milk. The SMA group received either SMA Gold Cap or White Cap formula milks and the CGOST group were given either Farley's Ostermilk (pre 1996 formulation) or Cow & Gate Premium (pre 1990 formulation). The essential fatty acid, α-linolenic acid, was present at a concentration of 1.5% of total fatty acids in the SMA group but at a low level of <0.5% in the CGOST group. A further smaller group (C&G) was fed Cow & Gate Premium or Cow & Gate Plus (post 1990 formulation) with an α-linolenic acid concentration of 2.2% of total fatty acids. At the time of the study none of the manufacturers' milks contained docosahexaenoic (DHA; C22:6n-3), arachidonic acid (AA;C20:4n-6) or nervonic acid (C24:1n-9).

Examination of cerebral cortex total phospholipid showed that breast fed infants had significantly greater concentrations of DHA than both formula fed groups. The corollary of this was an increased incorporation of n-6 series

LCPUFA, namely arachidonic, docosatetraenoic and docosapentaenoic acids. Examination of individual phospholipids of the cerebral cortex demonstrated similar significant differences in fatty acid compositions between feeding groups which were most marked in phosphatidylethanolamine and phosphatidylserine fractions as these neuronal membrane phospholipids contain the greatest percentages of these LCPUFA.

High performance thin layer chromatography (HPTLC) was employed to separate lipid classes in cerebral and cerebellar white tissues. Galactocerebrosides and sulphatides were absent at birth in cerebral white matter in comparison to cerebellar white matter in which both lipids were present in significant concentrations from birth. Individual phospholipid compositions did not vary markedly with age in either tissue.

Most of the fatty acid compositions of cerebral white matter were found to vary with age. There was a universal reduction in DHA concentrations in all feeding groups during the first year, being more marked in the formula fed infants and initially more rapid in the preterm infants. In the breast-fed group, concentrations of the major fatty acid of myelin, nervonic acid, increased logarithmically in the third month, from undetectable at birth to about 4% of total fatty acids at the end of the first year. This increase was not generally evident in the formula fed groups until the fourth month in both term and preterm infants.

Dietary related differences in DHA and the n-6 series LCPUFA in both cerebellar grey and white matter were similar to those found in cerebral cortex and cerebral white matter respectively. Cerebellar white matter, however, differed from cerebral white in that lignoceric (C24:0) and nervonic acid concentrations were present at about 1% at birth.

There may be long term health implications from lack of supply of DHA in the infant diet. The cerebral white matter results also point to the probable essentiality of nervonic acid, present in human milk but not in formula milks, especially as it is a major constituent of cerebrosides important in the formation of myelin.

Further studies are needed into the long-term effects of deficiency in DHA and nervonic acid in the diet of formula fed infants. The short term effect on efficiency of synaptic transmission and longer term effects on neuronal membrane integrity caused by these neuronal membrane fatty acid changes require investigation.

These results support both the hypothesis and maintenance of breast feeding for human infants.

CHAPTER 1

INTRODUCTION

1.1. History of infant feeding

The female of the human species evolved with the capacity to feed her infant. Breast feeding by either the infant's mother or by a "wet nurse" was nature's way of providing the infant with the all the nutrients required for development. Breast milk on its own, tended to be the infant's diet for 9 - 12 months but in some cases this could extend for up to 3 years.

There are references to the artificial feeding of babies as far back as ancient Roman times when animals' milk, particularly sheep or goats, with honey or sugar and bread added, was given as a substitute. However, this practice was thought to be dangerous.

A combination of factors brought about by the industrial revolution contributed to the mounting popularity of artificial feeding. For example, women began to take up employment, and families moved away from their traditional farming communities to an urban way of life. Also, glass bottles became available along with the first rubber teats and more importantly the development of the pasteurisation process made milk preparation much safer. Milk substitutes, manufactured from wheat flour, cow's milk, malt flour and potassium bicarbonate were developed during the latter part of the 19th Century. Most of these new formulas, however, were totally inadequate as a sole source of nourishment, and, infant death, due to contaminated artificial feeds, was a recognised problem.

By the beginning of the 20th Century, an estimated 80% of mothers were breast feeding at birth and approximately 50% of them continued to do so for at least three months (Fildes, 1988). The introduction of roller-dried milk powders significantly reduced mortality amongst artificially fed infants. New formulae with an altered protein and mineral composition and with added Vitamin D

and carbohydrate were developed in response to more sophisticated analyses of dietary needs and understanding of the physiological processes.

The introduction of the Welfare Food Scheme in 1940 resulted in free or cheap National Dried Milk for children and consequently an increase in the number of formula milks on the market. This convenient and safe way of feeding the baby was popular because it allowed young mothers to go on working and let fathers and other family members become involved in the feeding of their young children.

By the mid-1960s only 25% of mothers were breast feeding as artificial feeds became more convenient and refrigerators were available for storing pre-prepared feeds. Doctors were influenced by commercial claims of the increased nutritional value of formula feeds and recommended their use. Thus by 1970 the number of breast-feeding mothers reached a low of less than 20%. However, problems such as hypernatraemia, hypocalcaemia and hyperphosphataemia due to differences in the composition of human and cow's milk, and the ever present risk of infection in bottle-fed infants still remained to be resolved. After investigation revealed the presence of specific antibodies and growth factors in breast milk which could not be replicated in formula milks, doctors returned to recommending mothers' milk as the preferred method of infant feeding. Consequently the number of women breast feeding at birth rose to over 60%, with most continuing to breast feed for longer periods. However, when breast feeding was not possible there was a need for a safe, highly nutritious artificial substitute. Manufacturers have continued to develop formulas with biochemical compositions closer to mothers' milk using new food processing technology, for example the addition of the amino acid taurine. Nevertheless, in 1983, all manufacturers agreed a code of practice which ensured that breast feeding was promoted, on all their product labels, as the best form of infant feeding.

More recently, attention has focused on the fatty acid content of formula milks: not just because of their caloric content but more importantly, because it has been shown that certain fatty acids are essential components of neural membranes at this time of maximal growth and development of the human brain (Martinez, 1989).

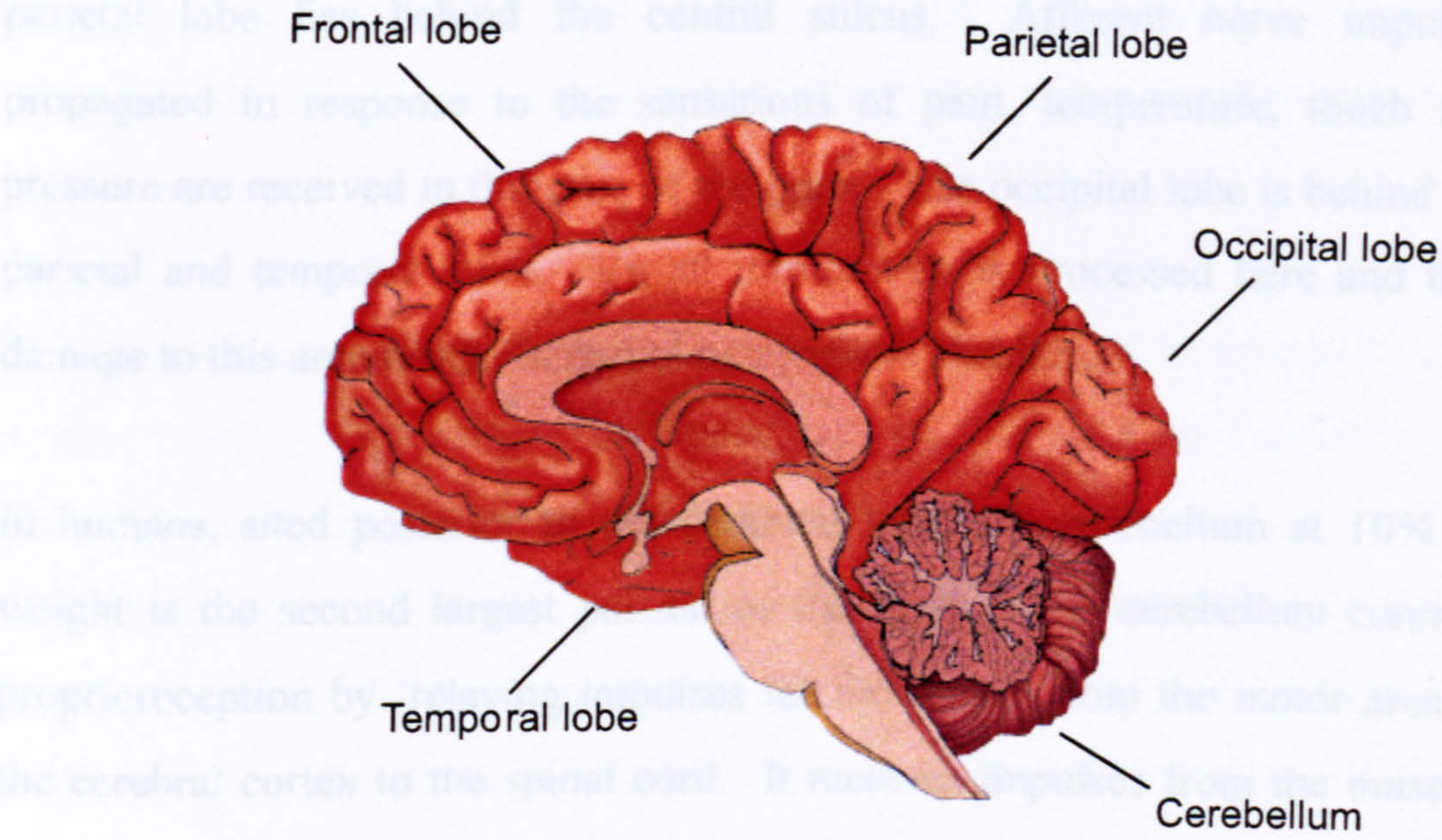
1.2 The human brain

The human brain (Fig 1.1) is the most complex organ in the body, a product of evolution and of its host's environment. It is unique to the individual and its development depends on external stimuli to that individual. Yet, it is unable to function without support from the other organs in the body.

1.2.1. Brain anatomy

The human brain can be divided into three main parts, namely the cerebral hemispheres, the cerebellum and the brain-stem. The two cerebral hemispheres comprise 85% of the brain (by weight). They are highly convoluted, consisting of ridges known as gyri and valleys called sulci. The surface layer of the hemispheres is known as the cerebral cortex. The cortex is about 2.5 mm thick with an approximate area of 2400cm^2 (adult brain) and consists of neuronal cell bodies, their dendrites and glial cells. The cortex is also referred to as grey matter. Axons entering the cortex as well as those leaving it form the white matter, which constitutes the major part of the sub-cortical tissue of the hemispheres.

Each hemisphere can be divided into four lobes, namely the frontal, parietal, temporal and occipital. The frontal lobe constitutes about one third of the hemispheric surface and is concerned with planning ahead, prediction, and programming for an individual's needs. The part of the hemisphere below the lateral fissure is the temporal lobe, the uppermost part of which is involved in the sense of hearing. Much of the remaining lobe may be involved with the



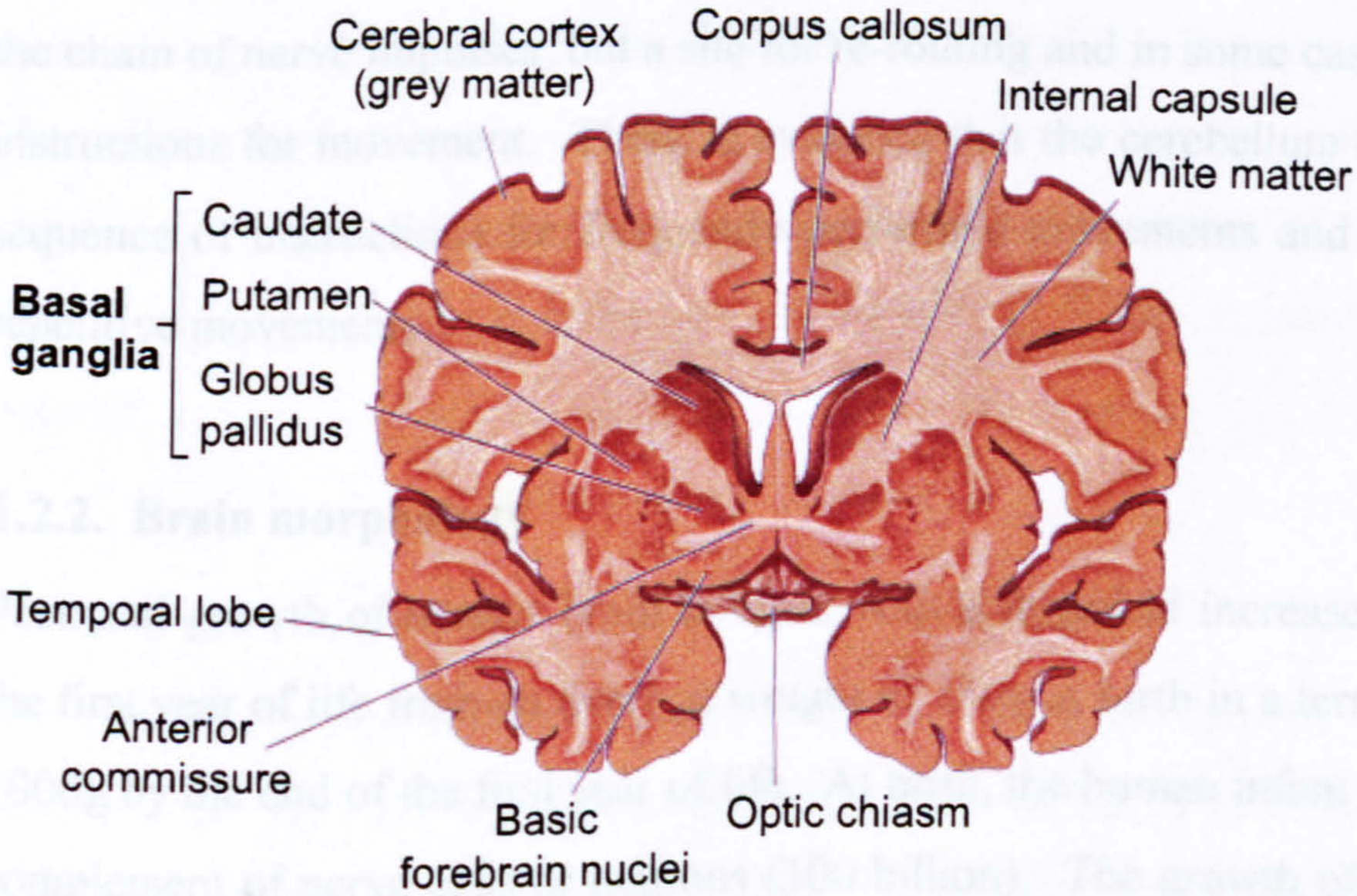


Figure 1.1 - Main structures of the human brain

integration of multiple sensory functions such as hearing, sight and touch. The parietal lobe lies behind the central sulcus. Afferent nerve impulses propagated in response to the sensations of pain, temperature, touch and pressure are received in this area of the brain. The occipital lobe is behind the parietal and temporal lobes. Visual information is processed here and thus damage to this area results in partial or complete blindness.

In humans, sited posterior to the occipital lobe, the cerebellum at 10% by weight is the second largest portion of the brain. The cerebellum controls proprioreception by relaying impulses for movement from the motor area of the cerebral cortex to the spinal cord. It receives impulses from the muscles and joints that are being activated and compares them with the instructions issued from the motor cortex, so that adjustments can be made. The cerebellum thus is neither the sole initiator of movement nor a simple link in the chain of nerve impulses, but a site for re-routing and in some cases refining instructions for movement. There is evidence that the cerebellum can store a sequence of instructions for frequently performed movements and for skilled repetitive movements.

1.2.2. Brain morphology

Postnatal growth of human brain is rapid with a threefold increase in size in the first year of life from an average weight of 350g at birth in a term infant to 1000g by the end of the first year of life. At birth, the human infant has its full complement of nerve cells or neurons (100 billion). The growth of the infant brain is, therefore, a result of growth of pre-existing neurons proliferation of neuroglial cells and the process of myelination.

A neuron has a cell body containing a nucleus, endoplasmic reticulum, mitochondria, Golgi body, and ribosomes. Neurons differ from other cells in the body by having axons and areas of dendritic branching protruding from the

cell body which enable electrical (or electrochemical) conduction. Myelination is the "insulation" of these axons with a fatty sheath. Dendrites act as sites for synaptic connections with other nerve cells and other body cells such as muscles.

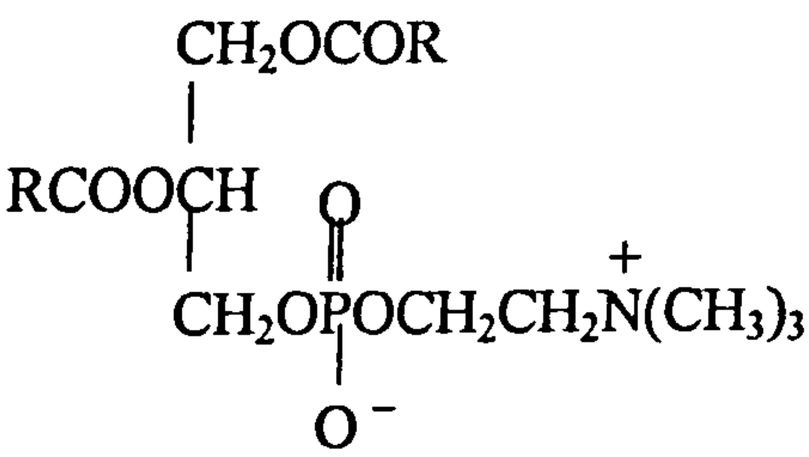
The other main group of cells present in the brain are known as neuroglial cells or glia. These cells differ from neurons in that they do not participate in electrical signalling. Glial cells outnumber neurons threefold and do not have axons or dendrites. In the developing brain these cells are thought to provide a support structure for the migrating neurons. There are three types of glial cells in the central nervous system. Astrocytes are characterised by a star-like appearance and maintain an appropriate chemical environment for neuronal signalling. Oligodendrocytes are the cells responsible for myelination of axons. Microglial cells are smaller and are thought to be a type of macrophage because they have been shown to proliferate after injury to the nervous system. The entire neuron including all its processes is bound by a thin plasma membrane which is selectively permeable to different chemical substances. This is important in the maintenance of the resting electrical potential of the neuron.

1.2.3. Brain composition

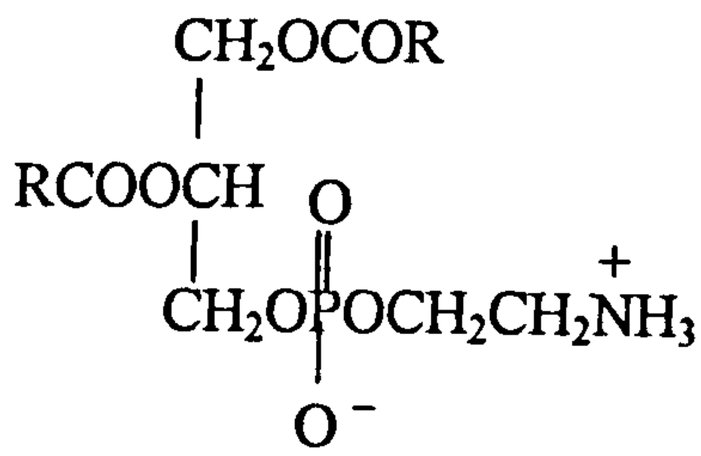
The chemical composition of the brain reflects the composition of its cellular components. At birth, approximately 90% of the whole brain is composed of water. The remaining ten percent, which represents the so called dry weight, is mainly lipid and protein with smaller amounts of carbohydrate, nucleic acids and hormones. The lipid is located in the cell membrane structures along with proteins which are also found in the membranes of intracellular organelles.

1.3.1. Brain lipids

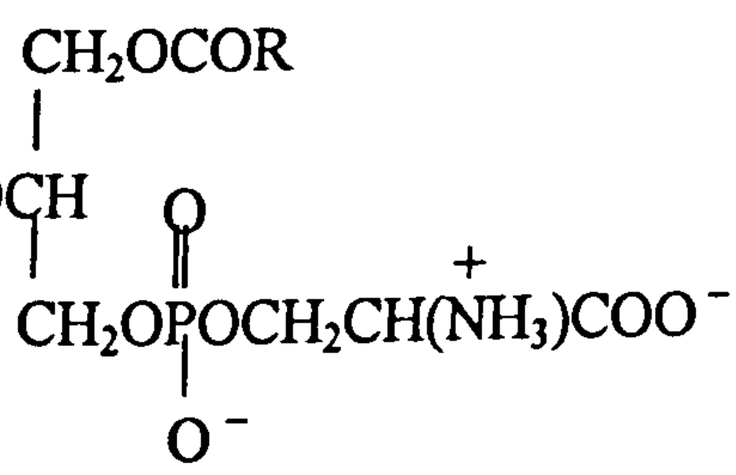
The brain has the second highest concentration of lipid after adipose tissue in the human body, (60% of the dry weight) which is important in the structure and function of brain cell membranes (Stubbs and Smith, 1984). There are three main classes of lipids present in brain tissue - phospholipids, glycolipids and neutral lipids.



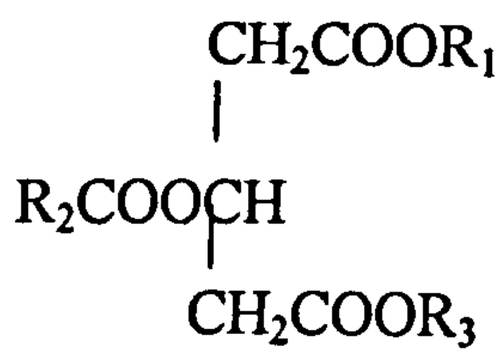
Phosphatidylcholine



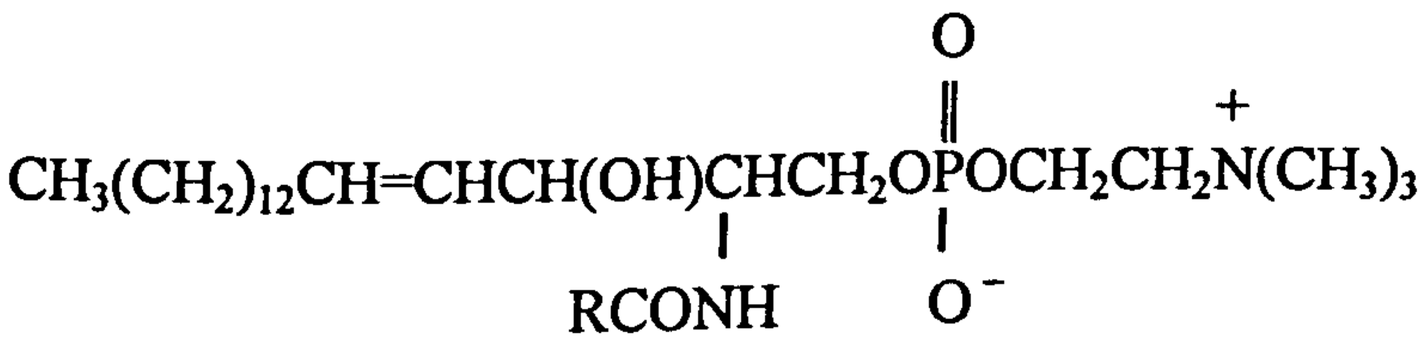
Phosphatidylethanolamine



Phosphatidylserine



Triacylglycerol



Sphingomyelin

Figure 1. 2 Lipid chemical structures

Phospholipids are the main constituents of membranes and are found in large quantities in the brain. These compounds have a glycerol backbone, two fatty acids esterified at positions 1 and 2, position 3 being occupied by phosphoric

acid esterified to the hydroxyl group and an alcohol base attached to the phosphate. The three which predominate in mammalian neuronal membranes are phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) (Fig 1.2). Others found in lesser amounts but which have important roles include phosphatidylinositol (PI), cardiolipin and phosphatidylglycerol. Sphingomyelin (SPH) (Fig 1.2), generally classified as a phospholipid, contains both phosphoric acid and a ceramide; a combination of the amino alcohol sphingosine and a fatty acid.

Glycolipids are widely distributed in all tissues including nervous tissue. They are the combination of a ceramide and carbohydrate. The most important glycolipid in brain is galactocerebroside (GalCer). The fatty acid composition of cerebrosides in glial cells differs from that found in neuronal cells (Abe and Norton, 1974). Neuronal cerebrosides contain more unsaturated and shorter chain fatty acids. Sulphation of the galactose moiety of galactocerebroside produces sulphatides (SU) which are also present in myelin.

Neutral lipids, which do not have a charge property, are acylglycerols, cholesterol and cholesterol esters. Acylglycerols (Fig 1.2) are esters of glycerol and fatty acids and are named according to the number of fatty acids esterified to the glycerol e.g. tripalmitin has three palmitic acid molecules attached to the glycerol backbone. While nervous tissue contains little triacylglycerol or cholesterol esters (McMurray,1983), cholesterol is a major and important constituent of brain membranes and plays an important role as a metabolic intermediate. It has a polycyclic ring structure and acts as a precursor for steroid hormones and bile acids.

1.3.2. Lipid composition of brain

Analysis of brain lipids in rats (Cuzner and Davison, 1968), in pigs (Dickerson and Dobbing, 1966) and in several vertebrate species (Rouser,

1971) showed that brain lipid composition changes significantly with the stage of development. Lipid composition of normal human brain was analysed by O'Brien and Sampson (1965) who demonstrated that absolute concentrations of all phospholipids increase during development. Svennerholm and Vanier (1972) studied the lipid composition of cerebral grey and white matter of fetal and infant brains up to two years of age. Absolute concentrations of cholesterol and total phospholipids increased rapidly in white matter after birth. In grey matter total phospholipids also increased with age but less dramatically, whereas cholesterol concentration was unchanged. Similarly they found that the concentrations of cerebrosides and sulphatides to increase in both grey and white matter. These glycolipids however were at much higher concentrations in the white matter than the grey at two years of age. Within the phospholipids, PC concentrations decreased in both grey and white matter during the first year of life. Conversely sphingomyelin increased and PE and PS concentrations remained constant over the time period.

1.3.3. Synthesis of brain lipids

Cholesterol is synthesised by brain tissue during development and myelination (Cuzner and Davison, 1968). Edmond *et al.* (1991) also using the rat model and exogenous deuterated cholesterol demonstrated that all cholesterol required by the developing brain is synthesised *de novo* in the brain.

The major pathways for the biosynthesis of phospholipids are illustrated in Fig 1.3. In the brain, the major route for PC synthesis is via the CDP-choline pathway. This is favoured over the alternative base-exchange conversion of PE to PC. Similarly, although four different pathways exist for the synthesis of PE, again the primary pathway is by *de novo* synthesis. The sole pathway for PS production in the brain is via base exchange with PE (Yavin and Zeigler, 1977). Porcellati demonstrated that in nervous tissue, membrane-bound base

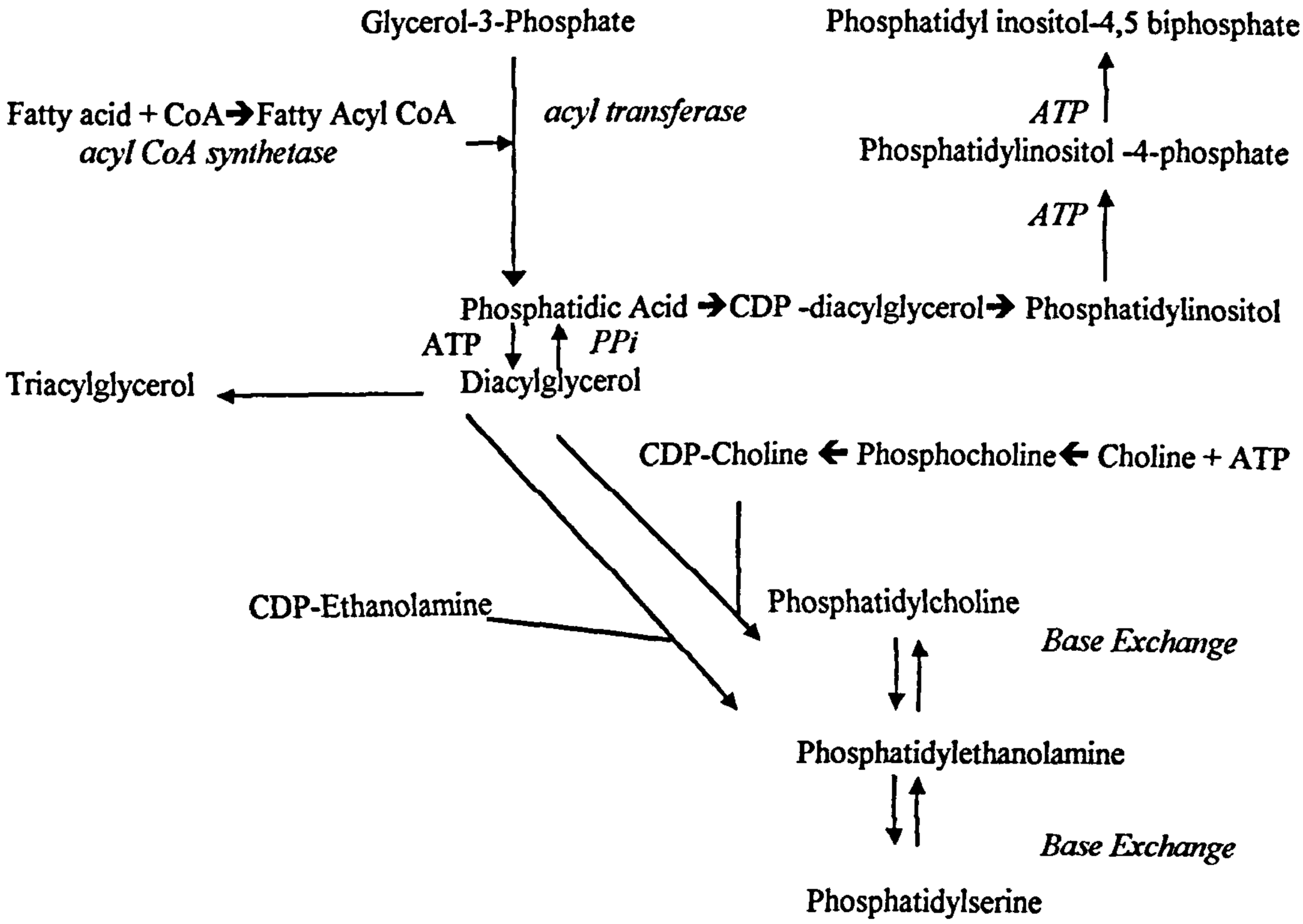


Figure 1.3 - Pathways of phospholipid synthesis.

exchange enzymes were responsible for the formation of the three main phospholipids by the energy independent incorporation of the appropriate alcohols. (Porcellati *et al.*, 1971). PE plasmalogens are also a major component of nervous tissue phospholipids. They differ from other phosphoglycerides in that the C_1 fatty acid esterified to glycerol is in the form of an alkyl chain with an ether linkage. The pathway of formation of the ether linkage in plasmalogens is not certain. It is thought that the most likely mechanism is for the fatty acid to be incorporated as an ester linkage which is then reduced to an ether group. The incorporation of the ethanolamine head group has been shown to proceed in a reaction similar to that already described for PE, namely the transfer from the CDP derivative (Ansell and Metcalfe, 1971).

Figure 1.4 summarises the pathways for synthesis of cerebrosides and sulphatides. Synthesis of these compounds occurs by sequential addition of glycosyl monomers transferred from sugar-nucleotide donors to the acceptor molecule. A sulphate group is then added to a galactocerebroside by transfer from the sulphate carrier, 3'-phosphoadenosine-5'-phosphosulphate (PAPS) to the 3'-hydroxyl group of the galactose.

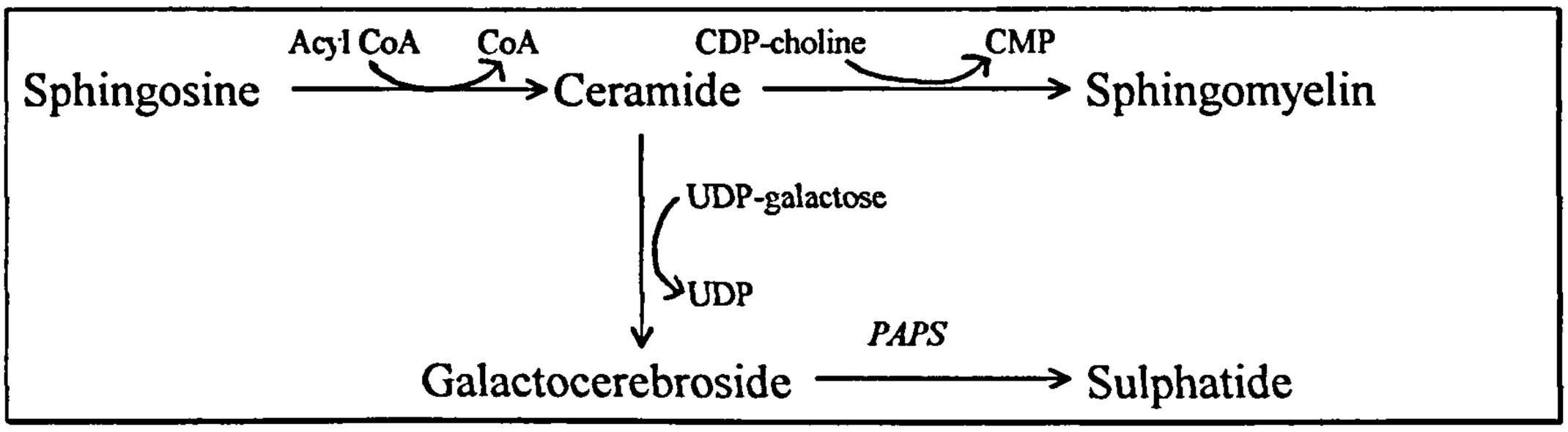


Figure 1.4 - Synthesis of cerebrosides and sulphatides.

1.3.4. Catabolism of brain lipids

Phospholipids present in cell membranes are not readily hydrolysed to release free fatty acids. However they can be catabolised in other tissues to provide energy in extreme circumstances. The breakdown of phospholipids involves a group of enzymes known as phospholipases which are widely distributed in animal tissues and each one acts at a specific site on the substrate molecule.

The fatty acid composition of intact lipids can however be modified by a process known as acyl exchange. Enzymes can exchange existing fatty acyl groups in phospholipids with free fatty acyl CoA derivatives. This method can enable the cell to adapt to different conditions. Fatty acids can also be released from triacylglycerols or phospholipids by positional specific lipases or phospholipases and then other esterifying enzymes can substitute a different fatty acid. Phospholipase A hydrolyses diacylphospholipids to give a free fatty acid and a monoacylphospholipid. The enzymes are further subdivided into A_1

and A_2 depending on whether they attack the fatty acid attached to the C_1 or C_2 glycerol carbon atom. All these reactions combine to determine the widely varying compositions of the various phospholipids in the cell membranes of different tissues.

1.4.1. Fatty acids

Fatty acids are major components in brain lipids. They consist of hydrocarbon chains of different length terminating in a methyl group at one end and a carboxyl group at the other. Most naturally occurring fatty acids consist of an even number of carbon atoms. The number of intermediate carbon atoms vary and accordingly they can be classified as short, medium or long chain fatty acids, with 2 to 4, 6 to 10 and greater than 10 carbon atoms respectively. Saturated fatty acids have no double bonds, mono-unsaturated one double bond and polyunsaturated two or more.

In general polyunsaturated fatty acids have a *cis* configuration at each double bond which produces "coiling" of the hydrocarbon backbone and resultant reduction in fatty acid length (Fig 1.5). Incorporation of *trans* double bonds establishes a linear structure of the fatty acid and endows it with properties similar to the corresponding saturated fatty acid. The most important unsaturated fatty acids can be categorised into three main families, namely n-3, n-6 and n-9. The number represents the position of the first double bond from the methyl end of the molecule. Double bonds are formed in desaturation reactions catalysed by a site specific enzyme, represented by the Greek letter delta (Δ). The numbering for the site of action of these enzymes is taken from the position of the double bond in relation to the carboxyl end of the molecule (Fig 1.6).

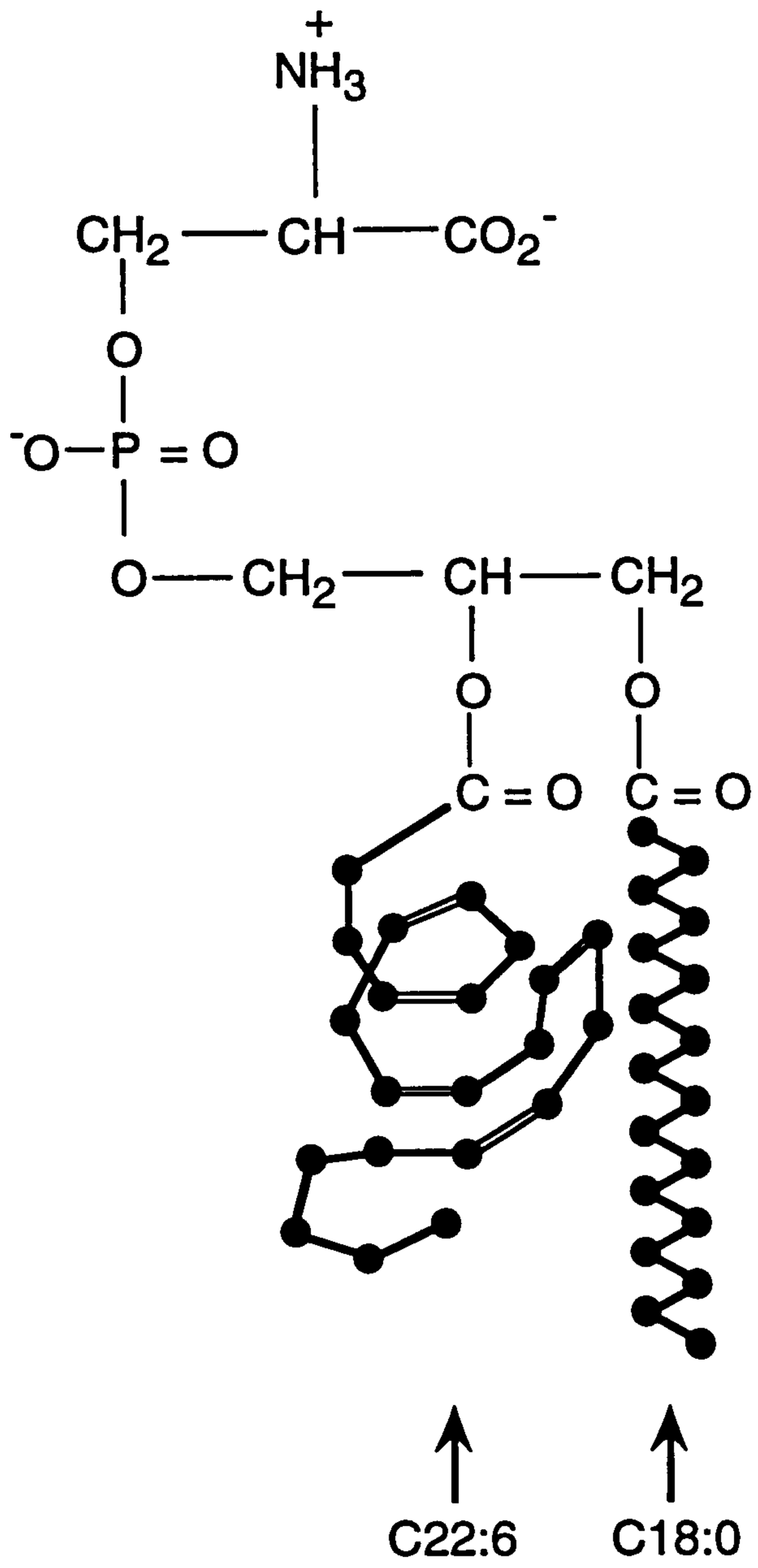


Figure 1.5 - Proposed molecular structure of a typical cerebral phospholipid

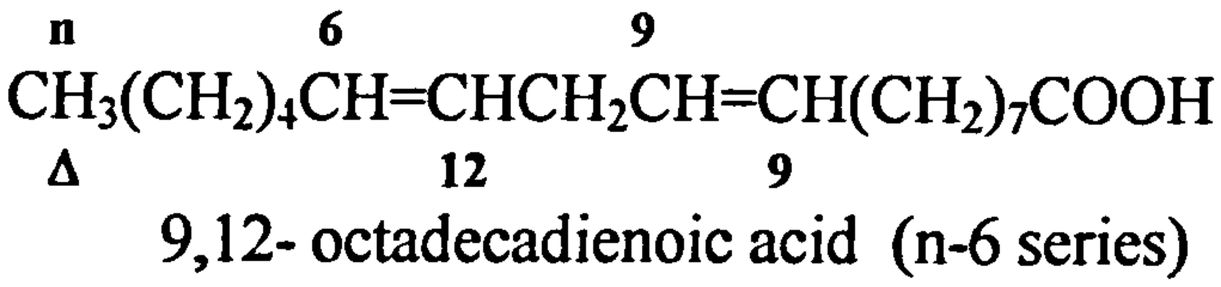


Figure 1.6 - Nomenclature of fatty acids.

Oleic acid (C18:1n-9), linoleic acid (C18:2n-6) and α-linolenic acid (C18:3n-3) are considered to be the parent molecules for the three different families. Oleic acid, however, is not an essential fatty acid as animals, including humans, can introduce a double bond, or desaturate at the Δ9 position in saturated stearic acid (C18:0). Animals, unlike plants, however are unable to desaturate beyond the Δ9 atom towards the methyl end (Rivers *et al.*, 1975). Linoleic acid and α-linolenic acid (Fig 1.7) are considered to be essential in humans and therefore must be provided in the diet. The fatty acids of each family are not inter-convertible, and synthesis of the longer chain polyunsaturates is directly dependent on the supply of the respective precursors.

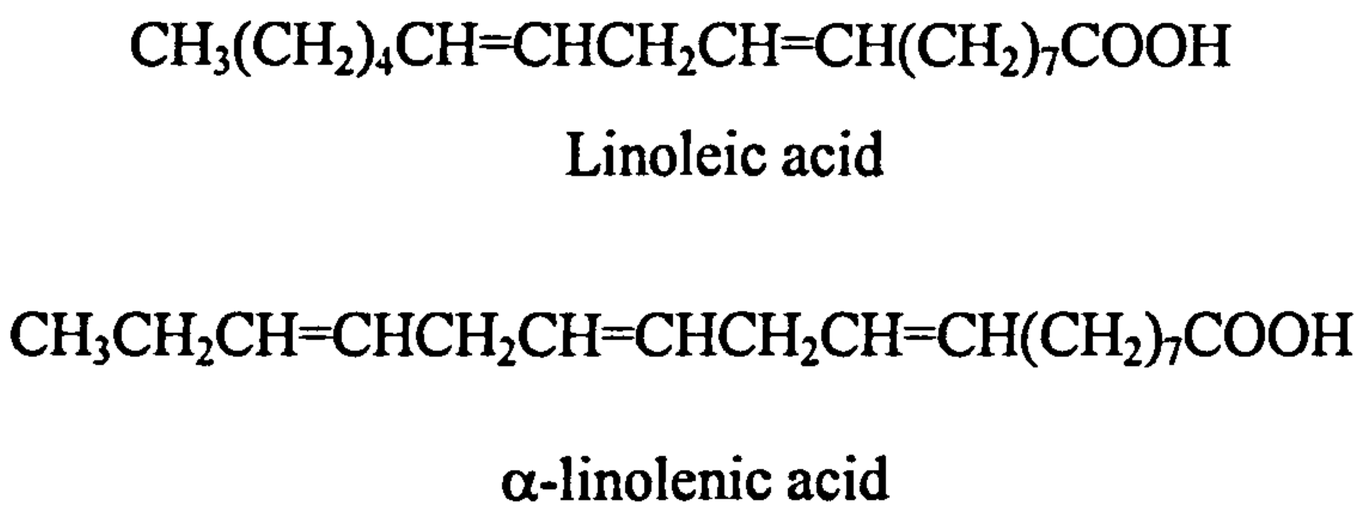


Figure 1. 7 - Chemical structures of the essential fatty acids.

1.4.2. Dietary sources of fatty acids

Linoleic acid and α-linolenic acid are synthesised in plants, fungi and bacteria. They are both found in abundance in plant leaves and seeds (Hitchcock and Nichols, 1971). Table 1.1 illustrates linoleic acid and α-linolenic acid

concentrations in various terrestrial and marine plants along with some fish. Also shown in the table are arachidonic acid and docosahexaenoic acid concentrations, the latter being especially rich in marine plants and animals (Cunnane *et al.*, 1993).

Table 1.1 - Polyunsaturated fatty acid composition (weight % of total fatty acids) of terrestrial plants, seed oils, marine plants and fish.

	Linoleic	Arachidonic	α-linolenic	Docosahexaenoic
Terrestrial Plants				
Leaves				
Rye Grass	14.3	-	33.8	-
Oak	12.2	-	57.1	-
Beech	15.9	-	51.9	-
Seeds				
Grass	49.0	-	0.4	-
Oak	52.7	-	11.6	-
Beech	54.4	-	1.9	-
Flax	15.0	-	58.2	-
Seed Oils				
Corn	50.0	-	2.0	-
Olive	11.5	-	0.8	-
Soyabean	52.0	-	7.0	-
Sunflower	52.0	-	0.2	-
Marine Plants				
Phytoplankton	0.8	0.7	0.3	10.5
Algae	1.2	12.4	2.6	0.8
Fish				
Mollusc	0.9	2.3	1.3	22.0
Squid	0.5	5.8	0.8	21.3
Herring	0.2	0.6	0.1	23.0

1.4.3. Dietary sources of fatty acids for the infant

In 1982, the European Society of Pediatric Gastroenterology and Nutrition (ESPGAN) committee report on guidelines for infant nutrition stated that "human milk is assumed to be the ideal food for the infant at least up to the age of 5 or 6 months, ensuring optimal growth and development." The composition of breast milk is unique in that it varies from day to day, with the time of day and with maternal diet as well as length of gestation. The infant's energy requirements must sustain adequate growth and optimal body composition. The average calorific content of human milk has been used in this assessment. These measurements have been determined using expressed breast milk, however Lucas *et al.* (1990a) reported that there may be an overestimation as the fat content may be greater in expressed milk than in suckled milk. Since milk manufacturers take account of expressed milk values when producing formula milks, this may explain why breast fed infants grow less rapidly than formula fed infants despite their lower total daily energy expenditure (Butte *et al.*, 1990; Heinig *et al.*, 1993).

The fat content of human milk is important not only because it provides more than 50% of the energy requirements and is the vehicle for fat soluble vitamins, but also because lipids are precursors of prostaglandins and hormones, and are essential constituents of all cell membranes. Lipids are the most variable component of human milk. Total lipid is present in lower concentration in colostrum than in mature milk (Bitman *et al.*, 1983) as well as within an individual feed (low in fore milk and high in hind milk). Conversely, cholesterol and phospholipids are high in colostrum and both decrease to lower levels in mature milk.

Fat in milk is contained within membrane-enclosed milk fat globules. The core of the globules consists of triacylglycerols (98 - 99% of total milk fat), whereas the globule membrane is composed mainly of phospholipids, cholesterol and

proteins. The major constituents of triacylglycerols are long chain fatty acids derived from the circulation. Short and medium chain fatty acids are synthesised by the fatty acid synthetase complex. Phospholipids are synthesised *de novo* within the mammary gland and cholesterol originates from both *de novo* synthesis and from the circulation. The amount and composition of fatty acids in milk fat is the final product of the processes of fat digestion, absorption, transport in the circulation, release from adipose tissue storage and uptake into the lactating mammary gland.

Fatty acids in breast milk triacylglycerols have a highly specific positional distribution (Martin *et al.*, 1993). Seventy to seventy-five percent of palmitic acid is esterified at the sn-2 position of the triacylglycerol whereas in infant formulas, palmitic acid derived from vegetable oils is esterified at the sn-1 and sn-3 positions (Tomarelli *et al.*, 1968). Stearic and oleic acids are mainly located at the sn-1 position in breast milk triacylglycerols, whereas linoleic acid is found at sn-1 and sn-3. LCPUFA are found primarily at the sn-2 and sn-3 positions (Martin *et al.*, 1993). The result of these stereospecific differences is that triacylglycerols derived from human milk are assimilated more readily than those from manufactured sources.

1.4.4. Fatty acid digestion and absorption

Dietary fat whether that from breast milk or formula milk is mostly in the form of triacylglycerol. The digestion and absorption of fat can be broken down into three steps. The luminal phase where triacylglycerols are hydrolysed into free fatty acids, monoacylglycerols and glycerol, before they are transferred to the intestinal mucosa : this involves solubilisation with bile salts. The mucosal phase involves the re-esterification of the free fatty acids in the enterocytes. Here they are combined with chylomicrons and very low density lipoproteins before release into the blood stream. In the transport and delivery phases, the lipoproteins are taken up selectively by individual tissues (Fig 1.8).

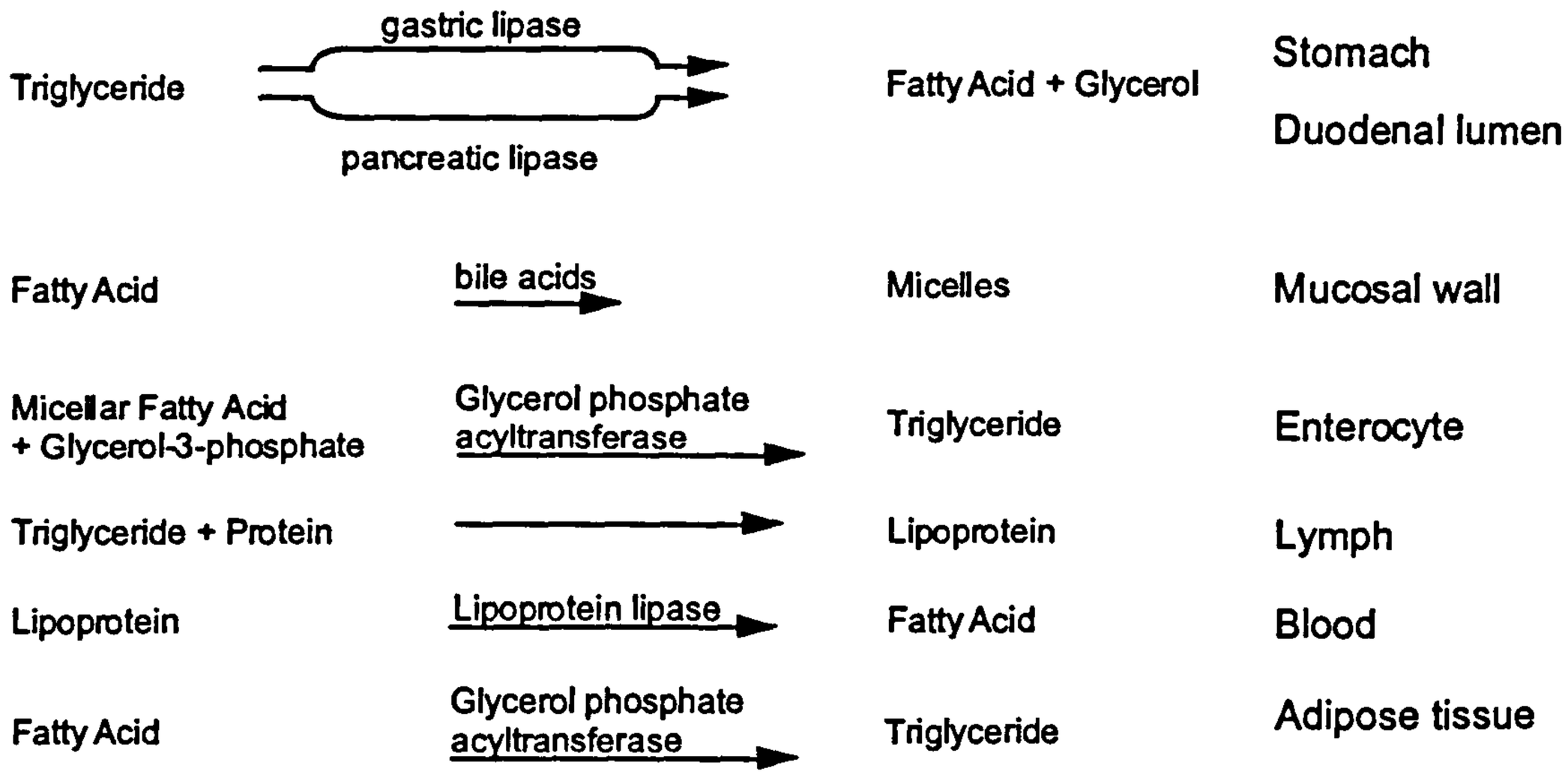


Figure 1.8 - Digestion and absorption of fatty acids

The hydrolysis of triacylglycerols in the luminal phase depends on a series of lipases. The first, lingual lipase, is secreted by the serous glands of the tongue (Hamosh and Burns, 1977). This enzyme degrades medium and short chain triacylglycerols in the stomach. Gastric lipase is also found in the stomach and acts similarly on medium and short chain triacylglycerols. Further digestion then takes place in the small intestine through the action of pancreatic lipase. This enzyme is relatively inactive in the new-born, especially in preterm infants (Thomson *et al.*, 1989). In addition, infants have reduced concentrations of bile salts necessary for emulsification of the digested fat (Watkins, 1975).

Breast-fed infants are uniquely provided with an additional digestive enzyme known as bile-salt stimulated lipase (BSSL) which hydrolysed triacylglycerols at all three fatty acid positions. Chen *et al.* (1994) demonstrated that without this enzyme there was incomplete hydrolysis of triacylglycerols containing arachidonic acid or DHA. All four lipases are necessary for complete digestion of milk triacylglycerols in infants (Bernback *et al.*, 1990).

In recent years workers have studied the digestion of dietary fats with respect to their fatty acid composition. Chen *et al.* (1987) investigated the uptake of n-3 fatty acids from triacylglycerol by introducing aqueous emulsions of oleic acid or different oil concentrates, namely corn oil, menhaden oil or fish oil directly into the duodenum of rats. The total recovery of fatty acids from n-3 rich oils was less than 50% of that obtained from n-6 rich maize oil. El Boustani *et al.* (1987) performed a similar study in humans where they compared the absorption of ethyl ester of eicosapentaenoic acid (EPA; C20:5 n-3) with the free fatty acid or other esterified forms. Lawson and Hughes (1988) compared the absorption of fish oil fatty acids in healthy human adults when given as triacylglycerol or free fatty acids. The fish oil was absorbed at a rate of 95% in the free acid form but the absorption of EPA and DHA was less

than 70% from triacylglycerol and only 20% from ethyl esters. This may be significant when considering which LCPUFA should be added to formula milks.

Long-chain fatty acids, bile salts and monoacylglycerols form micelles, which move easily into intestinal cells. They diffuse to the surface of mucosal cells and deliver the digested fat for absorption. The enterocyte contains Δ6 and Δ9 fatty acid desaturase activity. These enzymes may modify fatty acids transported out of the enterocyte (Garg *et al.*, 1988) to systemic tissues and the brain.

Transportation through the blood-stream requires that the fatty acids and monoacylglycerols are reassembled into triacylglycerols, combined with proteins and phospholipids to form lipoproteins. Lipoproteins can be classified according to composition, method of isolation or physical properties. Their composition differs in the ratio of total lipid to protein and also in the proportions of particular lipids, namely triacylglycerols, cholesterol, cholesterol ester and phospholipids. In general those lipoproteins thought to have a beneficial effect on human metabolism tend to contain the highest percentages of phospholipids (and protein) and hence long chain polyunsaturated fatty acids. Shorter chain fatty acids (less than 14 carbons) can also be bound to albumin in the plasma and transported as free acids in the circulation. Tissues in the body incorporate different lipids dependent on their specific requirements for structural and for "energy producing" fatty acids. As the brain is largely fuelled by glucose (and ketone bodies) structural fatty acids are preferentially accreted from phospholipids in contrast to the energy providing adipose tissue stores composed predominantly of triacylglycerols.

1.4.5. Fatty acid biosynthesis

Saturated fatty acids can be synthesised *de novo* from acetyl CoA and the liver is the most important organ for such synthesis. It has two systems for chain elongation. In the mitochondria, acetyl CoA is used in the presence of NADH or NADPH to elongate acetyl CoA derivatives. Elongation also takes place in the endoplasmic reticulum where 2-carbon units from malonyl CoA are made in the presence of NADH. These reactions are catalysed by a number of enzymes that form a multi-enzyme complex known as fatty acid synthetase. The end product of these reactions would normally be palmitic acid. However, in man there is little requirement for saturated fatty acid synthesis because of adequate dietary supply.

Mono-unsaturated fatty acids are formed by a desaturation reaction, usually at carbon 9, by a Δ9 desaturase enzyme. Desaturation is NAD dependent and the enzyme complex is found in liver microsomes, although it is also found in other body tissues. Its activity is decreased by fasting (i.e. protein and/or glucose deprivation) and can be restored by protein consumption or the administration of insulin (Brenner, 1989).

Plants, unlike animals, can insert more double bonds into oleic acid between the C-9 and the methyl end of the molecule. Therefore plants form linoleic acid under the influence of a 12-desaturase enzyme and α-linolenic acid by a 15-desaturase enzyme. Both of these systems are oxygen dependent. Marine algae have also the ability to effect these changes and therefore form PUFA with 5 and 6 double bonds.

Animals do not possess the above enzyme systems. They must have linoleic and α-linolenic acids supplied in their diet. From these two compounds plus oleic acid, animals produce more unsaturated fatty acids by chain elongation.

The Δ6 desaturase enzyme is responsible for the introduction of a double bond at position 6 in each of these three fatty acids. All three fatty acids can compete for the same enzyme (Brenner and Peluffo, 1968). The reaction rates, however, differ in that α-linolenic acid is preferred before linoleic acid and then lastly oleic acid. (Mohrhauer and Holman, 1963). The activity of the Δ6 desaturase varies between species and in individual tissues of one species, but is now not thought to be rate-limiting in the synthesis of PUFA in humans (Poisson *et al.*, 1993). Δ5 desaturase inserts a double bond between carbon atoms 5 and 6 and its distribution in tissues tends to be similar to that of Δ6 desaturase. The enzyme also responds to dietary changes and hormones similarly to those described for Δ6 desaturase (Brenner, 1989).

Until recently, a Δ4 desaturase system was thought to be responsible for the formation of docosapentaenoic acid (DPA; C22:5n-6) and DHA. However microsomal preparations and all culture preparations *in vitro* showed little Δ4 desaturase activity using appropriate fatty acid substrates.

Enzyme	**Fatty Acid Family**			
	n-9	**n-6**	**n-3**	
	18:0 ↓			
Δ-9 desaturase	18:1 ↓	18:2 ↓	18:3 ↓	
Δ-6 desaturase	18:2 ↓	18:3 ↓	18:4 ↓	
elongase	20:2 ↓	20:3 ↓	20:4 ↓	
Δ-5 desaturase	20:3 ↓	20:4 ↓	20:5 ↓	
elongase	22:3	22:4 ↓	22:5 ↓	→ 24:5 ↓ Δ-6
Δ-4 desaturase		22:5	22:6	← 24:6

Figure 1.9 - Desaturation-elongation pathways for synthesis of polyunsaturated fatty acids including alternative pathway.

Voss *et al.* (1991) using rat liver preparations demonstrated an alternative route for the formation of these compounds via chain elongation to C24:4n-6 and C24:5n-3 from C22:4n-6 and C22:5n-3 followed by a desaturation by a Δ6 desaturase enzyme to C24:5n-6 and C24:6n-3 (Fig 1.9). Subsequently this new pathway was confirmed using human skin fibroblasts (Moore *et al.*, 1995). These reactions have been shown to occur in the endoplasmic reticulum. In the peroxisomes a β-oxidation step then leads to the formation of C22:5n-6 and C22:6n-3. This to some extent explains the findings by Martinez (1992a) of low levels of DHA in tissues of adrenoleukodystrophy (ALD) patients who are known to have a defect in peroxisomal metabolism.

It is well known that chain elongation occurs more rapidly than desaturation and therefore it can be deduced that the regulation of fatty acid synthesis is dependent on desaturation reaction rates (Voss *et al.*, 1991).

1.4.6. Supply of fatty acids to the fetus in utero

The placenta is involved in providing nutrients for the developing fetus and also deals with the metabolic waste formed by the fetus. It is also capable of metabolic activities including glycolysis, gluconeogenesis, triglyceride synthesis, and fatty acid synthesis and oxidation (Hay, 1991). The placenta has been shown to be "permeable" to free fatty acids but not to phospholipids and triacylglycerols (Feldman *et al.*, 1992). Linoleic acid has been found in fetal plasma, and, as this can only be provided by diet, it would indicate a placental transfer of fatty acids (Robertson and Sprecher, 1968). This group of workers also demonstrated that the placenta was capable of synthesising most lipid classes *de novo*, as well as having the ability to alter the composition of certain lipids. The human placenta, however, has limited ability to elongate and desaturate linoleic and α-linolenic acids (Kuhn and Crawford, 1986). Placental membrane fatty acid binding protein has been shown to have a role in the preferential transfer of maternal LCPUFA across the human placenta.

Campbell *et al.* (1998) demonstrated that this protein had higher affinities and binding capacities for AA and DHA compared with linoleic and oleic acids.

Fetal brain concentrations of AA and DHA rise with increasing gestational age (Menon and Dhopeshwarkar, 1982; Martinez *et al.*, 1974). This could be due to the preferential transfer of these acids from maternal to fetal circulation for membrane incorporation. Crawford *et al.* (1976) proposed a process, which they referred to as biomagnification, in which there is a progressive increase in LCPUFA from maternal blood to fetal blood, fetal liver and fetal brain. Neuringer *et al.* (1984) demonstrated the effect in monkeys where the levels of AA and DHA were higher in foetal blood than in maternal blood : the converse was found for levels of linoleic acid and α-linolenic acid.

Clandinin *et al.* (1980) suggested that 80% of fetal brain DHA accrued between 26 and 40 weeks of gestation. They reported low levels of DHA in the brains of infants born before 32 weeks. This is an important finding in relation to the feeding of preterm infants.

1.5.1. Essential fatty acid deficiency disease states

The first report of a deficiency in essential fatty acids was described by Burr and Burr in 1929. An acute deficiency state in rats fed fat-free diets was characterised by skin lesions, where the skin became more porous to water, growth retardation and impairment of reproduction. Moreover, these deficiencies could be corrected when certain fatty acids were added to the diet and as a result they showed that linoleic acid was an essential fatty acid. In 1937, Turpeinen demonstrated that arachidonic acid was even more active in reversing the induced deficiency state than linoleic acid.

Evidence for a human requirement for linoleic acid was first described by Hansen *et al.* (1963). They showed that infants fed on different milk formulas

containing low levels of linoleic acid developed dermatitis which could be cured by the addition of linoleic or arachidonic acid. In the 1970s, two studies reported similar deficiencies in infants fed with low-fat intravenous nutrition (Caldwell *et al.*, 1972; White *et al.*, 1973). A case of n-3 fatty acid deficiency was reported by Holman *et al.* in 1982, in a six year old girl receiving parenteral nutrition for several months following gastrointestinal surgery. She subsequently developed severe neurological abnormalities including sensory, motor and visual defects. The initial dietary preparation contained very high amounts of linoleic acid and almost no α-linolenic acid. When an α-linolenic acid supplement was added the symptoms disappeared. In 1987, Norwegian workers (Bjerve *et al.*) reported that a group of elderly patients fed by gastric tube displayed symptoms associated with α-linolenic acid deficiency.

There have been other disease states linked with reduced levels of these and other polyunsaturated fatty acids. Menkes *et al.* (1962) described a disease known as "kinky hair disease" which was characterised by peculiar, stubby white hair, cerebral and cerebellar degeneration, early and severe mental retardation and severe neurological impairment starting within the first months of life. Analysis of the brains of these infants showed that the DHA levels were very low in PS and partially reduced in PE. Svennerholm *et al.* (1975) described a progressive neuropathy in which cerebral DHA decreased especially in PE and PS and arachidonic acid increased. Other workers (Gerstl *et al.*, 1965) described a decrease in total polyunsaturated fatty acids in white matter of patients with multiple sclerosis. McAlpine *et al.* (1972) suggested that there may be an inverse relationship between high dietary intake of n-3 fatty acids and multiple sclerosis, because in Japan where fish consumption is high, there is a low incidence of the disease. Also within Norway a similar effect is seen when comparing the population of coastal regions and urbanised centres. As previously stated, Martinez (1992a) has reported low levels of

DHA in the brains of patients with adrenoleukodystrophy, a metabolic disorder involving defective peroxisomal metabolism.

1.5.2. Animal studies using radio-labelled substrates

Radio-labelling studies using animal models were carried out in the early 1970s to elucidate fatty acid metabolic pathways. Mead and Dhopeshwarkar (1971) injected ^{14}C labelled acetate into rats of varying ages. Their results showed that synthesis of fatty acids in the brain is very rapid in the pre-myelination animal but falls off to a steady state in the adult rat. Hassam *et al.* (1974) studied the incorporation of orally fed linoleic acid and γ-linolenic acid showing that Δ6-desaturation of linoleic to γ-linolenic acid was a rate limiting step in the conversion to arachidonic acid. Subsequently Sinclair (1975) demonstrated using oral feeding of ^{14}C labelled precursor C18 fatty acids and AA and DHA, that dietary rather than synthesised LCPUFAs were preferentially incorporated into the developing rat brain.

More recently, similar studies were performed on near-term fetal rat brains, when ^{14}C labelled fatty acids were introduced by the intra-cranial route (Green and Yavin, 1993). It was concluded that the fetal brain had the capacity to take up, convert, and selectively esterify essential fatty acids and their long-chain derivatives into phospholipids. Pawlosky *et al.* (1996) examined different areas of the brain in neonatal rats and mice to study the uptake of radio-labelled linoleic and α-linolenic acids. They found a greater uptake of α-linolenic acid into the cerebellum as compared to the cortex whereas uptake of linoleic acid did not vary. Their results indicated that different regions within the brain may vary in their capacity to synthesise DHA and this may correlate with variation in regional growth rates.

1.5.3. Animal studies inducing EFA deficiency states

Following on from humans studies , several groups of workers returned to animal models to measure tissue levels of fatty acids with respect to EFA-deficient diets.

Essential fatty acid deficiency in animals has been shown to produce increased tissue concentrations of n-9 series PUFA synthesised from oleic acid in an attempt to "compensate" for the reduction in LCPUFA of the n-3 and n-6 series (Mead and Slaton, 1956). When n-9 series PUFA were identified in significant amounts, animals displayed clinical symptoms of fat deficiency (Mohrhauer and Holman, 1963). Clausen and Moller in 1967 reported that rat brains could be made susceptible to an induced autoimmune attack by depleting their membranes through dietary deficiency of essential fatty acids and that PUFA were essential for the integrity of the neural membrane. Galli *et al.* (1970) also noted reduced body and brain weight in EFA-deficient animals when compared with a control group and that the n-9 series PUFA were more abundant in the males. Sun (1972) examined brain myelin and microsomal phosphoglyceride fractions in rats given EFA deficient diets, confirming Galli's findings and concluding that such alterations to membrane structure could ultimately lead to demyelination in addition to disturbances in enzyme activity in the microsomal membranes.

In 1984, Bourre *et al.* presented their first in a series of publications using the rat as an animal model. In the initial study , several generations of rats were fed a diet deficient in n-3 fatty acids. Sunflower oil provided the n-3 deficient feed whereas n-3 rich soyabean (Table 1.1) or rapeseed oil were given to a control group. They measured the fatty acid composition of various neural cell types (neurons, astrocytes and oligodendrocytes) as well as subcellular fractions (myelin and synaptosomes) and showed a deficiency of DHA which was associated with an increase in n-6 series DPA concentrations.

They repeated the experiment by feeding the rats for only 60 days and the same findings were observed. The n-3 diet was then re-introduced, whereupon, it took several months for the cells to recover their normal levels of DHA at the expense of n-6 DPA. The authors also demonstrated a reduction in certain enzyme activities, namely 5'-nucleotidase and Na-K-ATPase, in n-3 deficient animals (Bourre *et al.*, 1989).

Anding and Hwang (1986) used coconut oil (low in n-3 fatty acids) to induce EFA deficiency in weanling rats. After 15 weeks, graded amounts of α-linolenic acid with constant linoleic acid were re-introduced to the diet. Increasing dietary α-linolenic acid resulted in a decrease in the n-6 series PUFA in brain lipid suggesting an inhibition of the conversion of linoleic acid to arachidonic acid.

Carlson *et al.* (1986) studied cerebrum and cerebellum fatty acids of PC and PE and also red cell membrane fatty acids from rats fed diets with varying ratios of linoleic and α-linolenic acids. These authors suggested that red cell membrane composition could be used as an indicator of neural accumulation of LCPUFA.

Connor *et al.* (1990) used rhesus monkeys as a primate model to study fatty acid metabolism. Initially the monkeys were fed n-3 deficient diets and were shown to have low levels of DHA in the cerebral cortex and retina with a compensatory rise in n-6 series DPA. Low levels of DHA were "corrected" by 12 weeks after fish oil was introduced to their diet.

Piglets have also been used as an animal model (Arbuckle and Innis, 1992) because the timing of their perinatal brain growth spurt resembles that observed in humans. In addition, the fatty acid composition of a sow's milk resembles human breast milk. The authors examined the effect of feeding

piglets from birth for 15 days with linolenate at two different levels or fish oil and low level linolenate in combination. They concluded that dietary n-3 PUFA from fish oil were more effective as a source of membrane n-3 fatty acids than their precursor, α-linolenic acid.

1.5.4. Red cell membrane fatty acids in relation to diet

Many workers have reported measurement of red cell membrane fatty acids and plasma fatty acids in relation to infant diet. The studies have been on both preterm and term children and diets have included breast milk, standard formulas and supplemented formulas.

As early as 1971, Olegard and Svennerholm showed that the red cell membrane fatty acids of breast-fed babies at 3 to 4 months of age had a greater proportion of n-3 to n-6 fatty acids when compared to formula fed infants. This work was confirmed by several other groups of workers. (Sanders and Naismith, 1979; Putnam *et al.*, 1982; Ponder and Innes., 1992) Moreover preterm infants fed standard preterm formulas had significantly lower DHA levels in both red cell membrane phospholipids and plasma lipids than infants fed breast milk (Carlson and Rhodes., 1986b; Koletzko *et al.*, 1989).

Various groups then began to study the effect of the addition of LCPUFA to standard formulas on red cell membrane fatty acid concentrations while other workers studied the effect of altered ratios of n-3 to n-6 fatty acids in the formula milks.

Carlson *et al.* (1991) showed that adding small amounts of fish oil to standard formula milk produced red cell membrane phospholipid DHA levels similar to those of breast fed infants. These authors also observed a reduction in the arachidonic acid concentration in the red cell membranes of infants fed the fish oil supplemented formula. This was a concern since arachidonic acid is

an important precursor for eicosanoid metabolism (Martinez, 1989) and also has been shown to be positively related to preterm growth in the first year of life (Carlson *et al.*, 1993).

Clark *et al.* (1992) examined the effect of varying the ratio of linoleic acid/ α-linolenic acid in infant formulas. They found that decreasing the ratio to <5:1 increased the LCPUFA incorporation into red cell membranes but the levels of DHA and AA still did not attain those found in breast fed infants.

1.5.5. Animal behavioural and visual studies

In animal studies several aspects of behaviour have been investigated in relation to fatty acid content of the diet. Rats fed a diet deficient in n-3 fatty acids showed poorer discriminatory learning between black and white stimuli compared to those fed an n-3 rich diet (Lamptey and Walker, 1976). The authors also showed that this correlated with reduced levels of DHA in the brain phospholipids of the n-3 deficient rats. Yamamoto *et al.* (1988) observed differences in the learning ability of rats fed diets either supplemented with safflower seed oil (rich in linoleic acid) or perilla seed oil (rich in α-linolenic acid). They used a brightness discrimination learning test and showed that the group fed the high n-6 fatty acid diet required a significantly longer time for extinction of the learning test than the group fed the high n-3 fatty acid diet or than a group fed a normal diet. Bourre *et al.* (1989) used a shuttle box test as a measure of learning in comparing rats fed low α-linolenic acid diets with rats fed a normal diet. They found that the group on the low n-3 fatty acid diet were significantly slower in their learning ability than the normally fed group.

This group of workers also studied electroretinogram measurements in relation to diet, as the retina is very rich in n-3 fatty acids, especially DHA. Deficiencies in PUFA result in modifications of membrane fatty acids in the retina, which can be measured as changes to the *a* and *b* waves of the

electroretinogram. The results obtained revealed substantial changes in the ERG of the n-3 deficient animals when compared to the control group. A similar finding had already been reported by Neuringer *et al.* (1986) who used rhesus monkeys as a study model and compared animals fed an n-3 deficient diet throughout pregnancy and the infants receiving a similar diet after birth. These workers observed not only low levels of DHA in the retina and cerebral cortex but also subnormal visual acuity at 4 - 12 weeks of age and impairment of ERG measured at 21 months of age when compared to a group of monkeys fed normally. These results were observed despite a compensatory rise in n-6 series DPA in the retina as well as the cortex.

1.6.1. Aim of this study

This study is designed to assess whether the fatty acid composition of brain membranes in infants dying in the first year of life is influenced by their diet. It is intended to study different areas of the brain namely cerebral and cerebellar cortex grey and white matter.

CHAPTER 2

MATERIALS AND METHODS

2.1. MATERIALS

In the following section all chemicals and standards used in the experimental procedures are listed along with their supplier.

2.1.1. Chemicals

a) Solvents

The following solvents were used in extraction procedures and thin layer chromatography. These were Analar grade unless otherwise specified and obtained from Merck Ltd., Poole, Dorset, UK.

Solvent	Catalogue No.
Butan-1-ol	10061 6J
Chloroform	10077 6B
Diethyl ether	10094 6B
n-Hexane	10387 4X
Methanol	10158 6B
Methyl Acetate(GPR grade)	29202 4E
Propan-2-ol	10224 4J
Petroleum spirit (40-60°C)	10178 4F

b) Additional chemicals

Reagent	Catalogue No.
Acetic acid	10001 BT
Copper acetate	10087 4C
2′7′ -dichlorofluorescein	20034 3J
Orthophosphoric acid	10173 AB
Potassium chloride	10198 4L
Sodium chloride	10241 4J
2,6-di-tert. butyl -p-cresol (BHT) (GPR)	28067 4T

Reagent	Catalogue No.
Boron trifluoride in methanol (12 - 14%w/v)	27419 4T
Methanolic HCl 3N (Supelco)	3-3051

c) Lipid standards

Lipid standards for TLC identification purposes were purchased from Sigma-Aldrich Company Ltd., Poole, Dorset, UK.

Standard	Catalogue No.
L-α-phosphatidylcholine (from bovine brain)	P6638
L-α-phosphatidylethanolamine (from bovine brain)	P9137
L-α-phosphatidylinositol (from bovine liver)	P2517
L-α-phosphatidyl -L-serine (from bovine brain)	P6641
Sphingomyelin (from bovine brain)	S7004
Tripalmitin	T5888
Cholesteryl stearate	C3549
Galactocerebrosides (from bovine brain)	C4905
Sulphatides (from bovine brain)	S1006

d) Fatty acid standards

Fatty acids methyl esters (FAME) were purchased from the same supplier.

Standard		Catalogue No.	Standard		Catalogue No.
Saturated Fatty Acids:-			**Monounsaturated Fatty Acids:-**		
C14:0	Myristic Acid	M3378	C14:1n-5	Myristoleic Acid	M3650
C16:0	Palmitic Acid	P5177	C16:1n-7	Palmitoleic Acid	P6087
C18:0	Stearic Acid	S5376	C18:1n-9	Oleic Acid	O4754
C20:0	Arachidic Acid	A3881	C20:1n-9	Eicosenoic Acid	E6885
C22:0	Behenic Acid	B3271	C22:1n-9	Erucic Acid	E3510
C24:0	Lignoceric Acid	L6766	C24:1n-9	Nervonic Acid	N6767

Standard		Catalogue No.
	Polyunsaturated Fatty Acids:-	
C18:2n-6	Linoleic Acid	L1876
C18:3n-6	α-Linolenic Acid	L2626
C20:2n-6	Eicosadienoic Acid	E7877
C20:3n-6	Eicosatrienoic Acid	E3511
C20:3n-9	Eicosatrienoic Acid (Mead Acid)	E6013
C20:4n-6	Arachidonic Acid	A9298
C20:5n-3	Eicosapentaenoic Acid	E2012
C22:4n-6	Docosatetraenoic Acid	D3534
C22:5n-6	Docosapentaenoic Acid	D5679
C22:6n-3	Docosahexaenoic Acid	D2659

An additional commercial standard was also purchased:

Supelco 37 Component FAME Mix 4-7885

Supelco UK, Sigma-Aldrich Chemical Company Ltd., Poole, Dorset, UK.

e) Miscellaneous items

Item	Catalogue No.
TLC plates 20cm x 20 cm Silica Gel 60 F_{254} 0.25mm.	5715

obtained from Merck Ltd., Poole, Dorset, UK.

HPTLC plates 10cm x 10 cm LHPK Silica Gel 60A	4805-410

obtained from Whatman International Ltd., Maidstone, Kent, UK.

2.1.2. Tissue samples

Tissue samples were taken from precise loci at post-mortem from 66 subjects who died from sudden infant death syndrome. In addition, the pathologist's examination excluded any other cause of death. Samples of cerebral parietal cortex, parietal central white matter (above the corpus callosum) and cerebellum were obtained. Where possible cerebellar tissue was also separated

Table 2.1 Infant details relating to age, sex, gestation, feeding regimen, birth, body and brain weights.

Tissue No.	Age (wk)	Sex	Gestation (wk)	Feed	Group	Birth Wt.(kg)	Wt. at death (kg)	Brain Wt.(mg)
19/85	38	F	40	Breast	Breast	3.60	9.19	NR
510/85	12	M	40	Ostermilk	CGOST	3.64	5.30	NR
547/85	14	M	37	Cow&Gate	CGOST	1.90	4.25	660
617/85	17	M	38	SMA	SMA	NR	8.42	710
642/85	11	M	39	Ostermilk	CGOST	2.50	4.45	545
85/86	24	F	40	SMA	SMA	3.23	5.96	810
181/86	10	F	40	Breast	Breast	3.24	5.61	652
217/86	8	F	42	Breast	Breast	2.95	3.54	530
219/86	7	M	40	SMA	SMA	3.12	4.52	NR
248/86	18	M	42	SMA	SMA	3.63	6.44	785
249/86	7	F	40	Breast	Breast	3.93	5.16	575
602/86	34	M	34	SMA	SMA Prem	2.33	6.69	1010
630/86	16	F	40	SMA	SMA	3.94	5.86	820
7/87	17	M	32	SMA	SMA Prem	0.91	3.06	540
38/87	13	M	37	SMA	SMA	2.98	5.78	750
39/87	4	M	40	SMA	SMA	3.35	4.14	560
277/87	18	M	37	SMA	SMA	2.21	5.60	670
291/87	43	F	40	SMA	SMA	2.74	7.37	NR
581/87	8	F	42	Breast	Breast	3.72	4.59	NR
593/87	22	M	40	SMA	SMA	3.39	7.52	900
657/87	10	M	30	SMA	SMA Prem	1.27	2.95	465
5/88	18	M	39	SMA	SMA	2.72	5.46	NR
17/88	9	M	40	Cow&Gate	CGOST	2.67	4.82	680
67/88	10	F	37	Ostermilk	CGOST	2.61	3.82	490
133/88	6	M	40	SMA	SMA	3.75	4.89	590
175/88	21	M	41	Ostermilk	CGOST	3.55	6.86	1030
189/88	40	F	37	Cow&Gate	CGOST	2.28	5.08	890
195/88	29	M	40	Cow&Gate	CGOST	3.35	8.02	930
225/88	11	F	39	Breast	Breast	2.50	4.95	NR
268/88	16	M	40	SMA	SMA	2.98	6.20	780
288/88	25	M	32	SMA	SMA Prem	1.76	7.48	840
472/88	6	M	40	Breast	Breast	3.07	5.34	640
2/89	5	F	37	Breast	Breast	2.47	3.15	530

Table 2.1 Infant details (continued)

Tissue No.	Age (wk)	Sex	Gestation (wk)	Feed	Group	Birth Wt.(kg)	Wt. at death (kg)	Brain Wt.(mg)
457/89	16	M	40	Breast	Breast	3.66	7.71	780
51/93	11	M	40	Ostermilk	CGOST	3.21	5.08	570
75/93	19	M	40	Breast	Breast	4.14	7.55	740
90/93	21	M	38	Cow&Gate II	C&G	3.18	4.85	780
103/93	6.5	F	37	Ostermilk	CGOST	2.50	3.58	520
163/93	9	M	39	SMA	SMA	2.58	4.59	645
197/93	19	M	40	SMA	SMA	3.55	6.73	NR
256/93	20	M	40	Ostermilk	CGOST	3.80	6.89	845
263/93	18	M	40	Ostermilk	CGOST	2.04	4.56	605
279/93	25	M	41	SMA	SMA	3.58	7.18	NR
51/94	7	M	38	Ostermilk	CGOST	2.53	4.17	565
81/94	15	M	39	Breast	Breast	2.90	4.17	660
110/94	18	M	40	Breast/SMA	Mix	3.97	7.40	905
164/94	33	F	38	Cow&Gate II	C&G	3.01	8.45	920
180/94	13	F	39	Cow&Gate II	C&G	3.01	4.65	600
193/94	11	M	37	SMA	SMA	2.81	5.02	605
249/94	9	F	38	Ostermilk	CGOST	3.29	4.34	577
275/94	5	M	39	Cow&Gate II	C&G	2.71	3.99	572
277/94	4	F	41	SMA	SMA	3.37	4.51	527
283/94	7	M	37	SMA	SMA	2.70	4.41	NR
333/94	19	F	34	Ostermilk	CGOST Prem	2.58	5.45	631
402/94	17	M	40	SMA	SMA	2.90	6.13	779
447/94	15	M	38	SMA	SMA	2.98	6.02	NR
48/95	9	F	41	Breast	Breast	3.97	5.40	570
72/95	7	M	32	Breast/SMA	Mix Prem	1.19	2.28	400
114/95	7	F	36	SMA	SMA Prem	2.04	3.01	470
251/95	3	F	40	Breast	Breast	3.21	3.16	455
266/95	0.5	M	38	SMA	SMA	2.80	2.56	380
276/95	2	F	38	SMA	SMA	3.20	3.53	412
382/95	1	F	40	Breast	Breast	3.09	2.55	425
419/95	12	M	35	SMA	SMA Prem	2.50	4.82	548
429/95	23	M	34	SMA	SMA Prem	2.27	6.86	802

into grey and white matter. Exclusion criteria were based on identification of significant neurological deficits prior to death (one case with hypotonia and aphasia) or brain abnormalities at necropsy. A pathological classification of minor gyral flattening in the parietal region (recorded in two term-born formula fed infants) was not seen as sufficient justification for exclusion. A full medical and dietary history was established by inspection of police, post-mortem and health visitors' reports. Other dietary information was obtained from the responses to a questionnaire completed at parental interview (see appendix). Individuals were classified as either term (≥37 weeks gestation; n=57) or preterm (<37 weeks gestation; n=9). A birthweight between the 3rd and 97th centiles for the appropriate gestational age defined a normally grown term infant (Keen and Pearse, 1985) and applied to all term-born subjects in this study. Whenever possible tissue samples were collected within 48 hours of death and stored in universal containers at -70°C. Table 2.1 lists all subjects included in the study and information obtained from sources as detailed above.

2.1.3. Milk feeds

Infants from whom brain tissues were collected, were grouped according to which type of milk feed they received during life. The characteristics of each group were as described below.

a) Breast feeding

The majority (n=14) of this group were exclusively breast fed. However the 7 week old preterm and 18 week old term "breast fed" infants had also received SMA formula supplementation during the last 3 and 8 weeks of life respectively. These were designated as mix-fed infants.

b) SMA SMA, Wyeth Laboratories, Maidenhead, U.K.

This group of infants (n=31) had received either SMA gold cap or SMA white cap exclusively or in some cases a mixture of both. These formulas contained identical blends of fatty acids derived from vegetable oils and animal fats but differed in casein:whey protein ratios and therefore amino acid compositions. The were devoid of long-chain PUFAs but the essential fatty acid precursor, α-linolenic acid was present at a concentration of 1.5% of total fatty acids.

c) CGOST

Infants (n=15) who received one of the undermentioned formula milks were combined into a single CGOST group because their linoleic and α-linolenic acid concentrations were similar. The milks did not contain any preformed long-chain PUFAs and the essential fatty acid precursor, α-linolenic acid, was present within a range of <0.1 - 0.4% of total fatty acids. The milks included in this group are listed below:

Cow and Gate Premium (pre 1990)	Cow&Gate Ltd., Trowbridge, U.K.
Cow and Gate Plus (pre 1990)	
Farley's Ostermilk 1	Heinz Ltd., Nottingham, U.K
Farley's Ostermilk 2	(formerly Crookes Healthcare)

d) C&G Cow&Gate Ltd., Trowbridge, U.K.

This group of infants (n=4) had received a new formulation of Cow & Gate milk with an increased α-linolenic acid content (2.2%) and reduced ratio of linoleic:α-linolenic acid at approximately 5:1. The infants were fed either of the milks listed below or a combination of both:

Cow and Gate Premium (post 1990)

Cow and Gate Plus (post 1990)

Seven individuals also received solids in their diet and were interspersed between all feeding groups.

In spite of the extended collection period of tissues, few significant changes occurred in any of the formula milks in respect of either the non-essential saturated or mono-unsaturated fatty acids during this time and in addition the provision of linoleic acid remained relatively constant at between 11 to 20% of total fatty acids. A summary of the manufacturers' quoted values for the fatty acid content of milks is presented in Table 2.2. This is followed by published ranges for human breast milk fatty acid concentrations (Table 2.3).

Table 2.2 Formula milk fatty acid compositions (weight % of total fatty acids) as quoted by manufacturers

Fatty Acid		*Milk Diet*			
		SMA	*CGOST (1)*	*CGOST (2)*	*C&G*
Myristic	C14:0	4.31	6.8	4.80	4.3
Palmitic	C16:0	21.70	21.0	16.01	17.7
Palmitoleic	C16:1n-7	0.12	0.5	trace	0.3
Stearic	C18:0	4.48	4.0	3.32	3.5
Oleic	C18:1n-9	35.62	29.5	46.45	45.5
Linoleic	C18:2n-6	18.92	17.1	11.87	11.4
α-Linolenic	C18:3n-3	1.80	0.4	trace	2.1
Dihomo-γ-linolenic	C20:3n-6	<0.05	<0.05	<0.05	<0.05
Arachidonic	C20:4n-6	<0.05	<0.05	<0.05	<0.05
Docosahexaenoic	C22:6n-3	<0.05	<0.05	<0.05	<0.05
Arachidic	C20:0	0.31	na	na	0.4
Eicosenoic	C20:1n-9	0.21	na	<0.05	0.5
Behenic	C22:0	0.18	na	na	0.4
Erucic	C22:1n-9	<0.05	na	<0.05	0.1
Lignoceric	C24:0	na	na	na	na
Nervonic	C24:1n-9	na	na	na	0.1

SMA(Wyeth, UK) Gold or White Cap
CGOST (1) - Cow & Gate Premium or Plus (pre 1990)
CGOST (2) - Farley's Ostermilk 2
C&G - Cow & Gate Premium or Plus (post 1990)
all formula fatty acid compositions represent manufacturers' data.
na - not quoted by manufacturer.

Table 2.3 Published breast milk fatty acid compositions (weight % of total fatty acids)

Fatty Acid		(1) n=5	(2) n=61	(3) n=14	(4) n=26
Myristic	C14:0	4.14-12.66	2.73-8.53	7.48	4.01-16.09
Palmitic	C16:0	13.58-22.82	18.80-26.08	23.29	21.44-32.28
Palmitoleic	C16:1n-7	1.72-4.68	2.49-5.09	3.90	1.87-6.47
Stearic	C18:0	2.64-8.96	6.34-12.06	8.61	2.75-5.43
Oleic	C18:1n-9	27.90-43.10	35.38-39.62	36.01	21.68-38.69
Linoleic	C18:2n-6	0.7-21.9	2.31-19.19	9.96	7.60-10.08
α-Linolenic	C18:3n-3	0.08-1.61	0.27-0.91	0.70	0.02-0.58
Dihomo-γ-linolenic	C20:3n-6	0.14-0.66	0.13-0.49	0.29	ud-0.55
Arachidonic	C20:4n-6	0.36-0.64	0.20-0.60	0.36	0.07-0.87
Docosahexaenoic	C22:6n-3	0.24-0.56	ud-0.66	0.15	0.32-1.48
Arachidic	C20:0	ud-0.36	0.43-1.07	<0.05	0.10-0.42
Eicosenoic	C20:1n-9	na	0.44-0.76	0.81	0.11-0.91
Behenic	C22:0	0.02-0.14	ud-0.10	<0.05	0.02-0.10
Erucic	C22:1n-9	na	ud-0.10	<0.05	ud-0.40
Lignoceric	C24:0	na	0.02-0.14	0.10	0.05-0.33
Nervonic	C24:1n-9	ud-0.15	0.01-0.05	0.14	ud-0.23

(1) *Clandinin et al.* (1981) - Fatty acid analysis of human milk at day 16 of lactation from Canadian mothers of 5 preterm infants. Results given as normal ranges.

(2) *Gibson and Kneebone* (1981) - Fatty acid analysis from an Australian population of 61 mature human milks. Results given as normal ranges.

(3) *Harzer et al.* (1983) - Fatty acid analysis of 14 breast milk samples from mothers at Day 22 postpartum. Results quoted as means.

(4) *Kneebone et al.* (1985) -Fatty acid analysis from a population of Malay mothers living in Penang. Results given as normal ranges.

na - not available ud - not detected.

These articles incorporate data from the analyses of both term and preterm breast milks, and from disparate geographical populations. The references

chosen were those which provided the most comprehensive ranges of milk fatty acids together with brief statistical analyses of the results. Most authors quote the essential fatty acid concentrations together with those of the polyunsaturated fatty acids in their breast milk analyses. As this thesis also examines very long chain saturated (lignoceric) and monounsaturated fatty acids (nervonic), it was necessary to consider publications which quoted values for these compounds.

2.2. METHODS

2.2.1. Preparation of chemicals

a) Solvents

Solvents for thin layer chromatography were prepared freshly before use and allowed to equilibrate in the chromatography tanks as described in the methodology section.

Chloroform/Methanol/Water (65:25:4) This was prepared by combining 130 ml. chloroform, 50 ml. methanol and 8 ml. distilled water in a measuring cylinder with a glass stopper. The reagents were mixed well before adding to a chromatography tank.

Butanol/Acetic acid/Water (60:20:20) This was prepared by combining 120 ml. chloroform, 40ml. butanol and 40 ml. acetic acid in a measuring cylinder with a glass stopper. The reagents were mixed well before adding to a chromatography tank.

Hexane/Diethyl ether This solution was used to rinse HPTLC plates before use and was prepared by combining 25ml hexane with 25 ml diethyl ether in a measuring cylinder with a glass stopper. The reagents were mixed before adding to a chromatography tank.

Methyl acetate/Propan-2-ol/Chloroform/Methanol/0.25% aqueous KCl (25:25:25:10:9) HPLC Solvent This was prepared by mixing 25 ml methyl acetate, 25 ml propan-2-ol, 25 ml chloroform, 10 ml methanol and 9 ml 0.25%

aqueous KCl in a 100 ml measuring cylinder with a glass stopper. From this, 40 ml of solvent was used in each chromatography tank. In general two tanks were used in each run to avoid waste of the solvent.

Chloroform with BHT 50 mg of butylated hydroxytoluene was added to one litre of chloroform and this was used in all extraction procedures.

Methanol with BHT 50 mg of butylated hydroxytoluene was added to one litre of methanol and this was used in all extraction procedures.

Methanol/Water To prepare this reagent, 500 ml of methanol containing BHT was combined with 500 ml distilled water.

The chloroform and methanol working solutions were stored in dark glass bottles at room temperature.

b) Chemicals

The following chemicals were used in either extraction of the tissues, derivatisation of the fatty acids or visualisation of TLC plates.

The chemicals were weighed using a Sartorius RC 210D balance. (Sartorius)

Sodium chloride Sodium chloride (1.8g) was dissolved in 200 ml distilled water and stored in a glass bottle at room temperature.

Potassium chloride Potassium chloride (0.25g) was dissolved in 100 ml distilled water and stored at room temperature in a plastic bottle.

Boron trifluoride in methanol This was stored at 4°C after the addition of 50 mg BHT to the 500 ml glass bottle.

3N Methanolic HCl This was stored at 4°C after the addition of 50 mg BHT to the 400 ml glass bottle.

2′7′ Dichlorofluorescein (Visualisation reagent for TLC) This was prepared by the addition of 200 mg of 2′7′-dichlorofluorescein to 100 ml methanol. This was then diluted further by the addition of 100 ml distilled water. The resulting solution was stored at room temperature in a dark glass bottle. This stock solution was diluted 1:5 with methanol immediately before use.

3% Copper acetate in 8% orthophosphoric acid (Charring reagent for HPTLC plates) 8 mls of orthophosphoric acid was added slowly to 92 ml

distilled water. Copper acetate (3g) was dissolved in this mixture and the resultant solution stored in a dark glass bottle at room temperature.

c) Lipid standards

Lipid standards were dissolved in chloroform:methanol (98:2, v/v) at a concentration of 5 mg/ml solvent and stored at -20°C in 2 ml glass trident vials.

d) Fatty acid methyl ester standards

Gravimetric stock solutions of each fatty acid methyl ester standard at a concentration of 30 mg/ml in hexane were prepared.

Working FAME solutions 100 µl of each individual stock solution was diluted to 1 ml with hexane (3 mg/ml). Individual working standards were used as GLC/GCMS positional standards.

Combined FAME standard A combined FAME standard was prepared from the stock solutions and contained the following fatty acids methyl esters :- C16:0, C16:1, C18:0, C18:1, C18:2, C20:3, C20:4, C22:6, C24:0 and C24:1. 20 µl of each standard was used and then made up to 2 ml with hexane (3mg/ml).

All stock and working solutions were stored at -70°C in 2 ml trident glass vials, when not in use, and allowed to equilibrate to room temperature immediately before use. This ensured that the fatty acid standards were completely solubilised.

2.3. Experimental procedures

In this section there is a description of all procedures in the preparation of total lipid fatty acid methyl esters from the various brain tissues, the thin layer chromatographic techniques involved in the separation of the individual phospholipids and the gas chromatography methods employed in the analysis of the fatty acid methyl esters.

2.3.1. Lipid extraction

Complex lipids such as those found in brain tissue are usually constituents of membranes where they are found in close association with proteins. The lipid molecules are linked to other cellular components by weak hydrophobic bonds, by hydrogen bonds and by ionic bonds.

When considering the extraction of lipids from membranes these associations have to be taken into consideration. Various solvent systems have been used to extract lipids from tissues. These include ethanol:ether:chloroform, ethanol:petroleum ether (Bloor, 1943) isopropanol:hexane (Hara and Radin, 1978). The method of Folch *et al.*, (1957) who used chloroform:methanol (2:1 v/v) is the most popular and frequently used extraction procedure. Bligh and Dyer (1959) adjusted the ratio of chloroform:methanol employed by Folch to produce a single phase solution taking account of water derived from the tissue. Their method was designed for the extraction of larger amounts of tissue and was dependent on estimating the amount of water in the tissue.

Lipid extracts of tissues processed by the above methods tend to contain appreciable amounts of non-lipid contaminants such as sugars, amino acids and salts. Ways and Hanahan (1964) introduced a modified Folch procedure which included a washing stage in which the chloroform/methanol extract was combined with ¼ of its volume of a saline solution. Once centrifuged, the mixture settles into two phases, the lower being predominantly chloroform and containing virtually all lipids while the upper phase consists mainly of methanol/water and contains most of the non-lipid contaminants. A second wash of methanol/water (1:1) can be used to remove any remaining contaminants again ensuring that the proportions of chloroform/methanol/water are appropriate to avoid selective loss of lipid material.

As part of the experimental procedure, the approximate weight of tissue to be extracted was recorded each time (it was observed that the degree of hydration of specimens was variable on receipt) together with the weight of lipid recovered at the end of the extraction procedure. This had a two-fold purpose. Firstly as an estimation of the recovery of lipid in the extraction procedure and secondly to calculate the amount of chloroform to be added to the lipid residue prior to chromatography or derivatisation.

All solvents contained the synthetic antioxidant BHT. Polyunsaturated fatty acids autoxidise rapidly when exposed to air, and the more double bonds present, the more rapid the rate of oxidisation. Tissues will contain natural antioxidants such as α-tocopherol but the levels of these may not be sufficient to prevent deterioration on storage. For the same reason, vials were flushed with nitrogen at various stages in the extraction procedure and before storage at -70°C.

The experimental procedure is now described:-

After removing the tissue from storage at -70°C, approximately 200 mg was removed by means of a scalpel and placed in a pre-weighed, polystyrene weighing boat. Extraneous water was carefully removed with a piece of filter paper and the exact weight of tissue noted. The weighed tissue was transferred to a glass hand homogeniser and 6ml chloroform containing BHT added. After homogenisation the solution was transferred to a 10 ml glass centrifuge tube, 3 ml methanol containing BHT added and the homogenisation process repeated. The methanol and chloroform extracts were combined, and shaken vigorously by hand for 60 seconds. 2 ml sodium chloride were added to the extract to aid removal of any non-lipid contaminants, shaken again for 20 seconds before centrifugation for 5 minutes at 3,000 r.p.m. (1000g; MSE centrifuge). The upper aqueous methanol layer was discarded and 4.5 ml methanol/water (1:1 v/v) added to the remaining chloroform phase followed

by gentle mixing and centrifugation as before. The lower chloroform layer containing the purified lipid was transferred using a glass pasteur pipette to a 10 ml glass tube and evaporated to dryness under nitrogen at 40°C using a Techne "Dri-Block" sample concentrator. (Model No. SD 300-10, SC3; Merck Ltd.) Evaporation of the final 1 ml of solvent was carried out in a pre-weighed 2 ml glass vial, which on completion was re-weighed. The weight difference was taken as total weight of lipid extracted and this was dissolved in chloroform to yield a final lipid concentration of 2mg/100µl and stored at -70°C.

2.3.2. Total lipid transesterification

Methyl esters are the most widely used derivatives formed from fatty acids prior to GLC analysis. The fatty acids on the various phospholipids or triacylglycerols can be directly transesterified to fatty acid methyl esters.

Various reagents have been used to carry our this reaction, namely diazomethane, sodium methoxide in anhydrous methanol, hydrochloric acid or sulphuric acid in anhydrous methanol and boron trifluoride in methanol. Non-polar lipids, such as triacylglycerols and cholesterol esters, are insoluble in reagents composed primarily of methanol, and therefore a small amount of another solvent such as chloroform has to be present for solubilisation.

Metcalfe *et al.* (1966) were the first group to use boron trifluoride in methanol but since then there have been many modifications by various groups of workers including changing the percentage composition of boron trifluoride in methanol. Morrison and Smith (1964) described the procedure including esterifying different phospholipids and using petroleum ether to extract them post derivatisation and this is the basis of the technique described below. It was however noted that esterification of galactocerebrosides and sulphatides

was not so readily achieved by the latter reagent and therefore methanolic hydrochloric acid was employed when derivatising white matter tissue.

For cerebral cortex and cerebellar grey matter, 100 µl (2 mg) of lipid extract was derivatised in a 5 ml "Reactivial" by adding 2 ml boron trifluoride in methanol. The vial was flushed with nitrogen and closed tightly before heating at 80°C for 30 minutes. For parietal white matter and cerebellar white matter, 100µl of lipid extract was added to 100 µl chloroform and 2 ml methanolic hydrogen chloride in a specially tapered 20 ml test tube. These test tubes were used to allow reflux of the solution without any loss of volume. A glass stopper and parafilm were used to seal the test tube. After mixing the tubes were transferred to a heating block for 2 hours at 90°C.

After removal from the heating block the solutions were allowed to cool to room temperature and transferred to 10 ml glass tubes containing 5 ml distilled water in order to remove any polar impurities produced by the derivatisation reaction. The FAME were extracted twice with 3 ml petroleum spirit, and after centrifugation at 3000 r.p.m for 5 minutes, the combined petroleum spirit layers were evaporated to dryness under nitrogen at 40°C. The FAME were then re-dissolved in 1 ml hexane, transferred to a 2 ml glass vial and again the solvent evaporated. The final extract was dissolved in 200µl hexane and stored at –70°C pending GLC analysis.

2.3.3. Thin layer chromatography

Thin layer chromatography (TLC) has been used for the last fifty years as a means of separating lipid mixtures into their various classes. There have been many different solvent systems developed in an attempt to separate as many compounds as possible in a single run. Chloroform/methanol/water (65/25/4) was initially employed as the standard method for separating phospholipids

(Svennerholm and Vanier,1972, Martinez et al., 1974). The results obtained in our laboratory using this system were unsatisfactory (Tables 3.1 and 3.2, Results chapter) most probably due to variations in environmental temperature and humidity. (Tsao, 1982) Several different solvents were thus assessed for use in a two dimensional TLC system to improve separation of the phospholipids of interest, namely PC, PE and PS. It was important to ensure that these major phospholipids were also separated from the less abundant ones (e.g. PI) and also from the cerebrosides and sulphatides. The solvent combinations tested included n-butanol / acetic acid / water, chloroform / methanol / ammonia, chloroform / acetone / methanol / acetic acid/water and tetrahydrofuran/methylal/methanol/ammonia. The combination of chloroform/ methanol/water followed by butanol/acetic acid/water (Rouser et al., 1967) proved to be the most satisfactory and was therefore employed as described below. The TLC plates used for both systems were commercially prepared Silica Gel 60 with a fluorescent indicator to allow detection of the individual phospholipids using a non-destructive chemical.

One dimensional high performance thin layer chromatography (HPTLC) (Vitiello and Zanetta, 1978) was used for separation of white matter lipids prior to quantitation by scanning densitometry (Olsen and Henderson, 1989). This method worked well for small quantities of lipid but would not have been suitable for the preparative stage. The HPTLC plates incorporated a concentration zone at one end of the plate. The adsorbent in this zone was a silica of very high pore diameter and small surface area. The sample applied to this area was concentrated by the developing solvent front into a narrow band and carried up on to the silica gel.

a) One dimensional chromatography

In this procedure, plates were activated for at least 1 hour at 100°C before use. Lipid extract (4 mg) was applied as a band about 7 cm long and two samples run on each plate. An additional narrow band of lipid standards was applied

adjacent to the test samples. The walls of the chromatography tanks were lined with filter paper and 200ml. of chloroform/methanol/water added, and the tank equilibrated for 1 hour at room temperature before introducing the plates. The plates were run for 90 minutes, dried in air on removal and developed using 2′7′-dichlorofluorescein.

b) Two dimensional TLC

Two plates per sample were run in order to obtain enough of the less predominant lipids for derivatisation and subsequent GLC analysis. Plates were activated for at least 1 hour at 100°C before use. Lipid extracts (1.5mg) were spotted in the left-hand corner of each plate. The walls of the chromatography tanks were lined with filter paper, 200ml of chloroform/ methanol/water added, and the tank equilibrated for 1 hour at room temperature before introducing the plates. The plates were run for 90 minutes, on removal dried at 40°C for 30 minutes before placing in the second tank containing butanol/acetic acid/water and run for a further four hours at ninety degrees to the first run.

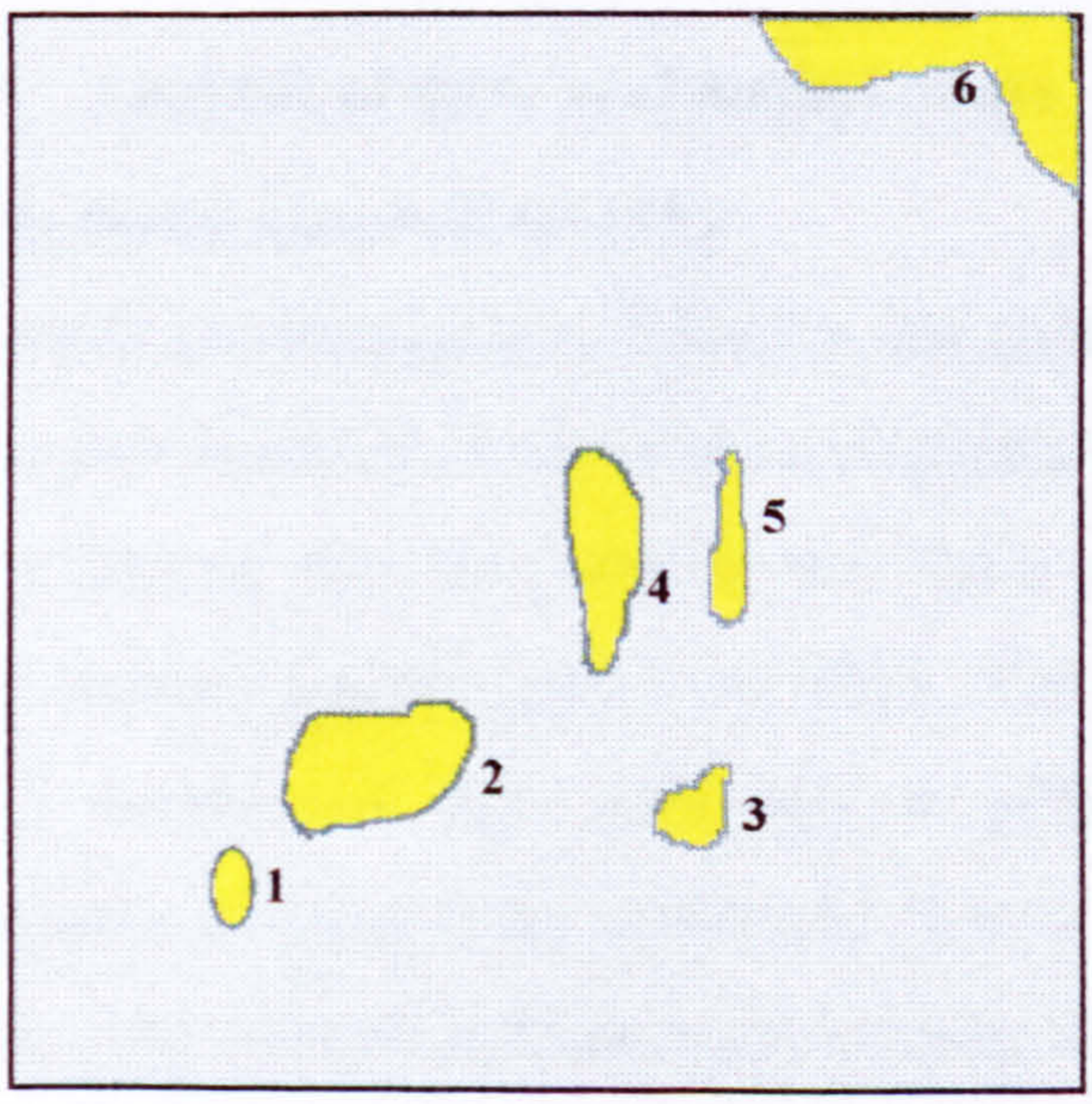


Figure 2.1 - Two dimensional TLC separation of cerebral cortex phospholipids.

The plates were dried for 30 minutes at 40°C, sprayed lightly with 2′7′ dichlorofluorescein and the lipids viewed as yellow spots under UV light at 254 nm (Spectroline CX-20 viewing cabinet, Supelco UK., Sigma-Aldrich Chemical Company Ltd., Poole, Dorset) The spots corresponding to PC, PE, PS and SPH were marked with a pencil (Fig 2.1). The silica gel from these marked areas on both plates were scraped off using a disposable scalpel and the total material from both was stored in 5ml "Reactivials" at -70°C pending further analysis.

2.3.4. Individual lipid transesterification

To each vial containing the silica gel removed from the PC, PE and PS spots, 2 ml boron trifluoride methanol containing BHT was added and the vials flushed with nitrogen, closed tightly and placed in the hot block for 30 minutes at 80°C. The extraction procedure following heating was as for the total lipid esterification but the final extracts were dissolved in 50 µl hexane prior to GLC analysis.

2.3.5. Analysis of fatty acid methyl esters

a) Gas liquid chromatography

Gas liquid chromatography (GLC) is the most widely used analytical method for the determination of the relative proportions of fatty acids in animal tissues. The technique was first used in the 1950s and initially packed columns of either stainless steel or glass were fitted. The average length of these columns was 2 metres and they were easy to prepare in the laboratory with an appropriate packing either purchased from a manufacturer or again prepared on site. The analysis of fatty acids on these columns was time consuming, run times could exceed 2 hours if the separation included longer chain fatty acids, and there was a lack of component resolution.

A further development was the introduction of glass capillary columns with the availability of commercial glass drawing machines. The glass columns had to be treated to ensure adhesion of the liquid phase. A variety of techniques were employed to achieve this. Fused silica was then introduced in the early 1980s as an alternative for capillary columns as it proved to be an inert material and was more flexible than glass. Liquid phases were bonded chemically to the surface of the column. This resulted in improved stability at high temperatures.

Wide bore capillary columns were also manufactured (0.53 mm and 0.75 mm ID) as they could be adapted to both injection and detector ends of GLC units for packed columns relatively simply with little additional cost. Thus direct injection without stream-splitting was possible. Using wide bore columns it was possible to reduce run times and yet resolve monoethylenic isomers.

A Pye Unicam 304 Gas Chromatograph with flame ionisation detector linked to a Philips single pen recorder PM8251 and a Pye Unicam CDP1 computing integrator was employed in the measurement of the fatty acid methyl esters. The flame was ignited using hydrogen from a Canberra Industries Hydrogen Generator (Model B7526) and air from a British Oxygen Company cylinder. Helium was used as carrier gas and also make-up gas.

The following conditions were set when running the FAME samples:-

Oven temperature - 130°C for 4 minutes then rising at 4°/minute to 200°C

Injection temperature - 250°C Detector Temperature - 280°C

Carrier gas flow rate - 5 ml/minute Make-up gas flow rate - 20 ml/minute

Column - A 25 metre fused silica, wide bore (0.53mm) capillary column with cyanopropyl siloxane stationary phase BPX70 (Scientific Glass Engineering, Milton Keynes, UK.)

Sample - A sample size of 0.1ul and detector attenuation setting 2 X 100 was used to determine the total lipid samples whereas for individual phospholipid samples 0.3 µl sample was injected and detector attenuation set at 8 X 10. A typical chromatographic tracing is illustrated in Figure 2.2.

b) Gas chromatography-mass spectrometry (GCMS)

A Hewlett-Packard GCMS was used from 1995 onwards. A mass spectrometer consists of :

a) sample inlet system.

b) source of ions

c) mass measuring system

d) means of ion detection, amplification and recording.

a) The Hewlett-Packard 5890 Gas Chromatograph was the sample introduction technique employed in this case. After separation of the fatty acids by capillary column, they are transferred to the mass spectrometer. The gas chromatograph was fitted with an autosampler (Part No. 3880). The introduction of this technology allowed batch analysis either during the working day or on occasion overnight.

b) The Hewlett-Packard 5972A Mass Detector was fitted with an electron ionisation ion source. The ion source fragments and ionises sample molecules and directs ions through a series of lenses into the mass filter. The mass filter parameters determine which ions can move from the ion source to the detector. It filters the ions according to their mass to charge ratio. The charged molecule fragments into a unique pattern which is characteristic of that specific molecule. This includes the molecular ion (M^+) which provides the molecular weight of the compound.

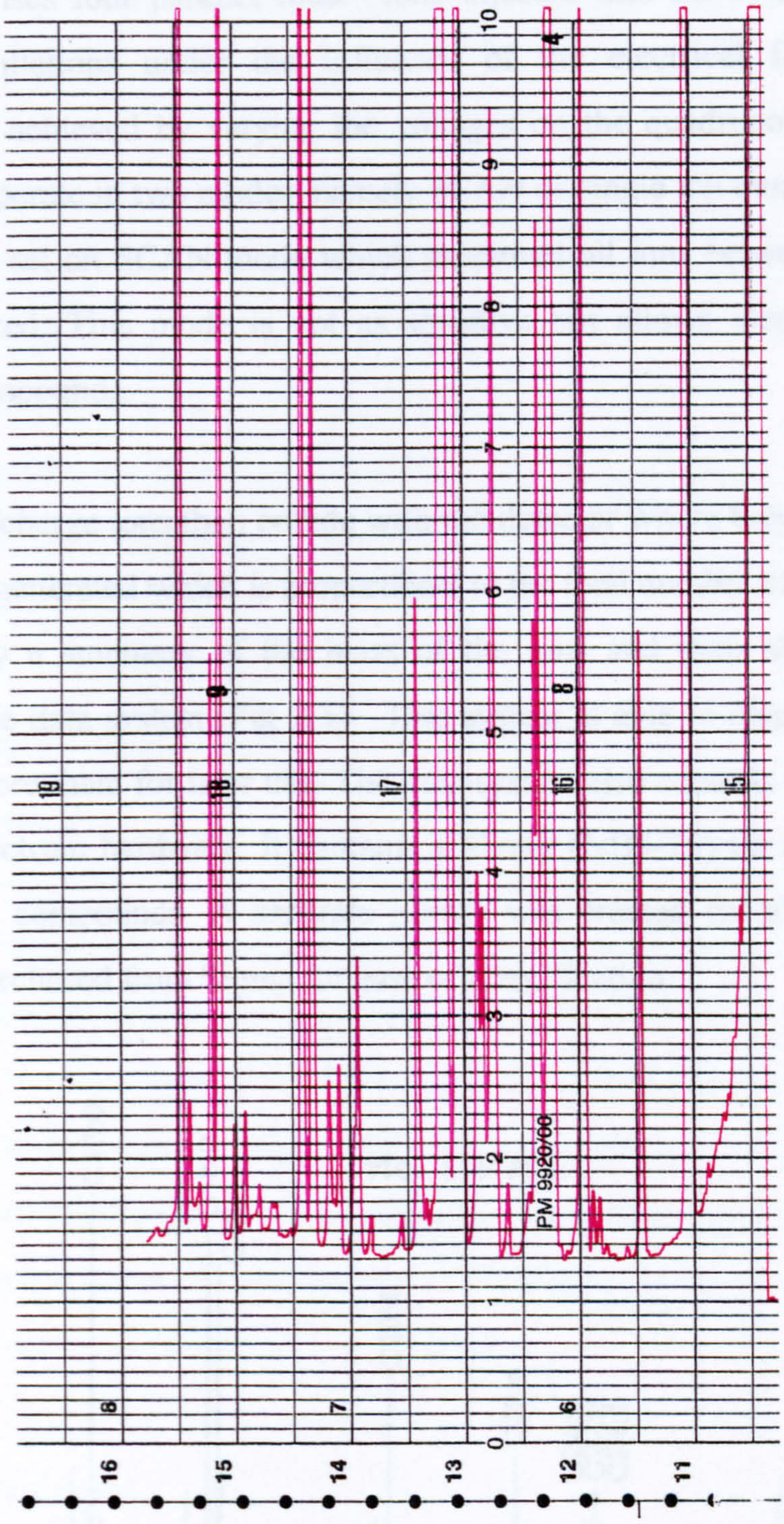


Figure 2.2 - GLC recording of cerebellar cortex phospholipid fatty acids

c) The measuring system used by Hewlett-Packard is a quadrupole analyser which comprises four parallel rods. Ions injected into the system undergo complex oscillations under the influence of the electrical field. Mass separation is achieved by varying the voltages on the quadrupole rods. The system can operate in two modes, namely SCAN or single ion monitoring. The analyser was set on SCAN mode which measured all ions between the mass ranges selected. This mode is not as sensitive but allows identification of unknown compounds.

d) The mass/charge ions then collide with the detector where they are counted and a signal generated which is proportional to the total number of ions of each mass. Finally a summary of the mass of the ions and their abundances is plotted by the data system (Fig 2.3). This system is able to display real time spectra or store them for later use. The data system also controls all aspects of the mass spectrum hardware. It contains a library (NBS-75) for identification of unknown compounds. A separate library was created for all the FAME standards purchased from Sigma for ease of identification.

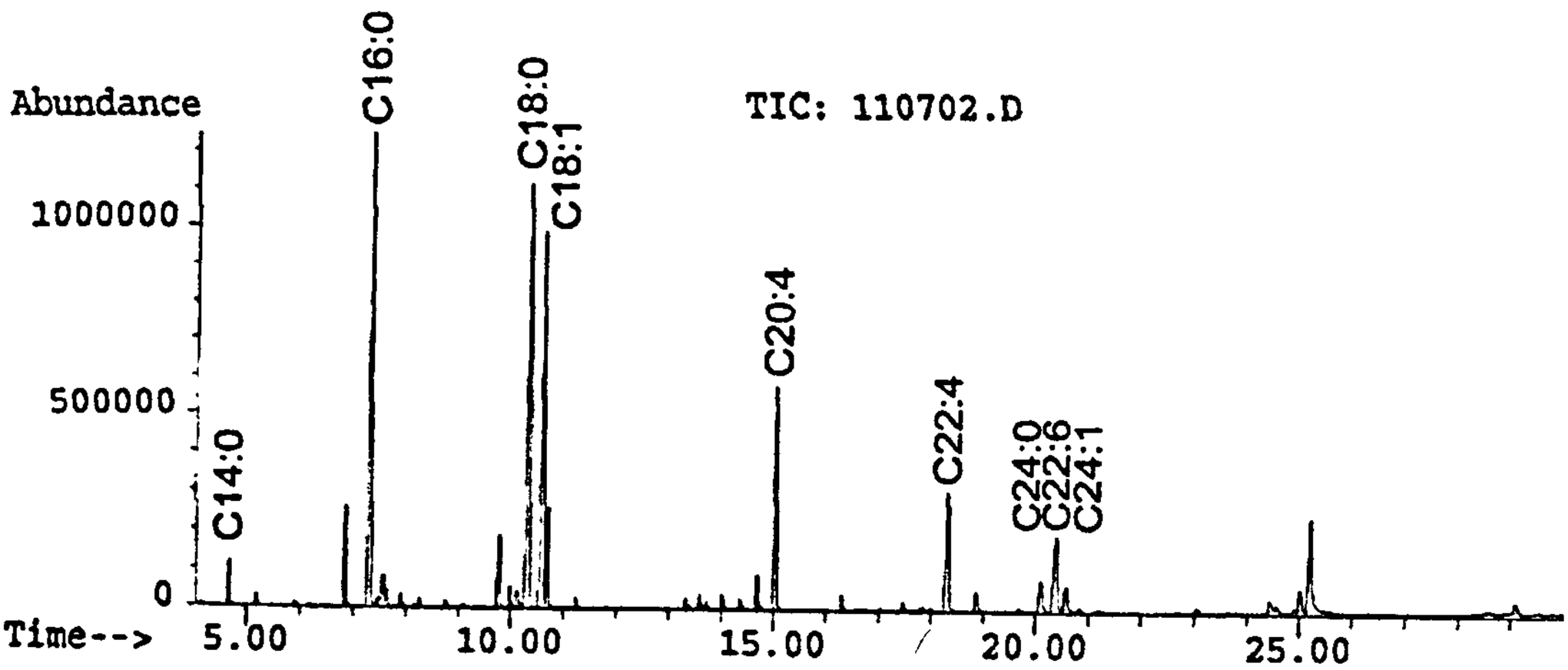


Figure 2.3 - GCMS recording of cerebral white matter total lipid fatty acids.

The Gas Chromatograph was fitted with a 30m x 0.32 mm capillary column with bonded PEG stationary phase (Supelco Omegawax 320) obtained from Supelco UK.

The following conditions were employed:-

Oven temperature - 150°C for 2 minutes then 5°/minute to 220°C followed by 10°/minute to 250°C for 5 minutes.

Injection temperature - 250°C

Detector temperature - 280°C

Carrier gas (Helium) flow rate - 1 ml/minute Split - 25:1

2 µl sample injected.

2.3.6. Method evaluation

a) Precision studies

The FAME standard was injected into both systems daily and this produced data for between batch reproducibility. To test for precision of the method as currently described, a large sample of cerebral cortex was extracted ten times in the same batch. This formed the basis for within batch analysis. For between batch precision studies, a separate piece of the same cerebral cortex tissue was extracted daily over a period of two weeks. A manufacturer's standard with quoted weight percentages of 37 fatty acids was injected into the GCMS as an accuracy check.

b) Recovery studies

For the method as a whole, recovery studies were carried out as previously described using radiolabelled fatty acid substrates (Abassi, PhD thesis) to assess the percentage of lipids extracted and therefore assess any losses at various stages of the extraction procedure.

c) Method comparison

Cerebellar cortex samples (n=30) were analysed by both GLC and GCMS systems and results compared. Regression analysis was carried out using the Microsoft Excel statistics program.

2.3.7. High performance thin layer chromatography

Chloroform extracts of cerebral parietal white and cerebellar white tissues were run on HPTLC plates. 1 µl (20 µg lipid) was spotted as a 1 cm long narrow band together with a mixed standard containing SPH, PC, PE, PS, PI, SU and GalCer prepared as follows. Each phospholipid standard was made up at a concentration of 5 mg/ml in $CHCl_3$ as were those of the cerebrosides and sulphatides but in $CHCl_3$/MeOH (1:1v/v). 100 µl of each solution were combined in a standard mixture and 2 µl (10 µg total lipid) of this run on each plate.

Plates were rinsed in hexane:diethyl ether and activated at 120°C for 1 hour. The TLC tank was lined with filter paper and allowed to equilibrate for at least 1 hour with 35 ml of solvent before introduction of the plates. These were run to 1 cm from the top of the plate (approximately 40 minutes), then removed from the tank and dried for 10 minutes at room temperature before heating in an oven at 110°C for 1 hour. After dipping in the developing reagent the plates were charred in an oven at 160°C for 20 minutes. A typical separation pattern is shown in Figure 2.4.

The resultant bands were quantified using a Shimadzu scanning densitometer at the following settings:-

Mode - reflectance Beam - Zigzag

Wavelength - 340 nm Band width - 8 mm

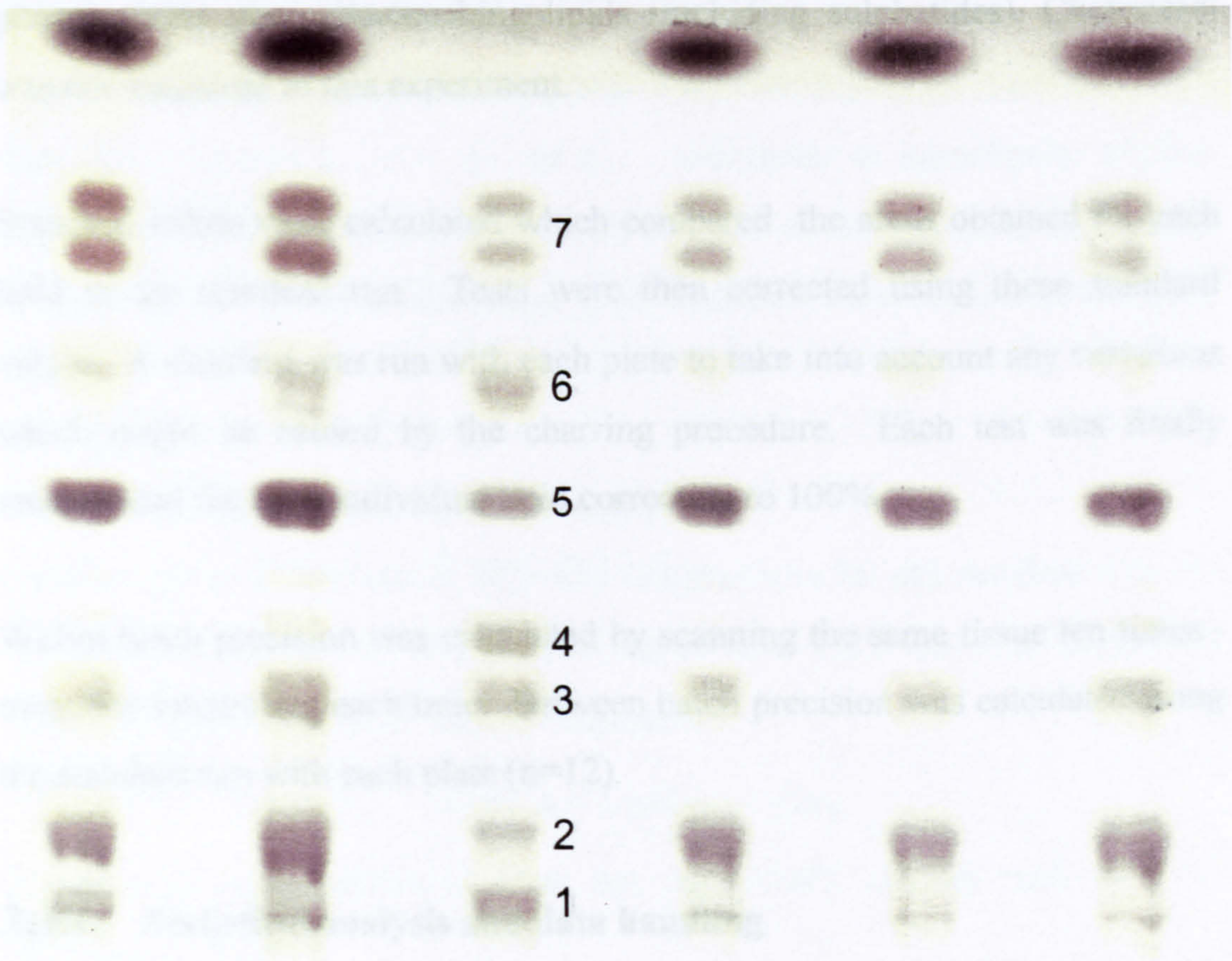


Figure 2.4 - HPTLC separation of phospholipids and glycosphingolipids

1 Sphingomyelin
2 PC
3 PS
4 PI
5 PE
6 Sulphatides
7 Galactocerebrosides

Baselines were drawn for each peak using manual integration. Peak areas for standards and tests were calculated for each individual lipid. Since it is known that the relationship between absorption and lipid concentration varies between different lipid classes, the total lipid was defined as the sum of the phospholipids plus galactosphingolipids (including sulphatides). Cholesterol was not measured in this experiment.

Standard values were calculated which compared the areas obtained for each lipid in the standard run. Tests were then corrected using these standard values. A standard was run with each plate to take into account any variations which might be caused by the charring procedure. Each test was finally recalculated for each individual lipid correcting to 100%.

Within batch precision was calculated by scanning the same tissue ten times - manually integrating each time. Between batch precision was calculated using the standard run with each plate (n=12).

2.3.8. Statistical analysis and data handling

The following statistical analyses were used in the evaluation of the results obtained in this study.

a) Students "t" test (two-tailed)

Between group differences in normally distributed fatty acid compositions were calculated by Students' t test where results were not age dependent.

$$\text{Values of } \mathbf{t} = \bar{x}_1 - \bar{x}_2 / \sqrt{SD_1^2/n_1 + SD_2^2/n_2}$$

where n_1 and n_2 are the group sizes and $\bar{x}_1$ and $\bar{x}_2$ group means.

Probabilities for each derived value of **t** were computed (Excel Version 7.0) for the appropriate degree of freedom ($df = n_1 + n_2 - 2$)

b) Non-parametric two tailed Mann Whitney U test

Between group differences in non-normally distributed fatty acid compositions (and which could not be transformed) were assessed by the Mann-Whitney U rank test.

$$U = (n_1 \times n_2) - c$$

where c = no. of times a value in group 1 is either higher (c^+) or lower (c^-) than that in group 2. $c = c^+$ or c^-, whichever is numerically smaller. Calculation is independent of group size (i.e. $n_1 \neq n_2$) and probabilities related to the calculated U values are found from statistical tables (Mann & Whitney, 1947).

c) Wilcoxon signed rank test (two-tailed)

Between group differences in fatty acid compositions for age-matched data (±1 week) were assessed by the Wilcoxon signed rank test. The sums of positive (T_+) and negative ranks (T_-) were calculated.

$T_+ + T_- = n(n+1)/2$ where n = number of pairs.

Probabilities were calculated from the numerically smaller rank sum (T) (Wilcoxon, 1945).

d) General linear model analysis of covariance (ANCOVA-GLM)

Between group differences in fatty acid compositions for non age-matched data were assessed by GLM analysis of covariance when results were both age dependent and not normally distributed. Application was restricted to log-transformed data and to dietary and age effects over narrow age ranges (<20 weeks). Age measurements as the independent variable (x) were considered error free. Significances were calculated using Minitab Version 10.

e) Bonferroni correction

Multiple group comparisons of normally distributed results were subject to the Bonferroni correction which essentially increased the requirement for

significance from the standard $p<0.05$ to $p<0.05/n$ where n = number of comparisons.

f) Chi-square test *

This test was applied to assess differences in dichotomous variables (sex ratio) between feeding groups. It was calculated using Microsoft Excel 7.0.

g) Correlation coefficient (Age)

Age was considered to be the only major confounding covariate in this dietary fatty acid study. Because of the relatively small group sizes, only where age was found to correlate strongly ($p<0.01$), either directly or inversely with the concentrations of any given fatty acid, no further between group statistical analysis was undertaken with unpaired data.

h) Regression analysis

This was used in the assessment of the GCMS and GLC tissue fatty acid compositions. Resultant regression equations were calculated using Microsoft Excel Version 7.0.

i) Graphical presentation - polynomials

Where fatty acid concentrations varied in a curvilinear manner with age, whenever appropriate, polynomial equations for best-fit curves were derived (Microsoft Excel Version 7.0) and presented in graphical form. Otherwise fatty acid concentrations with respect to age and dietary group were presented as non-delineated scattergrams.

* Assessment of group gender differences with respect to fatty acid compositions did not however come within the scope of this work.

CHAPTER 3

RESULTS

3.1. Method evaluation

This section presents the results of method evaluations carried out during the course of this study.

3.1.1. One dimensional chromatography

The results for the cerebral cortex tissues analysed by this technique are shown in Tables 3.1 & 3.2. The SEM for the individual fatty acids in the different groups were variable and unacceptably large in a few cases. It was observed that the technique was not reproducible and did not always achieve complete separation of individual phospholipids.

Table 3.1 Cerebral cortex phosphatidylcholine fatty acid results by 1-D TLC

PC	*Feed*				*Fatty*	*Acid*			
		C14:0	C16:0	C16:1	C18:0	C18:1	C20:4	C22:4	C22:6
	Breast								
Mean	n=5	1.6	39.7	5.3	15.6	27.9	5.4	1.5	2.1
SEM		0.33	4.33	0.47	3.33	1.49	0.26	0.43	0.56
	SMA								
Mean	n=5	1.7	41.7	5.0	16.9	22.6	5.9	2.3	3.1
SEM		0.18	0.60	0.38	0.69	1.18	0.35	0.11	0.35
	CGOST								
Mean	n=5	1.5	42.6	5.4	13.7	25.6	5.9	1.9	1.9
SEM		0.45	2.40	0.34	1.70	1.82	0.35	0.64	0.68

Table 3.2 Cerebral cortex phosphatidylethanolamine fatty acid results by 1-D TLC

PE	*Feed*		*Fatty*	*Acid*			
		C16:0	C18:0	C18:1	C20:4	C22:4	C22:6
	Breast						
Mean	n=5	5.0	15.4	12.9	9.6	12.4	10.4
SEM		0.65	3.60	1.71	1.95	0.95	1.25
	SMA						
Mean	n=5	7.2	22.1	11.1	15.5	10.6	9.8
SEM		0.46	2.05	1.45	0.56	0.85	0.82
	CGOST						
Mean	n=5	5.5	19.0	15.7	15.7	13.0	8.1
SEM		0.17	1.88	0.73	0.73	0.81	0.60

3.1.2. Recovery studies

Table 3.3 presents the results for recovery studies carried out by Abassi (1989). These were performed to assess losses at each stage of the extraction procedure.

Table 3.3 Recovery of labelled L-3-PC, 1-stearyl, 2(1-^{14}C) arachidonyl after chloroform:methanol extraction

Tube	Activity (cpm)	Recovery (%)
Before extraction	**527896**	
1st $CHCl_3$ extract	**440018**	**83.4**
2nd $CHCl_3$ extract	**26740**	**5.1**
overall	**466758**	**88.4**

Results from Abassi, PhD thesis, 1989.

3.1.3. Precision studies

Within batch and between batch recovery studies for tissue extraction and derivatisation are given in Tables 3.4 & 3.5 respectively. FAME were subsequently analysed by GCMS. The coefficients of variation for all fatty acids are in general below an acceptable 5% level. On the only occasions in which the CV exceeded 5% the fatty acid concentration represented less than 2% of total fatty acids.

Table 3.4 Within batch precision results (GCMS analysis)

Fatty Acid	*C14:0*	*C16:0*	*C16:1*	*C18:0*	*C18:1*	*C20:3*	*C20:4*	*C22:4*	*C22:5*	*C22:6*
Mean (n=10)	1.17	30.38	2.78	25.24	16.12	0.59	9.23	5.25	1.76	7.66
S.D.	0.078	0.444	0.109	0.312	0.473	0.021	0.076	0.061	0.105	0.112
C.V.	6.67	1.46	3.99	1.24	2.93	3.56	0.81	1.16	5.97	1.45

Table 3.5 Between batch precision results (GCMS analysis)

Fatty Acid	*C14:0*	*C16:0*	*C16:1*	*C18:0*	*C18:1*	*C20:3*	*C20:4*	*C22:4*	*C22:5*	*C22:6*
Mean (n=10)	1.49	30.01	3.21	22.54	15.44	0.65	10.70	5.57	1.82	8.56
S.D.	0.060	0.519	0.156	0.427	0.703	0.037	0.138	0.137	0.069	0.355
C.V.	4.03	1.73	4.86	1.89	4.64	5.67	1.29	2.46	3.79	4.15

Table 3.6 summarises a similar within batch experiment where the analysis was performed by GLC. In most cases, the CV values are higher than those for the GCMS more especially again for those fatty acids which are present at low concentration.

Table 3.6 Within batch precision results (GLC analysis)

Fatty Acid	*C14:0*	*C16:0*	*C16:1*	*C18:0*	*C18:1*	*C20:3*	*C20:4*	*C22:4*	*C22:5*	*C22:6*
Mean (n=7)	0.52	27.97	2.06	21.57	17.30	0.36	12.43	6.17	2.47	8.63
S.D.	0.086	0.818	0.079	0.431	0.245	0.074	0.315	0.330	0.111	0.450
C.V.	16.54	2.92	3.83	2.00	1.42	20.56	2.54	5.35	4.49	5.21

Between batch studies for sampling precision only were also carried out by GC and GCMS, using the FAME standard which was injected daily into both systems and the results obtained during one month (n=20) are illustrated in Table 3.7.

Table 3.7 FAME standard precision studies

Fatty Acid	*C16:0*	*C16:1*	*C18:0*	*C18:1*	*C20:4*	*C22:6*	*C24:0*	*C24:1*
C.V. (GLC)	2.91	2.83	1.77	1.92	2.34	3.53	-	-
C.V. (GCMS)	1.54	1.47	1.56	0.88	1.62	1.01	1.27	1.00

The FAME standard may illustrate improved precision associated with GCMS automatic sampling. The FAME standard was also used to calculate detector response factors for each fatty acid by both GLC and GCMS analysis.

A gravimetric standard with known weight percentages of 37 fatty acids (Supelco 37) became available through the latter part of the experimental work and was run on the GCMS as a test of accuracy of the system. The results (wt %) obtained are in close agreement with the manufacturer's quoted values and both are given in Table 3.8.

Table 3.8 Analysis of Supelco 37 standard by GCMS

Fatty Acid	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C20:3	C20:4	C22:6	C24:0	C24:1
GCMS	3.87	5.99	1.98	4.20	3.96	1.97	1.90	1.95	1.96	4.28	2.05
Supelco	4	6	2	4	4	2	2	2	2	4	2

Results for Supelco standard and GCMS values given as weight %.

3.1.4. Method comparison

Regression analysis was used to assess results of GLC and GCMS fatty acid compositions. Figure 3.1 comparing cerebellar grey docosahexaenoic acid gives an example of the graph obtained, including the curve equation and regression coefficient. The equations and regression coefficients for the remaining fatty acids are given in Table 3.9.

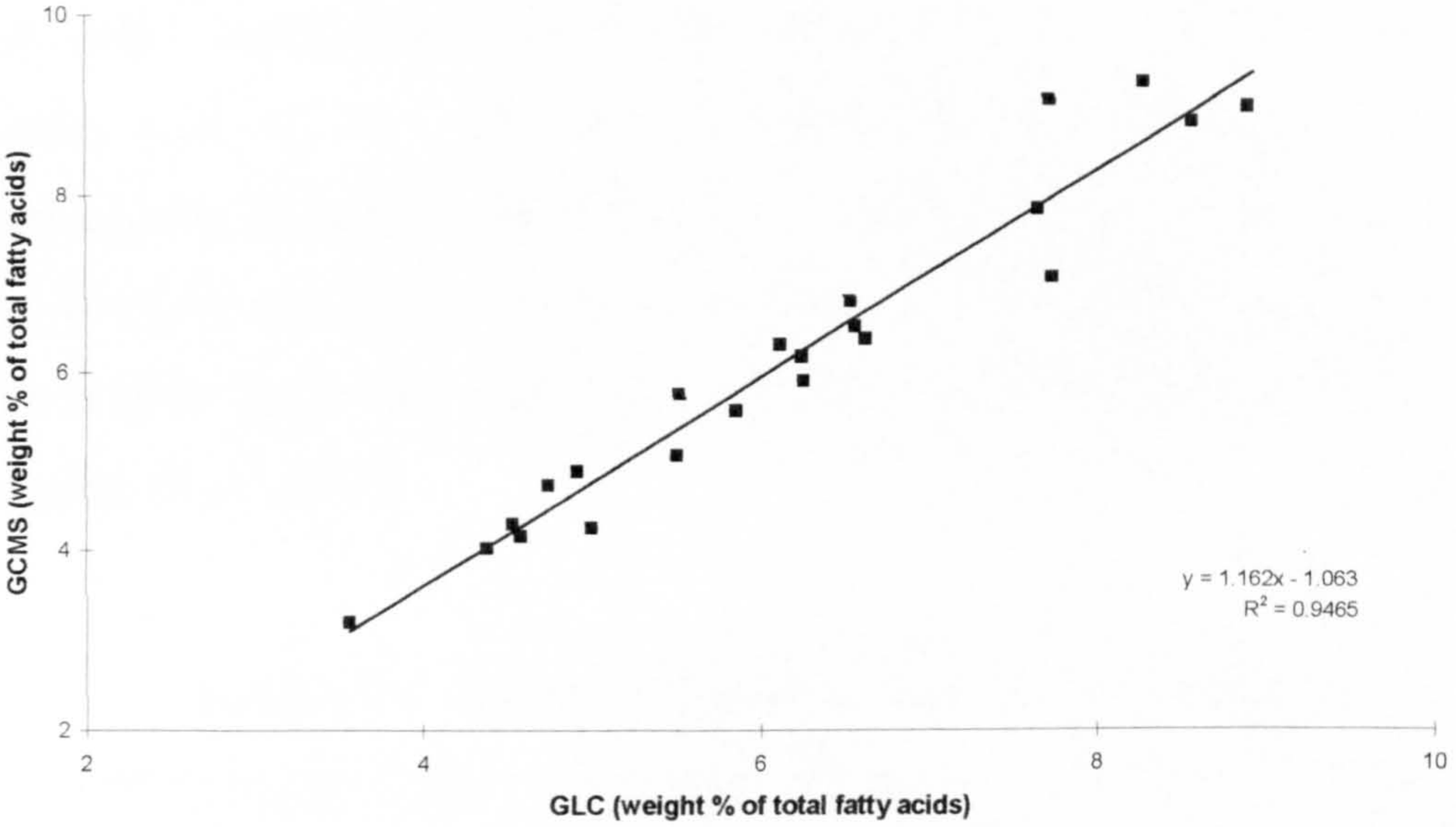


Figure 3.1 -Regression analysis of cerebellar cortex docosahexaenoic acid

Table 3.9 Regression analysis for cerebellar cortex tissues

Fatty Acid	Equation of the line	R^2
C16:0	y = 0.6526x + 11.135	0.5476
C16:1	y = 0.8525x – 0.2528	0.8877
C18:0	y = 0.8200x + 4.2048	0.6638
C18:1	y = 0.6388x + 6.3687	0.8200
C18:2n-6	y = 0.7958x – 0.1132	0.9517
C20:3n-6	y = 1.0623x – 0.3284	0.9690
C20:4n-6	y = 0.7038x + 3.5729	0.8044
C22:4n-6	y = 0.7084x + 1.3365	0.7960
C22:5n-6	y = 0.8260x – 0.1625	0.9485
C22:6n-3	y = 1.1621x – 1.0630	0.9465

y = GCMS values ; x = GC values.

3.1.5. HPTLC precision studies

The within batch and between batch precision study results are shown in Tables 3.10 and 3.11. The between batch analysis in which the mixed lipid standard was analysed on each plate gave satisfactory CVs. The results for the within batch studies where the same tissue was scanned several times were also in good agreement with a CV >5% only for PI which was present in low concentration (~2%).

Table 3.10 Between Batch HPTLC Precision Results

	SPH	*PC*	*PS*	*PI*	*PE*	*SU*	*GalCer A*	*GalCer B*
Mean (n=12)	13.80	10.30	13.36	13.44	13.03	15.20	10.64	9.91
S.D.	0.471	0.440	0.762	0.354	0.497	0.296	0.692	0.508
C.V.	3.41	4.27	5.70	2.63	3.81	1.95	6.50	5.13

Table 3.11 Within Batch HPTLC Precision Results

	SPH	*PC*	*PS*	*PI*	*PE*	*SU*	*GalCer A*	*GalCer B*
Mean (n=10)	6.57	21.82	14.63	2.17	26.87	7.53	10.87	9.06
S.D.	0.112	0.332	0.361	0.123	0.348	0.137	0.152	0.385
C.V.	1.70	1.52	2.47	5.67	1.30	1.82	1.40	4.25

Note that GalCer A and GalCer B represent hydroxy and non-hydroxy galactocerebrosides.

3.2. Milk analysis results

The breast milk fatty acid results from a local population of mothers together with those of the formula milks are given in Table 3.12. These were analysed as a comparison to published ranges. (Table 2.3 - Methods chapter)

Table 3.12 Milk fatty acid compositions in relation to diet (weight % of total fatty acids)

Fatty Acid		*Milk Diet*			
		Breast Milk	*SMA*	*CGOST*	*C&G*
Myristic	C14:0	3.8-11.5	4.49	4.50	4.68
Palmitic	C16:0	20.3-29.0	22.03	18.25	19.64
Palmitoleic	C16:1n-7	1.5-3.9	0.18	0.09	0.23
Stearic	C18:0	4.6-9.5	3.86	3.60	4.05
Oleic	C18:1n-9	31.3-43.4	38.98	43.32	46.52
Linoleic	C18:2n-6	6.3-15.7	18.15	14.93	11.35
α-Linolenic	C18:3n-3	0.5-1.2	1.72	0.07	2.12
Dihomo-γ-linolenic	C20:3n-6	0.20-0.77	<0.05	<0.05	<0.05
Arachidonic	C20:4n-6	0.31-0.79	<0.05	<0.05	<0.05
Docosahexaenoic	C22:6n-3	0.12-0.50	<0.05	<0.05	<0.05
Arachidic	C20:0	0.15-0.32	0.35	0.89	0.36
Eicosenoic	C20:1n-9	0.40-0.74	0.18	0.67	0.34
Behenic	C22:0	0.10-0.22	0.31	1.45	0.26
Erucic	C22:1n-9	0.08-0.23	<0.05	<0.05	<0.05
Lignoceric	C24:0	<0.05-0.19	0.12	0.65	0.12
Nervonic	C24:1n-9	<0.05-0.32	<0.05	<0.05	<0.05

Breast milk results are quoted as weight % ranges for 13 mature milks from a local population of mothers.
SMA(Wyeth, UK) White Cap, C&G - Cow & Gate Premium (post 1990), CGOST - Farley's Ostermilk 2 - results represent laboratory analysis of manufacturers' milks and are given as means of duplicate analysis.

3.3. Study results

The following section details the study results and has been divided into subsections for each individual tissue.

3.3.1. Cerebral cortex total phospholipid fatty acids

Table 3.13 illustrates the infant feeding group characteristics. Between group differences for the continuous variables were assessed by Student's t test and those for dichotomous variables (male/female ratios) were estimated by the Chi-square test. No significant differences between feeding groups in birth weight was found although the mean birth weights of the formula fed infants were noted to be lower than that of the breast fed group. However there were differences observed in male/female ratios between the breast-fed group and both the SMA and CGOST groups ($p<0.02$) with a preponderance of males in the formula groups and females in the breast fed group.

Table 3.13 Infant details for cerebral cortex tissues

Infant Data	*Milk Diet*				
	Breast Fed	***SMA***	***CGOST***	***C&G***	***SMA Prem***
Birth weight (g)	3324 (530)	2997 (397)	2902 (530)	2978 (169)	1968 (427)
Gestational age (wk)	39.9 (1.4)	39.1 (1.3)	38.8 (1.6)	38.5 (0.5)	33.4 (2.1)
Age (wk)	11.2	14.4	16.4	18	15.4
Age range (wk)	1-38	0.5-43	6.5-40	5-33	7-25
Male/Female	4/9	12/4 $^{\alpha}$	7/4 $^{\alpha}$	2/2	4/1

Results are shown as means with standard deviations in parenthesis.
Dichotomous variables were analysed by χ^2 test and applied only to male/female ratios.
$\alpha = p<0.02$ for significant differences between the breast feed and formula fed (SMA and CGOST) groups.

Table 3.14 lists the cerebral cortex total phospholipid fatty acid results for each individual in the study (See appendix). Each fatty acid concentration (wt %) was recalculated after correcting for the detector response and the total

rounded to 100%. As individuals could not be age-matched between groups, the degree of correlation of each fatty acid with age was estimated. Significant correlations were taken as $p<0.01$ (Table 3.15) and in the case of palmitic acid and palmitoleic acid no statistical analysis was possible. Results showing means and standard errors for total phospholipid fatty acids in the cerebral parietal cortex in relation to feeding groups are given in Table 3.16. Student's t test was performed to compare values for the breast fed group with the SMA and the CGOST groups. In addition a similar comparison was made between the SMA group and the CGOST and SMA preterm groups. Values for "p" of <0.02 were taken to imply significant differences between feeding regimens after application of the Bonferroni correction for multiple group comparisons. The results for the C&G group (n=4) are also provided for information without statistical analysis due to the group size. In figures 3.2 - 3.11, each individual fatty acid is plotted with respect to diet and age.

Table 3.15 Correlation coefficients for cerebral cortex fatty acid concentration vs age in relation to feeding group

Fatty Acid	Breast	SMA	CGOST	C&G	SMA Prem
	r	r	r	r	r
C16:0	-0.6953 $^{\delta}$	-0.8014 $^{\delta}$	-0.7387 $^{\delta}$	-0.9499 $^{\delta}$	-0.8901 $^{\delta}$
C16:1 n-7	-0.9196 $^{\delta}$	-0.7801$^{\delta}$	-0.8855 $^{\delta}$	-0.6028	-0.7087
C18:0	-0.0099	0.4145	0.3983	0.2788	0.6245
C18:1n-7+n-9	0.4758	0.5844	0.4747	0.9577 $^{\delta}$	-0.0009
C18:2 n-6	0.7719 $^{\delta}$	0.2662	0.6504	0.4501	0.3782
C20:3 n-6	0.6625 $^{\delta}$	0.3257	-0.1092	0.2795	-0.0916
C20:4 n-6	0.4980	0.6015	0.5425	0.1367	0.6369
C22:4 n-6	-0.1995	0.3859	0.4210	0.4782	0.7954
C22:5 n-6	-0.3848	0.6079	0.7553 $^{\delta}$	0.1592	0.9734 $^{\delta}$
C22:6 n-3	0.5327	-0.4680	-0.3162	-0.2524	-0.3436

δ = Significant correlation of individual fatty acid concentrations (wt %) with age. ($p<0.01$)

In this study when statistical analysis was possible, neither of the saturated (stearic acid; Fig 3.4) nor monounsaturated (oleic acid; Fig 3.5) fatty acids showed any significant differences between feeding groups (Table 3.16). As previously stated however, relative amounts of the equivalent C16 palmitic (Fig3.2) and palmitoleic acids (Fig 3.3) were found to correlate negatively with age; palmitic acid falling from 31% to 26% over the first forty weeks and palmitoleic acid falling from 3% to less than 1%.

Table 3.16 Cerebral cortex phospholipid fatty acid compositions

Fatty Acid	***Milk Diet***				
	Breast n=13	***SMA n=16***	***CGOST n=11***	***C&G n=4***	***SMA Prem n=5***
C16:0	29.02 (0.23)	28.59 (0.45)	27.27 (0.73)	28.35 (1.35)	27.99 (0.89)
C16:1n-7	2.12 (0.18)	1.94 (0.26)	1.70 (0.26)	1.75 (0.33)	2.06 (0.48)
C18:0	22.09 (0.24)	21.93 (0.28)	21.97 (0.37)	22.66 (0.59)	21.71 (0.48)
C18:1n-7+n-9	15.95 (0.42)	15.63 (0.33)	16.61 (0.39)	16.62 (1.23)	16.92 (0.71)
C18:2n-6	0.44 (0.06)	0.58 (0.04)	0.58 (0.05)	0.52 (0.06)	0.67 (0.11)
C20:3n-6	1.23 (0.11)	1.57 (0.11)	1.84 (0.11)	1.57 (0.18)	1.60 (0.17)
C20:4n-6	11.29 (0.28)	12.26 (0.28)	12.48 [b] (0.35)	11.56 (0.48)	12.66 (0.54)
C22:4n-6	6.24 (0.17)	6.69 (0.11)	6.90 [b] (0.13)	6.24 (0.14)	6.47 (0.38)
C22:5n-6	2.03 (0.14)	2.85 [a] (0.16)	3.85 [a,c] (0.37)	2.14 (0.31)	3.10 (0.53)
C22:6n-3	9.66 (0.22)	7.96 [a] (0.22)	6.85 [a,c] (0.38)	8.60 (0.50)	6.57 (1.04)

Fatty acid results are expressed as weight percentages of total fatty acids and are given as means and standard errors. Significant between group differences calculated by Student's t test are given by:- a = $p<0.001$; b = $p<0.01$ for BF vs formula fed (SMA and CGOST) groups and c = $p<0.02$ for SMA vs CGOST. No significant differences were found between the SMA and SMA preterm group.

Although linoleic acid concentrations (Fig 3.6) were in general lower in the breast fed infants than those fed formula milk, neither essential fatty acid, linoleic nor α-linolenic, was a prominent feature of the infant cerebral cortex.

Examination of the n-6 series fatty acids derived from linoleic acid showed that dihomo-γ-linolenic acid concentrations (DGLA; C20:3n-6) (Fig 3.7) were greater in both SMA-fed and CGOST-fed infants compared with those breast fed. A positive correlation of fatty acid concentrations with age in the breast fed group ($r=0.6625$, $p<0.01$) however precluded statistical analysis of the data. In addition no significant difference was found between the SMA group and the SMA preterm group.

Arachidonic acid concentrations (Fig 3.8) were significantly lower in the breast fed than CGOST group ($p<0.01$) and although also lower in the SMA group, the difference was not significant ($p=0.0238$). No significant differences existed between the SMA and CGOST formula groups nor the term and preterm SMA fed infants. The arachidonic acid compositions of the small C&G group tended to align more closely with the breast fed infants. The relative amount of docosatetraenoic acid (DTA; C22:4n-6) (Fig 3.9) was significantly greater in the CGOST-fed than the breast-fed group ($p <0.01$), and although also higher in the SMA-fed group than breast fed the difference was not significant. Here again none of the preterm infants showed obvious differences when compared to their term contemporaries.

Although CGOST docosapentaenoic acid concentrations (Table 3.15) were found to correlate strongly with age, nevertheless, the results were considered to represent skewed data resulting primarily from dietary rather than age-related effects and are discussed in that context.

Docosapentaenoic acid values (Fig 3.10) were significantly greater in both SMA-fed ($p<0.001$) and CGOST-fed babies ($p<0.001$) when compared with those breast-fed. As the data in the CGOST group were skewed, results were also compared by the non-parametric Mann-Whitney U test. Similar levels of significance between groups were confirmed (BF vs CGOST; $p<0.001$). Also the CGOST-fed infants had higher DPA concentrations than the SMA-fed ($p<0.02$). Again similar C22:5n-6 results were obtained for the C&G group ($\bar{x}= 2.14$) as for the breast fed group ($\bar{x}= 2.03$). This finding was also evident in C22:5n-6 concentrations of both partially breast fed infants. Results of the SMA group were not significantly different from those of the SMA preterm group and that of the CGOST fed preterm infant comparable to that of its term born counterparts (Fig 3.10).

Docosahexaenoic acid values were greater in the breast-fed group than in the SMA ($p<0.001$) and CGOST group ($p<0.001$) (Fig 3.11). DHA concentrations were also significantly greater in the SMA-fed than CGOST-fed infants ($p<0.02$). In the youngest infants the DHA concentrations in both the C&G fed and mixed-fed infants were adjacent to those of exclusively breast fed infants although it was noted that the lowest DHA concentration in the C&G group was found in the 33 week old infant (7.4%). DHA concentrations were lower in the SMA preterm than the SMA group but did not attain significance. The lowest DHA concentration was found in the 19 week old CGOST preterm infant (4.23%) although similarly "low" levels were encountered in the 7 week old CGOST term infants of relatively low birth weights of 2.50 kg and 2.53 kg respectively. The SMA fed preterm infant born at 30 weeks gestation and who died at 10 weeks had a DHA concentration of 4.27% together with detectable concentrations of Mead acid (1.3%) and dihomo Mead acid (0.9%). These two fatty acid were not found in other infant tissues.

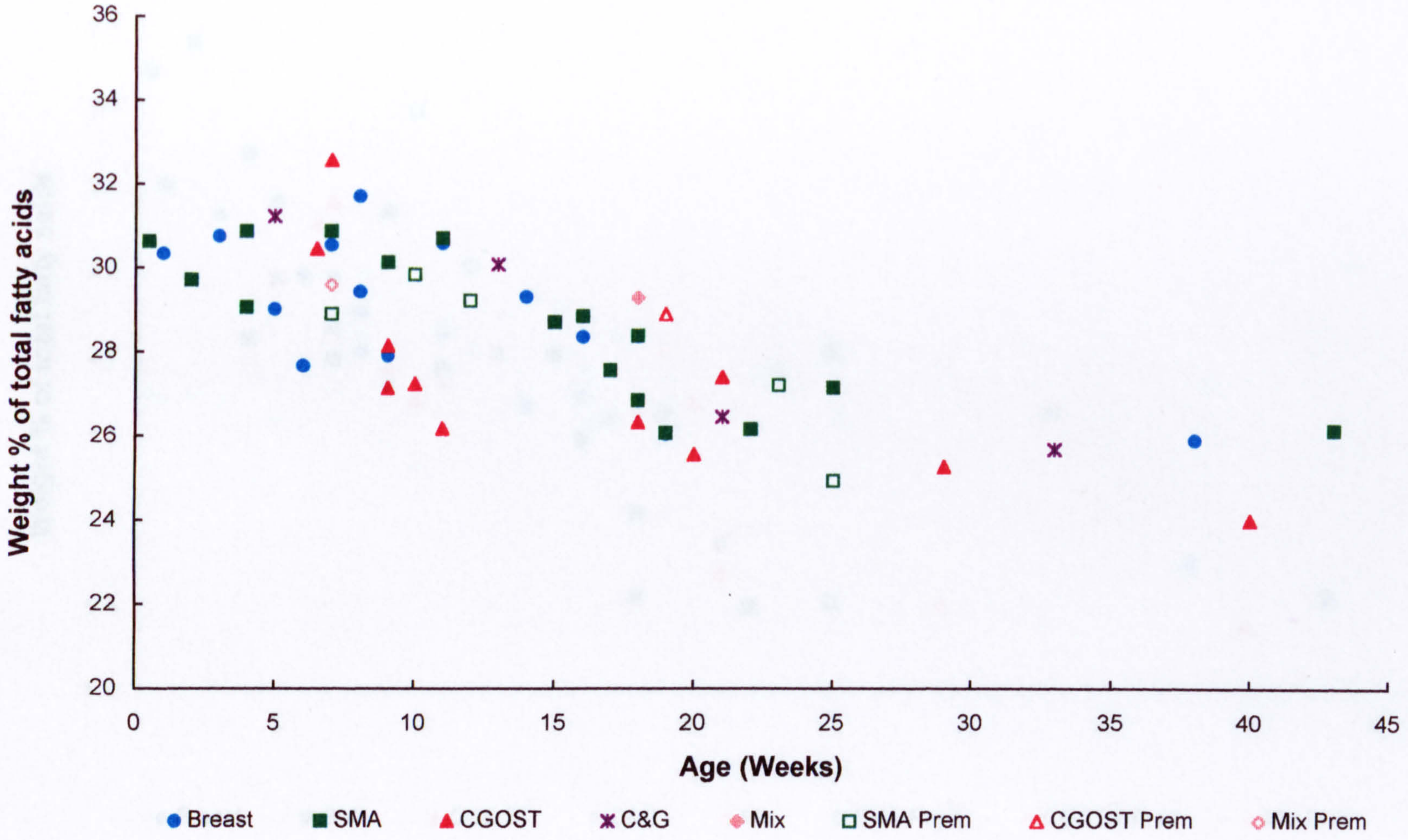


Figure 3.2 - Cerebral cortex phospholipid palmitic acid (C16:0) in relation to infants' diet and age.

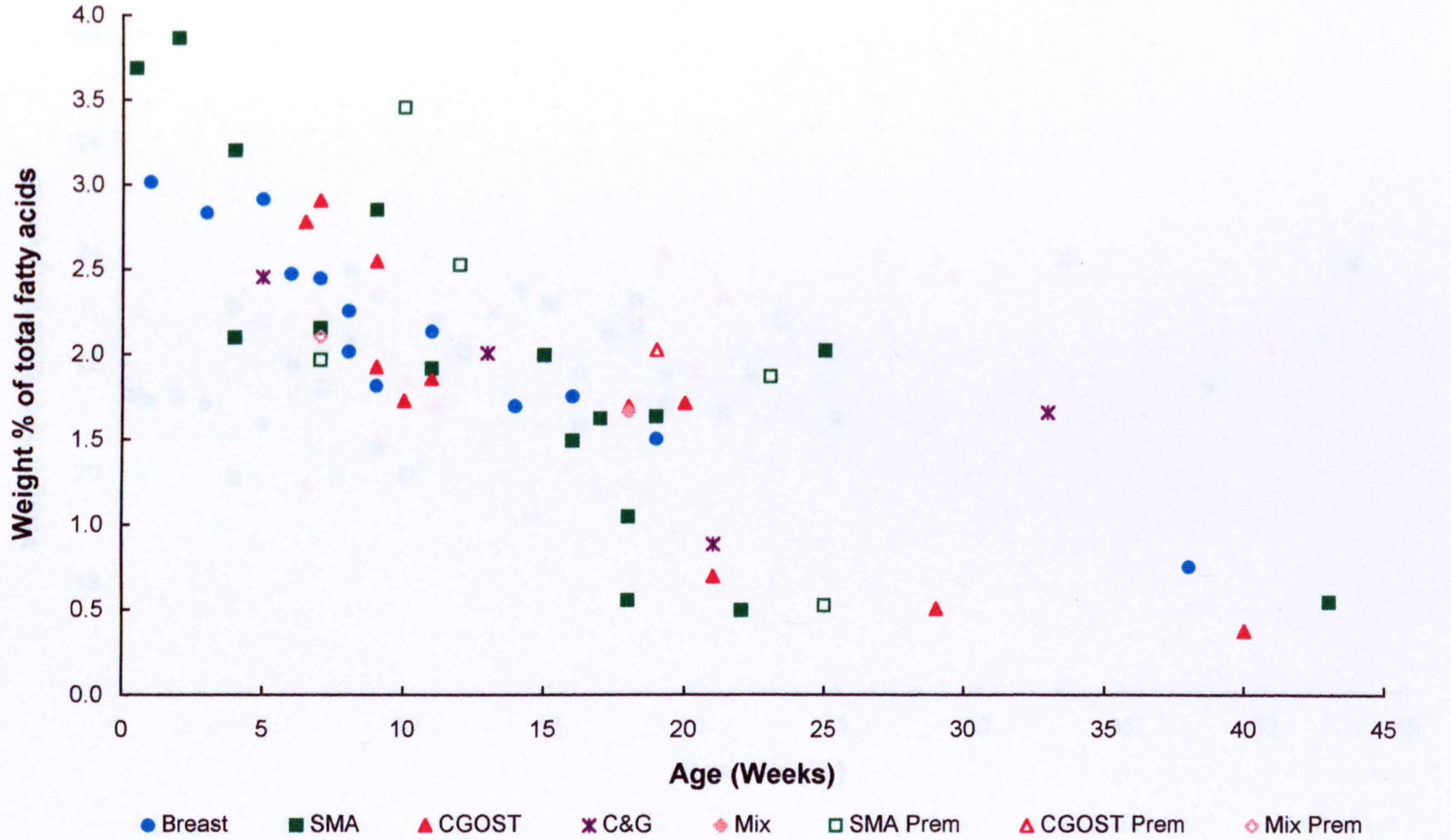


Figure 3.3 - Cerebral cortex phospholipid palmitoleic acid (C16:1n-7) in relation to infants' diet and age.

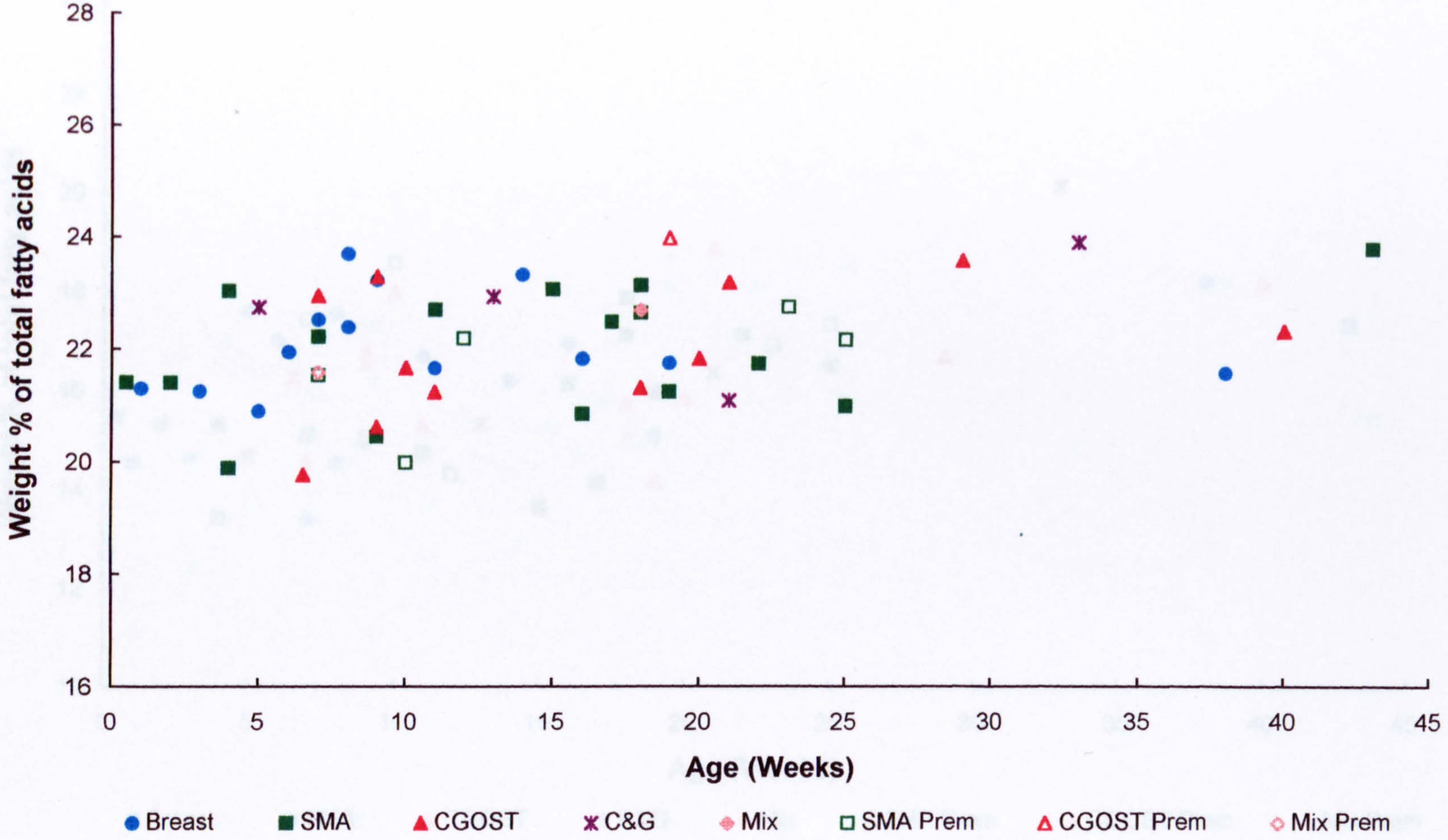


Figure 3.4 - Cerebral cortex phospholipid stearic acid (C18:0) in relation to infants' diet and age.

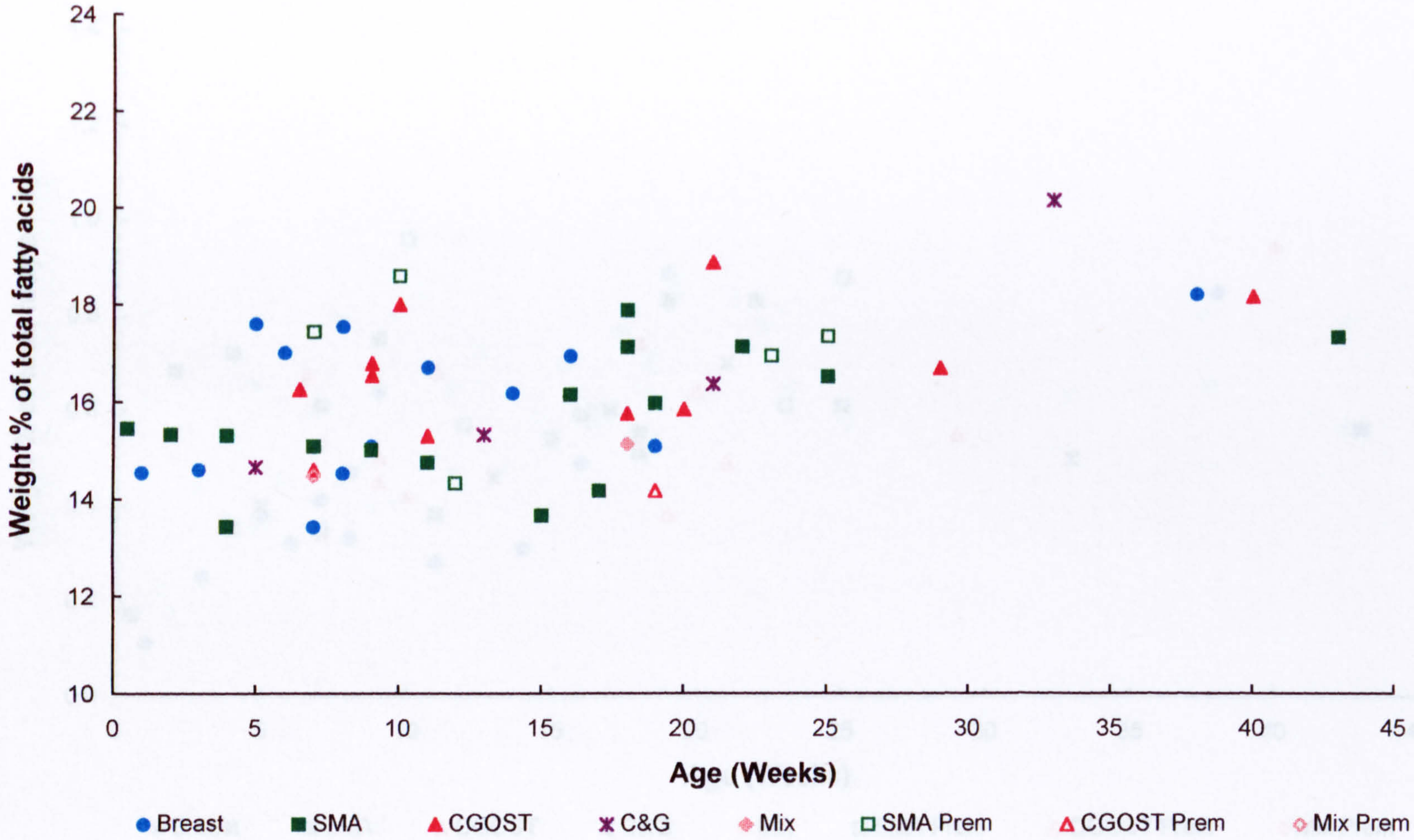


Figure 3.5 - Cerebral cortex phospholipid oleic acid (C18:1n-7+n-9) in relation to infants' diet and age.

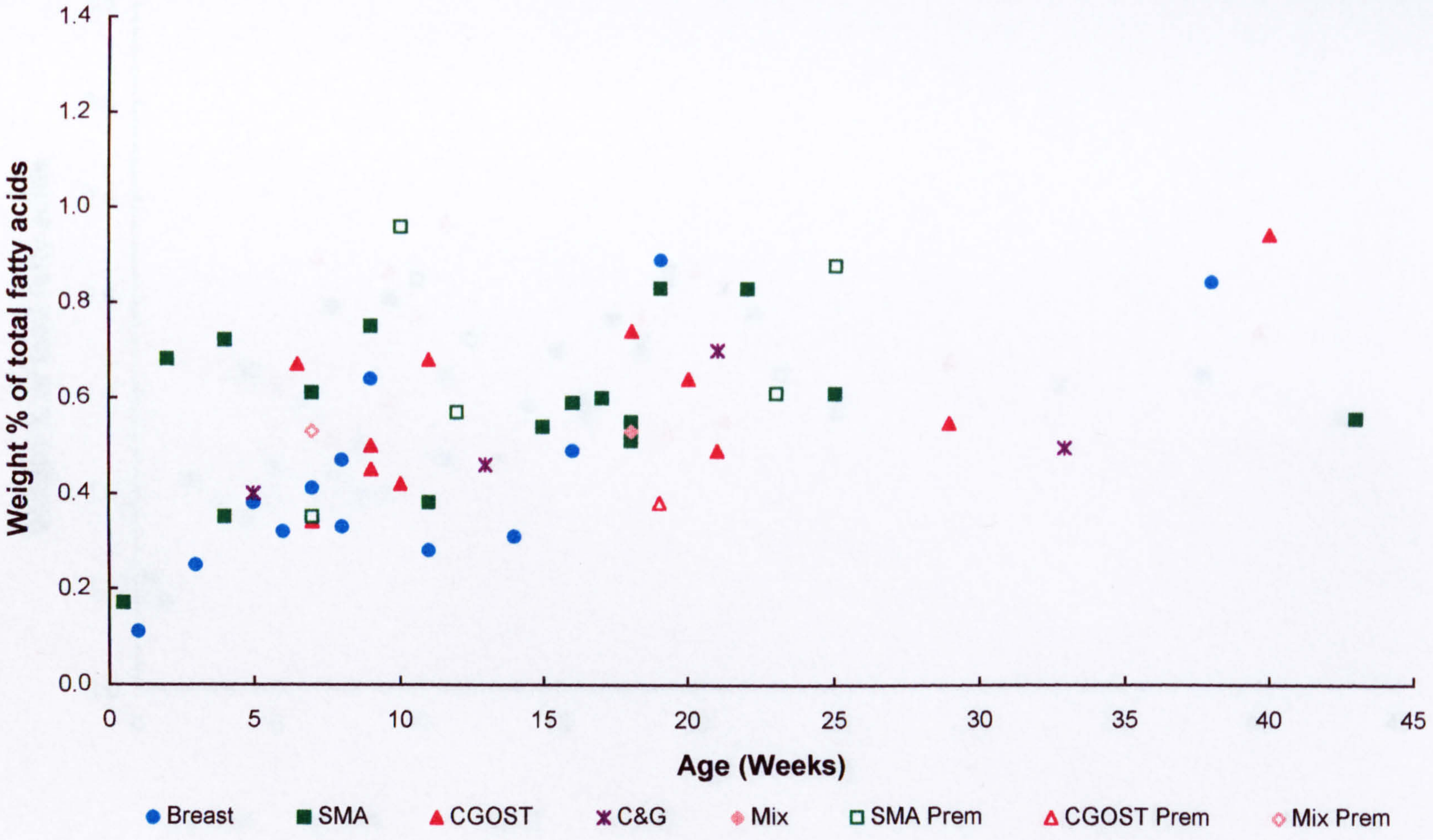


Figure 3.6 - Cerebral cortex phospholipid linoleic acid (C18:2n-6) in relation to infants' diet and age.

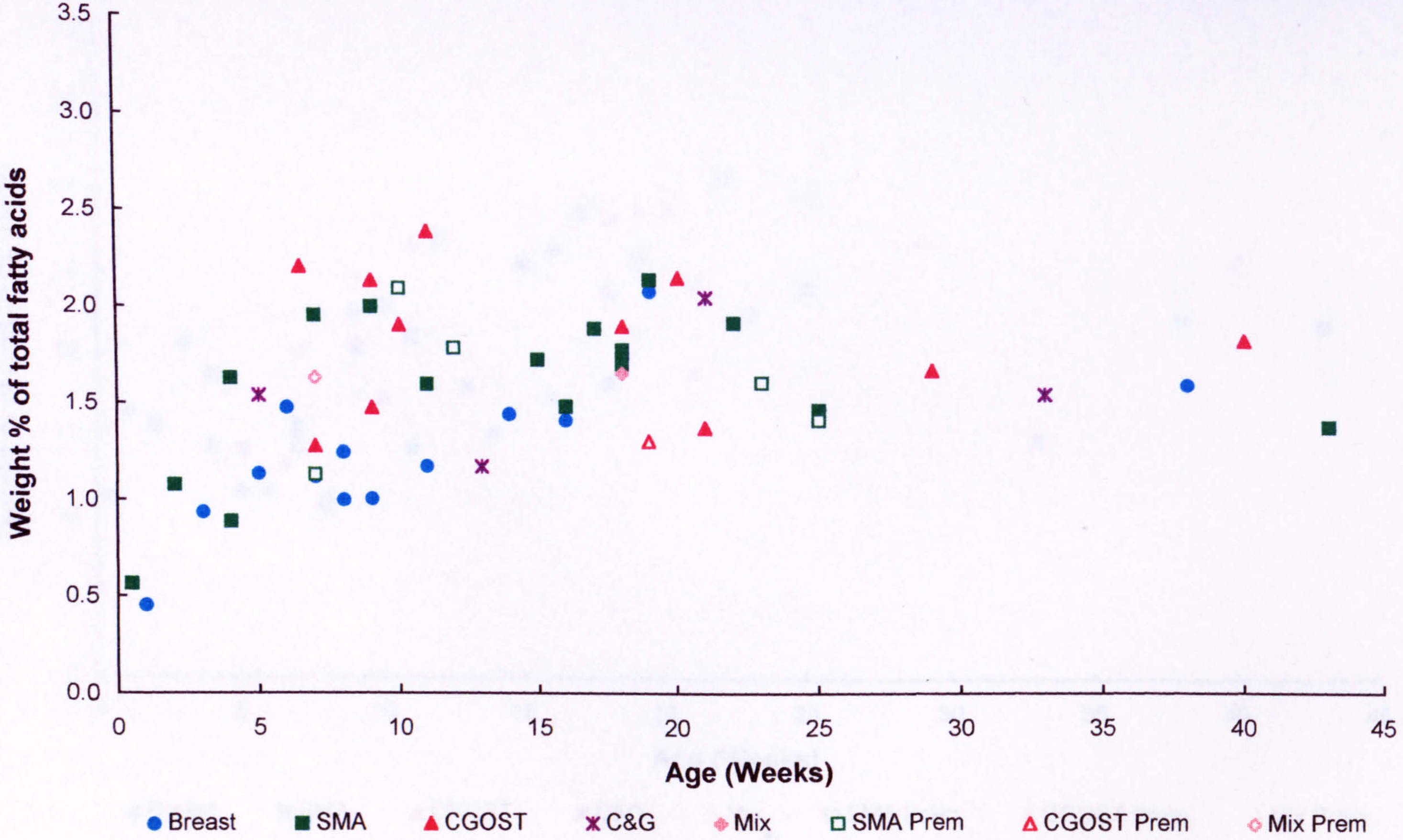


Figure 3.7 - Cerebral cortex phospholipid dihomogammalinolenic acid (C20:3n-6) in relation to infants' diet and age.

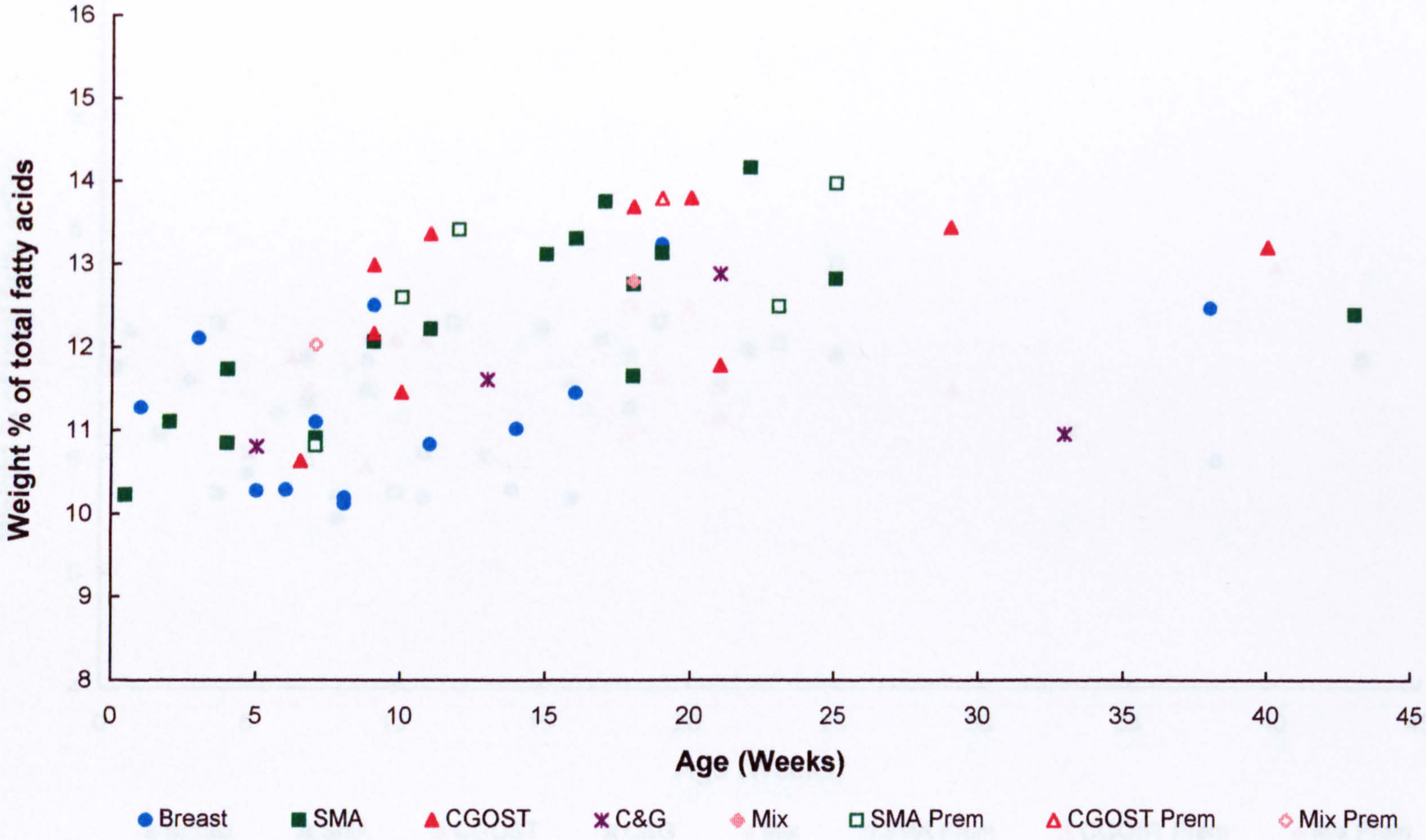


Figure 3.8 - Cerebral cortex phospholipid arachidonic acid (C20:4n-6) in relation to infants' diet and age.

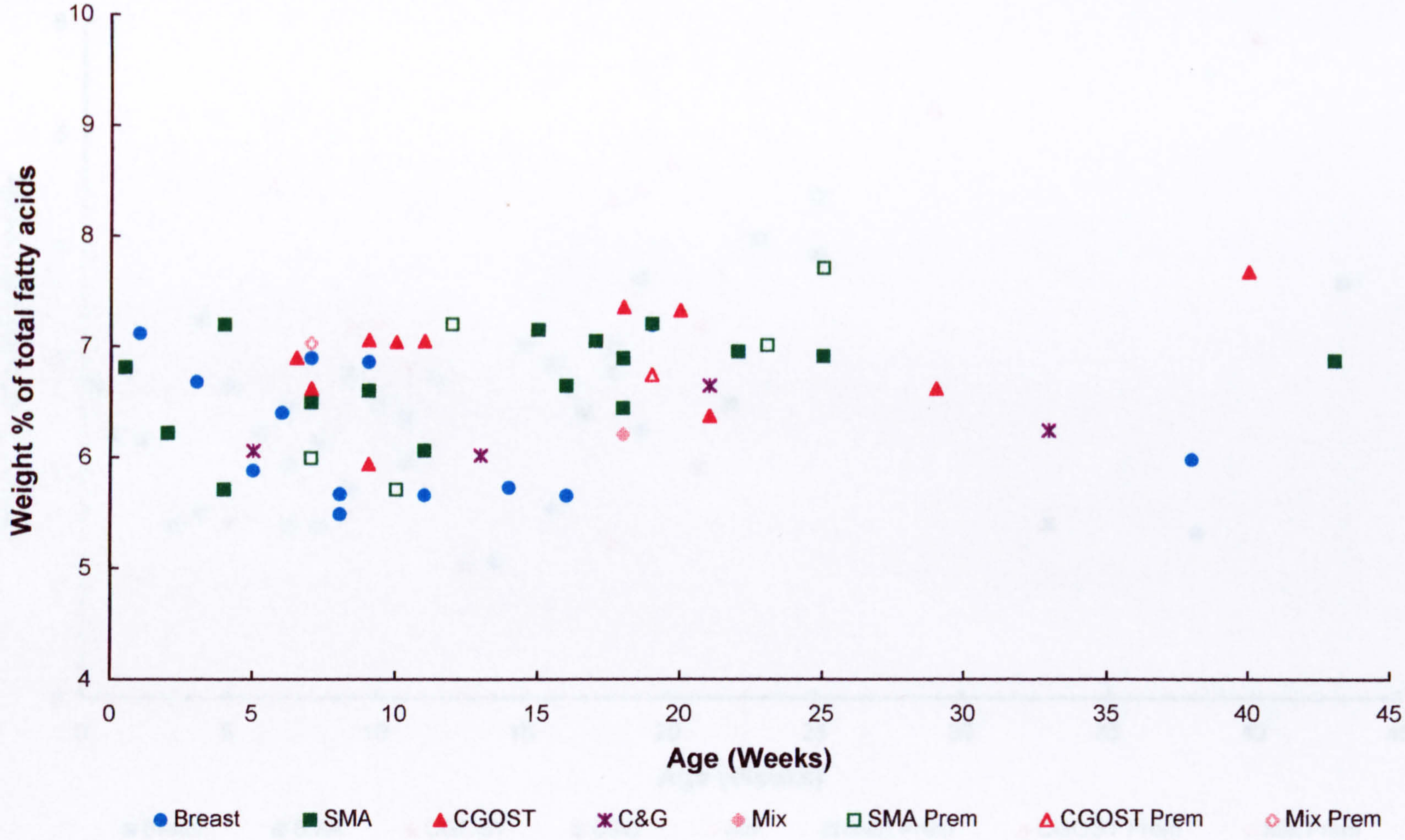


Figure 3.9 - Cerebral cortex phospholipid docosatetraenoic acid (C22:4n-6) in relation to infants' diet and age.

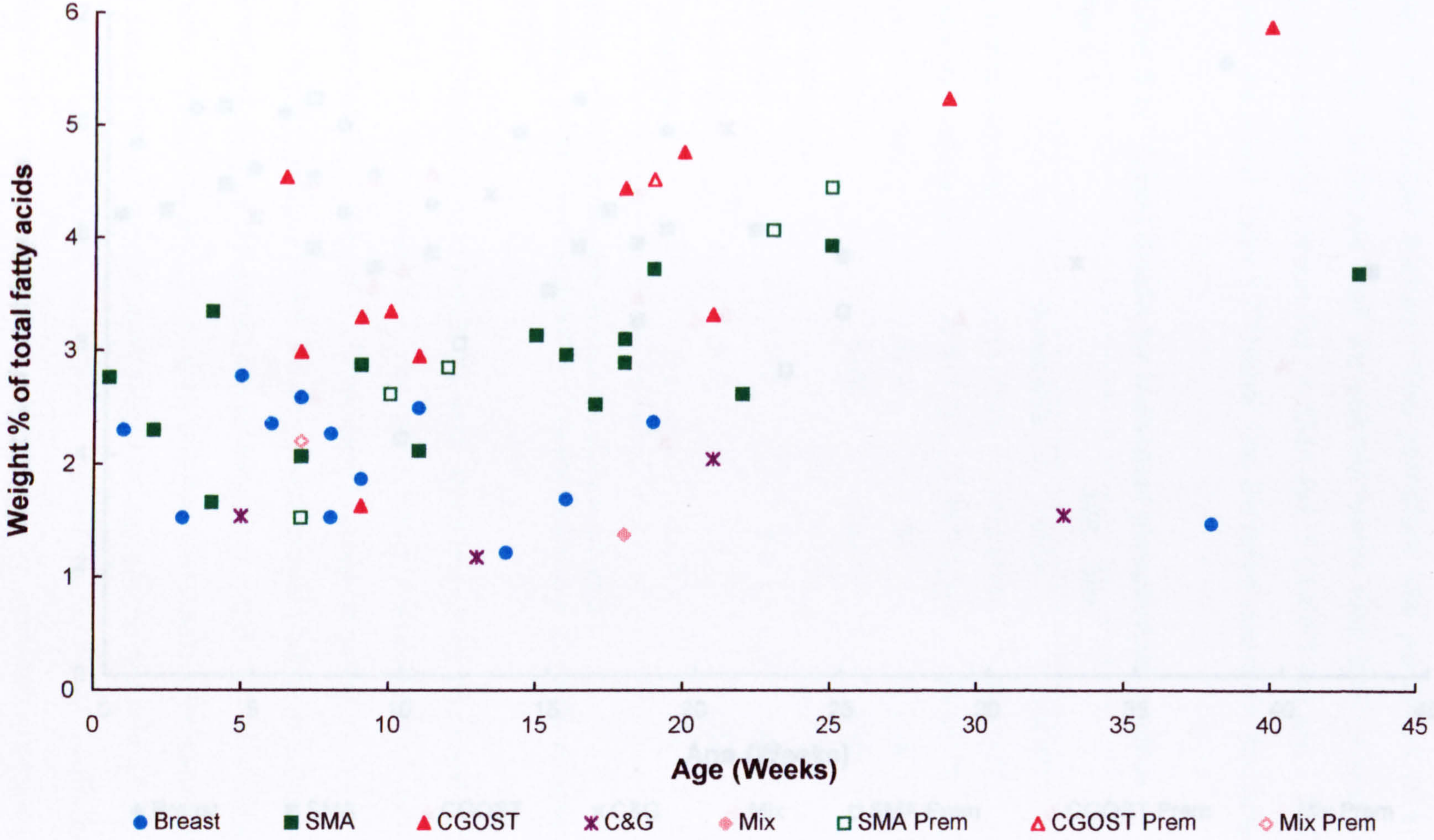


Figure 3.10 - Cerebral cortex phospholipid docosapentaenoic acid (C22:5n-6) in relation to infants' diet and age.

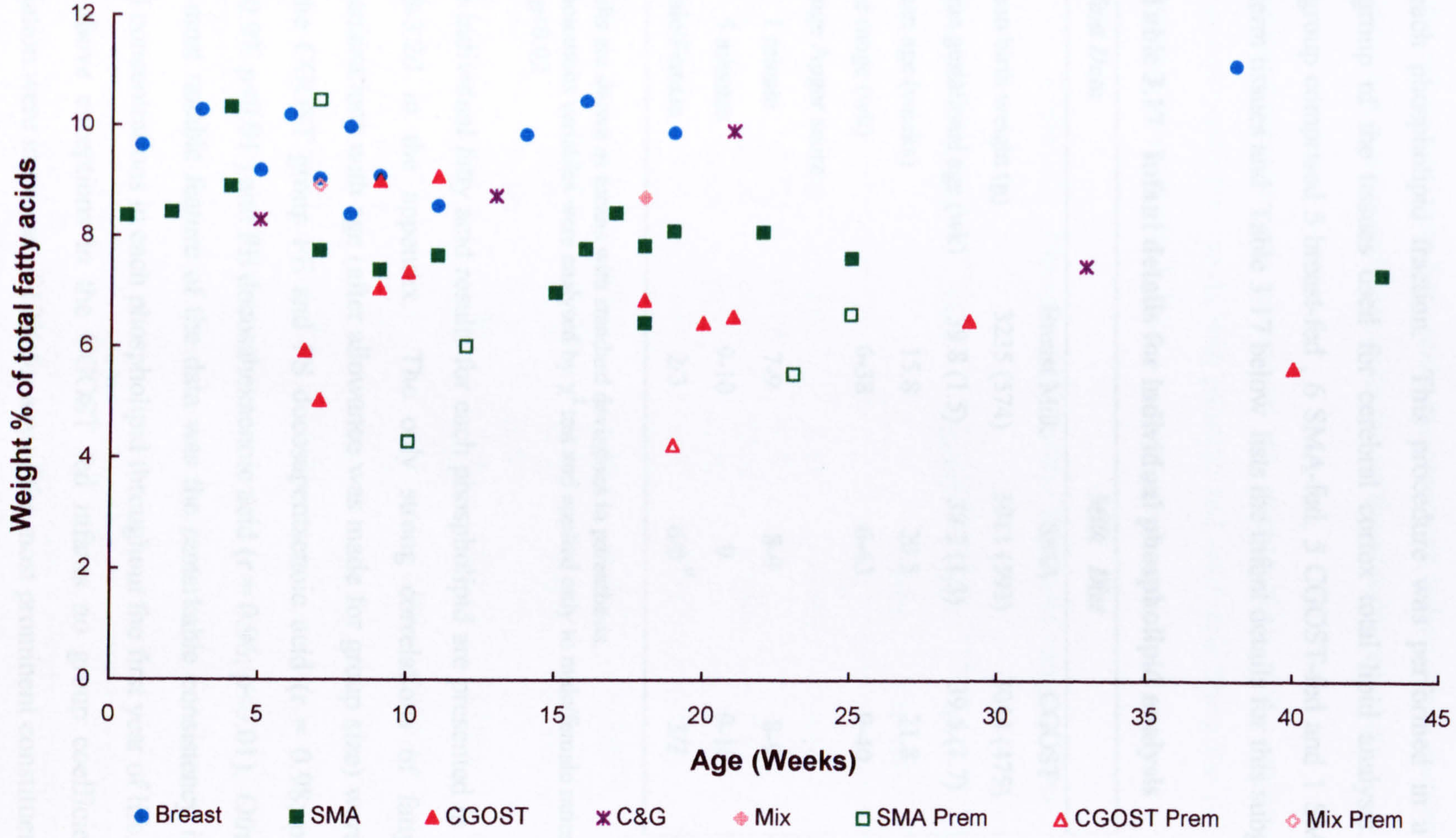


Figure 3.11 - Cerebral cortex phospholipid docosahexaenoic acid (C22:6n-3) in relation to infants' diet and age.

3.3.2. Cerebral cortex individual phospholipid fatty acids

Three major phospholipids (PC, PE and PS) were separated by thin layer chromatography as described in the methods section and fatty acids measured in each phospholipid fraction. This procedure was performed in a small subgroup of the tissues used for cerebral cortex total lipid analysis. The subgroup comprised 5 breast-fed , 6 SMA-fed, 5 CGOST-fed and 1 SMA-fed preterm tissues and Table 3.17 below lists the infant details for this subgroup.

Table 3.17 Infant details for individual phospholipid analysis

Infant Data	*Milk Diet*		
	Breast Milk	SMA	CGOST
Mean birth weight (g)	3235 (574)	3011 (593)	3044 (475)
Mean gestational age (wk)	39.8 (1.5)	39.2 (1.3)	39.5 (1.7)
Mean age (weeks)	15.8	20.5	21.8
Age range (wk)	6-38	6-43	9-40
Range Apgar score			
1 minute	7-9	8-9	8-9
5 minutes	9-10	9	9-10
Male/Female	2/3	6/0 $^{\alpha}$	3/2

Results are shown as means with standard deviations in parenthesis.
Dichotomous variables were analysed by χ^2 test and applied only to male/female ratios.
$\alpha = p<0.02$

The individual fatty acid results for each phospholipid are presented in Tables 3.18-3.20 in the appendix. The only strong correlations of fatty acid concentrations with age (after allowance was made for group size) were those of the CGOST group PE and PS docosapentaenoic acid ($r = 0.98$; $p<0.01$; $r = 0.97$: $p<0.01$) and PE docosahexaenoic acid ($r = 0.96$; $p<0.01$). Otherwise the most notable feature of the data was the remarkable consistency in fatty acid concentrations in each phospholipid throughout the first year of life. With the above exceptions in the CGOST fed infants no group coefficients of variation were in excess of 10% for any of the most prominent constituent fatty

acids. Tables 3.21, 3.22 and 3.23 summarise the means, standard errors and t test values for each phospholipid fatty acid. Figures 3.12 - 3.35 show the fatty acids for each phospholipid plotted with respect to diet and age. The graphs for the polyunsaturated fatty acids are presented below while the remaining saturated and unsaturated fatty acids can be found in the appendix.

Phosphatidylcholine was composed of greater than 90% saturated and monounsaturated fatty acids with palmitic and oleic acid being the two major fatty acids found. (Table 3.21)

Table 3.21 Phosphatidylcholine fatty acid compositions

Fatty Acid	*Breast*	*SMA*	*CGOST*	*BF/ SMA*	*BF/ CGOST*	*SMA/ CGOST*
				p	p	p
C14:0	1.88 (0.17)	2.02 (0.19)	1.82 (0.29)	0.5943	0.8680	0.5640
C16:0	53.78 (0.84)	54.40 (0.72)	53.33 (1.26)	0.5744	0.7722	0.4588
C16:1n-7	4.31 (0.60)	4.37 (0.18)	4.42 (0.59)	0.9143	0.8953	0.9310
C18:0	9.42 (0.46)	9.74 (0.50)	9.88 (0.70)	0.6529	0.5995	0.8730
C18:1 n-7+n-9	25.80 (0.32)	24.50 (0.54)	24.38 (0.82)	0.0820	0.1451	0.9008
C18:2 n-6	0.74 (0.10)	0.86 (0.05)	0.96 (0.09)	0.3240	0.1461	0.3351
C20:3 n-6	0.87 (0.07)	1.03 (0.09)	1.12 (0.05)	0.2261	0.0258	0.4541
C20:4 n-6	3.15 (0.30)	3.31 (0.58)	3.91 (0.26)	0.8265	0.0940	0.4043

Fatty acids are expressed as weight percentages and are given as means with standard errors in parenthesis.
p values represent between group differences as calculated using Student's t test.

Arachidonic acid (Fig 3.19) was the only polyunsaturated fatty acid present at levels greater than 2%. There were no significant differences found in any of the fatty acids in PC between different feeding groups. "Outliers" identified

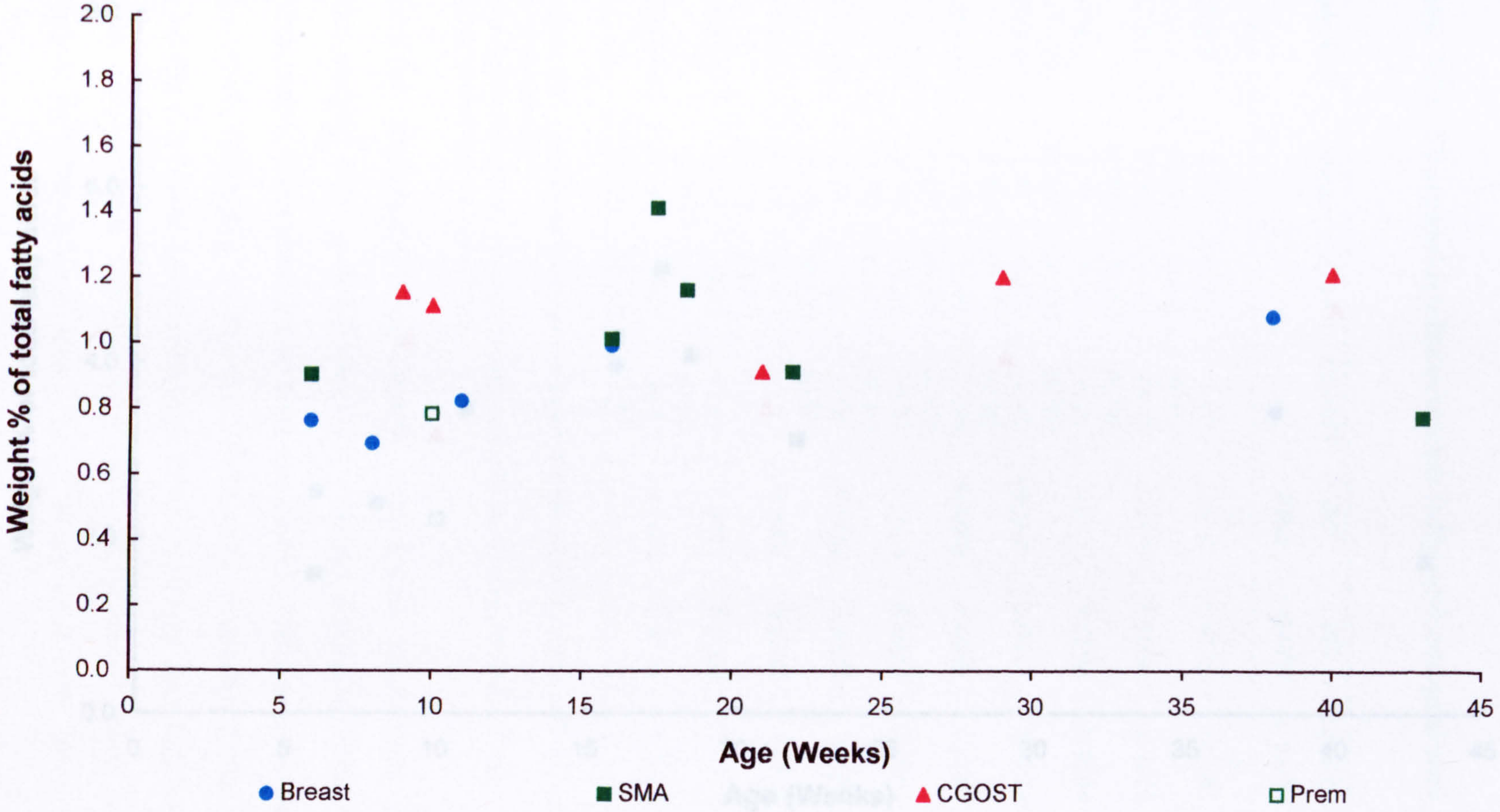


Figure 3.18 - Cerebral cortex phosphatidylcholine dihomogammalinolenic acid (C20:3n-6) in relation to infants' diet and age.

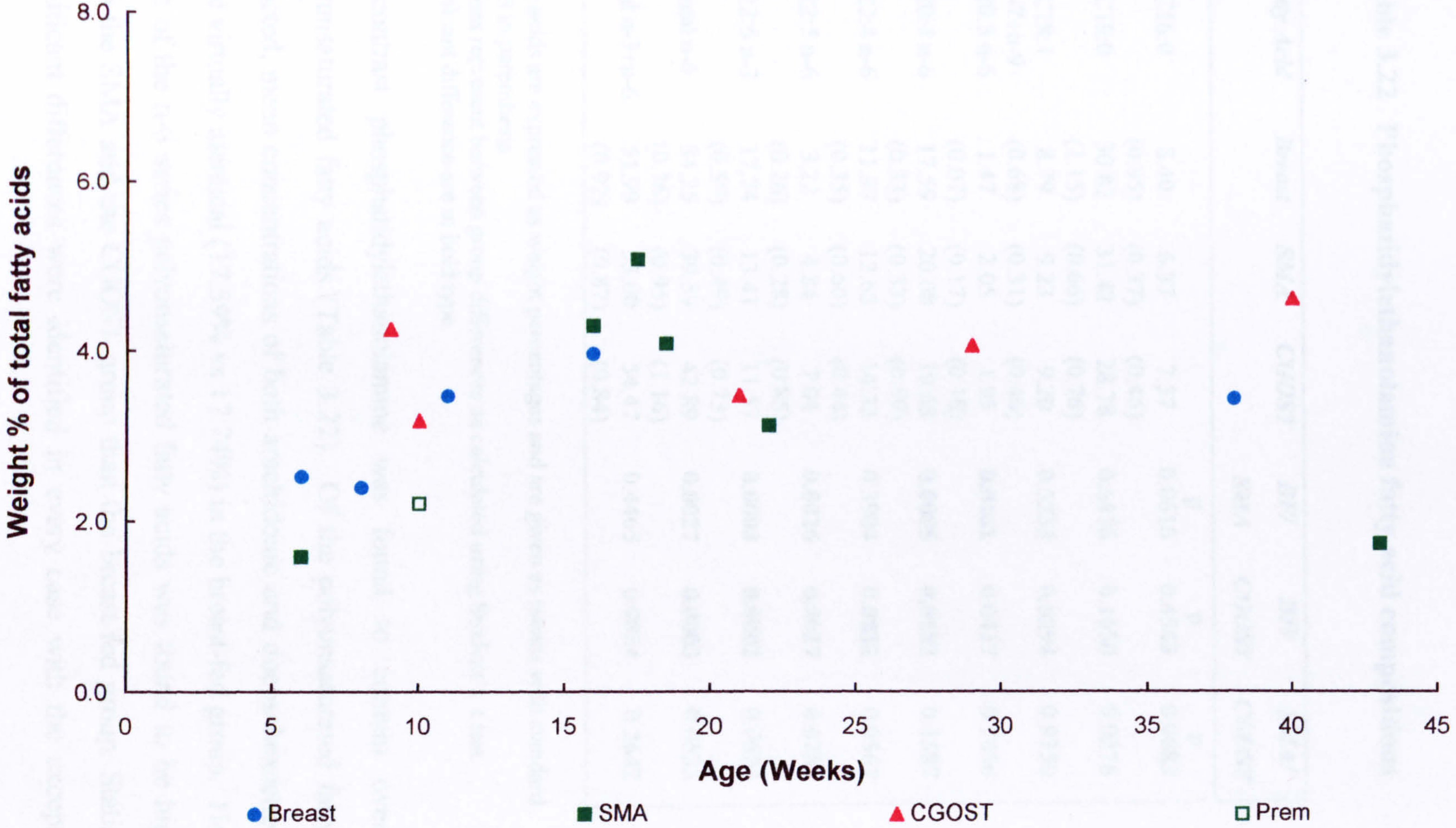


Figure 3.19 - Cerebral cortex phosphatidylcholine arachidonic acid (C20:4n-6) in relation to infants' diet and age.

tended to be associated with the preterm SMA fed infant as can be seen with palmitoleic acid (Fig 3.14) and to a lesser extent, stearic (Fig 3.15) and linoleic acid (Fig 3.17).

Table 3.22 Phosphatidylethanolamine fatty acid compositions

Fatty Acid	*Breast*	*SMA*	*CGOST*	*BF/ SMA*	*BF/ CGOST*	*SMA/ CGOST*
				p	p	p
C16:0	8.40 (0.95)	6.37 (0.37)	7.57 (0.46)	0.0610	0.4543	0.0683
C18:0	30.82 (1.15)	31.42 (0.66)	28.78 (0.76)	0.6476	0.1650	0.0276
C18:1 n-7+n-9	8.79 (0.68)	9.23 (0.31)	9.20 (0.46)	0.5333	0.6294	0.9350
C20:3 n-6	1.47 (0.07)	2.05 (0.17)	1.95 (0.18)	**0.0161**	0.0417	0.7056
C20:4 n-6	17.59 (0.33)	20.08 (0.33)	19.58 (0.59)	**0.0005**	**0.0182**	0.4587
C22:4 n-6	11.97 (0.35)	12.63 (0.60)	14.32 (0.44)	0.3904	**0.0032**	0.0567
C22:5 n-6	3.22 (0.26)	4.84 (0.28)	7.04 (0.85)	**0.0026**	**0.0027**	0.0263
C22:6 n-3	17.74 (0.59)	13.41 (0.49)	11.57 (0.73)	**0.0003**	**0.0002**	0.0623
Total n-6	34.25 (0.76)	39.59 (0.95)	42.89 (1.16)	**0.0027**	**0.0003**	0.0533
Total n-3+n-6	51.99 (0.92)	53.00 (0.87)	54.47 (0.84)	0.4465	0.0834	0.2642

Fatty acids are expressed as weight percentages and are given as means with standard errors in parenthesis
p values represent between group differences as calculated using Student's t test.
Significant difference are in bold type.

In contrast phosphatidylethanolamine was found to contain over 50% polyunsaturated fatty acids (Table 3.22). Of the polyunsaturated fatty acids detected, mean concentrations of both arachidonic and docosahexaenoic acid were virtually identical (17.59% vs 17.74%) in the breast-fed group. However each of the n-6 series polyunsaturated fatty acids was found to be higher in both the SMA and the CGOST group than the breast-fed group. Statistically significant differences were identified in every case with the exception of

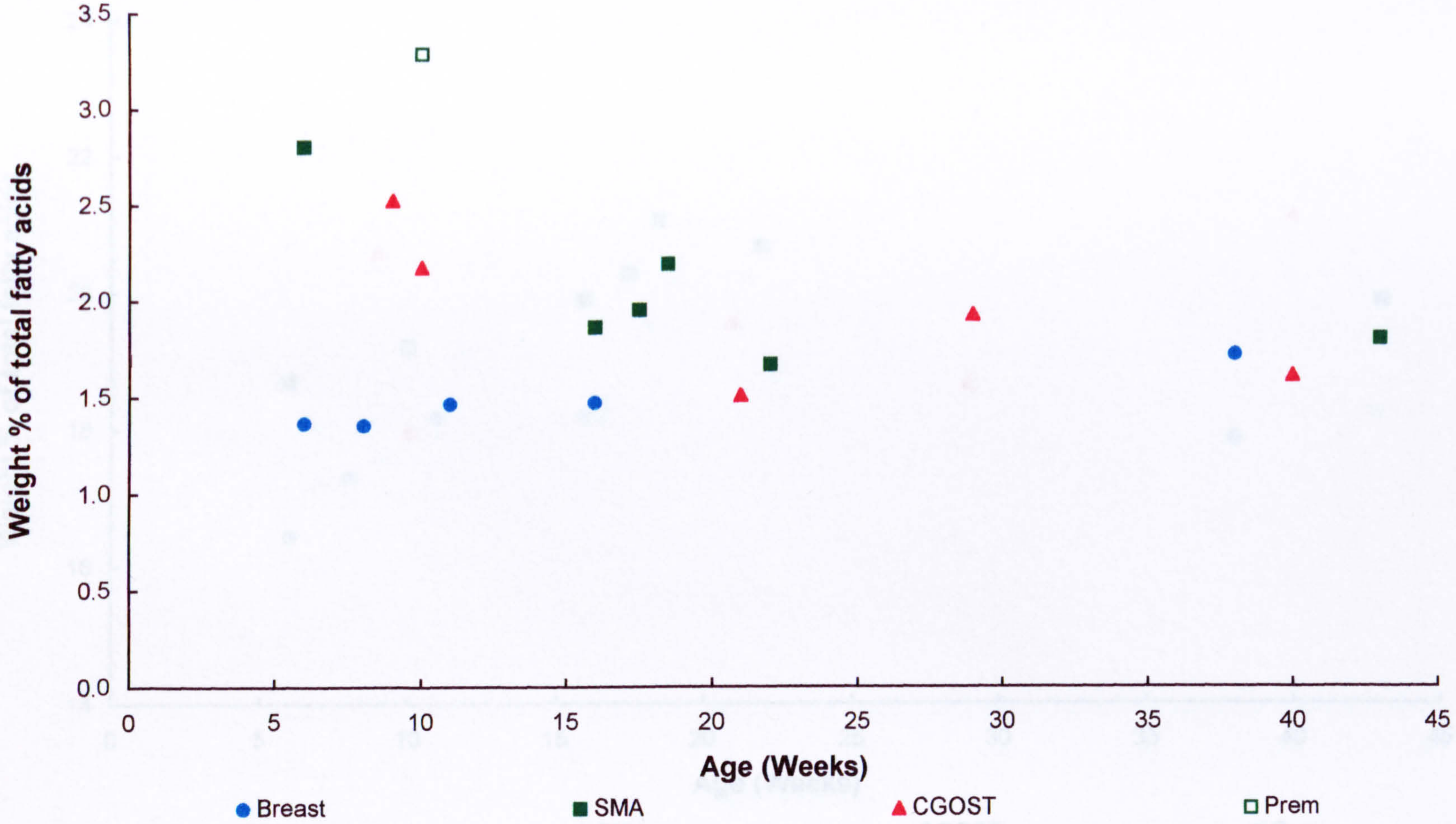


Figure 3.23 - Cerebral cortex phosphatidylethanolamine dihomogammalinolenic acid (C20:3n-6) in relation to infants' diet and age.

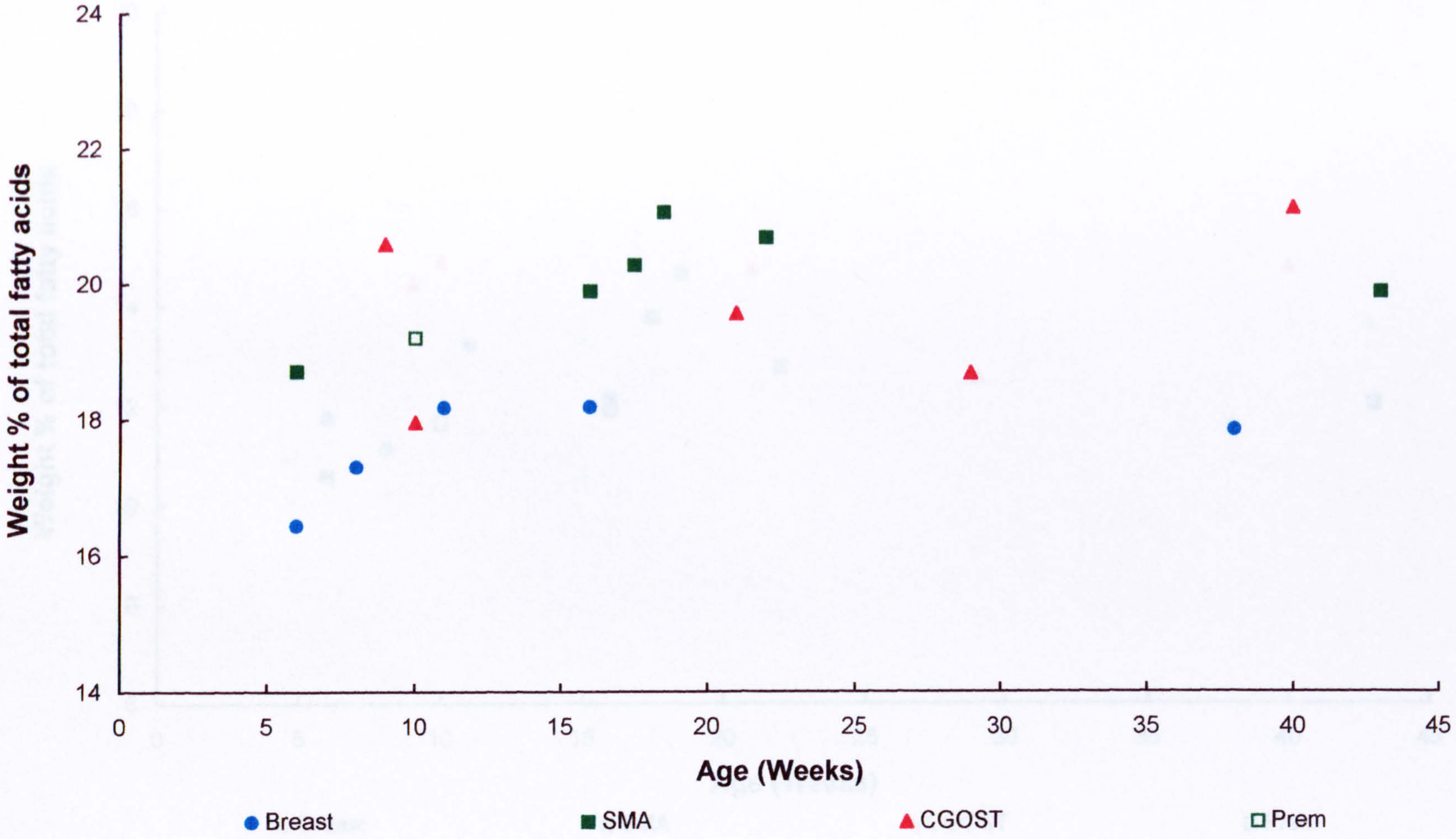


Figure 3.24 - Cerebral cortex phosphatidylethanolamine arachidonic acid (C20:4n-6) in relation to infants' diet and age.

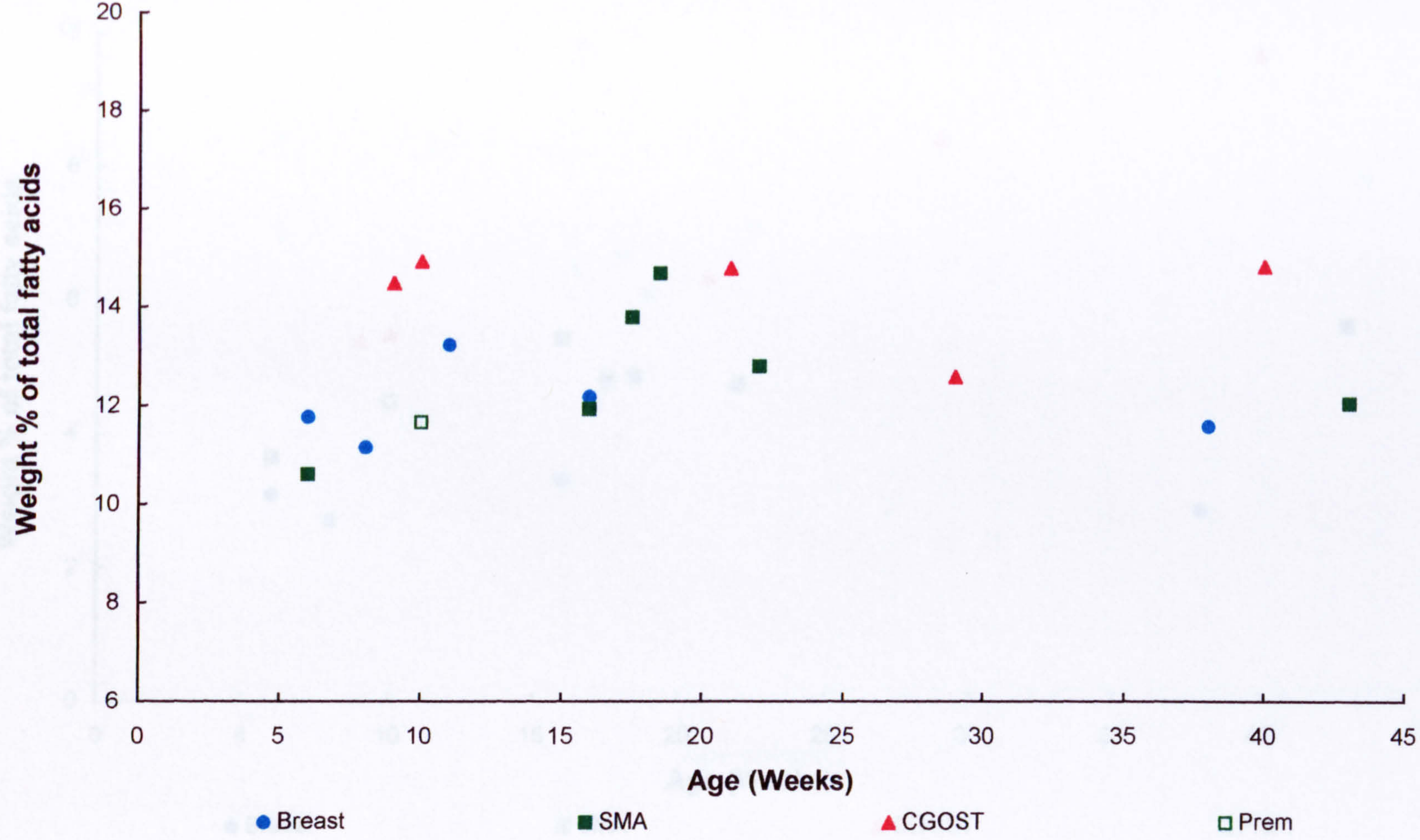


Figure 3.25 - Cerebral cortex phosphatidylethanolamine docosatetraenoic acid (C22:4n-6) in relation to infants' diet and age.

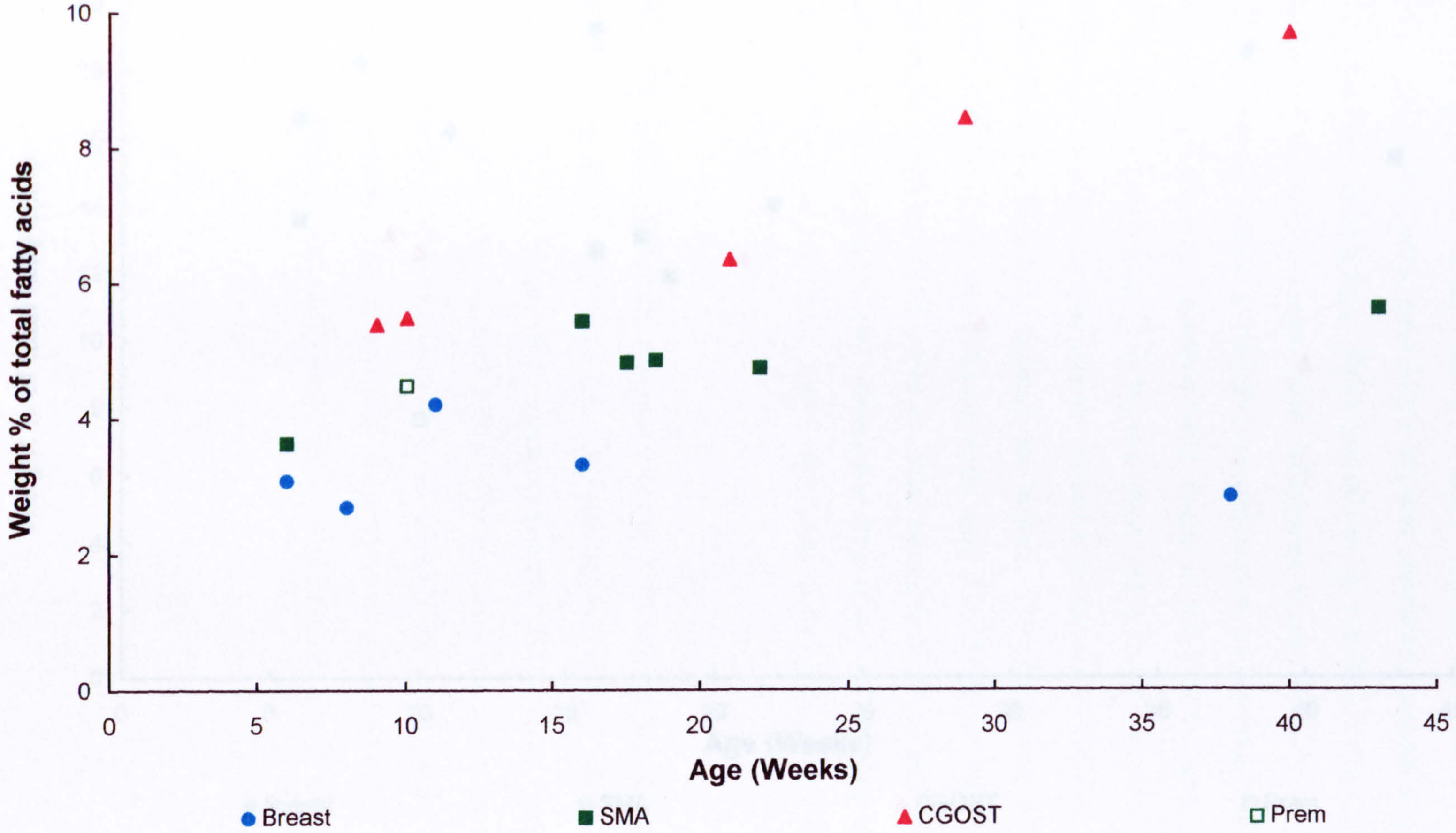


Figure 3.26 - Cerebral cortex phosphatidylethanolamine docosapentaenoic acid (C22:5n-6) in relation to infants' diet and age.

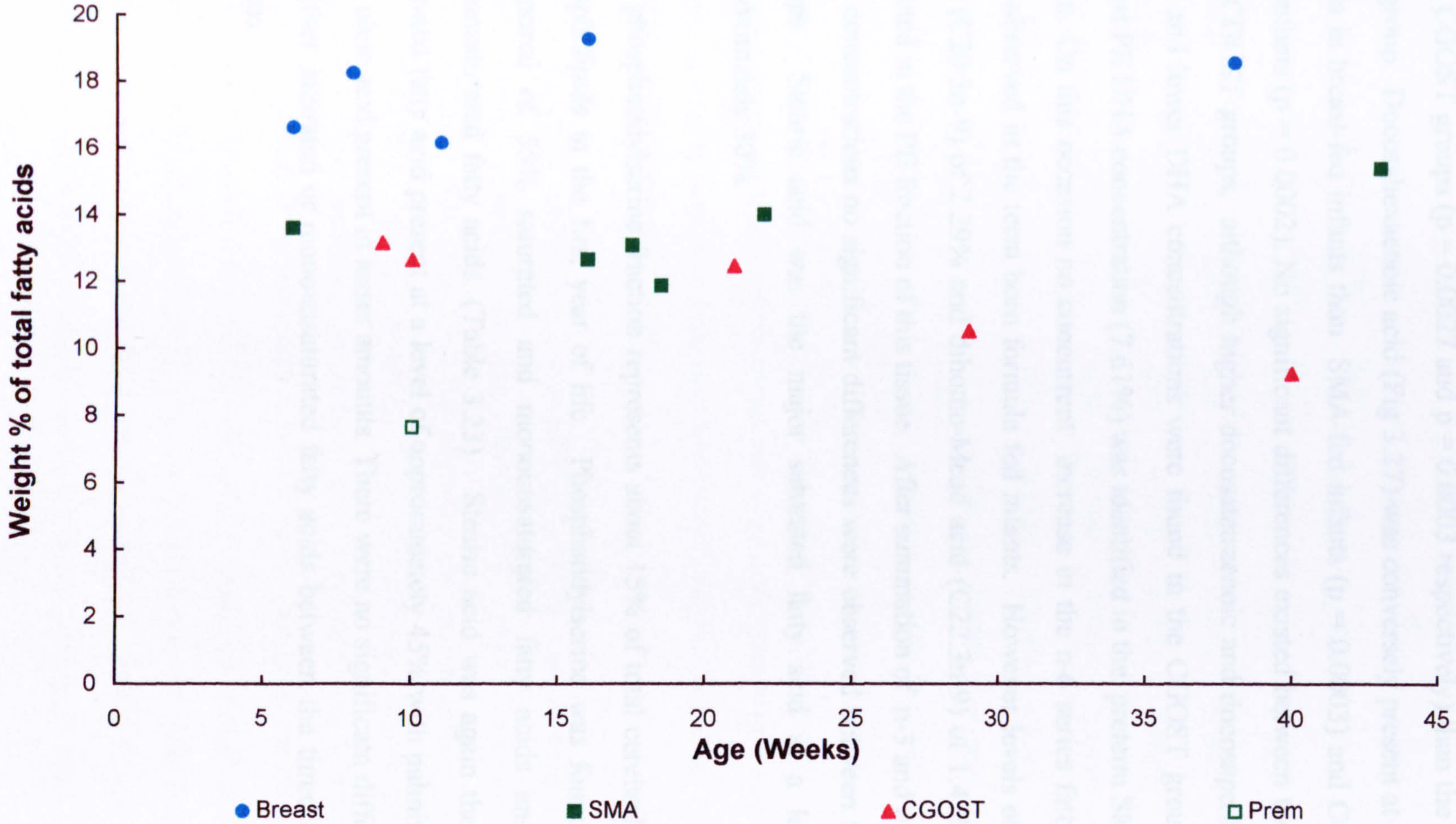


Figure 3.27 - Cerebral cortex phosphatidylethanolamine docosahexaenoic acid (C22:6n-3) in relation to infants' diet and age.

docosatetraenoic acid (Fig 3.25) which although higher in the SMA than breast-fed group did not reach a level of significance (Table 3.22). As a result the sum of the n-6 series fatty acids was significantly greater in both the SMA and CGOST groups ($p = 0.0027$ and $p = 0.0003$ respectively) than the breast-fed group. Docosahexaenoic acid (Fig 3.27) was conversely present at greater levels in breast-fed infants than SMA-fed infants ($p = 0.0003$) and CGOST-fed infants ($p = 0.0002$). No significant differences existed between the SMA and CGOST groups, although higher docosatetraenoic and docosapentaenoic acid and lower DHA concentrations were found in the CGOST group. The lowest PE DHA concentration (7.61%) was identified in the preterm SMA-fed infant. On this occasion no concurrent increase in the n-6 series fatty acids was observed in the term born formula fed infants. However levels of Mead acid (C20:3n-9) of 2.29% and dihomo-Mead acid (C22:3n-9) of 1.47% were detected in the PE fraction of this tissue. After summation of n-3 and n-6 fatty acid concentrations no significant differences were observed between feeding groups. Stearic acid was the major saturated fatty acid at a level of approximately 30%.

The phosphatidylserine fraction represents about 15% of total cerebral cortex phospholipids in the first year of life. Phosphatidylserine was found to be composed of 55% saturated and monounsaturated fatty acids and 45% polyunsaturated fatty acids. (Table 3.23) Stearic acid was again the major saturated fatty acid present at a level of approximately 45% with palmitic acid and oleic acid present in lesser amounts. There were no significant differences in either saturated or monounsaturated fatty acids between the three feeding groups.

Table 3.23 Phosphatidylserine fatty acid compositions

Fatty Acid	*Breast*	*SMA*	*CGOST*	*BF/ SMA*	*BF/ CGOST*	*SMA/ CGOST*
				p	p	p
C16:0	2.67 (0.47)	2.16 (0.36)	2.76 (0.56)	0.4049	0.9102	0.3798
C18:0	44.64 (0.76)	44.60 (0.62)	43.67 (0.45)	0.7063	0.2947	0.4572
C18:1 n-7+n-9	6.31 (1.14)	6.80 (0.32)	8.92 (0.90)	0.6681	0.2741	0.2223
C20:3 n-6	1.68 (0.12)	2.02 (0.18)	2.30 (0.23)	0.1801	0.0491	0.3695
C20:4 n-6	7.90 (0.73)	9.01 (0.57)	8.97 (0.49)	0.2520	0.2566	0.9597
C22:4 n-6	7.90 (0.47)	8.46 (0.38)	9.30 (0.53)	0.3989	0.0906	0.2168
C22:5 n-6	5.33 (0.65)	7.66 (0.39)	10.42 (1.39)	**0.0109**	**0.0105**	0.0661
C22:6 n-3	23.55 (0.60)	19.32 (0.74)	14.41 (0.66)	**0.0019**	**0.0000**	**0.0009**
Total n-6	22.84 (1.30)	27.15 (0.76)	30.99 (1.55)	**0.0154**	**0.0038**	0.0433
Total n-3+n-6	46.39 (1.27)	46.47 (0.38)	45.40 (1.38)	0.9122	0.6288	0.4364

Fatty acids are expressed as weight percentages and are given as means with standard errors in parenthesis.
p values represent between group differences as calculated using Student's t test.
Significant differences are in bold type.

PS also displayed higher n-6 series LCPUFA in the two formula milk fed groups when compared to the breast milk group. However only docosapentaenoic acid (Fig 3.34) was significantly greater in both the SMA ($p = 0.0109$) and CGOST group ($p = 0.0105$) than the breast fed group. Application of non-parametric, two tailed Mann Whitney U test statistics to the phosphatidylserine DPA concentrations confirmed that both the formula fed groups, SMA ($p<0.05$) and CGOST ($p<0.02$) contained higher percentage contents than the breast-fed group. Similarly total n-6 series fatty acids were significantly greater in both the SMA group ($p=0.0154$) and the CGOST group ($p=0.0038$) when compared to the breast fed group. Although total n-6 fatty

acid concentrations were greater in the CGOST (30.99%) than SMA group (27.15%) the difference did not reach significance ($p = 0.043$) for multi-group comparison. Docosahexaenoic acid (Fig 3.35) concentrations were found to be greater in the breast-fed infants than both the SMA ($p=0.0019$) and CGOST-fed groups ($p<0.0001$). At greater than 20% of total fatty acids the cerebral cortex phosphatidylserine DHA compositions of the breast-fed infants (with the exception of certain retinal structures) are not surpassed in any other human tissue. The highest DHA concentration (25.79%) was found in the 16 week old breast fed infant. It was also noted that whereas the DHA concentrations in the term born SMA-fed group appeared to remain stable at about 20% a continual drift downwards in DHA compositions was witnessed in the older CGOST-fed infants, to a level only 50% of that of the age-comparable breast-fed infant (Fig 3.35). Docosahexaenoic acid concentrations were also significantly greater in the SMA-fed than CGOST-fed infants ($p = 0.0009$). At 12.39% of total fatty acids the SMA-fed preterm infant had a lower level of DHA compared to term born infants of comparable age. 1.04% of Mead acid and 1.58% of dihomo-Mead acid were also found in the PS fraction of this tissue.

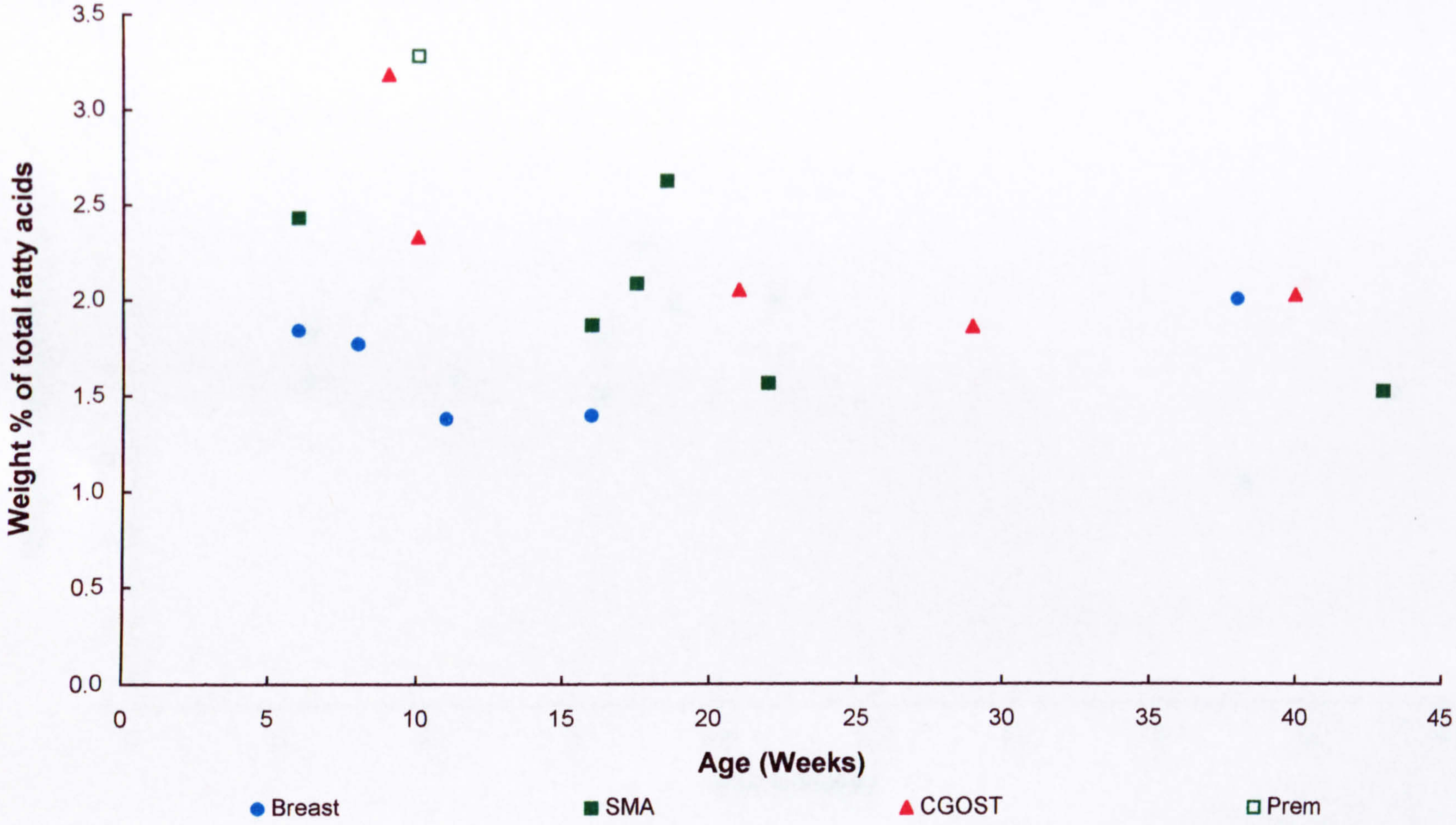


Figure 3.31 - Cerebral cortex phosphatidylserine dihomogammalinolenic acid (C20:3n-6) in relation to infants' diet and age.

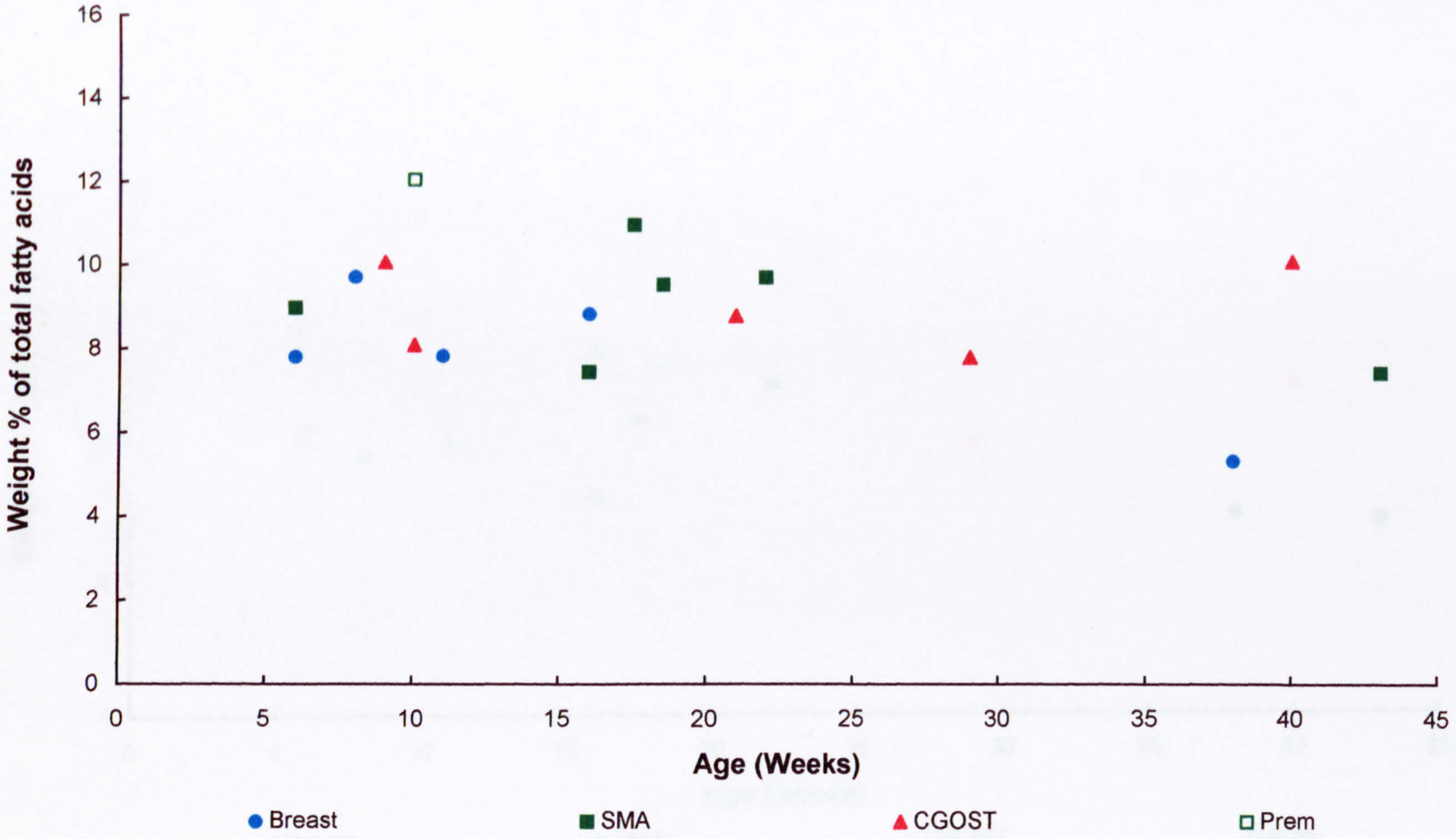


Figure 3.32 - Cerebral cortex phosphatidylserine arachidonic acid (C20:4n-6) in relation to infants' diet and age.

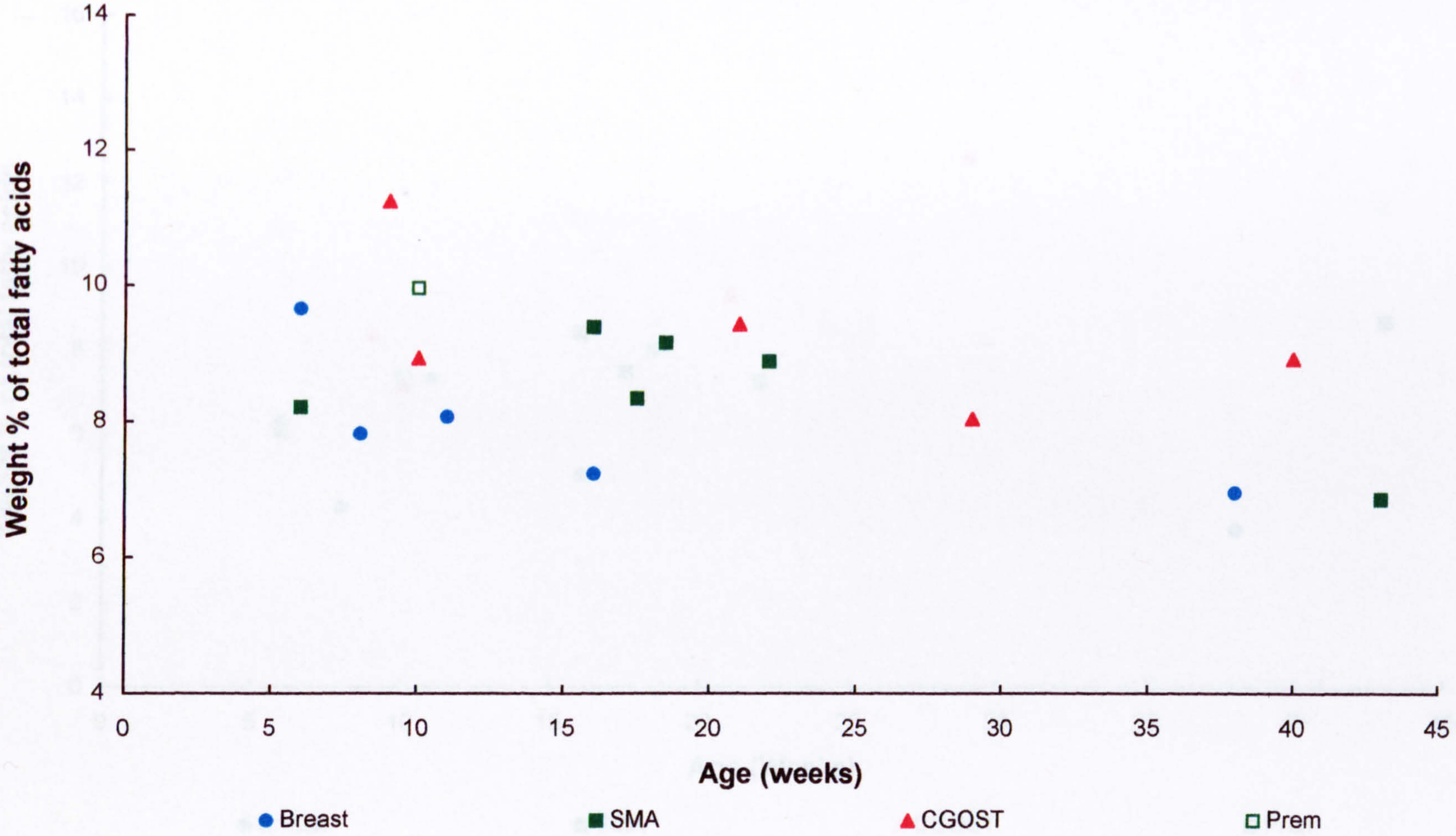


Figure 3.33 - Cerebral cortex phosphatidylserine docosatetraenoic acid (C22:4n-6) in relation to infants' diet and age.

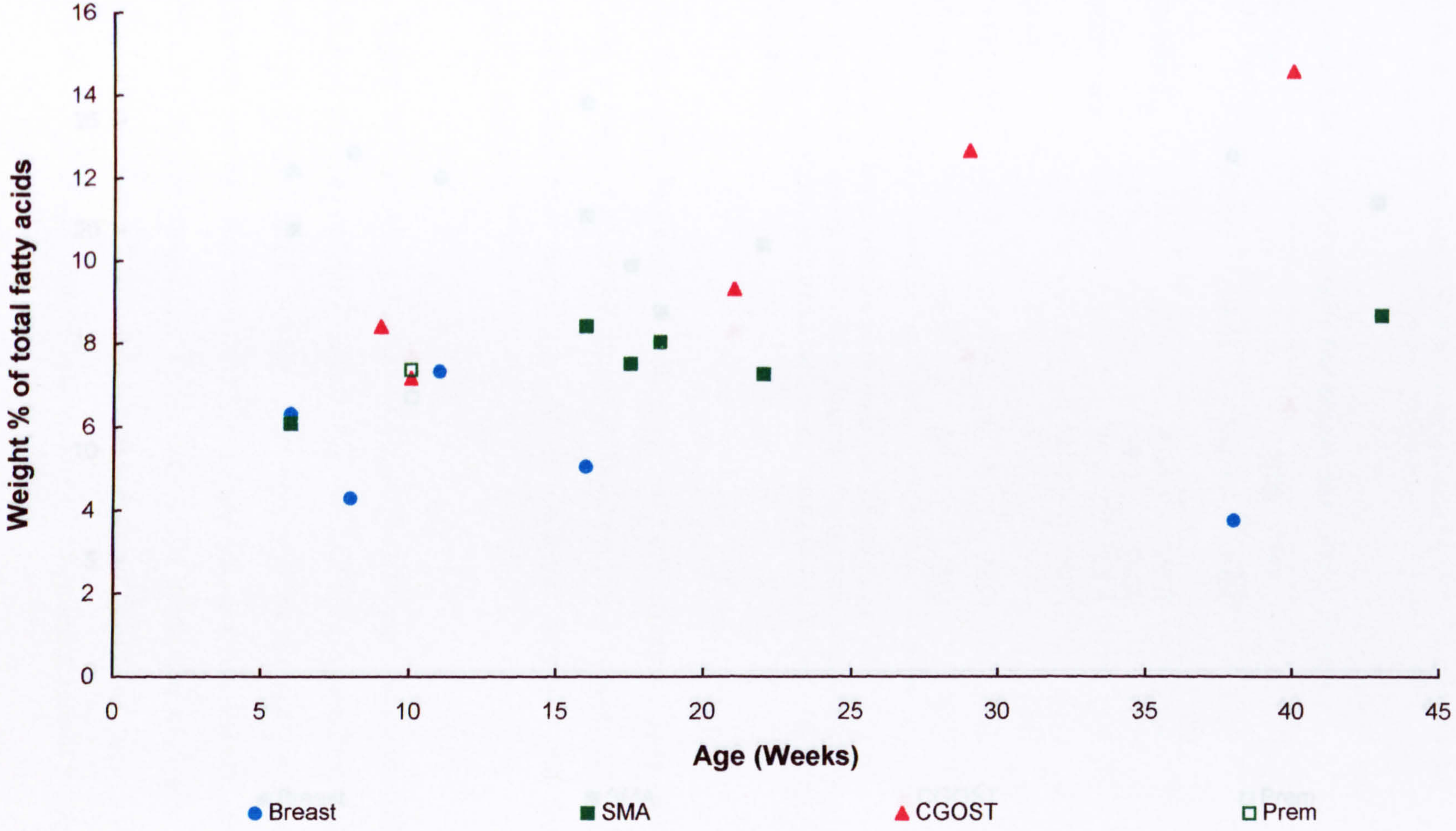


Figure 3.34 - Cerebral cortex phosphatidylserine docosapentaenoic acid (C22:5n-6) in relation to infants' diet and age.

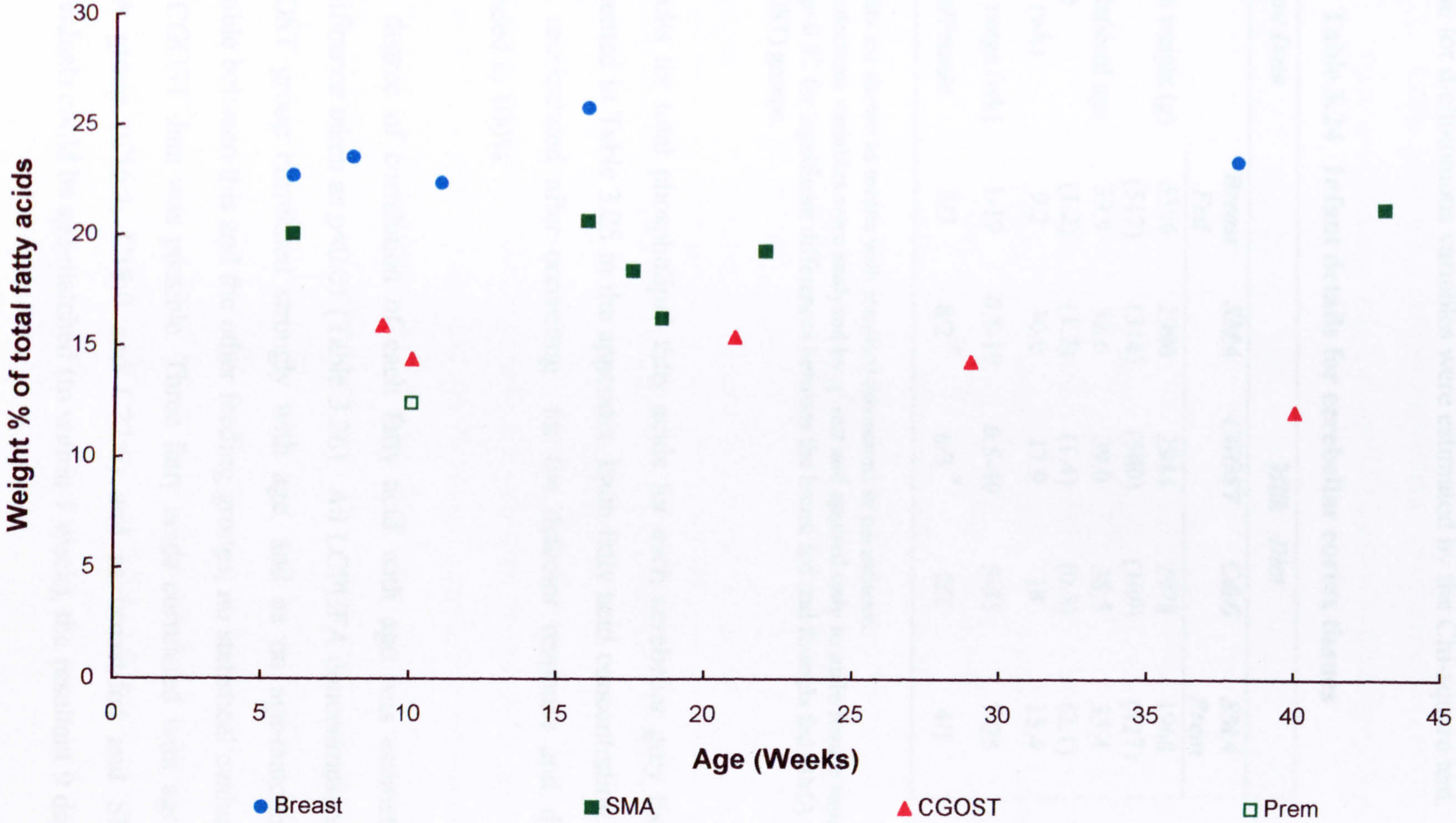


Figure 3.35 - Cerebral cortex phosphatidylserine docosahexaenoic acid (C22:6n-3) in relation to infants' diet and age.

3.3.3. Cerebellar cortex total phospholipid fatty acids

Table 3.24 illustrates the infant group characteristics. Between group differences for the continuous variables were assessed by Student's t test and those for dichotomous variables were estimated by the Chi-square test.

Table 3.24 Infant details for cerebellar cortex tissues

Infant Data	*Milk Diet*				
	Breast Fed	***SMA***	***CGOST***	***C&G***	***SMA Prem***
Birth weight (g)	3356 (517)	2996 (314)	2944 (580)	2978 (169)	1968 (427)
Gestational age (wk)	39.9 (1.2)	38.6 (1.2)	39.0 (1.4)	38.5 (0.5)	33.4 (2.1)
Age (wk)	9.2	10.0	17.9	18	15.4
Age range (wk)	1-19	0.5-19	6.5-40	5-33	7-25
Male/Female	3/7	8/2 $^{\alpha}$	6/3 $^{\alpha}$	2/2	4/1

Results are shown as means with standard deviations in parenthesis.
Dichotomous variables were analysed by χ^2 test and applied only to male/female ratios.
$\alpha = p<0.02$ for significant differences between the breast fed and formula fed (SMA and CGOST) groups.

Results for total phospholipid fatty acids for each cerebellar grey tissue are presented in Table 3.25 in the appendix. Each fatty acid concentration (wt %) was recalculated after correcting for the detector response and the total rounded to 100%.

The degree of correlation of each fatty acid with age was estimated and significance taken as $p<0.01$ (Table 3.26). All LCPUFA concentrations in the CGOST group correlated strongly with age, and as no age-matching was possible between this and the other feeding groups, no statistical evaluation of the CGOST data was possible. Three fatty acids correlated with age in the SMA group (C16:1, C18:0 and C22:6) and as breast-fed and SMA-fed individuals could be age-matched (to within 1 week), the resultant 9 data pairs

were assessed for each fatty acid using the Wilcoxon signed rank test. This resulted in the exclusion of one 8 week old breast-fed and one 15 week old SMA-fed infant.

Table 3.26 Correlation coefficients for cerebellar cortex fatty acid concentration vs age in relation to feeding group

Fatty Acid	Breast	SMA	CGOST	C&G	SMA Prem
	r	r	r	r	r
C16:0	-0.1919	-0.5496	-0.3932	-0.8261	0.4878
C16:1	-0.8643 $^{\delta}$	-0.9831$^{\delta}$	-0.8985 $^{\delta}$	-0.9212 $^{\delta}$	-0.9157 $^{\delta}$
C18:0	0.3578	0.8186 $^{\delta}$	0.5897	0.9153	0.7933
C18:1	0.0070	0.4468	-0.5773	0.7894	-0.3025
C18:2	0.5743	0.4336	0.5860	0.9341$^{\delta}$	-0.1124
C20:3	0.5941	0.5202	-0.8001$^{\delta}$	-0.8758	-0.3188
C20:4	0.5110	0.5340	0.7979 $^{\delta}$	0.0140	0.7169
C22:4	-0.2475	-0.1937	0.8279 $^{\delta}$	0.6601	-0.0428
C22:5	-0.2531	0.6518	0.8457 $^{\delta}$	0.1072	0.5752
C22:6	-0.3071	-0.8648 $^{\delta}$	-0.7533 $^{\delta}$	-0.8375	-0.6723

δ = Significant correlation of individual fatty acid concentrations (wt %) with age. ($p<0.01$)

Results showing means and standard errors for total phospholipid fatty acids in the cerebellar cortex together with the Wilcoxon signed rank test significances for the paired breast-fed and SMA groups are given in Table 3.27. The results for the CGOST group, C&G group and the SMA preterm group are also provided for information without statistical analysis. In figures 3.36 - 3.45, each individual fatty acid is plotted with respect to diet and age. As with the cerebral cortex results, included are results from two mixed fed infants (aged 7 and 18 weeks), both initially breast fed for 4 and 10 weeks respectively, then given SMA formula.

Table 3.27 Cerebellar cortex phospholipid fatty acid compositions

Fatty Acid	*Milk Diet*				
	Breast n=10	***SMA n=10***	***CGOST n=9***	***C&G n=4***	***SMA Prem n=5***
C16:0	28.50 (0.34)	29.77 (0.42)	28.94 (0.30)	28.74 (0.82)	29.44 (0.30)
C16:1n-7	3.21 (0.23)	3.13 (0.22)	2.78 (0.30)	2.91 (0.49)	3.32 (0.23)
C18:0	18.19 (0.37)	18.17 (0.31)	19.04 (0.53)	18.25 (0.70)	18.05 (0.35)
C18:1n-7+n-9	20.04 (0.33)	19.52 (0.37)	20.45 (0.29)	21.05 (1.17)	20.41 (0.61)
C18:2n-6	0.87 (0.14)	1.07 (0.09)	0.92 (0.06)	1.04 (0.06)	1.35 (0.11)
C20:3n-6	2.02 (0.21)	2.43 [c] (0.21)	2.47 (0.17)	2.53 (0.13)	2.70 (0.22)
C20:4n-6	11.15 (0.19)	11.10 (0.22)	11.63 (0.39)	11.11 (0.40)	11.35 (0.35)
C22:4n-6	4.74 (0.17)	5.12 [b] (0.19)	5.59 (0.27)	4.67 (0.12)	5.04 (0.16)
C22:5n-6	0.69 (0.19)	1.08 [c] (0.08)	1.95 (0.16)	1.05 (0.13)	1.38 (0.19)
C22:6n-3	8.44 (0.25)	6.54 [a] (0.34)	4.68 (0.36)	7.15 (0.36)	6.16 (0.57)

Fatty acids are expressed as weight percentages and are given as means with standard errors in parenthesis.
Significant differences in fatty acid concentrations between feeding groups (BF vsSMA only) were calculated by the Wilcoxon signed rank test for paired data (n=9)
a = $p<0.01$; b = $p<0.02$; c = $p<0.05$

As already observed in the study of cerebral cortex tissues, none of the saturated or monounsaturated fatty acids showed any differences between feeding groups (Table 3.27). However relative amounts of palmitoleic acid (Fig 3.37) decreased with age as confirmed by their correlation coefficients (Table 3.26).

The mean dihomo-γ-linolenic acid concentration (Fig 3.41) for breast fed infants was significantly lower than those for the SMA-fed group ($p<0.05$) as

shown in Table 3.27. Perinatal cerebellar dihomo-γ-linolenic acid concentrations (Fig 3.41) at about 1% increased rapidly to approximately 3% of total fatty acids by 8 weeks of age and then appeared to decline from 20 weeks onwards.

No statistical difference was observed in arachidonic acid compositions between breast and SMA groups (Fig 3.42). Arachidonic acid had a mean concentration of about 11% of total fatty acids which was maintained in each feeding group throughout early life. The increasing trend in arachidonic acid concentrations seen in the CGOST group could not be confirmed (or refuted) in the other feeding groups due to lack of data from older infants (Fig 3.42).

After perhaps an initial fall in docosatetraenoic acid concentrations in the first month of life (Fig 3.43), the distribution of results was similar to that for arachidonic acid with a mean concentration of about 5%. The docosatetraenoic acid composition of the SMA group was significantly greater than the breast-fed ($p<0.02$). Again the pattern of CGOST results pertaining with arachidonic acid was repeated in the direct correlation of docosatetraenoic acid concentrations with age ($r = 0.843$; $p<0.01$). Docosatetraenoic acid concentrations were generally at their highest in the CGOST group. Again no obvious trend in results from the preterm infants was evident (Fig 3.43).

Docosapentaenoic acid (Fig 3.44) was present in significantly lower concentrations in the breast fed group than the SMA group. As for DTA, the highest DPA concentrations were found in the CGOST group. The two mixed fed infants displayed DPA concentrations similar to those of breast-fed infants of a corresponding age. It was observed that the highest concentration of this fatty acid (3.06%) was found in the CGOST preterm infant. It should be stated that although concentrations of DGLA, AA and DTA (as weight %) were very

similar in both the cerebellar cortex and cerebral cortex (Figs 3.41-3.43 and 3.16-3.18) the increase in cerebellar cortex DPA in the term born CGOST group infants, to a maximum of <3% of total fatty acids is far less pronounced than that seen in the cerebral cortex (Figs3.44 and 3.19).

Docosahexaenoic acid concentration (Fig 3.45) was significantly greater in the breast fed than SMA fed group ($p<0.01$). Although no statistical analysis was possible, with the exception of one 9-week old infant, all CGOST DHA concentrations were lower than those for infants for equivalent age in other feeding groups. Observation of the term 18 week old mix fed infant showed a level of DHA similar to that of the 19 week old exclusively breast fed infant, whereas the DHA concentration in the 7 week old preterm mix fed infant was more closely matched with the term SMA-fed group values (Fig 3.45). SMA group DHA concentrations were higher than those of the SMA preterm group. In consideration of the CGOST group compositions a parallel with the cerebral cortex results seemed evident in the 7 week old infants of low birth weights (2.50 and 2.53 kg) who exhibited relatively reduced DHA concentrations (4.88 % and 4.73%) for their age. Overall, cerebellar cortex DHA concentrations were slightly lower in each feeding group than in the corresponding cerebral cortex groups. The cerebellar DHA "deficit" in formula fed infants was again apparently greatest in the CGOST group and the compensatory effect of increased incorporation of n-6 series LCPUFA seemed more evenly spread throughout all of these fatty acids in contrast to the cerebral cortex in which ultimately incorporation of n-6 series DPA predominated (Figs 3.44 and 3.19).

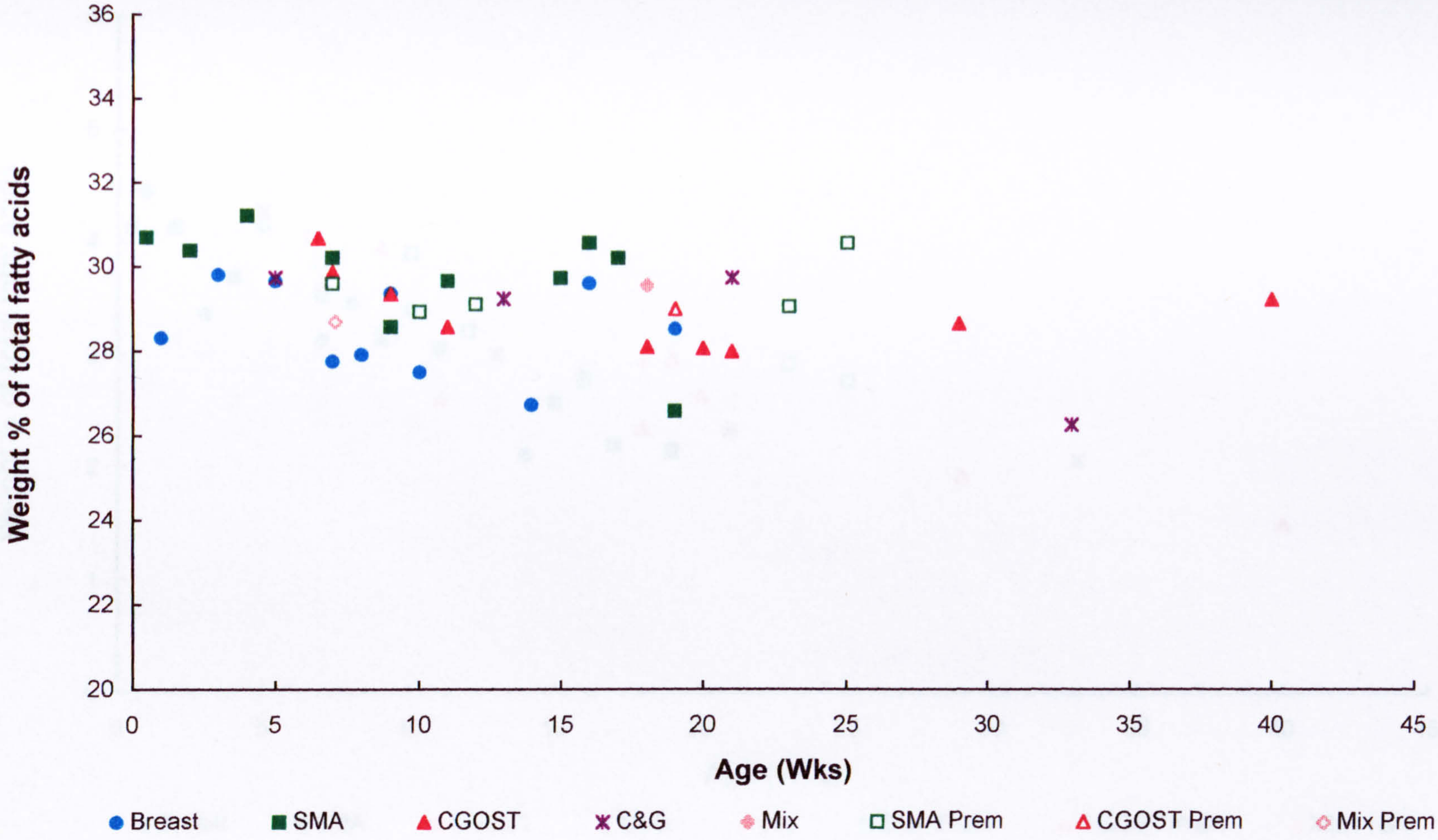


Figure 3.36 - Cerebellar cortex phospholipid palmitic acid (C16:0) in relation to infants' diet and age.

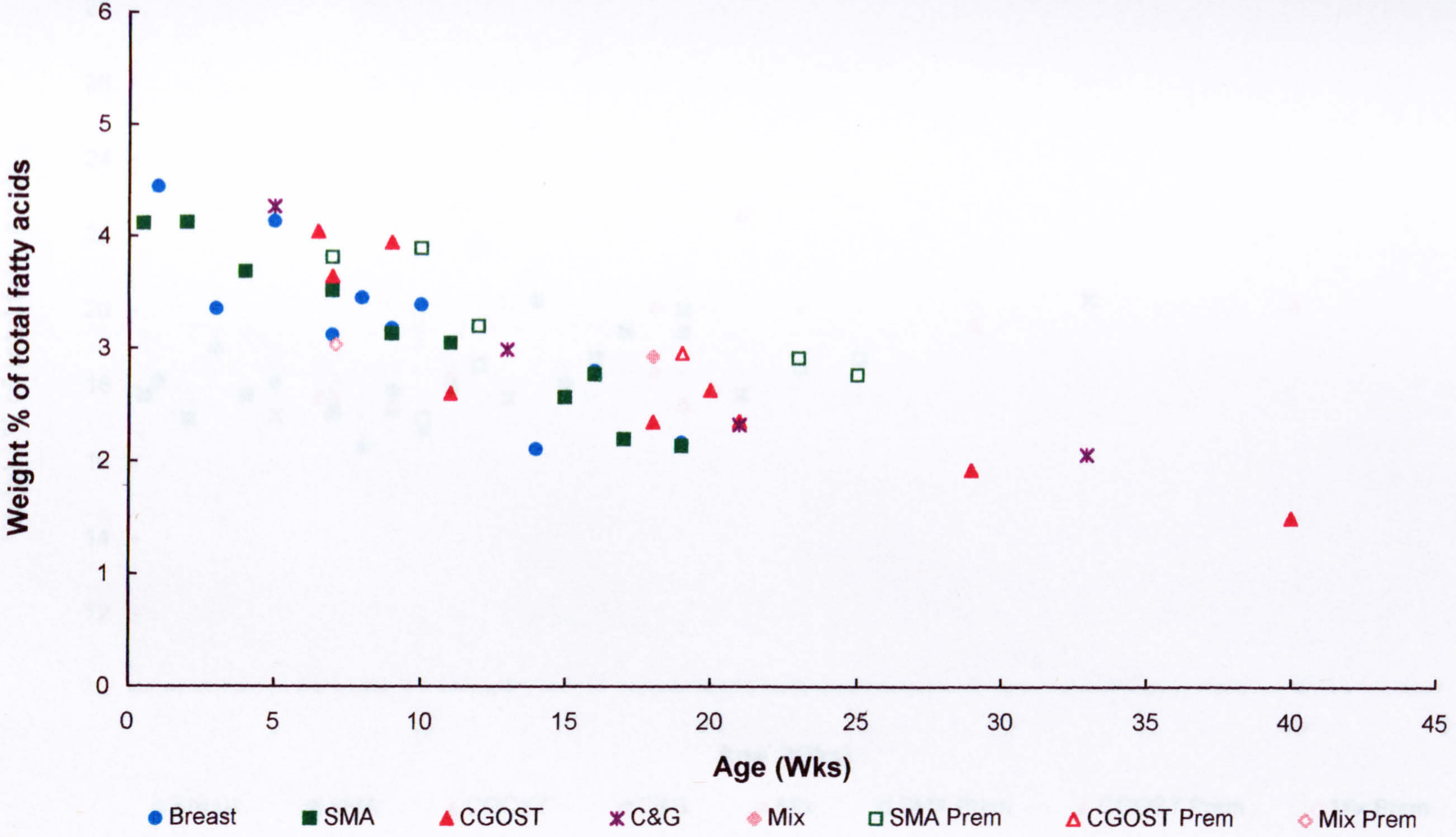


Figure 3.37 - Cerebellar cortex phospholipid palmitoleic acid (C16:1 n-7) in relation to infants' diet and age.

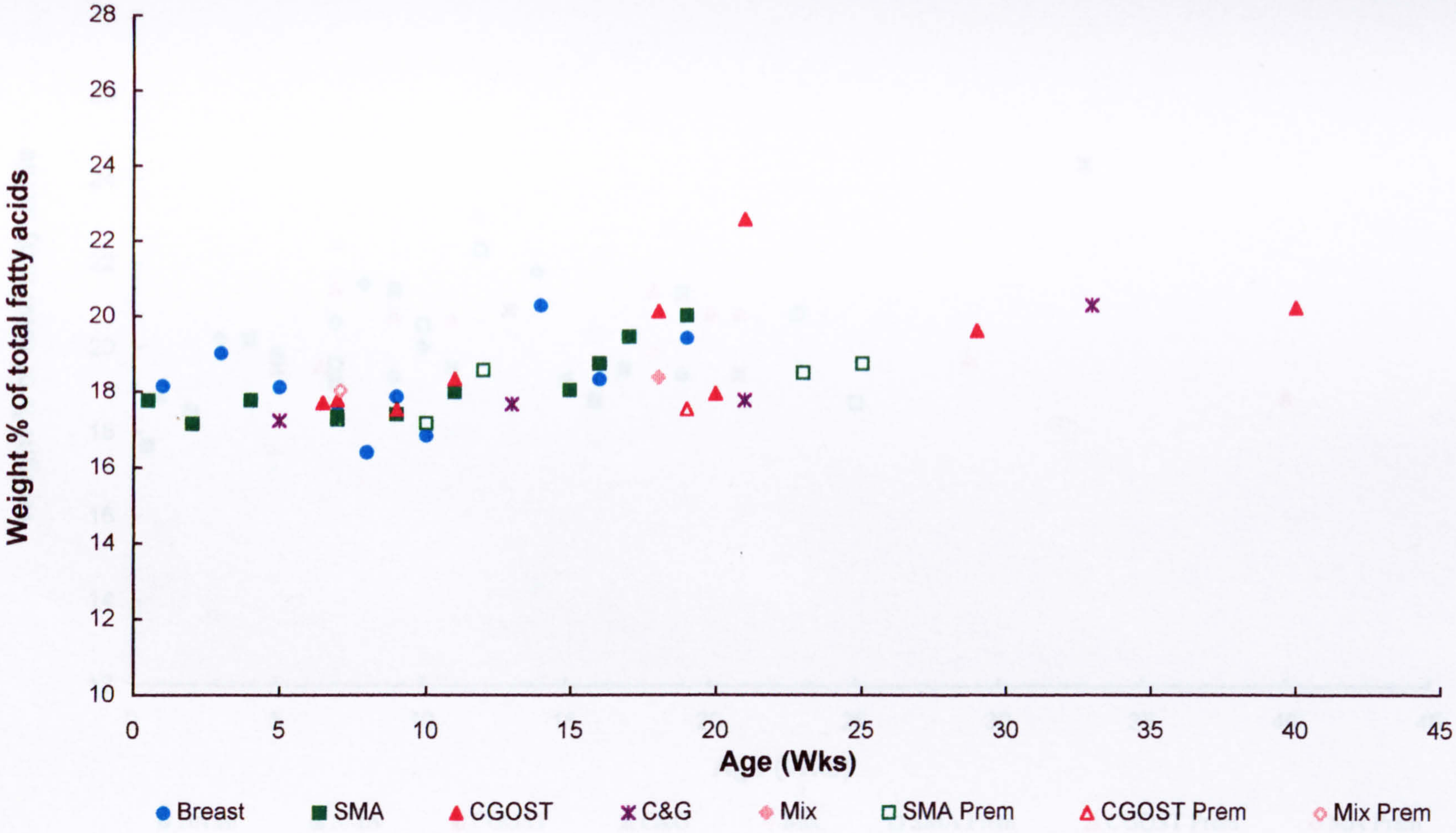


Figure 3.38 - Cerebellar cortex phospholipid stearic acid (C18:0) in relation to infants' diet and age.

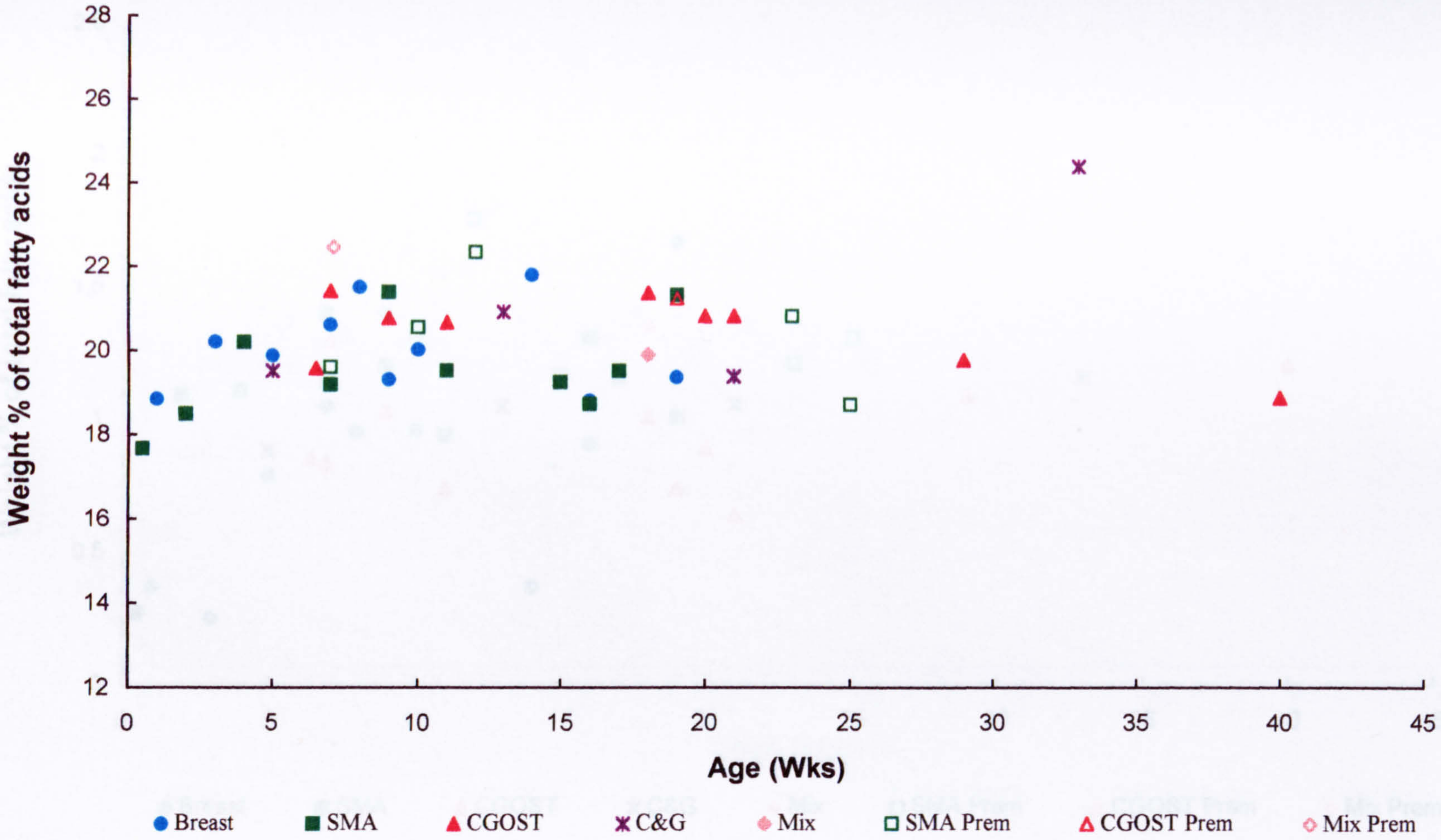


Figure 3.39 - Cerebellar cortex phospholipid oleic acid (C18:1n-7+n-9) in relation to infants' diet and age.

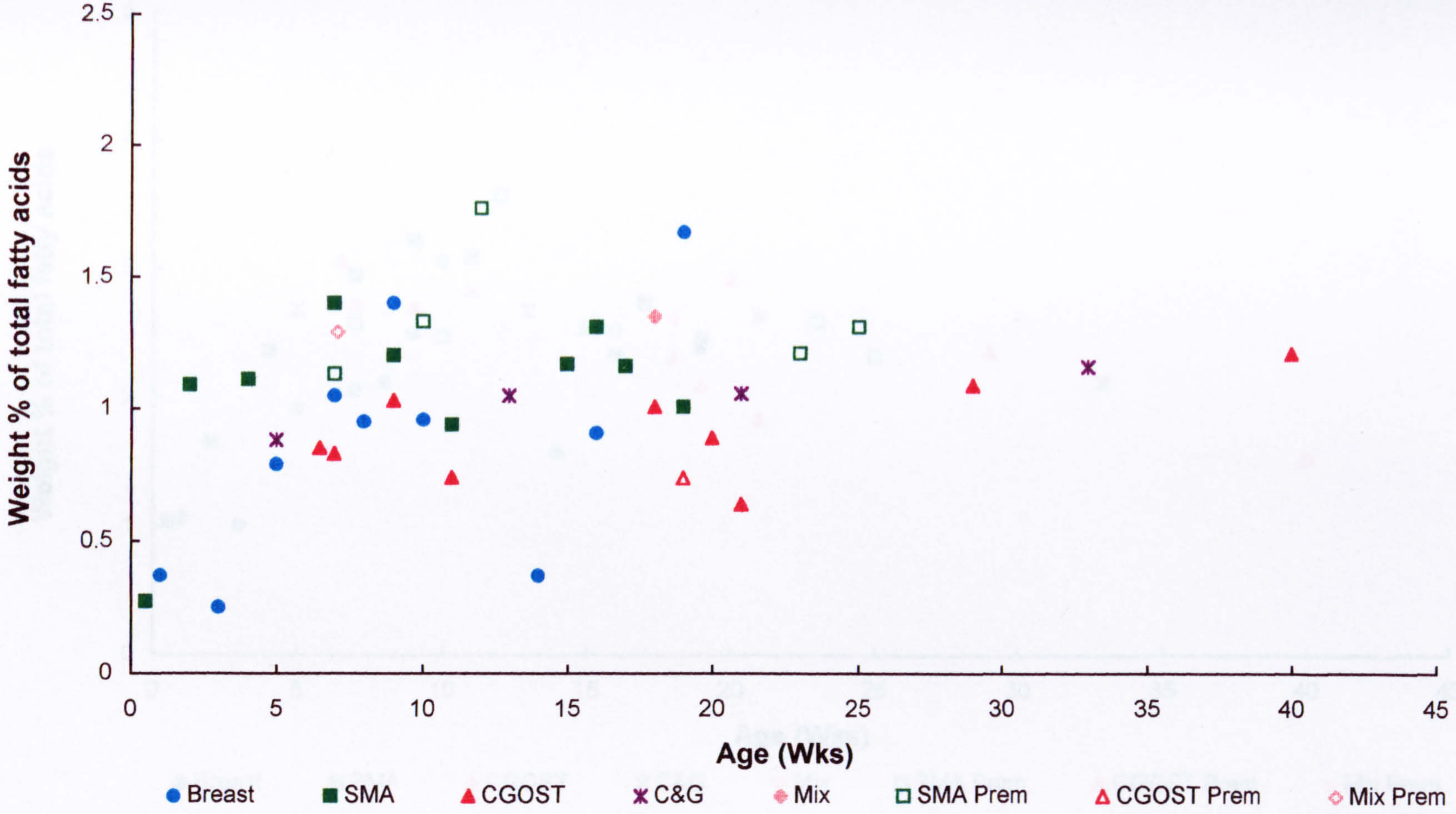


Figure 3.40 - Cerebellar cortex phospholipid linoleic acid (C18:2n-6) in relation to infants' diet and age.

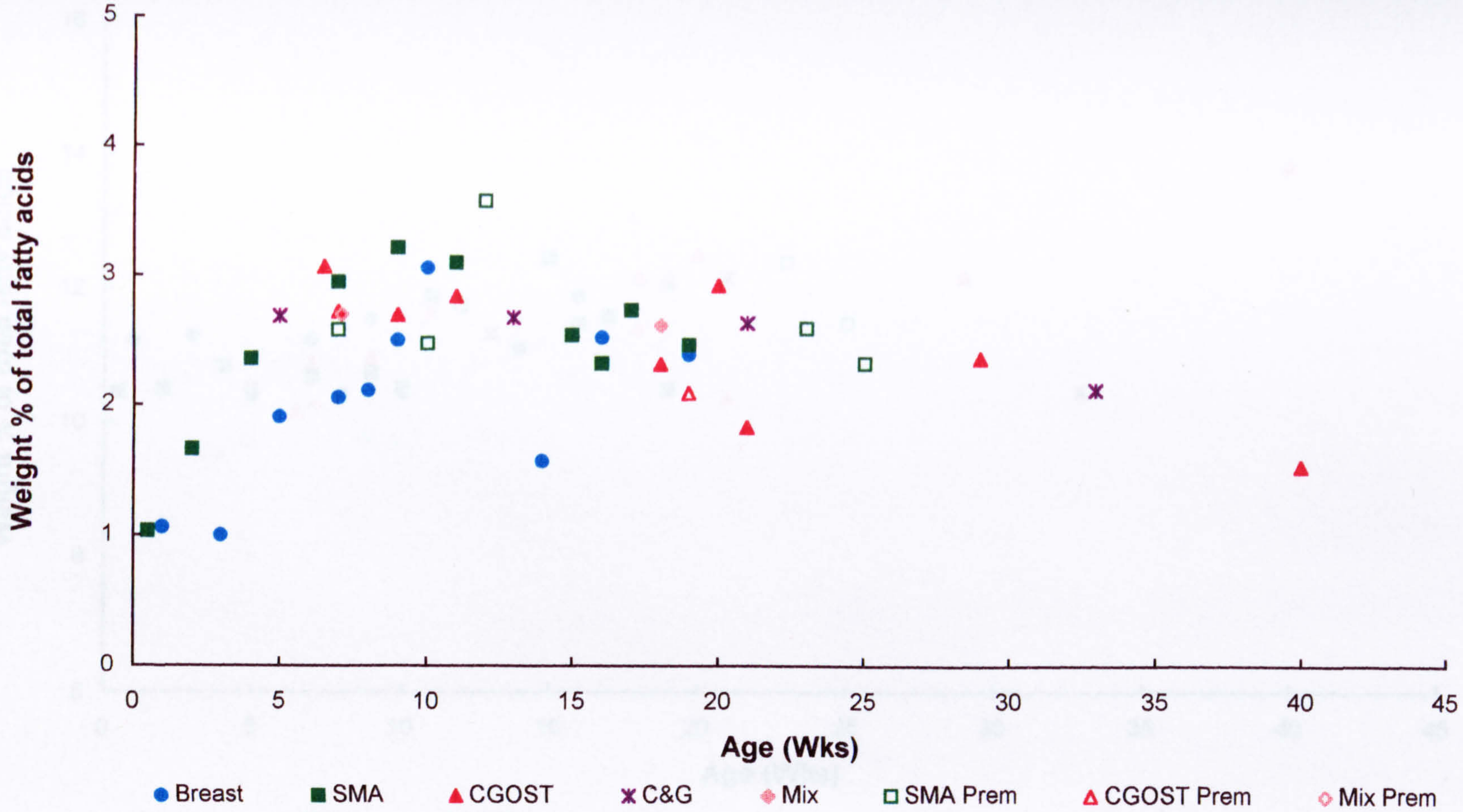


Figure 3.41 - Cerebellar cortex phospholipid dihomogammalinolenic acid (C20:3n-6) in relation to infants' diet and age.

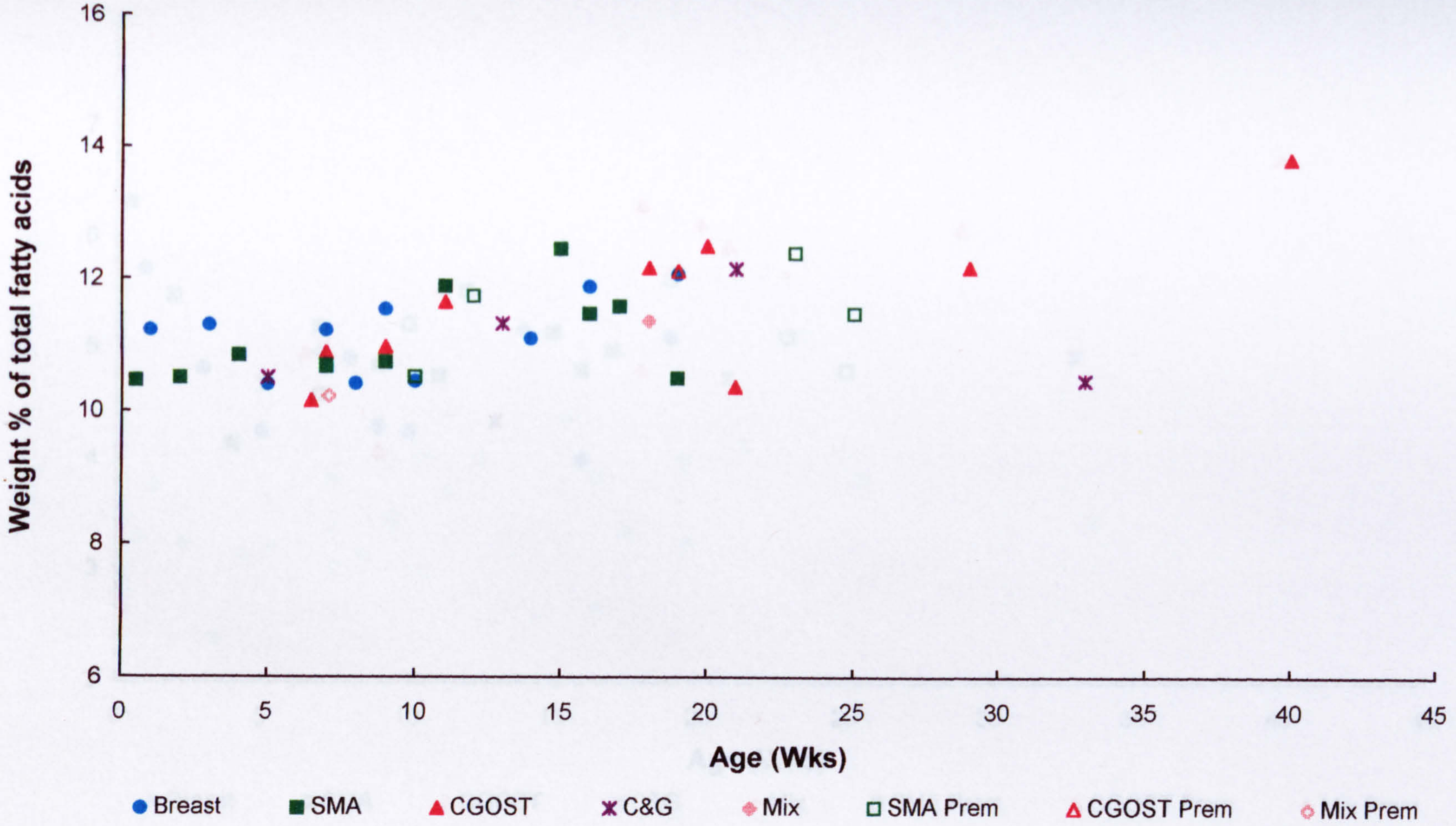


Figure 3.42 - Cerebellar cortex phospholipid arachidonic acid (C20:4n-6) in relation to infants' diet and age.

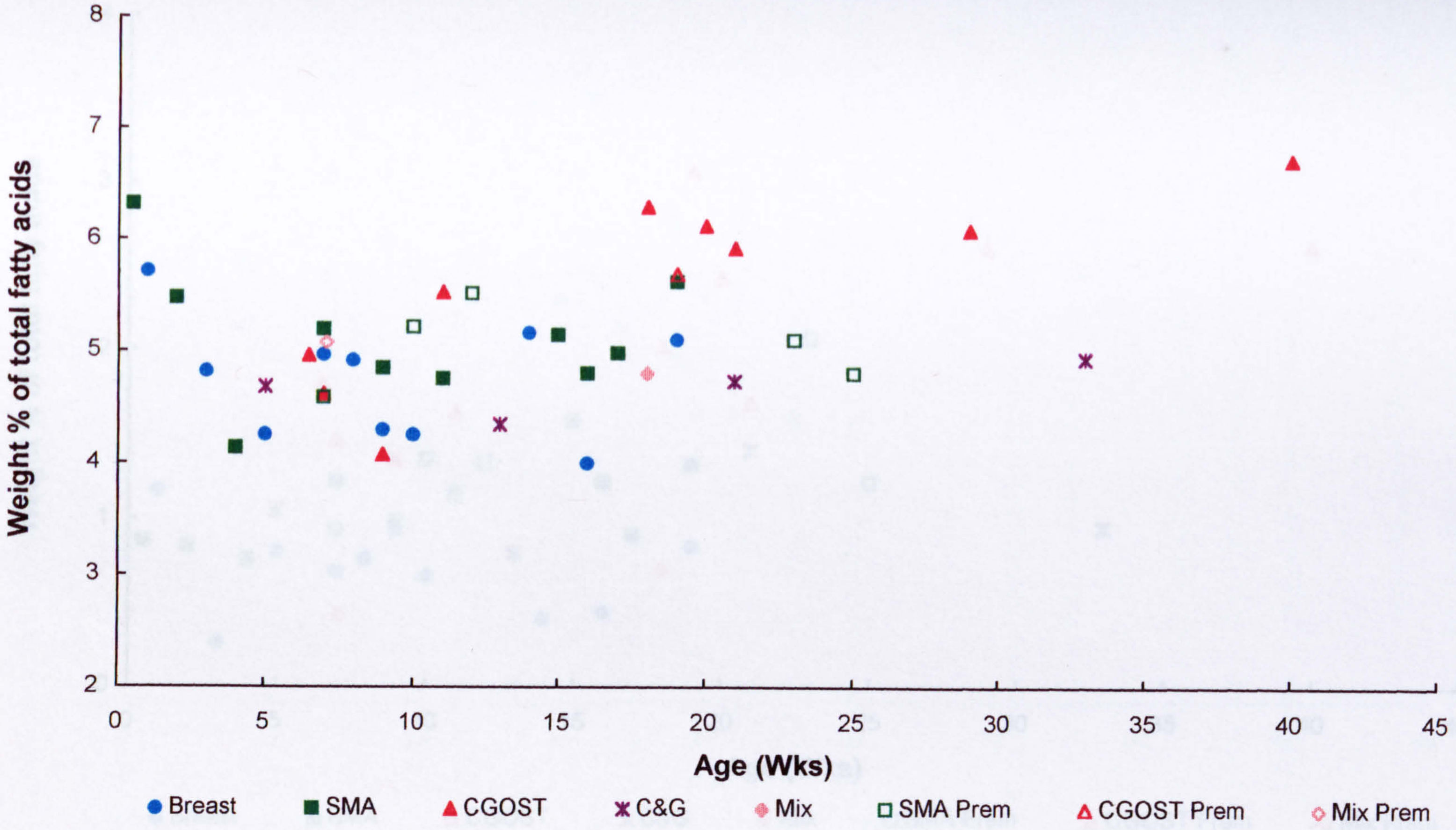


Figure 3.43 - Cerebellar cortex phospholipid docosatetraenoic acid (C22:4n-6) in relation to infants' diet and age.

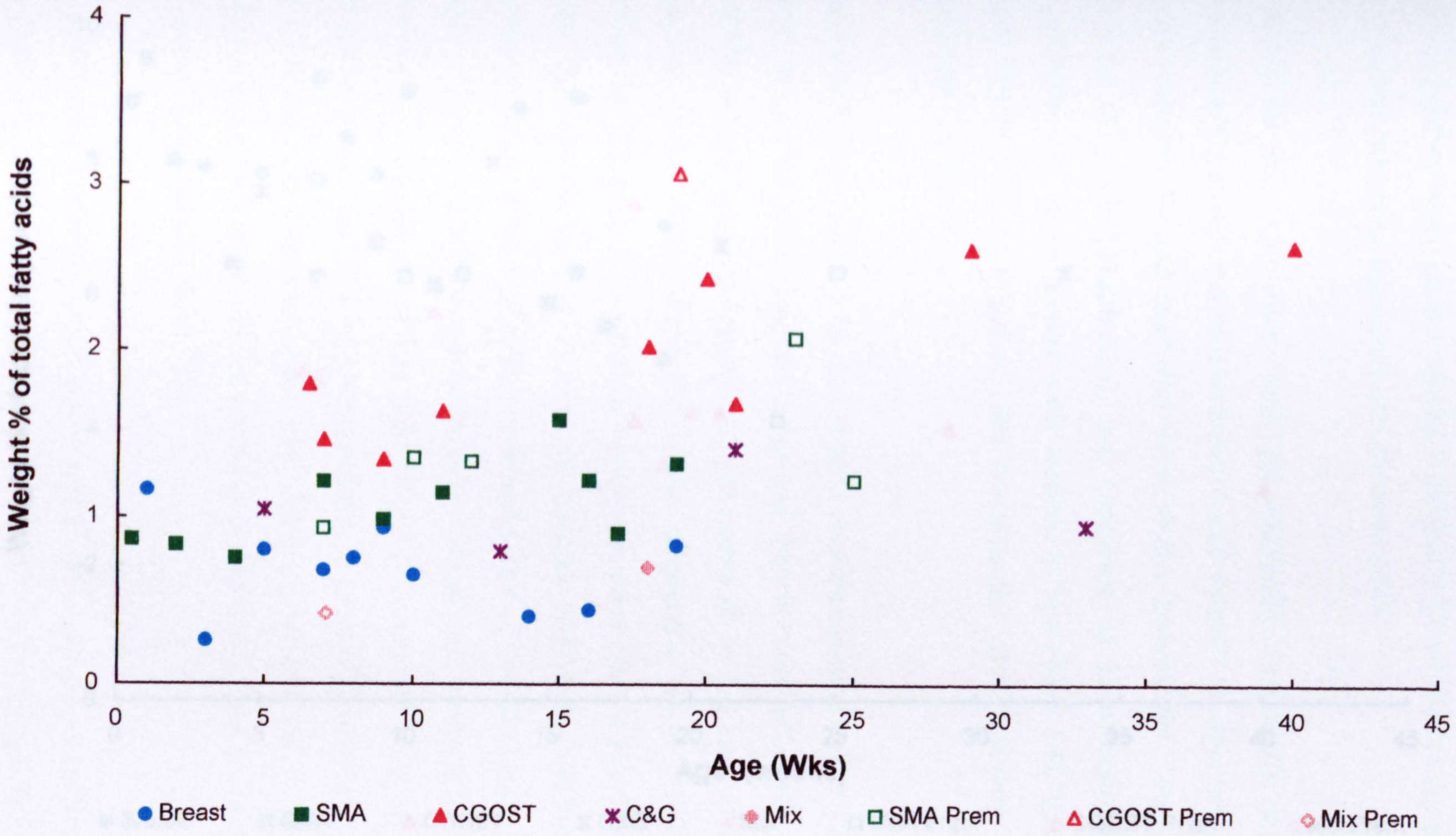


Figure 3.44 - Cerebellar cortex phospholipid docosapentaenoic acid (C22:5n-6) in relation to infants' diet and age.

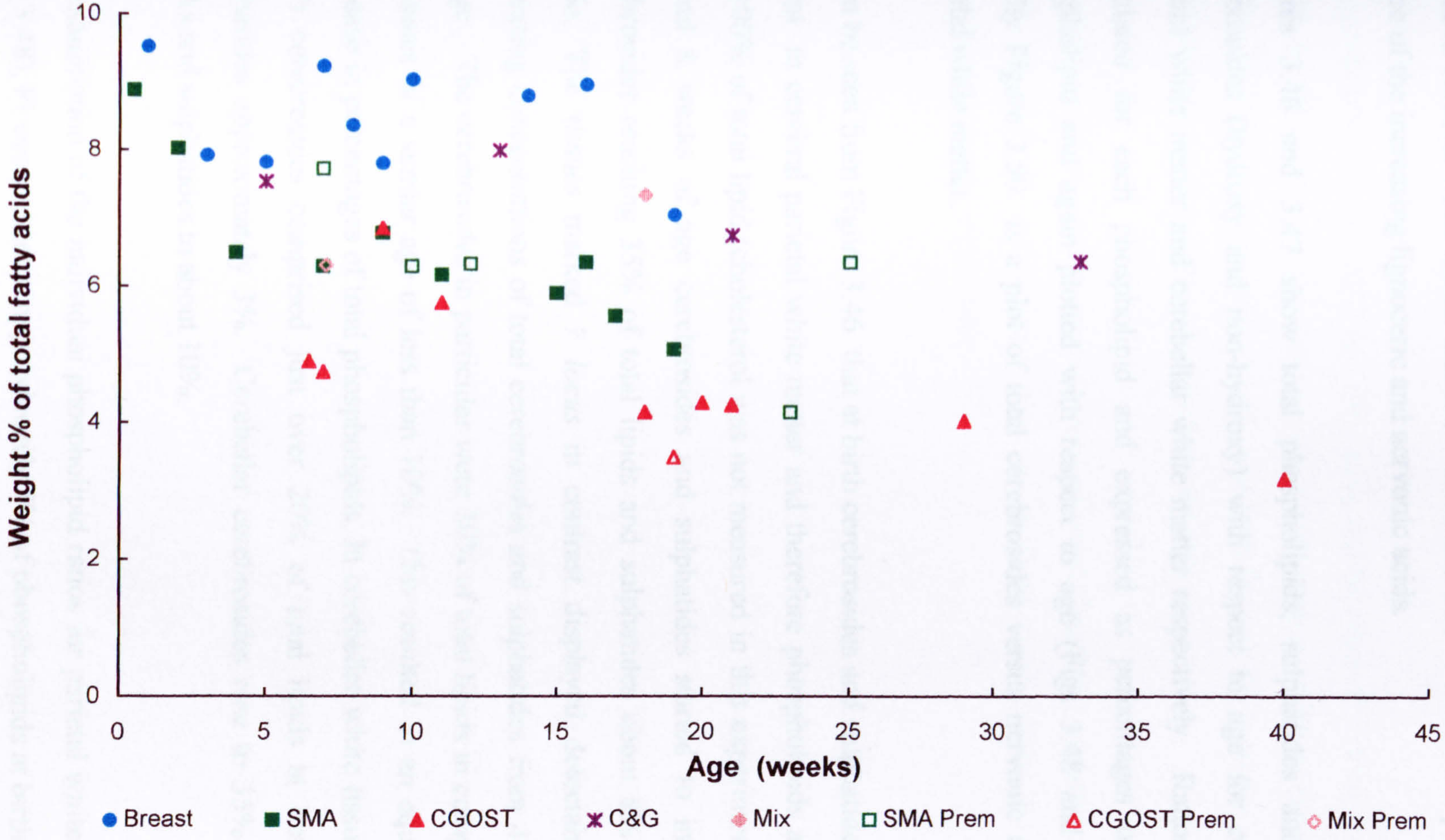


Figure 3.45 - Cerebellar cortex phospholipid docosahexaenoic acid (C22:6n-3) in relation to infants' diet and age.

3.3.4. Phospholipids and cerebrosides

The procedure of HPTLC followed by densitometry was employed in the case of cerebral and cerebellar white matter to elucidate the lipid composition of tissues in cerebral white matter marked *? locus* and also to define the probable source of the increasing lignoceric and nervonic acids.

Figures 3.46 and 3.47 show total phospholipids, sulphatides and total cerebrosides (hydroxy and non-hydroxy) with respect to age for cerebral parietal white matter and cerebellar white matter respectively. Ratios were calculated for each phospholipid and expressed as percentages of total phospholipid and again plotted with respect to age (Figs 3.48 and 3.49). Finally Figure 3.50 is a plot of total cerebrosides versus nervonic acid in parietal white matter.

It can be seen from Figure 3.46 that at birth cerebrosides and sulphatides were absent in cerebral parietal white matter and therefore phospholipids account for 100% of total lipid (cholesterol was not measured in this experiment). At around 8 weeks of age cerebrosides and sulphatides started to increase, cerebrosides reaching 35% of total lipids and sulphatides about 8% by 40 weeks. The tissues marked *? locus* in contrast displayed detectable and increasing concentrations of total cerebrosides and sulphatides from 4 weeks of age . The cerebrosides in particular were 30% of total lipids in comparison to tissues of a similar age of less than 10%. This resulted in an equivalent decrease in percentages of total phospholipids. In cerebellar white tissues (Fig 3.47) cerebrosides comprised just over 20% of total lipids at birth and sulphatides approximately 3%. Cerebellar cerebrosides rose to 35% by 40 weeks and sulphatides to about 10%.

On examination of the individual phospholipid ratios for parietal white matter (Fig 3.48), PI was present at approximately 5% of phospholipids at birth falling

to 2% at 40 weeks. Sphingomyelin was also present at 5% at birth but this climbed to 10% by 40 weeks. PS remained fairly constant at about 15% of phospholipids. PE rose about 5% over the period of study from between 30-35% to 35-40% whilst PC fell similarly from 40-45% to 35-40%.

The cerebellar white tissues (Fig 3.49) gave similar ratios PI falling from 5% at birth to 2% at 40 weeks, sphingomyelin slightly higher at 8% at birth rising to 14%, PS fairly constant around 18%, and both PC and PE between 30-40% but no obvious trend in either phospholipid evident.

The plot of total cerebrosides versus nervonic acid (Fig 3.50) in parietal white matter showed an exponential rise in nervonic acid with increasing percentages of total cerebrosides.

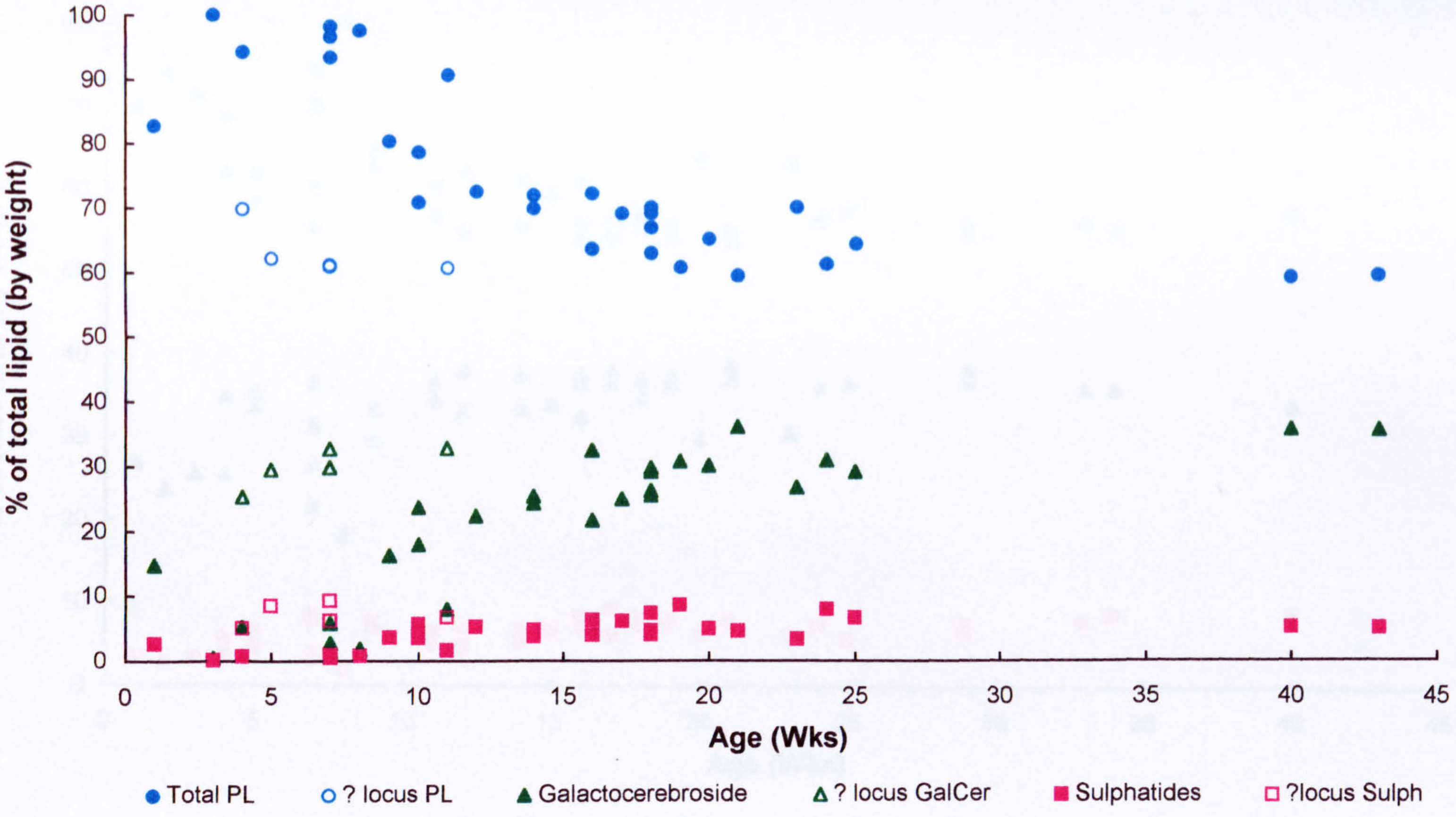


Figure 3.46 - Infant cerebral parietal white matter lipid compositions with respect to age.

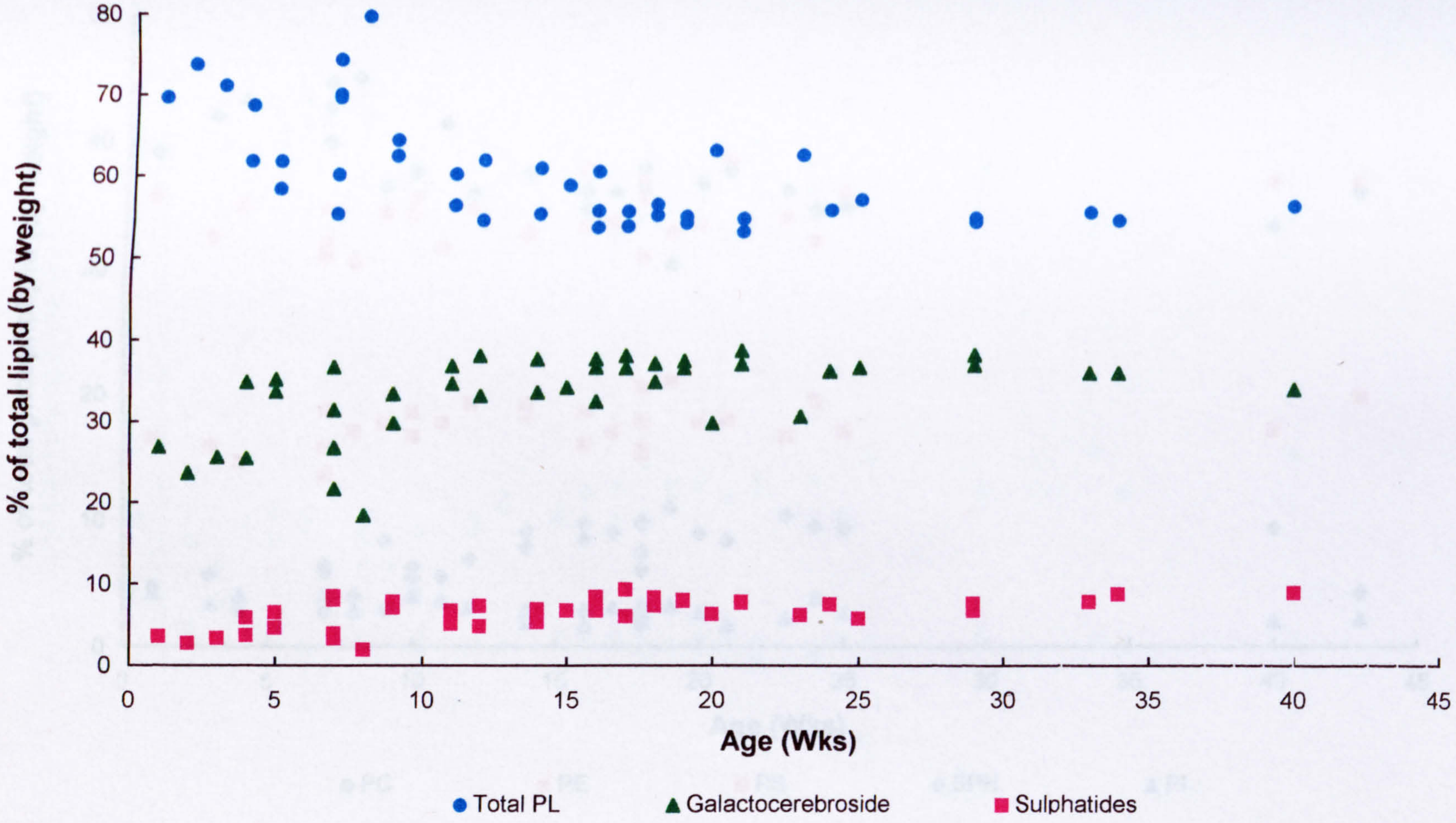


Figure 3.47 - Infant cerebellar white matter lipid compositions with respect to age.

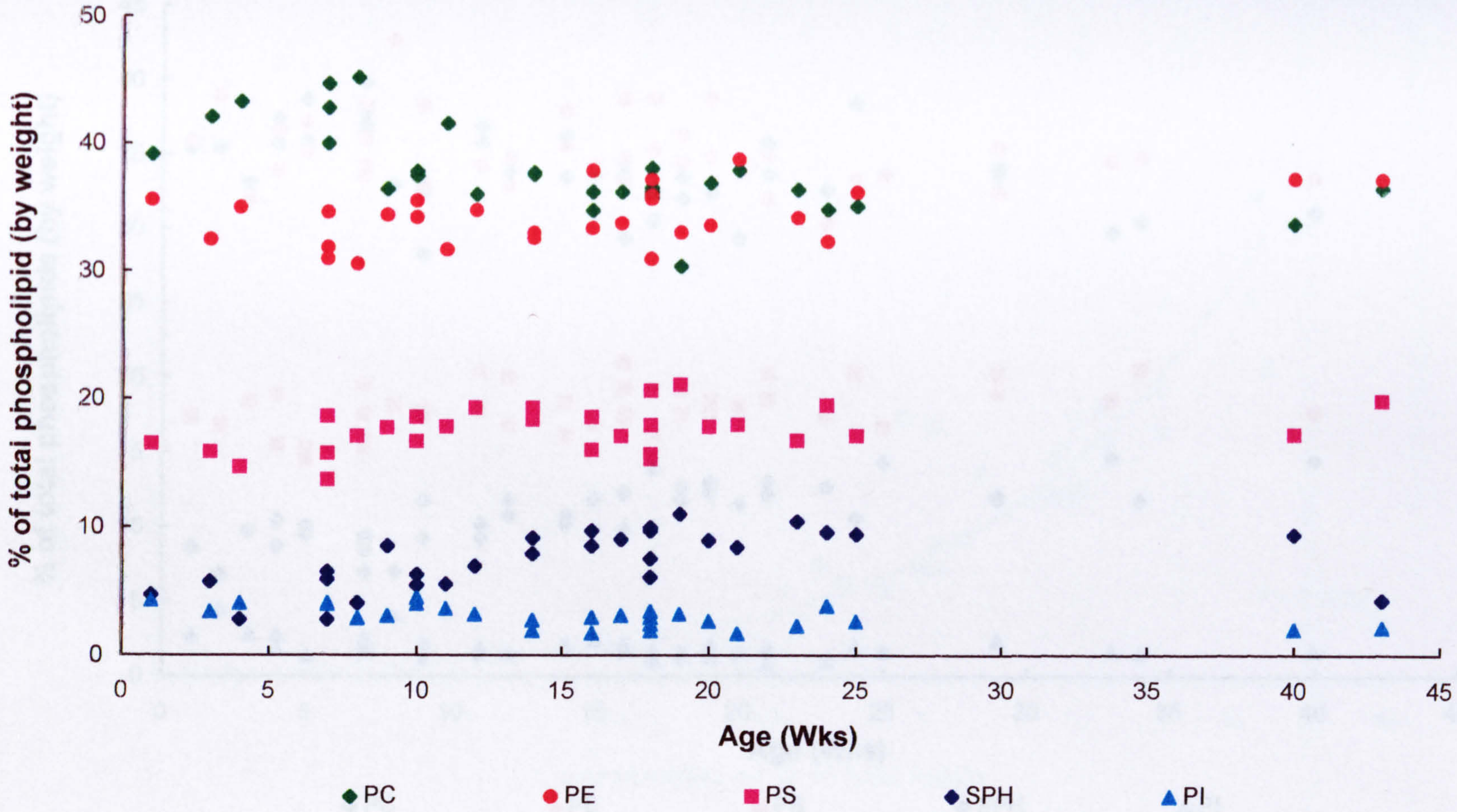


Figure 3.48 - Infant cerebral parietal white matter phospholipid compositions with respect to age.

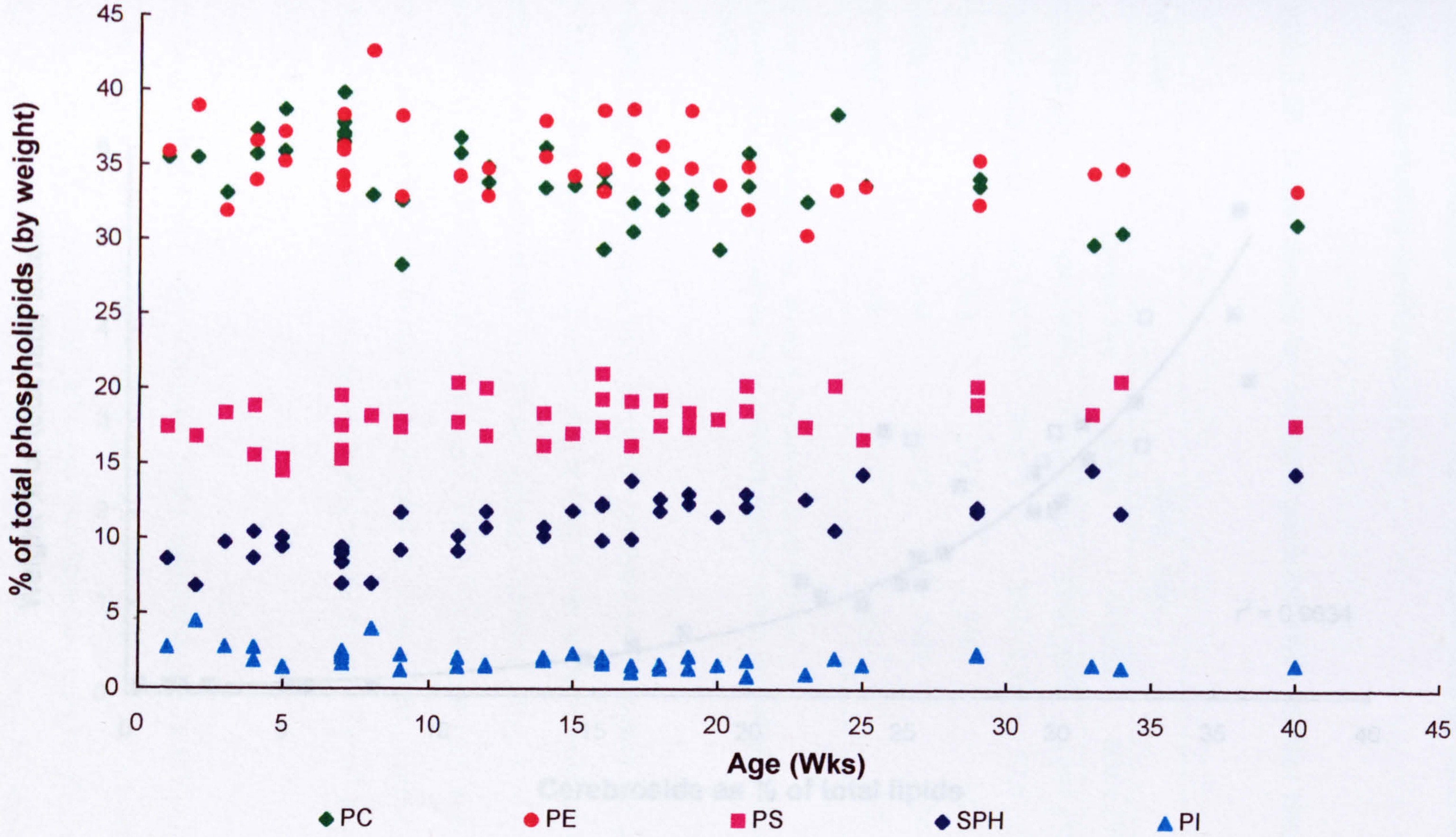


Figure 3.49 - Infant cerebellar white matter phospholipid compositions with respect to age.

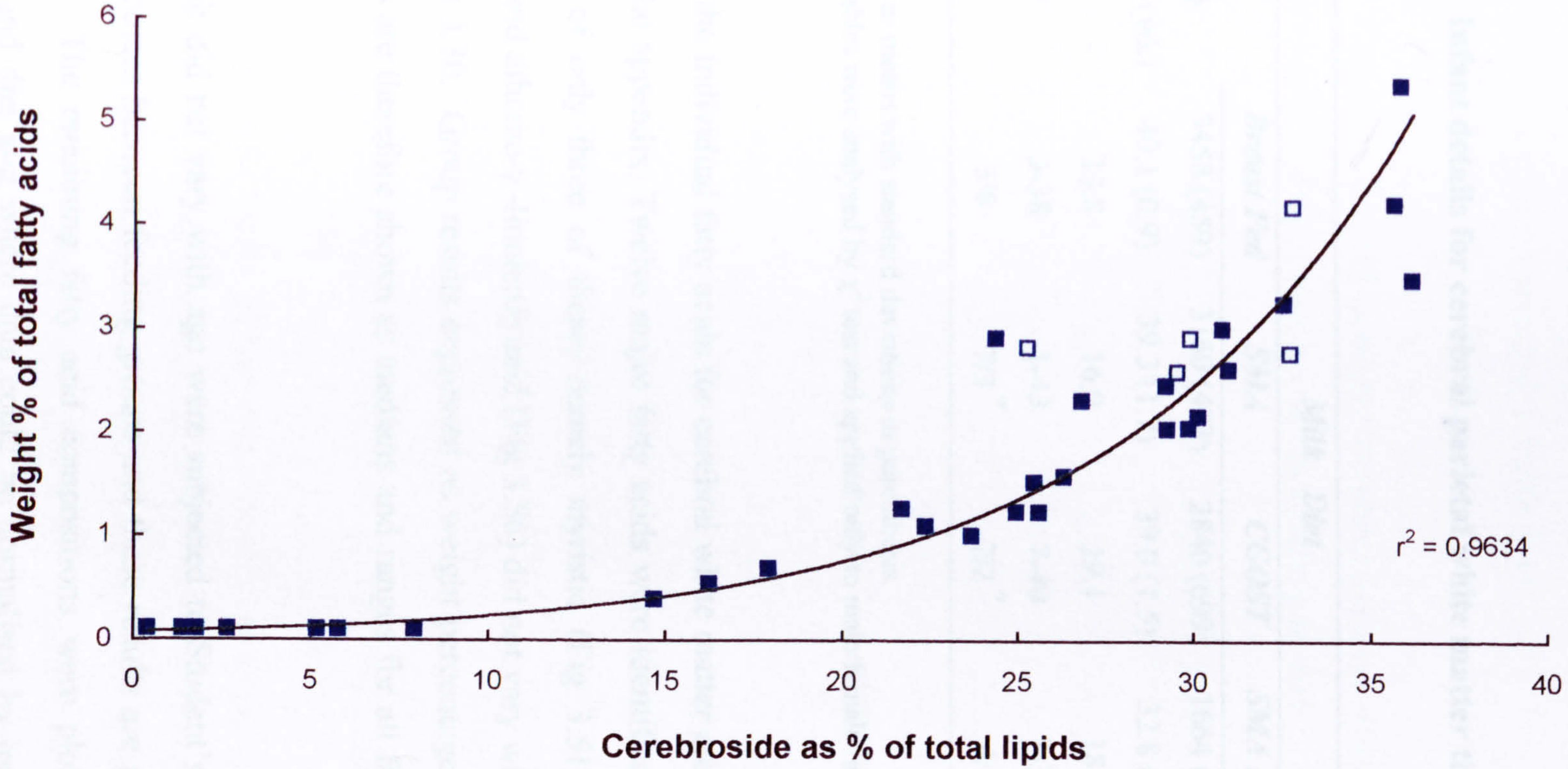


Figure 3.50 - Nervonic acid concentration (wt %) in relation to galactocerebroside in cerebral parietal white matter.

3.3.5. Cerebral parietal white matter total lipid fatty acids

Table 3.28 illustrates the infant group characteristics. Between group differences for the continuous variables (birth weights and gestational ages) were assessed by Student's t test and those for dichotomous variables (sex ratio) were estimated by the Chi-square test.

Table 3.28 Infant details for cerebral parietal white matter tissues

Infant Data	*Milk Diet*			
	Breast Fed	***SMA***	***CGOST***	***SMA Prem***
Birth weight (g)	3458 (459)	3160 (493)	2840 (699)	1664 (572)
Gestational age (wk)	40.1 (0.9)	39.3 (1.7)	39.0 (1.5)	32.8 (2.3)
Age (wk)	13.8	16.0	19.1	18.6
Age range (wk)	3-38	1-43	7-40	7-34
Male/Female	3/6	7/3 $^{\alpha}$	7/2 $^{\alpha}$	4/1

Results are shown as means with standard deviations in parenthesis.
Dichotomous variables were analysed by χ^2 test and applied only to male/female ratios.
$\alpha = p<0.02$

The results for the individual fatty acids for cerebral white matter are given in Table 3.29 in the appendix. Twelve major fatty acids were identified and the concentrations of only three of these, namely myristic (Fig 3.51), stearic acid(Fig 3.54) and dihomo-γ -linolenic acid (Fig 3.56) did not vary with age as shown in Table 3.30. Group results expressed as weight percentages of total lipid fatty acids are therefore shown as medians and ranges for all fatty acids (Table 3.31).

The three which did not vary with age were subjected to Student's t test to check for differences between feeding groups and these results are presented in Table 3.32. The remaining fatty acid compositions were plotted with respect to age and diet and where data could be normalised by logarithmic

transformation, this was performed and the derived curves presented for lignoceric (Fig 3.61) and nervonic acid (Fig 3.62). Equations for the curves are given for each term born group and the effect of diet and age determined by application of the general linear model analysis of covariance over the restricted age range 9-24 weeks inclusive (Minitab 10). The significance level was set at $p<0.02$ and the results presented in Table 3.34.

Table 3.30 Correlation coefficients for cerebral parietal white matter fatty acid concentration vs age in relation to diet

Fatty Acid	Breast	SMA	CGOST	SMA Prem
	r	r	r	r
C14:0	0.8105 $^{\delta}$	0.0363	-0.6290	0.3306
C16:0	-0.8734 $^{\delta}$	-0.8877 $^{\delta}$	-0.8553 $^{\delta}$	-0.9581 $^{\delta}$
C16:1 n-7	-0.3740	-0.9152 $^{\delta}$	-0.7601$^{\delta}$	-0.8963 $^{\delta}$
C18:0	-0.6494	-0.1807	-0.1801	0.9138 $^{\delta}$
C18:1	0.8650$^{\delta}$	0.9433 $^{\delta}$	0.9304 $^{\delta}$	0.9079 $^{\delta}$
C20:3 n-6	0.2999	0.2975	-0.3758	-0.4294
C20:4 n-6	-0.8243 $^{\delta}$	-0.8357 $^{\delta}$	-0.9046 $^{\delta}$	-0.4770
C22:4 n-6	0.8971$^{\delta}$	0.8468 $^{\delta}$	0.8296 $^{\delta}$	0.8085
C22:5 n-6	-0.8435 $^{\delta}$	-0.7129 $^{\delta}$	-0.7413 $^{\delta}$	-0.7841
C22:6 n-3	-0.9099 $^{\delta}$	-0.8831 $^{\delta}$	-0.8545 $^{\delta}$	-0.7386
C24:0	0.8111 $^{\delta}$	0.7814 $^{\delta}$	0.8191 $^{\delta}$	0.9369 $^{\delta}$
C24:1 n-9	0.7920 $^{\delta}$	0.9725 $^{\delta}$	0.9369 $^{\delta}$	0.7944

δ = Significant correlation of individual fatty acid concentrations (wt %) with age. ($p<0.01$)

Otherwise data points for each group were merely analysed by visual inspection for general trends without application of statistical analysis, again due to lack of age-matching of individuals. This analysis applied to palmitic (Fig 3.52), oleic (Fig 3.55), arachidonic (Fig 3.57), docosatetraenoic (Fig

3.58), docosapentaenoic (Fig 3.59) and docosahexaenoic acid (Fig 3.60) concentrations.

Table 3.31 Cerebral parietal white matter and myelin total lipid fatty acid compositions

Fatty Acid	*Milk Diet*			
	Breast n=9	***SMA n=10***	***CGOST n=9***	***SMA-Prem n=5***
C14:0	1.40 (0.92-2.48)	1.36 (1.12-1.97)	1.20 (1.02-1.76)	1.48 (1.22-1.77)
C16:0	24.50 (18.43-29.43)	24.65 (19.66-29.66)	21.63 (19.94-27.97)	27.95 (21.99-29.60)
C16:1n-7	2.38 (2.19-3.22)	2.37 (1.52-3.11)	2.19 (1.26-2.97)	2.54 (1.73-3.03)
C18:0	22.50 (21.15-23.69)	23.10 (20.91-24.71)	22.56 (20.67-24.15)	22.57 (21.74-23.72)
C18:1n-7+n-9	19.43 (14.12-26.49)	18.02 (14.79-28.03)	20.66 (14.07-28.50)	16.33 (14.46-21.48)
C20:3n-6	1.47 (0.95-1.66)	1.53 (0.73-1.95)	1.56 (1.11-1.75)	1.75 (1.28-2.62)
C20:4n-6	9.53 (7.00-11.90)	10.50 (6.95-11.37)	10.09 (6.15-11.81)	10.55 (9.76-12.19)
C22:4n-6	6.96 (6.11-9.34)	7.23 (6.20-8.45)	8.24 (7.12-9.26)	6.80 (6.14-9.10)
C22:5n-6	1.62 (0.48-2.49)	2.20 (1.24-3.06)	2.93 (1.84-4.03)	2.69 (2.18-3.42)
C22:6n-3	7.23 (3.67-9.24)	6.22 (3.24-8.76)	3.81 (1.78-6.93)	4.42 (3.24-7.25)
C24:0	1.46 (<0.10-3.14)	1.31 (<0.10-2.68)	1.35 (<0.10-2.72)	0.36 (<0.10-2.19)
C24:1n-9	1.01 (<0.10-3.93)	1.25 (<0.10-4.22)	2.06 (<0.10-4.30)	0.69 (<0.10-2.47)

Fatty acids are expressed as weight percentages and are given as medians and ranges

The five points displayed on the graphs marked *"? Locus"* refer to tissues collected consecutively over a four month period (March - June 1994) and as can be seen most of their fatty acid compositions were markedly different from those of infants of the same age. These tissues were reanalysed and similar results obtained. With their relatively "increased" levels of lignoceric and nervonic acids, it would appear that they may have been obtained from a site

outwith the white matter parietal lobe and in which the myelination process had occurred at an earlier age. This assertion could not be confirmed by our pathology colleagues. However as the results were obtained only during this short period of time, the derived data was considered separately.

Table 3.32 "t" test p values for cerebral white fatty acids

Feed	C14:0	C18:0	C20:3
BF/SMA	0.9364	0.03746	0.5133
BF/CGOST	0.2960	0.7490	0.2291
SMA/CGOST	0.1763	0.6341	0.7799

There were no significant differences between feeding groups in myristic acid (Fig 3.51) as determined using Student's "t" test and as already mentioned the concentration of this fatty acid did not vary with age being maintained between 1 and 2%. In the parietal central white matter neonatal palmitic acid concentrations represented around 30% of total fatty acids. Thereafter a downward cascade in results was apparent in all the term born infant feeding groups. Subsequently palmitic acid levels stabilised at about 20% after 5 months. It appeared that there was an approximate 4 week "delay" in this palmitic acid reduction in the preterm SMA infants with respect to the term SMA group and an equivalent delay in the latter group when comparison was made with infants in the breast-fed group. The CGOST group palmitic acid concentrations tended to occupy intermediary positions between those of the SMA and breast-fed infants of similar age (Fig 3.52).

A reversal of this pattern of results was encountered on examination of the oleic acid concentrations (Fig 3.55). From neonatal compositions at 15% of total fatty acids a rapid increase occurred to a maximum of between 25 and

30% towards the end of the first year of life in the main feeding groups. It was apparent that the rise in oleic acid concentrations was instigated about one month earlier in the breast fed than formula fed infants and that a subsequent further "delay" in oleic acid accretion may exist between the term and preterm SMA fed infants respectively. Whilst the highest palmitic acid level was found in the 7 week old mixed fed preterm (32 weeks gestation) infant, in contrast this individual also displayed the lowest oleic acid concentration.

Palmitoleic acid concentrations (Fig 3.53) fell sharply in the first two months and from hence more gradually. After transformation of data, results were subjected to ANCOVA analysis (Table 3.33). No between group dietary differences were revealed ($p>0.02$).

Table 3.33 ANCOVA statistics for palmitoleic acid

Feed	Diet	Age	Diet×Age
Breast/ SMA	0.234	<0.001	0.538
Breast/ CGOST	0.435	<0.001	0.613
SMA/ CGOST	0.556	<0.001	0.365

There were no significant differences between groups in stearic acid (Fig 3.54) as calculated by Student's "t" test and no concentrations outwith a narrow band from 20 to 25% of total fatty acids. After a slight rise in neonatal dihomo-γ-linolenic acid (Fig 3.56) subsequent concentrations were fairly static at about 1.5% of total fatty acids. There were no significant differences between feeding groups.

Arachidonic acid concentrations remained relatively constant at about 11 % of total fatty acids in all feeding groups during the first two months of life (Fig 3.57). This was immediately followed by a steady decline in arachidonic acid compositions initially exclusively in the breast-fed group. After a further 6 weeks of life this pattern of results was repeated in the term born SMA and CGOST formula fed infants. At 40 weeks of age AA concentrations in all feeding groups appeared to converge at values between 6 and 7% of total fatty acids. In the small preterm SMA fed group, AA results may be indicative of a further "postponement" in their reduction with respect to their term born equivalents and indeed the highest AA concentration encountered (12.2%) was that of the 17 week old preterm SMA fed infant (Fig 3.57).

In contrast to the cerebral cortex analysis in which docosatetraenoic acid concentrations merely mirrored those of AA, albeit at about half of the composition (12% vs 6%), in the cerebral white matter DTA concentrations commenced at between 6 and 7% but, uniquely among the polyunsaturated fatty acids, increased in concentration with time until by 40 weeks they were ultimately in excess of those of AA at about 9% of total fatty acids in each feeding group. Docosatetraenoic acid (Fig 3.58) concentrations did not however appear to be significantly different between feeding groups, with all groups displaying a similar rising trend over the 40 week period.

Docosapentaenoic acid was never prominent in any feeding group with concentrations seldom higher than 4% of total fatty acids and declining universally to under 3% by 20 weeks of age. The initial reduction in the breast fed infants in advance of the formula fed groups (by a minimum of 5 weeks) was again a notable feature of the results (Fig 3.59).

Docosahexaenoic acid concentrations at birth represented ca 9% of total fatty acids and underwent a similar fall in values as those of AA, however the

earlier reduction in the breast fed infants was here reversed with the initial steep decline noted in the SMA preterm and CGOST fed groups marginally in advance of that detected in both the SMA and breast-fed groups (Fig 3.60). By about 20 weeks, all results have tended to coalesce at a minimum of 4% of total fatty acids in all feeding groups (although a further overall reduction in the CGOST fed group may be evident).

Examination of the age-related nervonic acid and lignoceric acid compositions (Figs 3.61 and 3.62 respectively) demonstrated "parallel" logarithmic curves in which the increase for breast-fed and formula-fed infants was evident at the end of approximately two and three months respectively. Although insufficient data for the preterm infants precluded statistical analysis, there appeared to be in general a further delay in their accretion relative to their term counterparts.

Table3.34 ANCOVA statistics for lignoceric and nervonic acids

	Lignoceric acid			Nervonic acid		
Feed	**Diet**	**Age**	**Diet×Age**	**Diet**	**Age**	**Diet×Age**
Breast/ SMA	<0.001	<0.001	0.193	<0.001	<0.001	0.839
Breast/ CGOST	0.007	<0.0.01	0.788	0.007	<0.001	0.961
SMA/ CGOST	0.027	<0.001	0.282	0.005	<0.001	0.805

Although present in the figures (3.61 and 3.62) the lignoceric and nervonic concentrations which were undetected (<0.1% of total fatty acids) were excluded from the statistical analysis. As a result logarithmic curves could be derived for lignoceric and nervonic acids only for the term infant groups. A significant dietary effect on both lignoceric and nervonic acid concentrations

was found between the breast-fed and both SMA-fed ($p<0.001$) and CGOST-fed infants ($p=0.007$) by ANCOVA analysis over the age range 9 - 24 weeks. It was less clear that a significant dietary effect existed between the SMA and CGOST groups in cerebral white lignoceric acid ($p = 0.027$) although the difference was more emphatic in nervonic acid ($p = 0.005$).

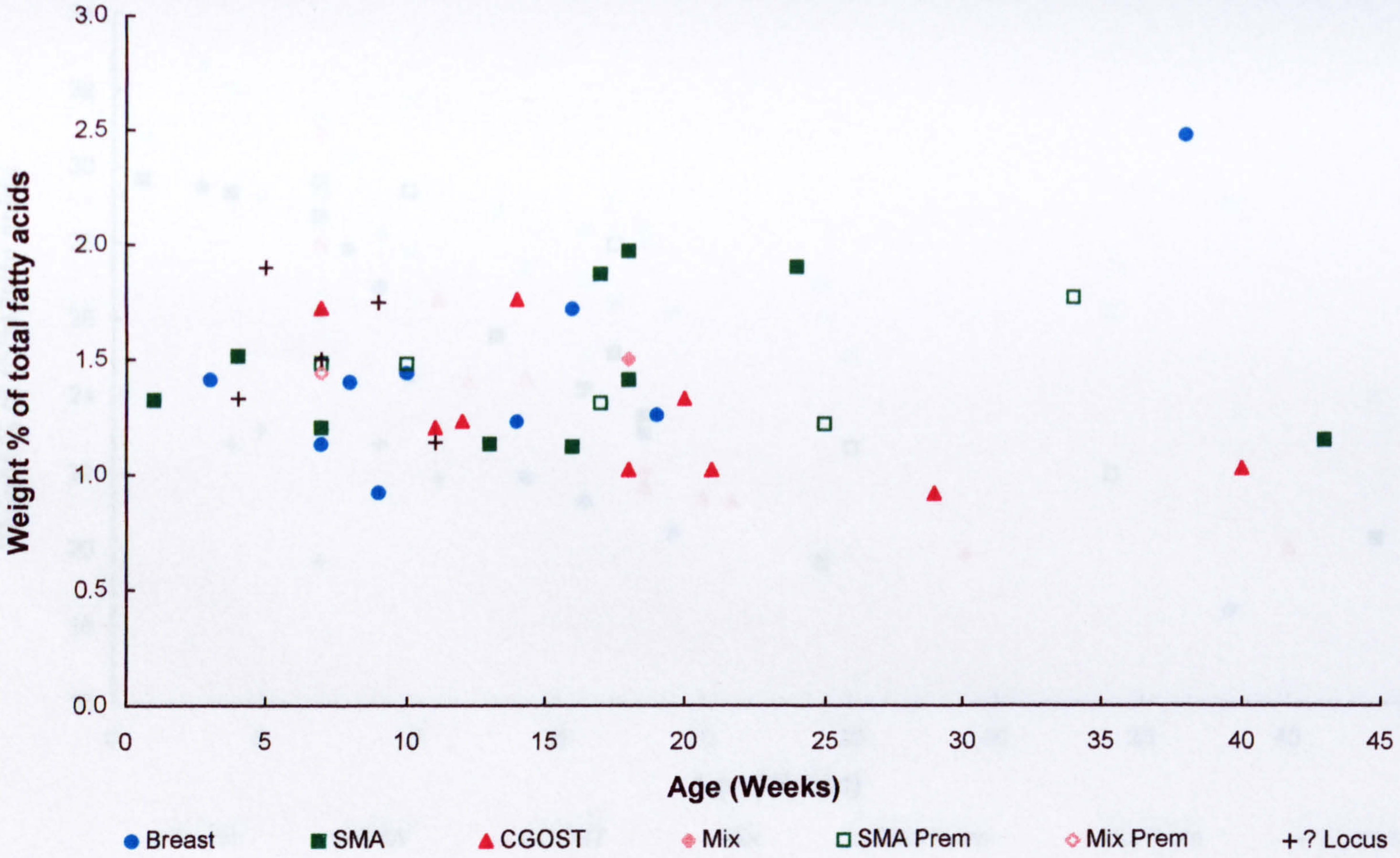


Figure 3.51 -Cerebral parietal white matter total lipid myristic acid (C14:0) in relation to infants' diet and age.

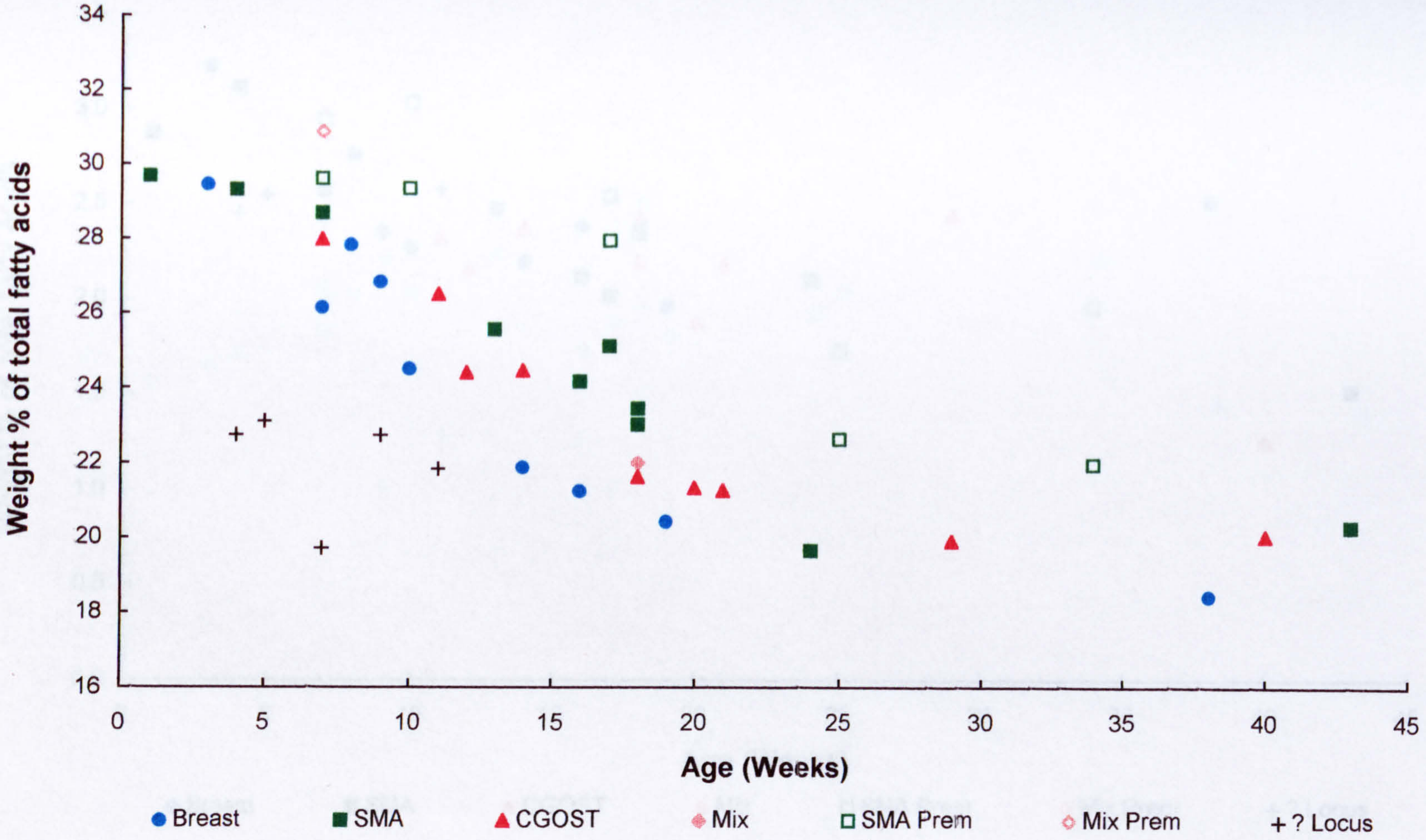


Figure 3.52 - Cerebral parietal white matter total lipid palmitic acid (C16:0) in relation to infants' diet and age.

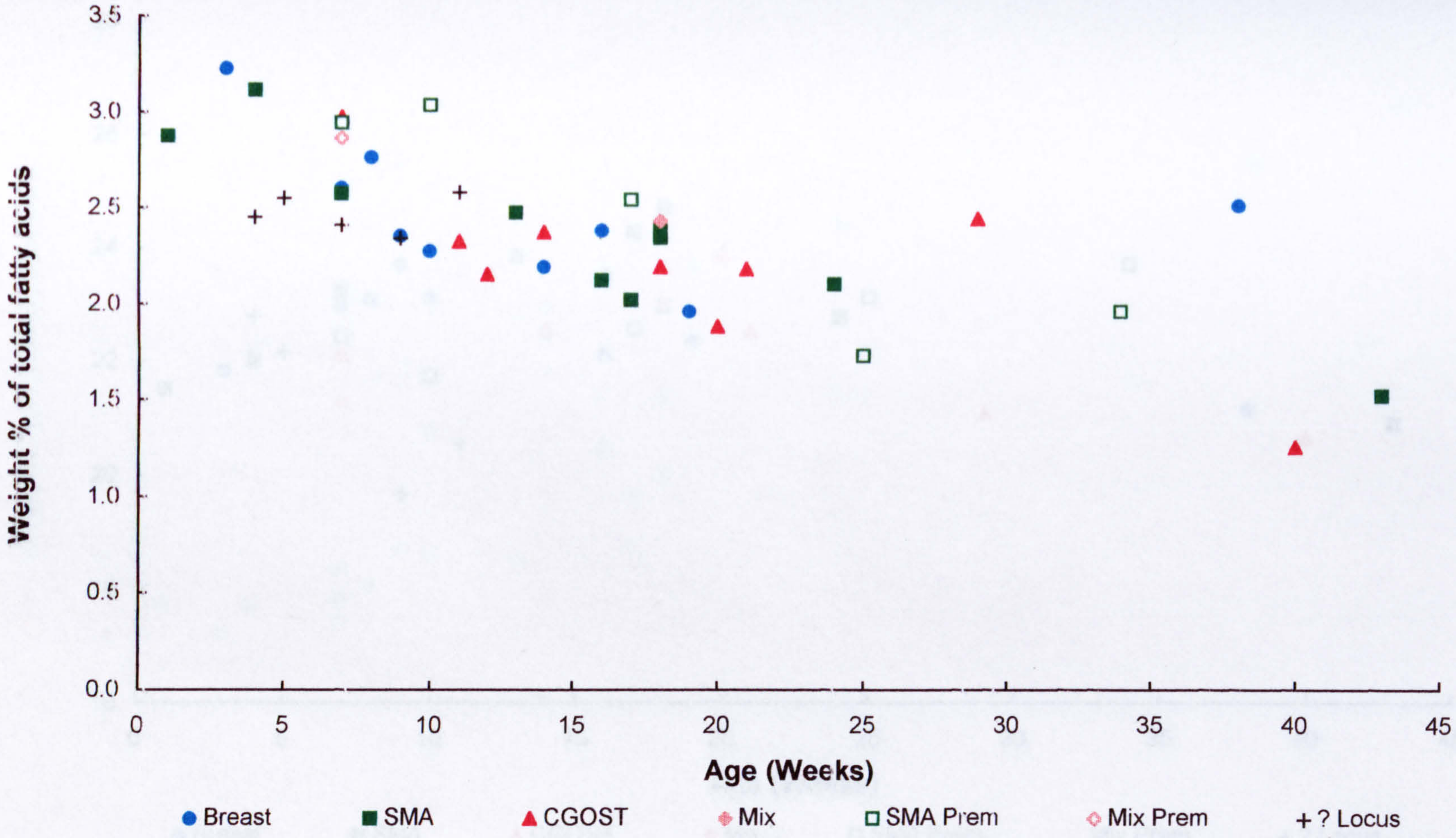


Figure 3.53 - Cerebral parietal white matter total lipid palmitoleic acid (C16:1n-7) in relation to infants' diet and age.

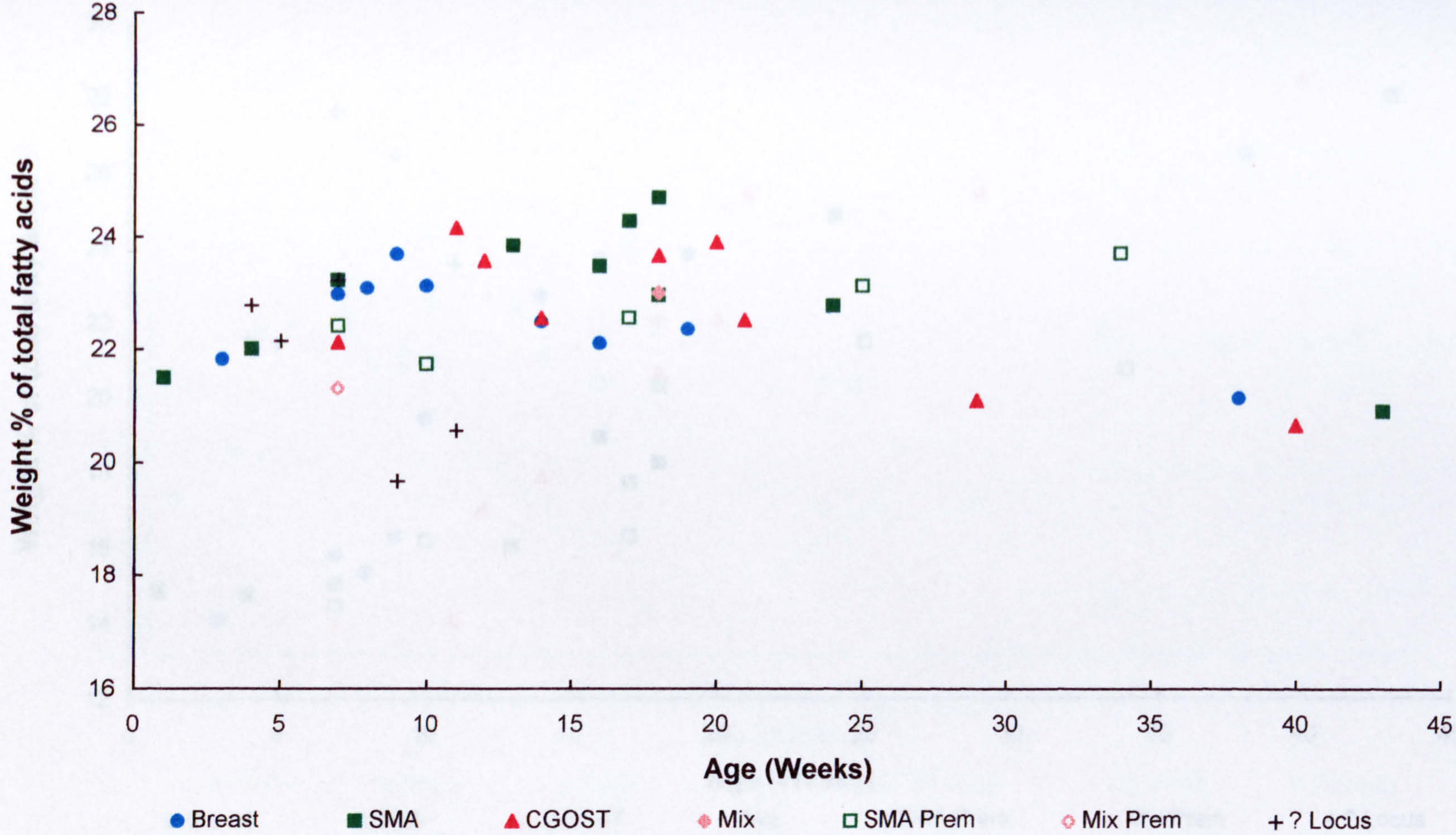


Figure 3.54 - Cerebral parietal white matter total lipid stearic acid (C18:0) in relation to infants' diet and age.

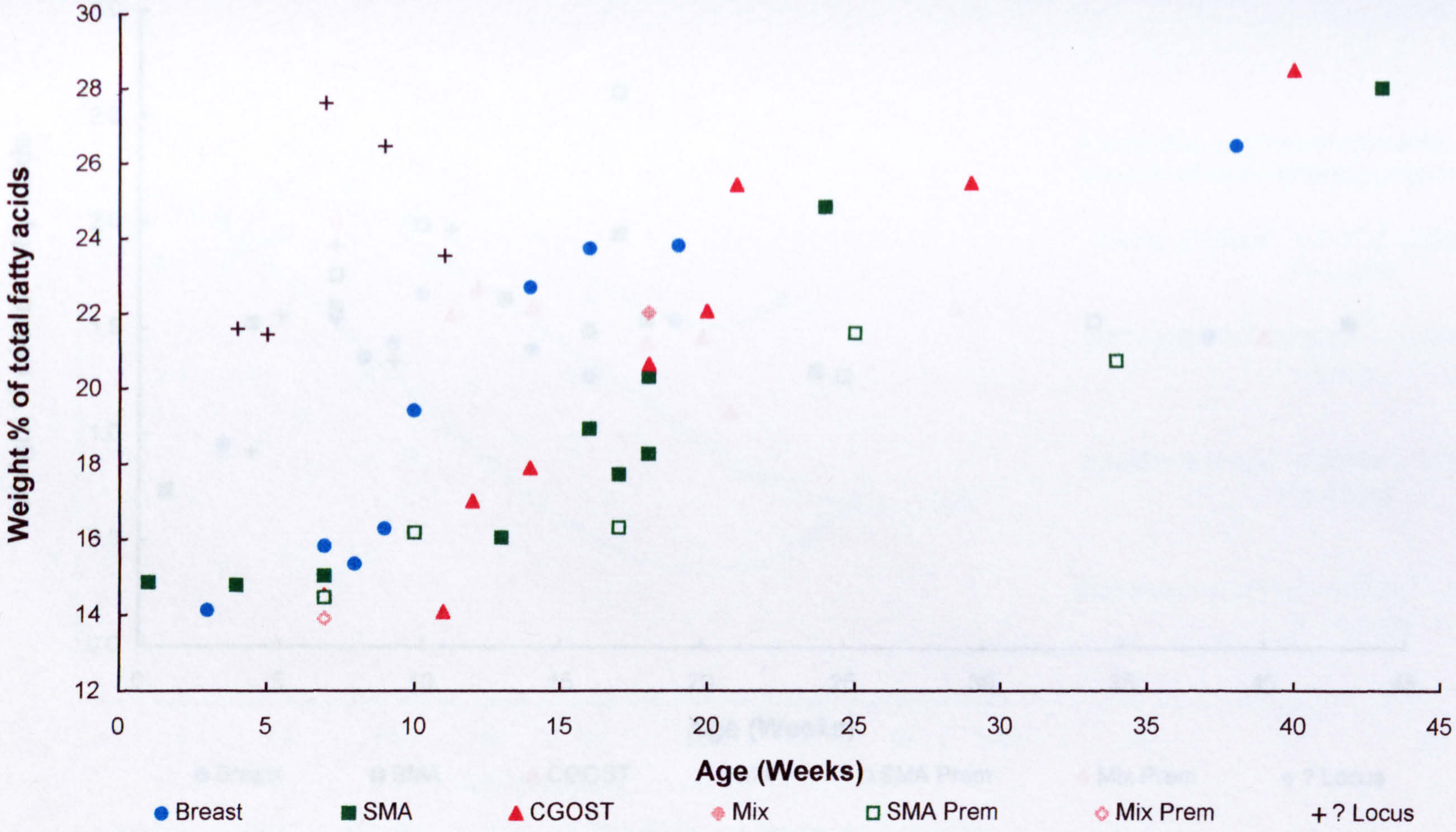


Figure 3.55 - Cerebral parietal white matter total lipid oleic acid (C18:1n-7+n-9) in relation to infants' diet and age.

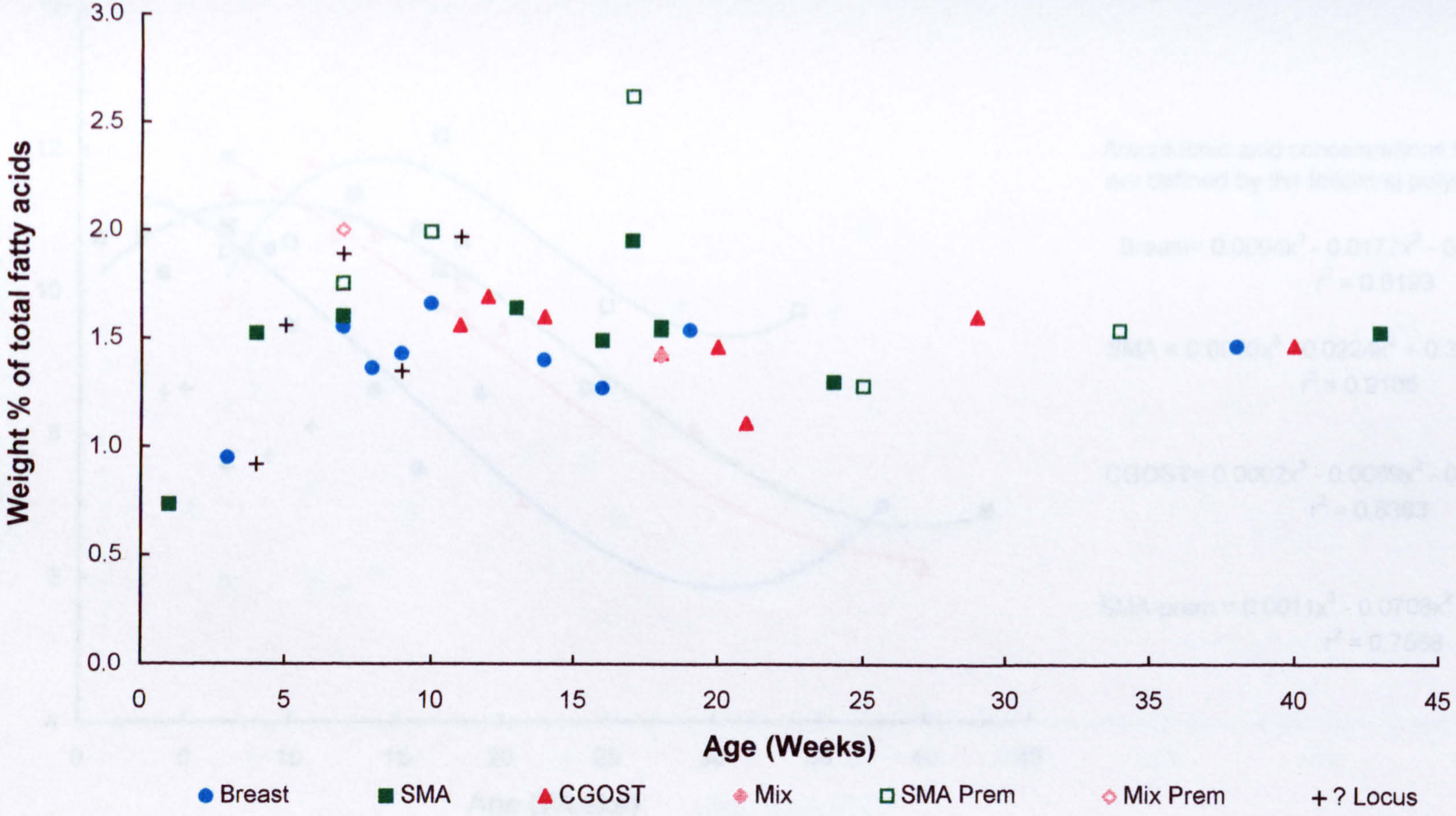
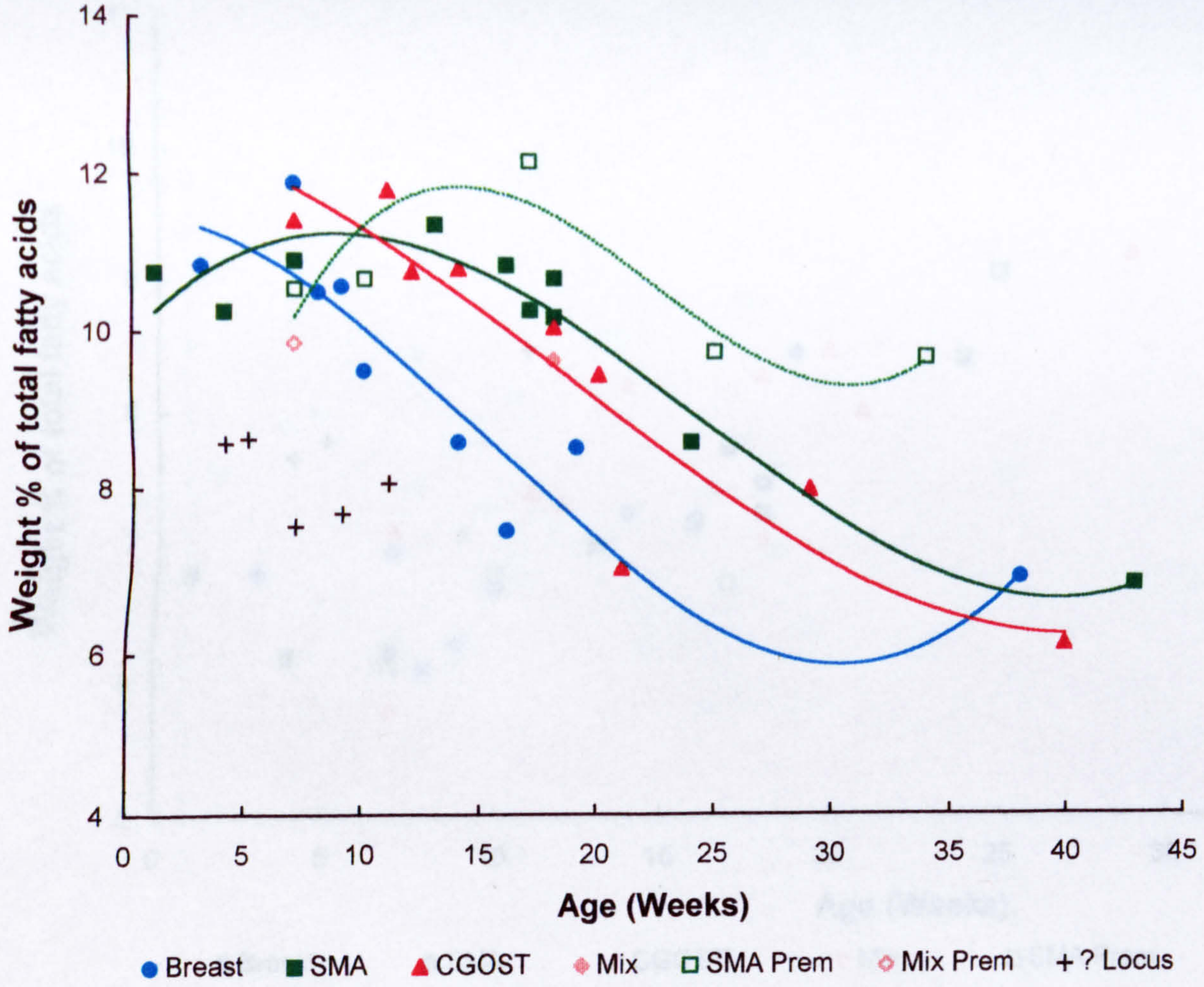


Figure 3.56 - Cerebral parietal white matter total lipid dihomogammalinolenic acid (C20:3n-6) in relation to infants' diet and age.



Arachidonic acid concentrations for a given age (x) are defined by the following polynomial equations:

Breast= $0.0004x^3 - 0.0177x^2 - 0.0064x + 11.506$
$r^2 = 0.8193$

SMA = $0.0003x^3 - 0.0224x^2 + 0.3219x + 9.9456$
$r^2 = 0.9185$

CGOST= $0.0002x^3 - 0.0089x^2 - 0.0596x + 12.638$
$r^2 = 0.8363$

SMA-prem = $0.0011x^3 - 0.0708x^2 + 1.3616x + 3.7723$
$r^2 = 0.7568$

Figure 3.57 - Cerebral parietal white matter total lipid arachidonic acid (C20:4n-6) in relation to infants' diet and age.

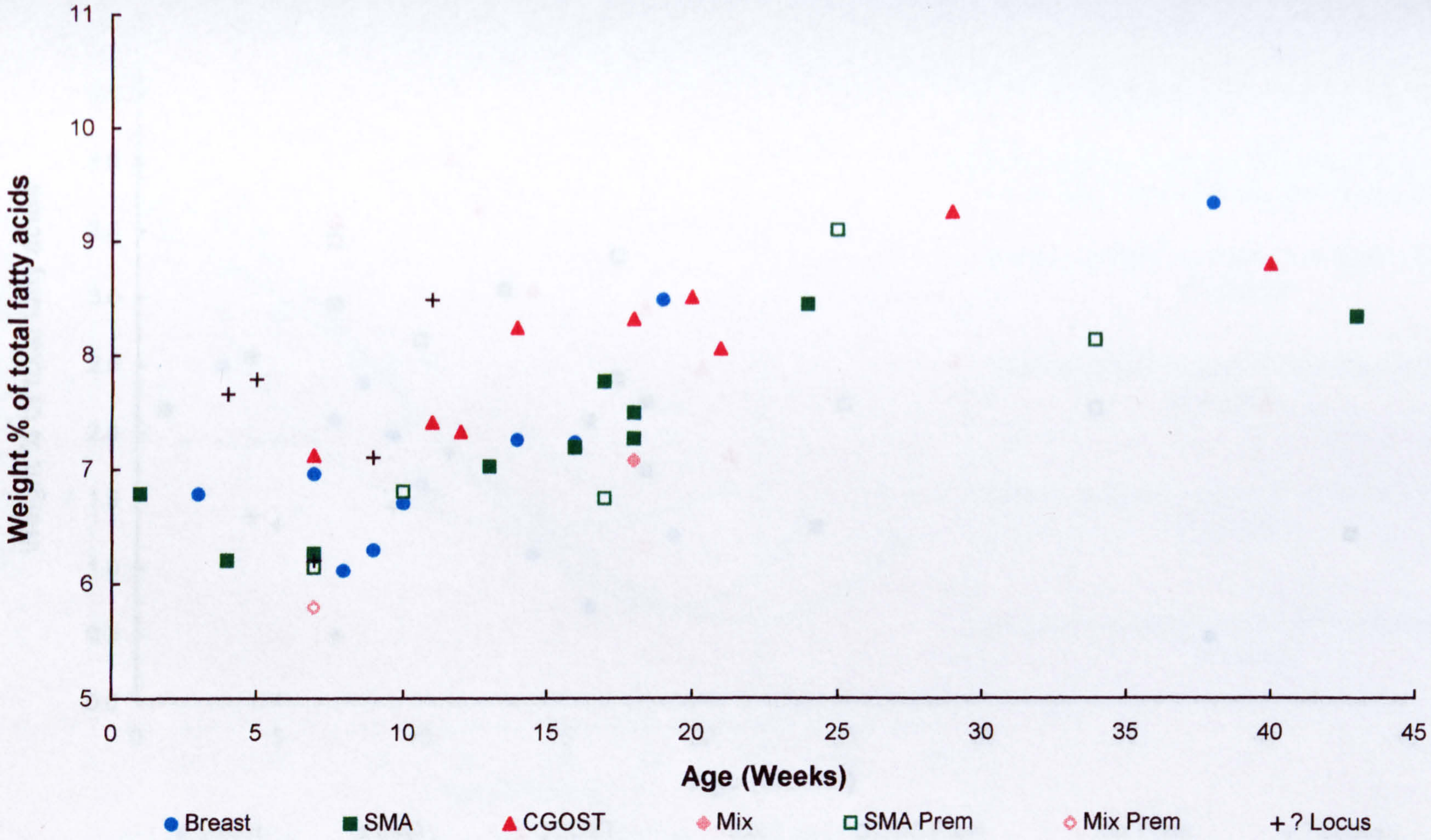


Figure 3.58 - Cerebral parietal white matter total lipid docosatetraenoic acid (C22:4n-6) in relation to infants' diet and age.

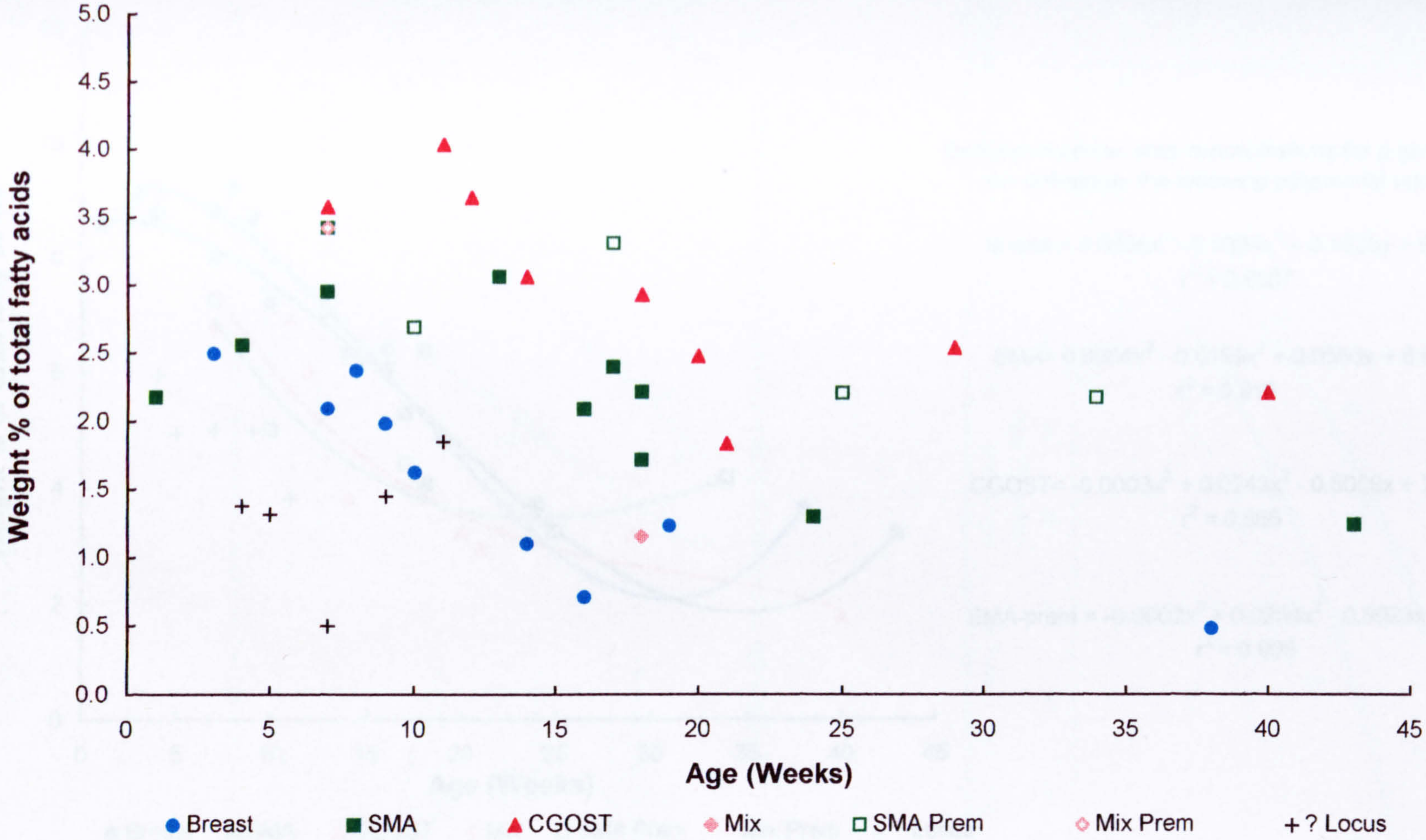
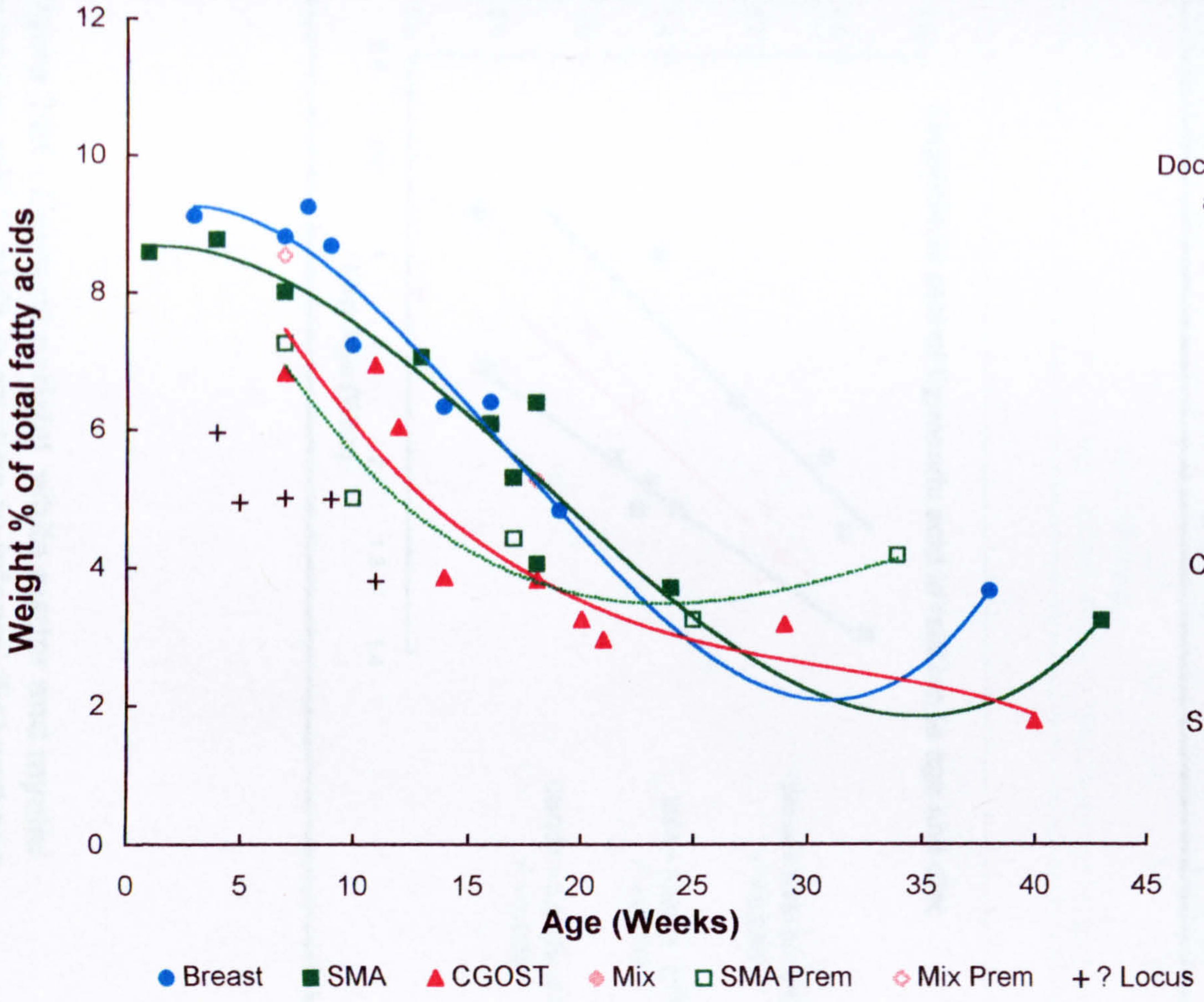


Figure 3.59 - Cerebral parietal white matter total lipid docosapentaenoic acid (C22:5n-6) in relation to infants' diet and age.



Docosahexaenoic acid concentrations for a given age (x) are defined by the following polynomial equations:

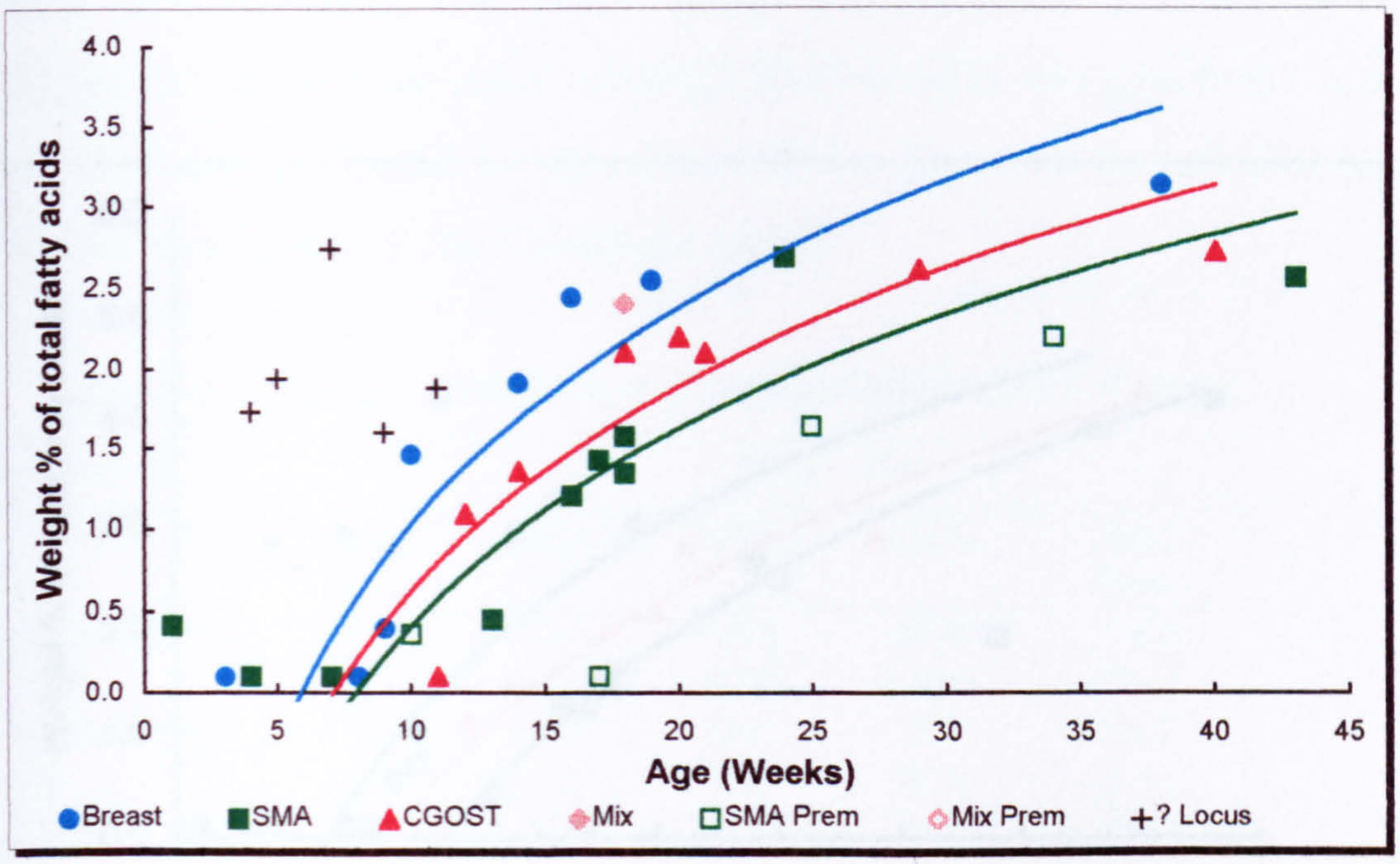
Breast = $0.0006x^3 - 0.0324x^2 + 0.1625x + 9.0363$
$r^2 = 0.9507$

SMA= $0.0004x^3 - 0.0199x^2 + 0.0558x + 8.6276$
$r^2 = 0.916$

CGOST= $-0.0003x^3 + 0.0243x^2 - 0.8059x + 11.997$
$r^2 = 0.866$

SMA-prem = $-0.0002x^3 + 0.0254x^2 - 0.8023x + 11.369$
$r^2 = 0.906$

Figure 3.60 - Cerebral parietal white matter total lipid docosahexaenoic acid (C22:6n-3) in relation to infants' diet and age.



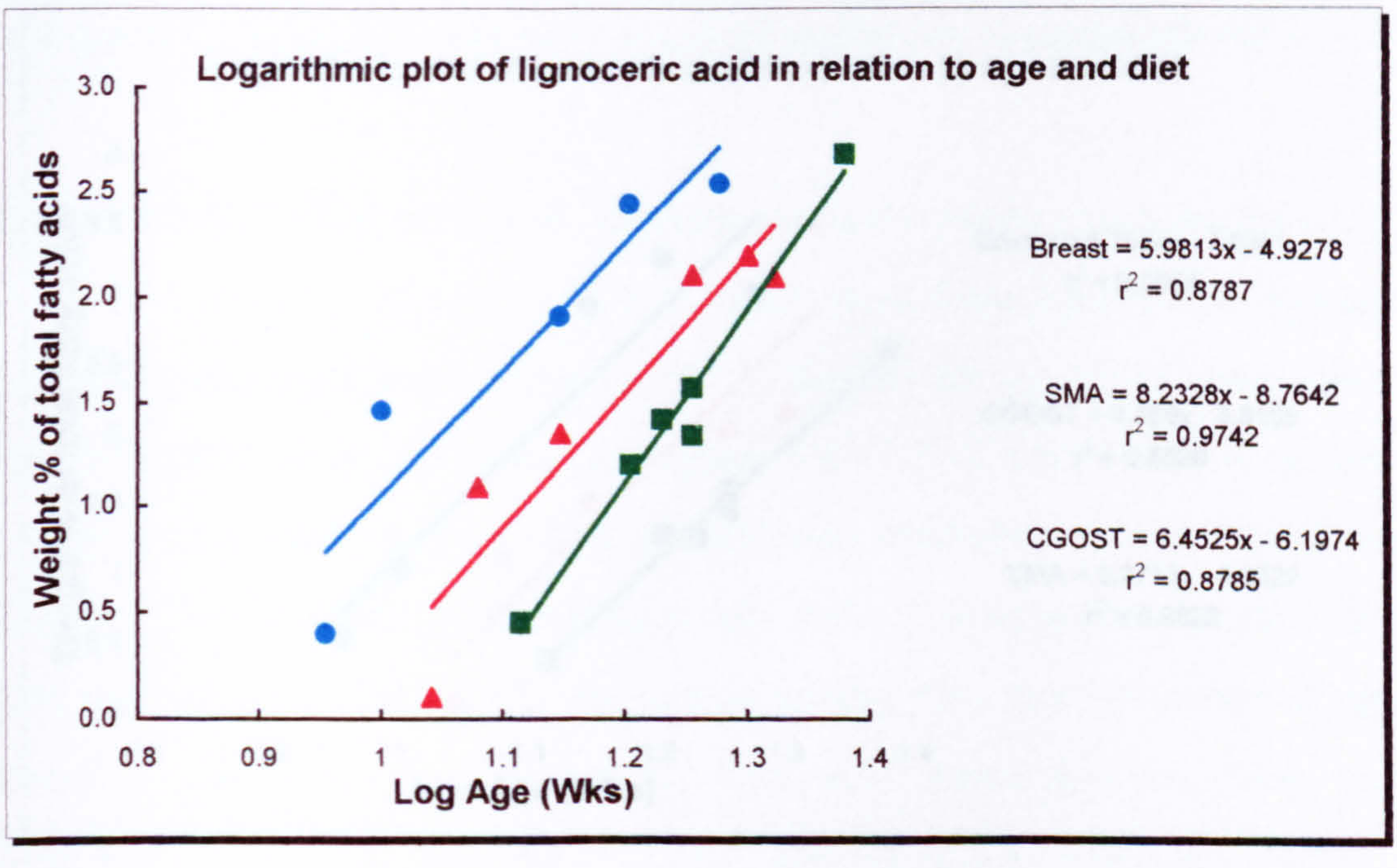
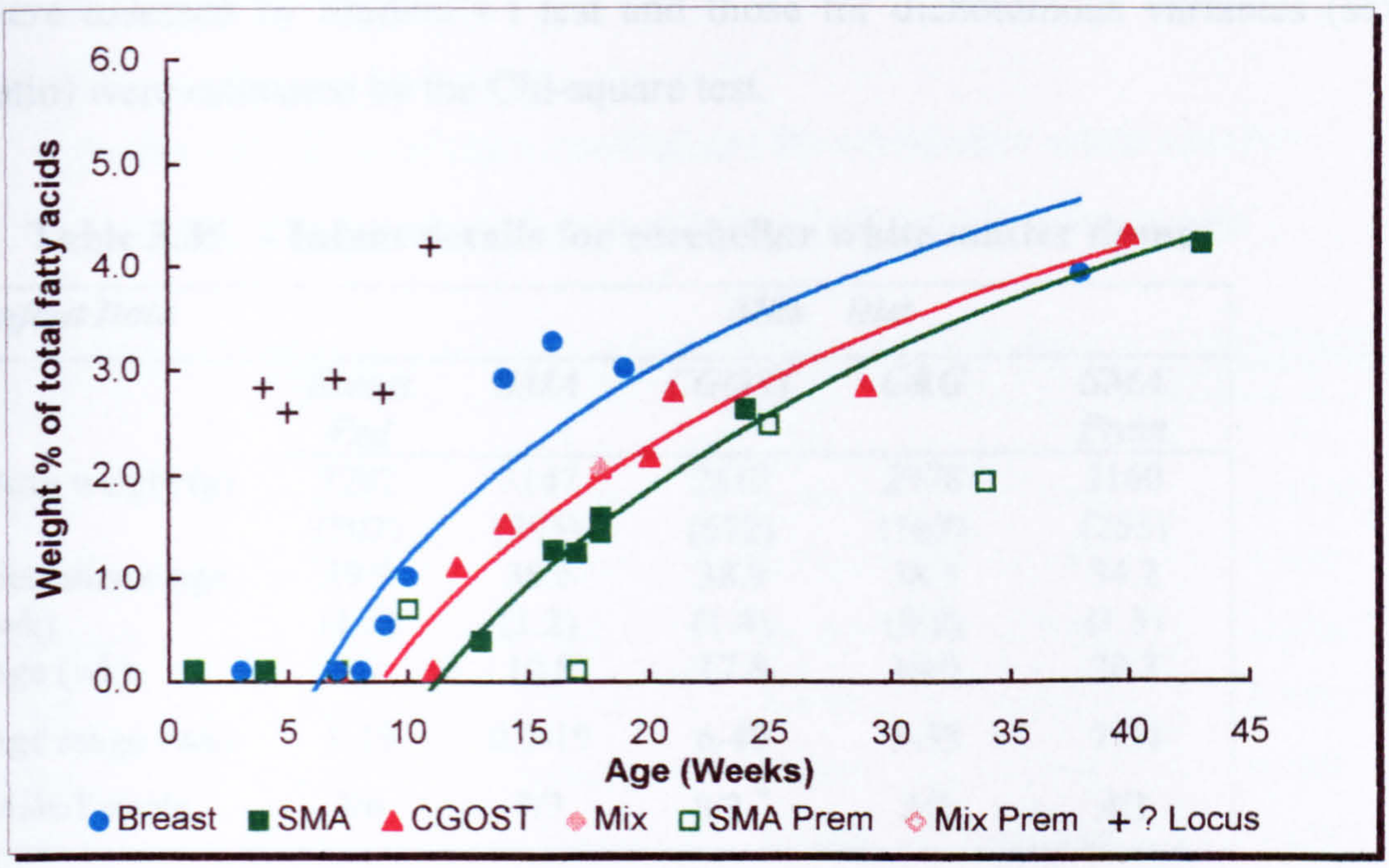


Figure 3.61 - Cerebral parietal white matter and myelin lignoceric acid (C24:0) in relation to infants' diet and age.



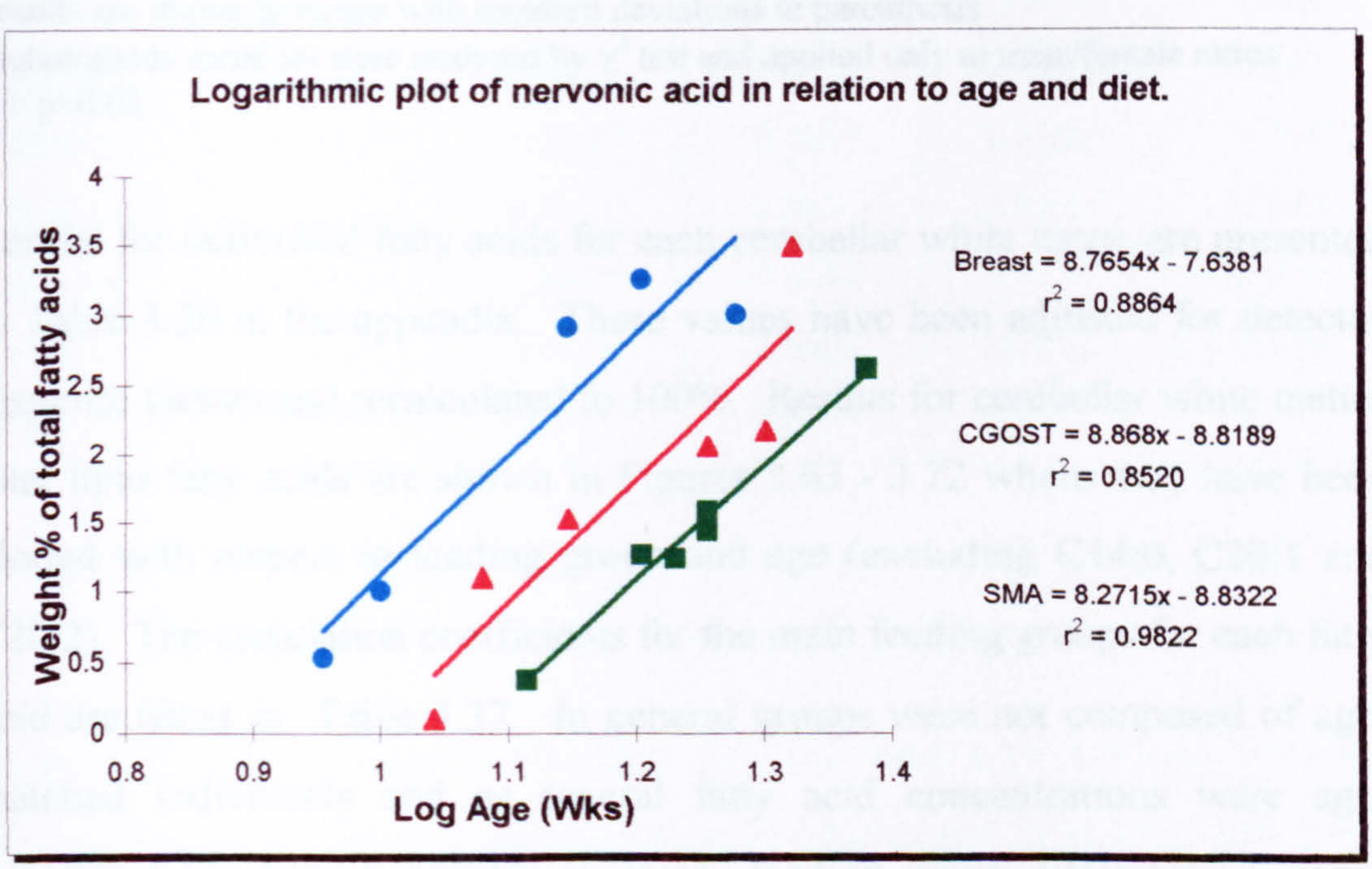


Figure 3.62 - Cerebral parietal white matter and myelin nervonic acid (C24:1) in relation to infants' diet and age.

3.3.6. Cerebellar white matter total lipid fatty acids

Table 3.35 illustrates the infant group characteristics. Between group differences for the continuous variables (birth weights and gestational ages) were assessed by Student's t test and those for dichotomous variables (sex ratio) were estimated by the Chi-square test.

Table 3.35 - Infant details for cerebellar white matter tissues

Infant Data	*Milk Diet*				
	Breast Fed	***SMA***	***CGOST***	***C&G***	***SMA Prem***
Birth weight (g)	3292 (507)	3147 (395)	2812 (672)	2978 (169)	2160 (253)
Gestational age (wk)	39.9 (1.3)	38.6 (1.2)	38.9 (1.4)	38.5 (0.5)	34.2 (1.3)
Age (wk)	9.4	10.8	17.8	18.0	20.2
Age range (wk)	1-19	0.5-19	6-40	5-33	7-34
Male/Female	3/6	7/3	8/2 $^{\alpha}$	2/2	4/1

Results are shown as means with standard deviations in parenthesis.
Dichotomous variables were analysed by χ^2 test and applied only to male/female ratios.
$\alpha = p<0.02$.

Results for individual fatty acids for each cerebellar white tissue are presented in Table 3.36 in the appendix. These values have been adjusted for detector response factors and recalculated to 100%. Results for cerebellar white matter total lipid fatty acids are shown in Figures 3.63 - 3.72 where they have been plotted with respect to feeding group and age (excluding C14:0, C20:1 and C20:2). The correlation coefficients for the main feeding groups for each fatty acid are listed in Table 3.37. In general groups were not composed of age-matched individuals and as several fatty acid concentrations were age-dependent it was not possible to assess feeding group differences using a standard parametric analytical procedure. However breast-fed and SMA-fed infants could be age-matched (to within 1 week) and the resultant 8 data pairs were assessed using the Wilcoxon signed rank test. Note that this required exclusion of one 9 week old breast-fed and two 17 week old SMA-fed infants.

No such age matching was possible with the CGOST group as this tended to be populated by "older" individuals than either the breast or SMA-fed groups (Table 3.35). Neither did the results' distributions with respect to age, allow for normalisation of the data.

Table 3.37 Correlation coefficients for cerebellar white matter fatty acid concentration vs age in relation to feeding group

Fatty Acid	Breast	SMA	CGOST	C&G	SMA Prem
	r	r	r	r	r
C14:0	0.0171	-0.0870	-0.3058	-0.5960	--0.6180
C16:0	-0.9529 $^{\delta}$	-0.9764 $^{\delta}$	-0.5326	-0.4982	-0.8432
C16:1 n-7	-0.9167 $^{\delta}$	-0.9603 $^{\delta}$	-0.5845	-0.8528	-0.6382
C18:0	0.7482 $^{\delta}$	0.3016	0.0701	0.9636 $^{\delta}$	0.5983
C18:1	0.8180 $^{\delta}$	0.9480 $^{\delta}$	0.5033	0.9772 $^{\delta}$	0.7905
C20:1	0.9215 $^{\delta}$	0.9545 $^{\delta}$	0.6409	0.6546	0.7719
C20:2	0.8224 $^{\delta}$	0.8589 $^{\delta}$	0.1662	0.1977	0.7773
C20:3 n-6	0.6228	0.3333	-0.1766	-0.3699	-0.5025
C20:4 n-6	-0.8250 $^{\delta}$	-0.9258 $^{\delta}$	-0.2617	-0.9280 $^{\delta}$	-0.7845
C22:4 n-6	0.5065	0.3150	0.5446	-0.9509 $^{\delta}$	0.7450
C22:6 n-3	-0.7227	-0.8891 $^{\delta}$	-0.6046	-0.7291	-0.8970 $^{\delta}$
C24:0	0.6634	0.7306	0.0663	0.3211	0.6350
C24:1	0.8869 $^{\delta}$	0.9439 $^{\delta}$	0.4299	0.9622 $^{\delta}$	0.7771

$^{\delta}$ = Significant correlation of individual fatty acid concentrations (wt %) with age. ($p<0.01$)

Table 3.38 lists medians and ranges for each fatty acid together with the Wilcoxon signed rank test significances for the paired breast-fed and SMA groups. In addition a non-statistical visual inspection of results for all groups was undertaken to assess the general trends in fatty acid incorporation into infant cerebellar white matter.

Table 3.38 Cerebellar white matter and myelin total lipid fatty acid compositions

Fatty Acid	*Milk Diet*				
	Breast n=9	***SMA n=10***	***CGOST n=10***	***C&G n=4***	***SMA Prem n=5***
C14:0	1.38 (1.05-2.22)	1.32 (0.72-1.58)	0.98 (0.79-1.60)	1.27 (0.83-1.56)	1.53 (0.92-2.51)
C16:0	19.82 (16.74-22.93)	19.93 (16.68-24.12)	19.13 (15.62-23.80)	19.94 (16.44-22.86)	19.27 (15.67-26.10)
C16:1 n-7	2.70 (2.03-3.69)	2.52 (1.78-3.43)	2.07 (1.20-3.36)	2.40 (2.00-2.96)	2.65 (1.77-3.43)
C18:0	21.50 (19.38-22.84)	22.32 (21.13-23.64)	22.96 (19.36-25.55)	22.67 (20.53-25.09)	21.06 (20.43-22.55)
C18:1 n-7+n-9	27.10 (22.74-31.41)	26.83 (21.36-30.69)	28.97 (24.30-31.49)	28.39 (25.35-31.17)	27.93 (25.35-31.50)
C20:1 n-9	1.59 (0.99-2.55)	1.59 (0.60-2.31)	1.84 (1.11-2.54)	2.14 (1.17-3.77)	2.12 (0.65-2.89)
C20:2 n-6	0.74 (0.49-0.97)	0.80 (0.42-1.07)	0.78 (0.49-1.09)	0.88 (0.76-1.59)	1.03 (0.51-1.11)
C20:3 n-6	1.49 (0.98-1.96)	1.39 (0.97-1.80)	1.47 (1.11-2.05)	1.63 (1.23-1.92)	1.75 (1.38-2.23)
C20:4 n-6	7.60 (5.81-9.55)	7.32 (5.94-9.89)	7.11 (5.34-9.86)	6.20 (4.68-7.17)	7.11 (5.47-8.93)
C22:4 n-6	5.72 (5.00-6.58)	6.47 [a] (5.23-7.20)	5.80 (4.56-7.54)	5.91 (4.73-6.66)	6.67 (4.88-7.76)
C22:6 n-3	6.55 (4.32-10.24)	4.76 [b] (2.64-8.16)	3.43 (2.06-5.56)	3.44 (1.99-4.25)	4.03 (2.32-6.28)
C24:0	1.88 (1.29-2.48)	2.23 [a] (1.60-2.96)	2.23 (1.18-3.27)	2.59 (2.21-3.08)	2.23 (0.75-3.04)
C24:1 n-9	2.25 (1.12-3.12)	2.62 (0.91-4.14)	2.91 (0.70-4.65)	3.90 (2.29-5.66)	3.24 (0.70-4.15)

Fatty acids are expressed as weight percentages and are given as medians and ranges
Significant differences in fatty acid concentrations between feeding groups (BF vs SMA only) were calculated by the Wilcoxon signed rank test for paired data (n=8)
a = $p<0.02$; b = $p<0.01$

As represented by the medians (and ranges) there would appear to be little variation in the fatty acid concentrations between groups in cerebellar white matter. Palmitic acid (Fig 3.63) decreased in concentration from around 24% at birth to less than 20% by 5 months of age in all feeding groups. Absence of data for older (>20 weeks) infants in both the breast-fed and term born SMA-

fed groups precluded a comparative between group analysis beyond this age. The median concentration for palmitic acid was approximately 20% for all feeding groups and within a range of 16 to 24% in the term born infants. Higher concentrations were revealed however in the 7 week old preterm SMA (26.10%) and mixed-fed (28.37%) infants. Although a similar trend in results was noted in cerebral white matter for both the preterm and term infants, the median concentrations were about 5% higher in this tissue (Fig 3.52). Palmitoleic acid fell sharply in the first 10 weeks of life and then stabilised at about 2% of total fatty acids (Fig 3.64). It was observed that for stearic acid cerebellar white matter medians and ranges (Table 3.38) were very similar to those of cerebral white matter (Table 3.31). At approximately 23% at birth, oleic acid concentrations (Fig 3.66) increase rapidly over the first 5 months to >30% of total fatty acids by 25 weeks. Whilst as discussed the lowest brain total lipid palmitic acid concentrations were present in the cerebellar white matter, the converse is true for oleic acid. No significant differences were found in saturated and monounsaturated fatty acids between the breast-fed and term born SMA-fed groups by the Wilcoxon test.

Dihomo-γ -linolenic acid (Fig 3.67) was present at fairly low concentrations (about 1.5% of total fatty acids) in all feeding groups. The remaining polyunsaturated fatty acids, AA, DTA and DHA, all exhibited lower group medians and ranges than in the equivalent cerebral white matter tissues. Arachidonic acid concentrations (Fig 3.68) fell from around 10% of total fatty acids at birth to about 7% at 10 weeks of age and thereafter remained relatively constant. No overt between group differences were apparent and the results from the preterm infants appeared to be confined within the ranges established for the term born groups. Again no statistically significant difference existed between the paired breast and SMA-fed infants. Concentrations of docosatetraenoic acid (Fig 3.69) were significantly higher in the age-matched SMA-fed infants than those breast-fed ($p<0.02$). In stark contrast to its

behaviour in the cerebral white matter in which its concentration increased by 50% over the first 6 months, here docosatetraenoic acid did not display any significant positive correlation with age. Median concentrations were maintained at about 6% of total fatty acids in all groups and on no occasion could the results of the preterm infants be classified as outliers. Interestingly n-6 series docosapentaenoic acid was never a prominent feature in the cerebellar white matter fatty acids accounting for <0.5% of total fatty acids and not considered in this analysis. Docosahexaenoic acid concentrations were significantly greater in the breast-fed infants than their age-matched SMA-fed counterparts ($p<0.01$). Neonatal DHA compositions varied widely (Fig 3.70) but decreased to <5% of total fatty acids in all formula fed infants by 10 weeks. A subsequent further decline to about 3% was evident in the older (>20 weeks) infants. As defined by the statistical analysis (Wilcoxon) DHA concentrations in the breast-fed infants were generally greater than those of age-equivalent formula fed infants, but they too declined inexorably to around 5% of total fatty acids before 20 weeks of life.

Whereas both lignoceric and nervonic acids increased with age in cerebral white matter, the former did not show a correlation with age in cerebellar white matter in any feeding regimen with lignoceric acid concentrations appearing randomly distributed with age. Lignoceric acid (Fig 3.71) was however significantly greater in the SMA group than the breast-fed group ($p<0.02$). Unlike the polyunsaturated fatty acids, however, in which the results from the preterm infants were indistinguishable from their term born contemporaries, here the youngest (7 weeks old) preterm infants displayed the lowest concentrations of lignoceric acid (SMA fed-0.75%;mixed-fed - 0.67%), the corollary of that in the precursor palmitic acid (Fig 3.63). Nervonic acid concentrations(Fig 3.72) were approximately 1% of total fatty acids at birth increasing to 4% at about 5 months. This was in contrast to cerebral parietal white matter where both nervonic acid and lignoceric acid were undetected at

birth and for the first 8 weeks of life. No significant differences in nervonic acid existed between the age-matched breast and SMA-fed individuals. The youngest preterm individuals were again identified with the lowest nervonic acid concentrations (Fig 3.72).

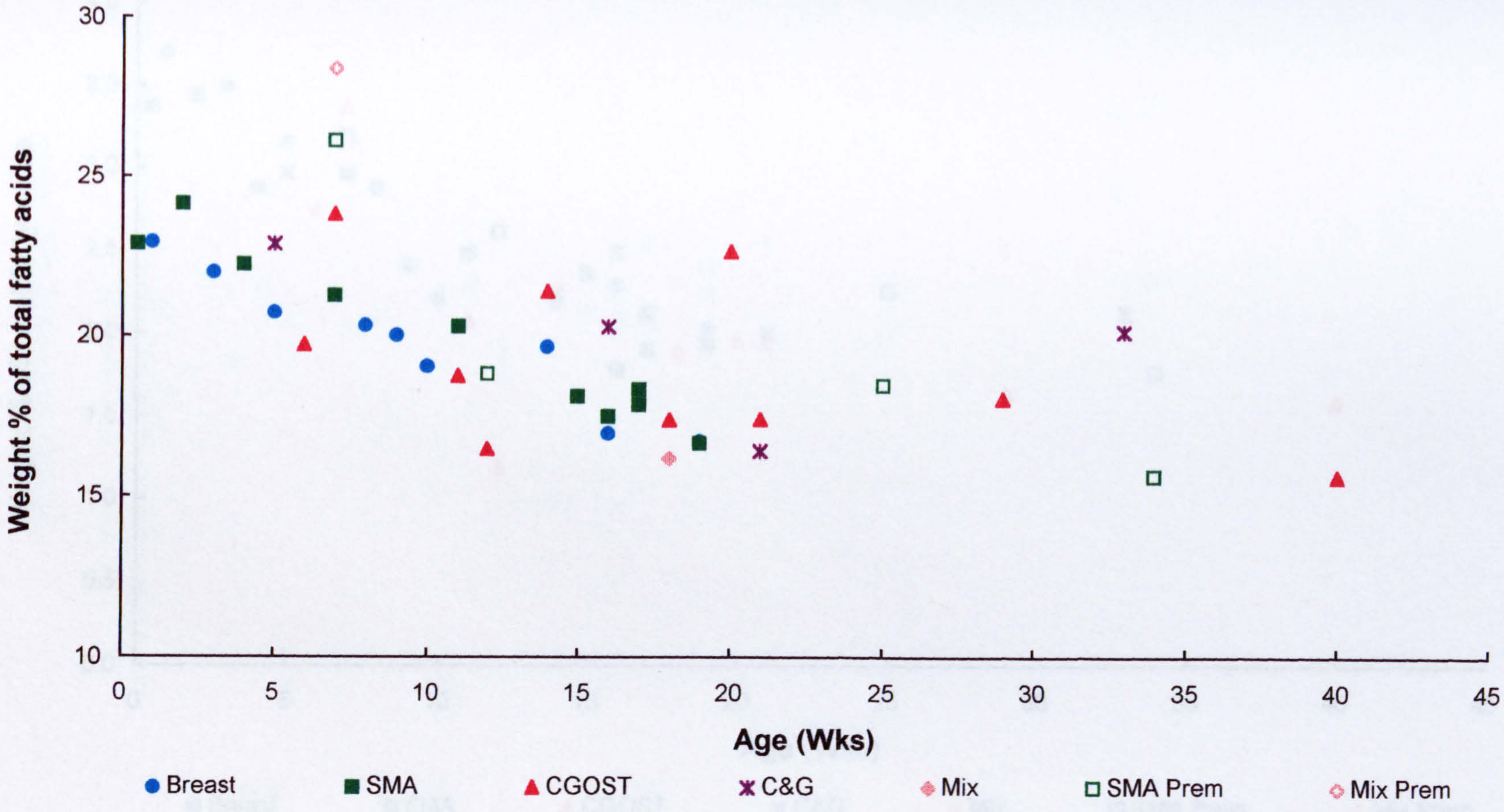


Figure 3.63 - Cerebellar white matter total lipid palmitic acid (C16:0) in relation to infants' diet and age.

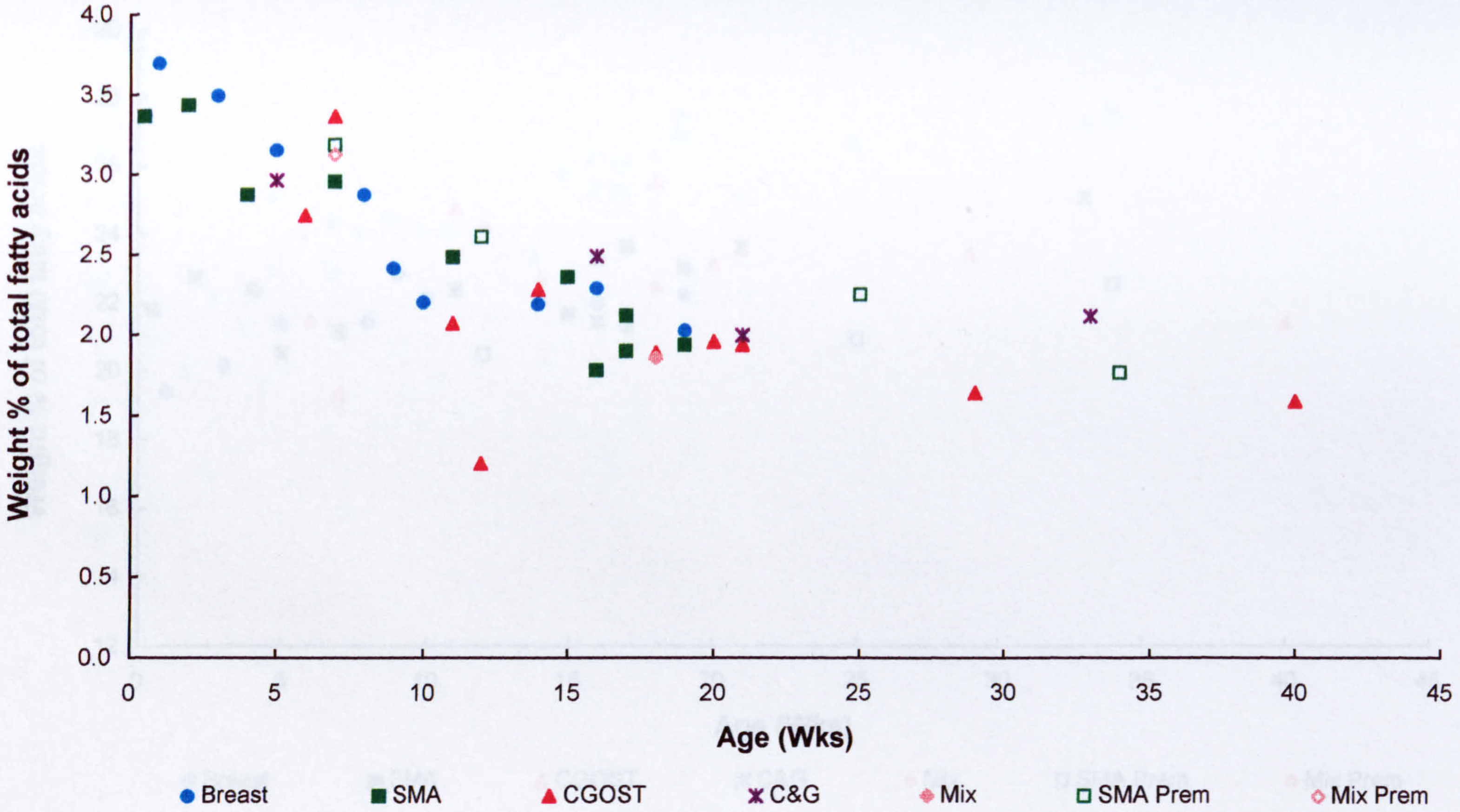


Figure 3.64 - Cerebellar white matter total lipid palmitoleic acid (C16:1n-7) in relation to infants' diet and age.

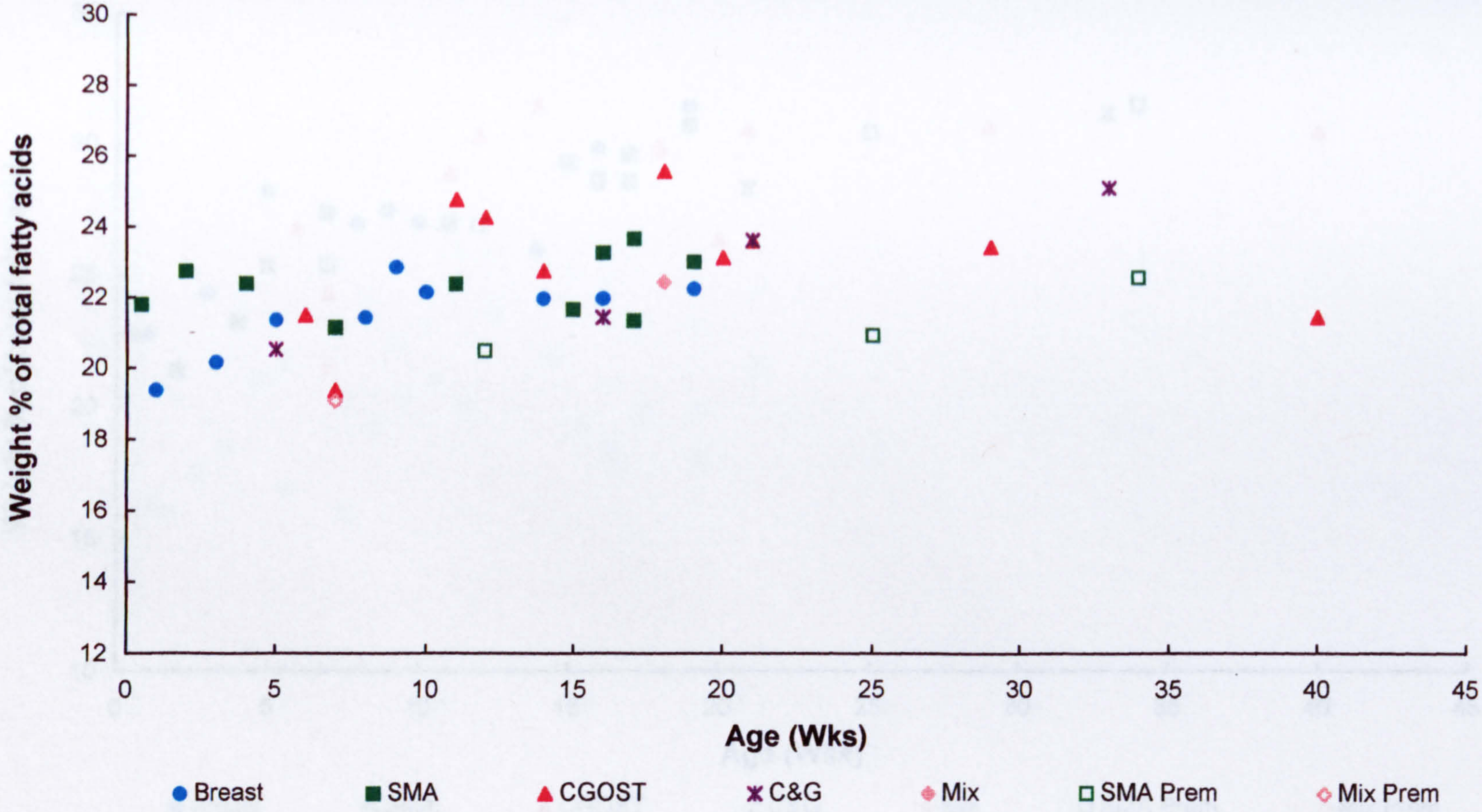


Figure 3.65 - Cerebellar white matter total lipid stearic acid (C18:0) in relation to infants' diet and age.

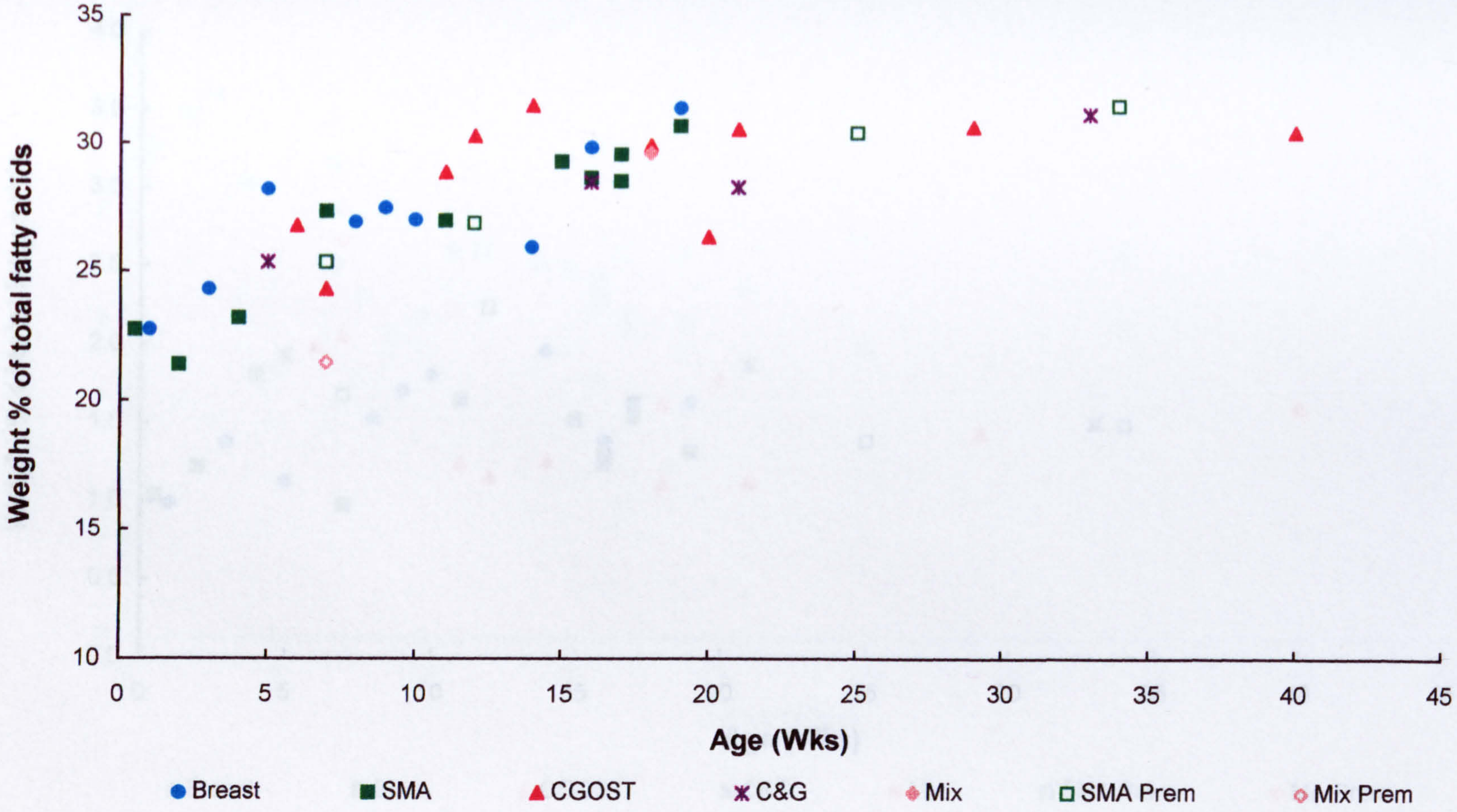


Figure 3.66 - Cerebellar white matter total lipid oleic acid (C18:1n-7+n-9) in relation to infants' diet and age.

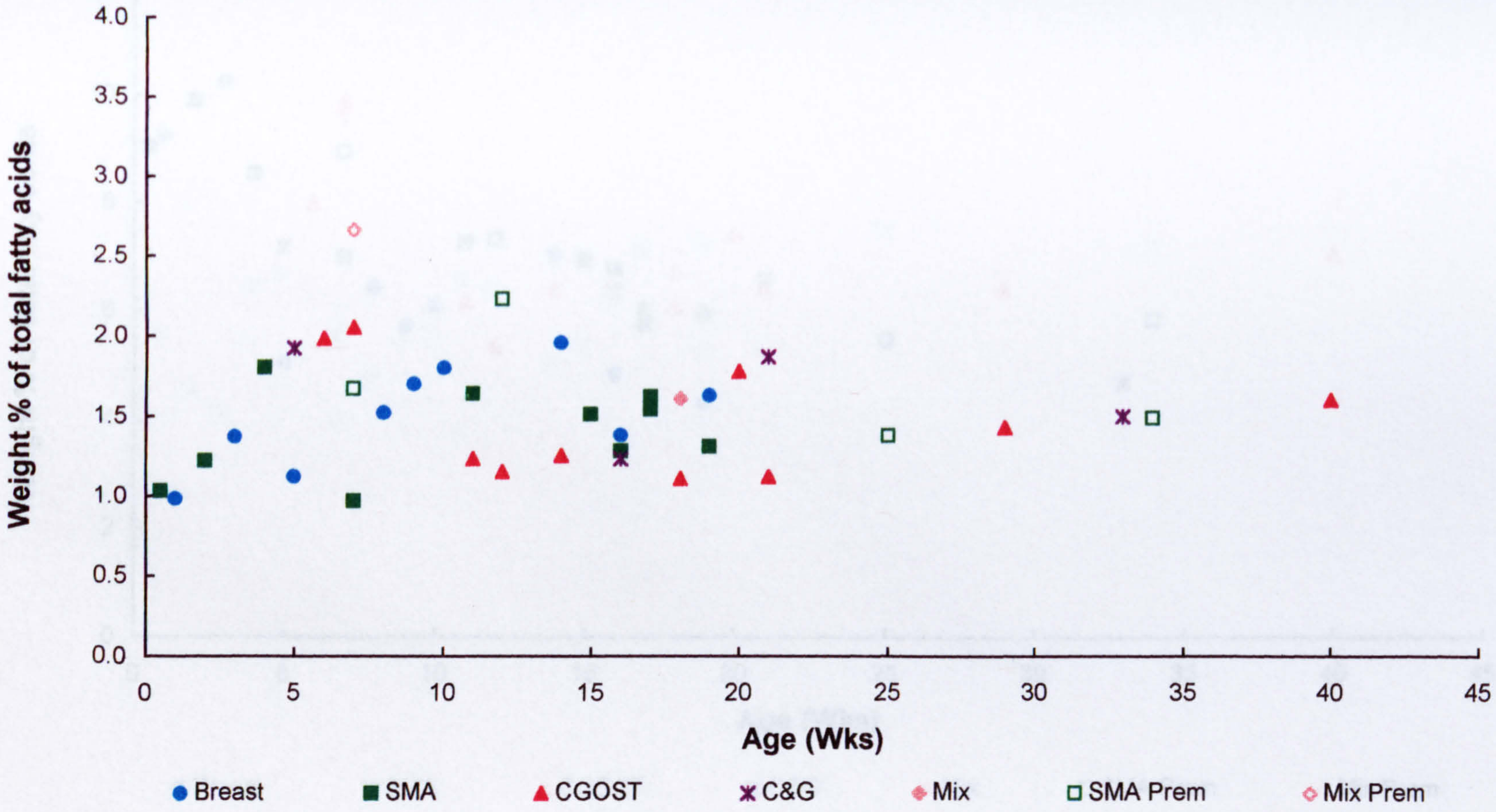


Figure 3.67 - Cerebellar white matter total lipid dihomogammalinolenic acid (C20:3n-6) in relation to infants' diet and age.

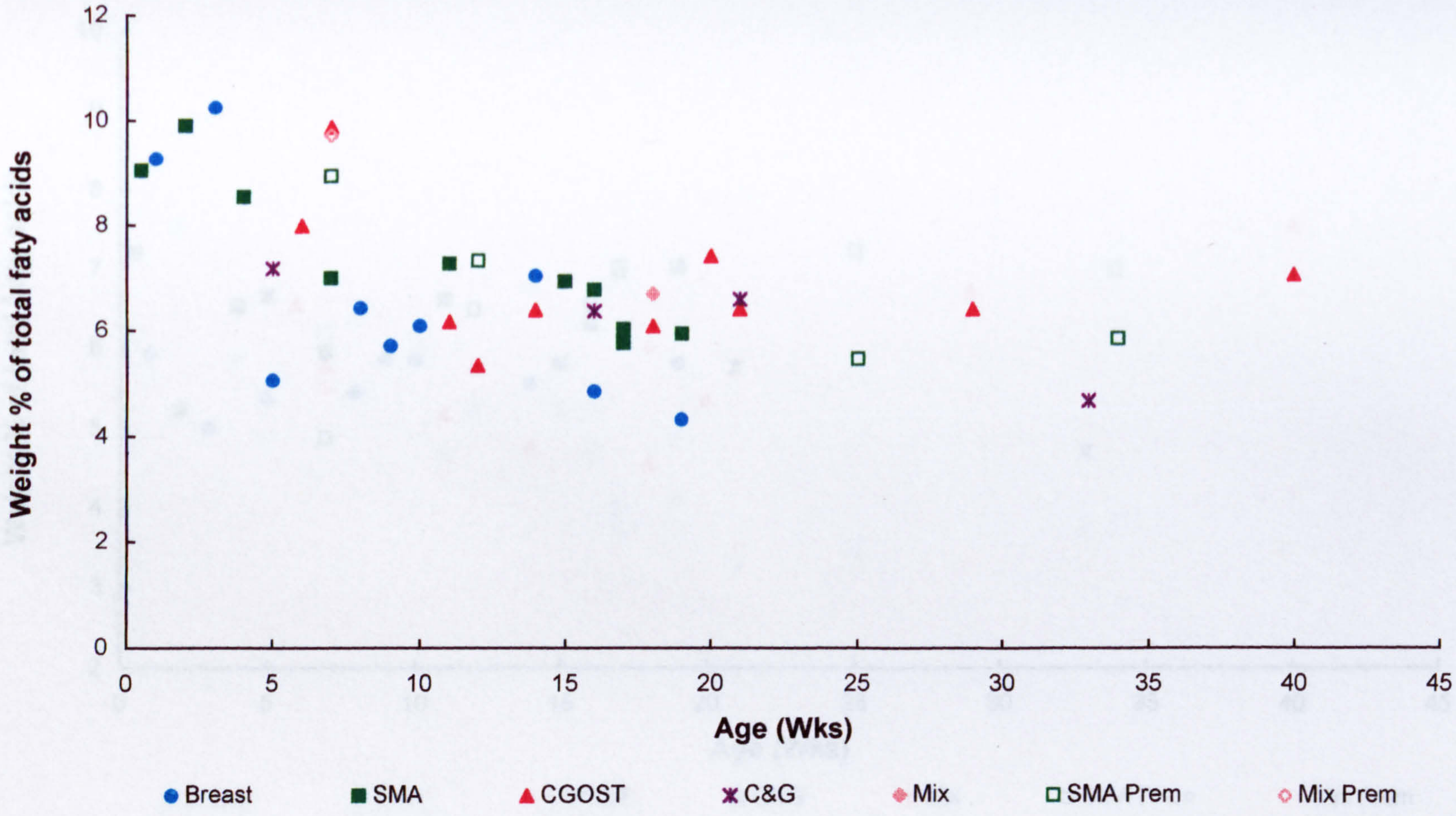


Figure 3.68 - Cerebellar white matter total lipid arachidonic acid (C20:4n-6) in relation to infants' diet and age.

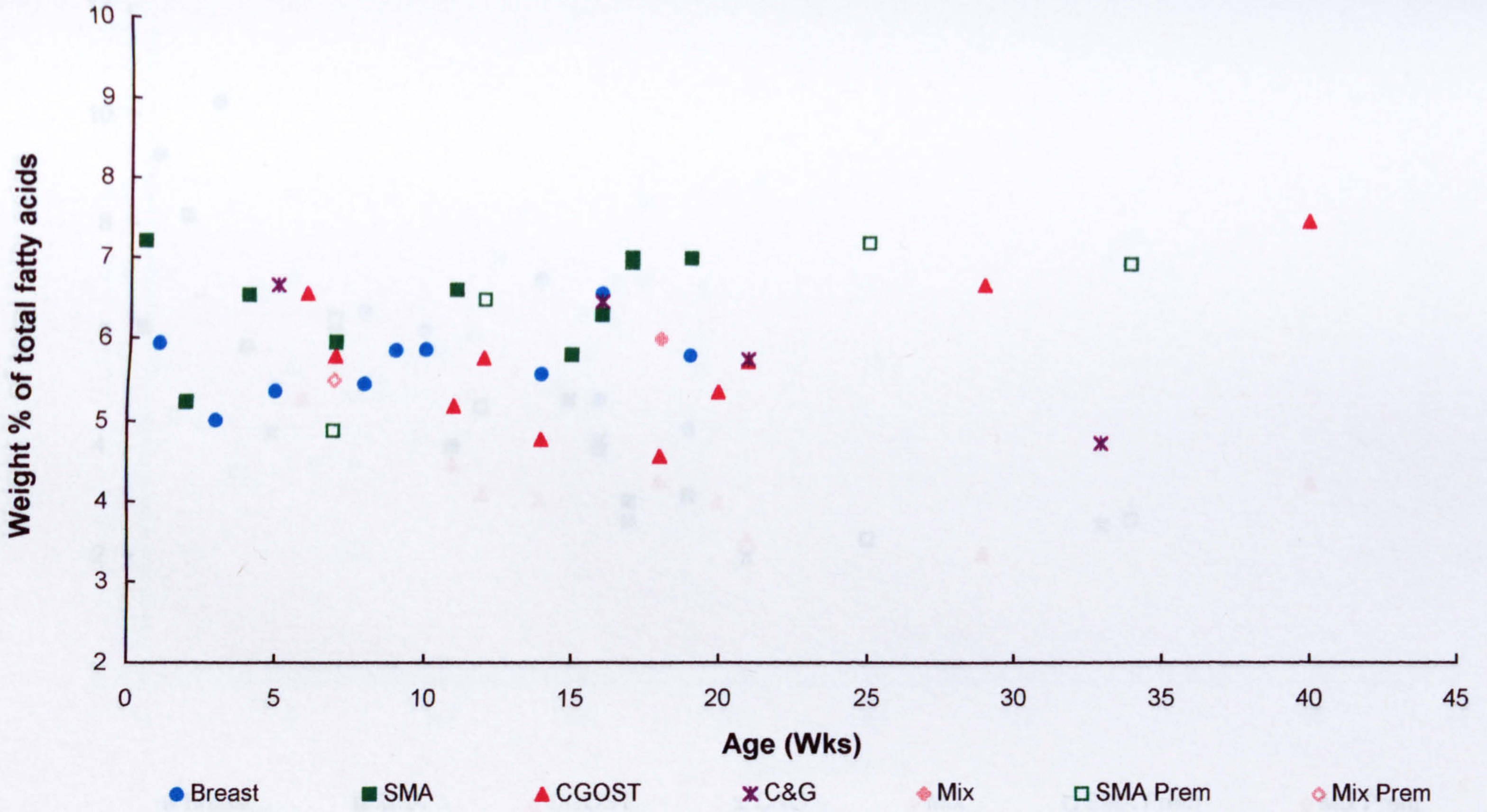


Figure 3.69 - Cerebellar white matter total lipid docosatetraenoic acid (C22:4n-6) in relation to infants' diet and age.

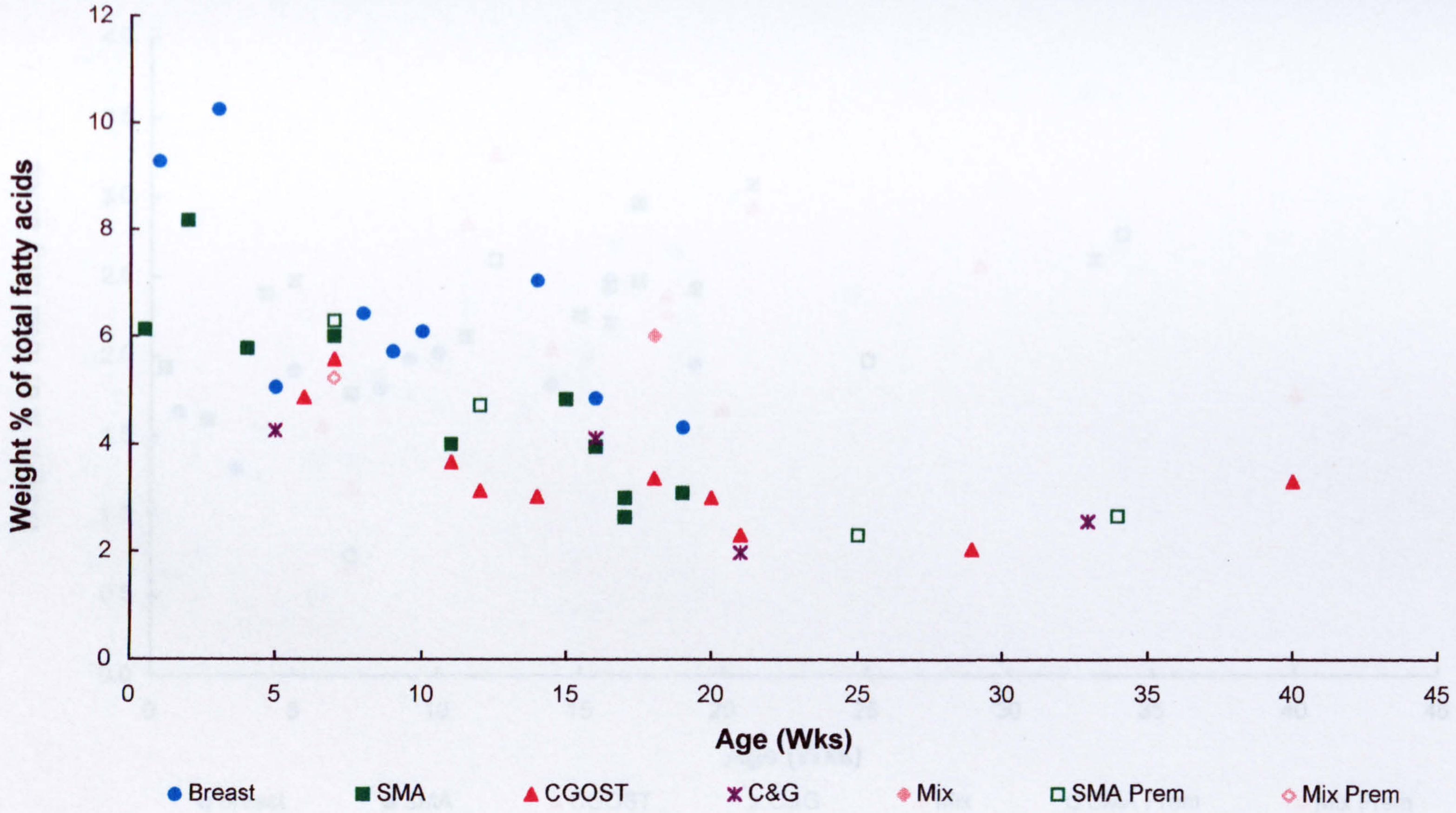


Figure 3.70 - Cerebellar white matter total lipid docosahexaenoic acid (C22:6n-3) in relation to infants' diet and age.

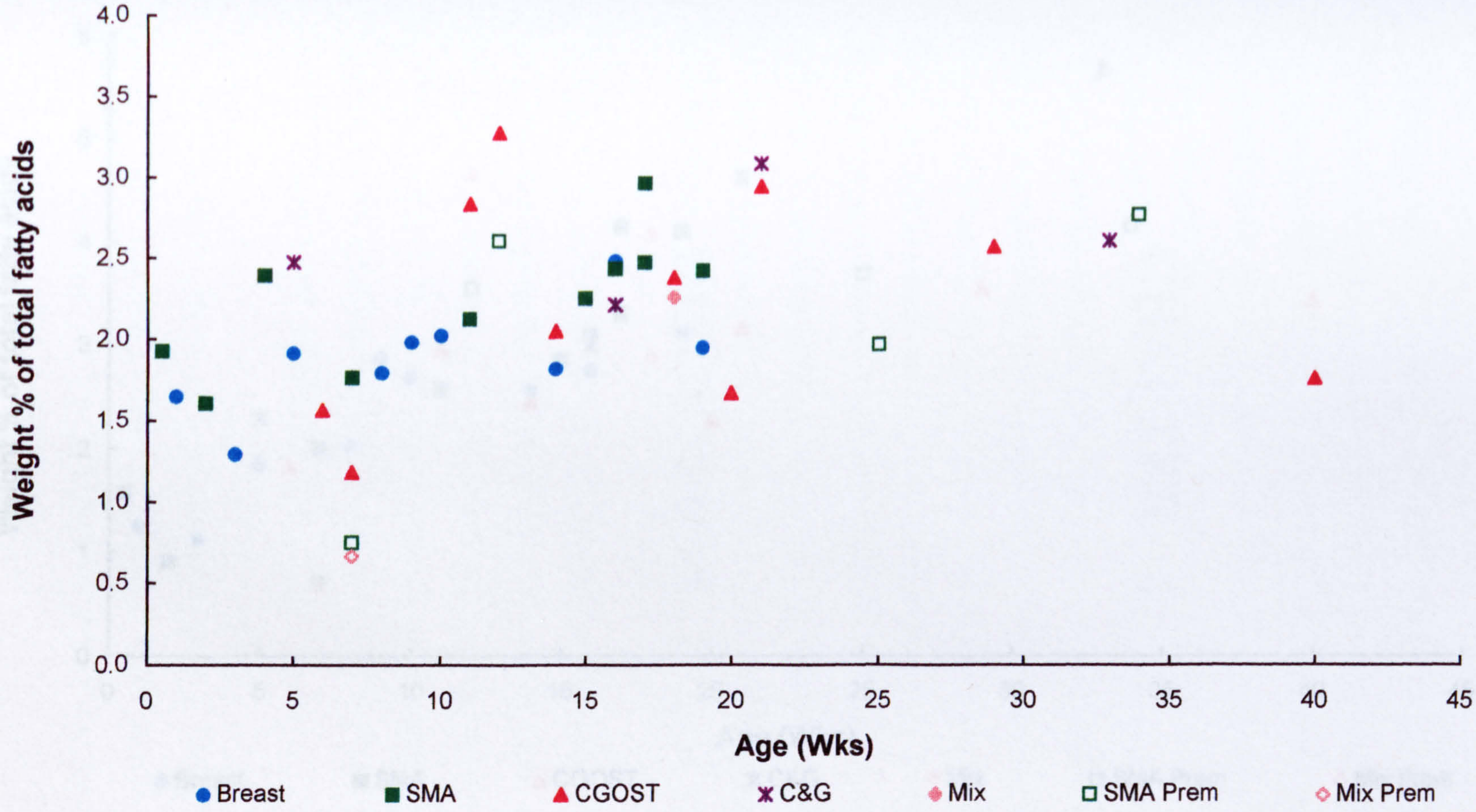


Figure 3.71 - Cerebellar white matter total lipid lignoceric acid (C24:0) in relation to infants' diet and age.

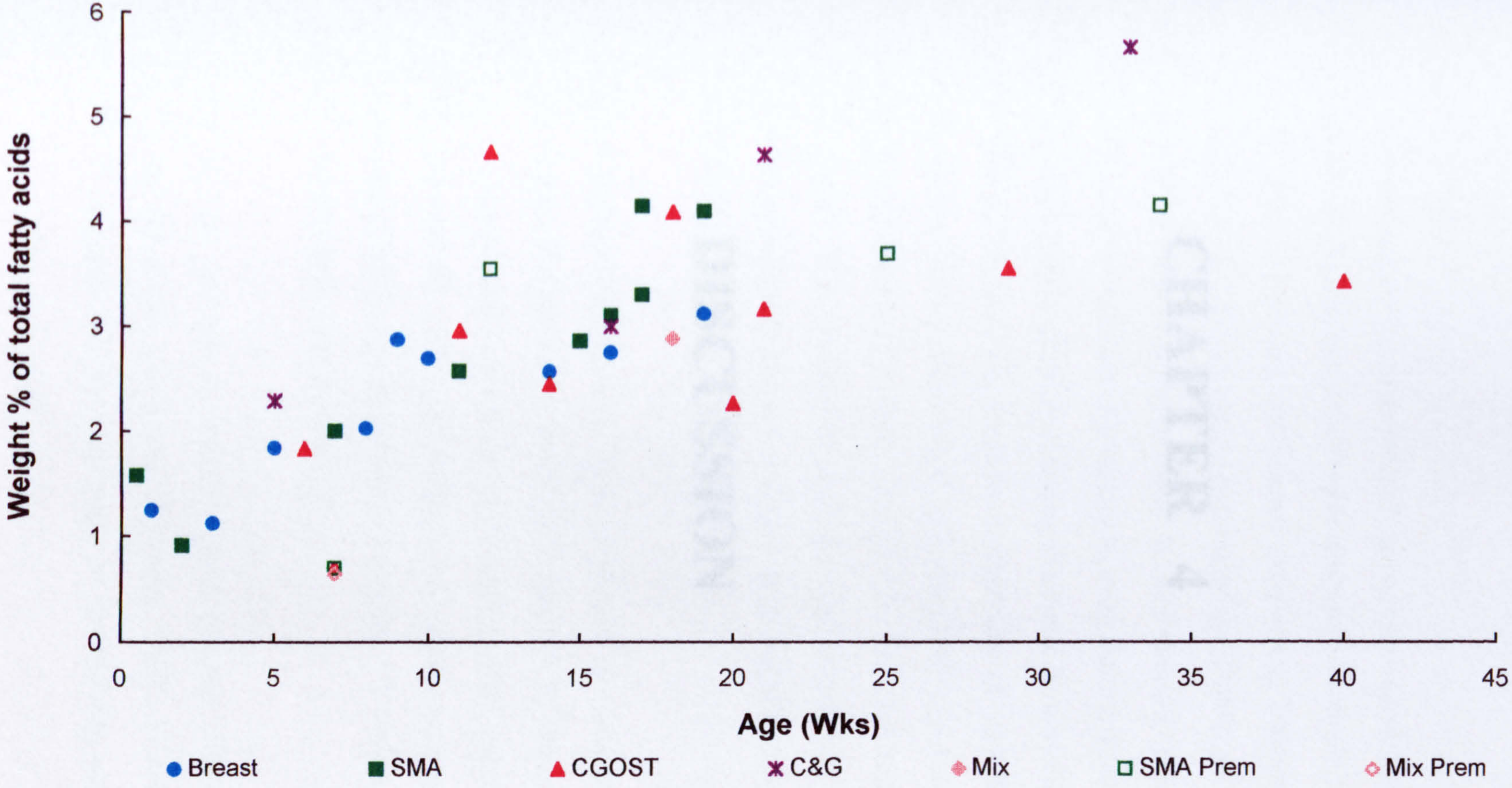


Figure 3.72 - Cerebellar white matter total lipid nervonic acid (C24:1n-9) in relation to infants' diet and age.

CHAPTER 4

DISCUSSION

4.1. Method Evaluation

There follows a brief discussion of the method evaluations followed by consideration of patient results.

4.1.1. Chromatography

One-dimensional chromatography is preferred as a preparative step for the separation of phospholipids owing to the larger amount of sample which can be applied to each plate. Working conditions, particularly temperature and humidity, must however be fairly rigidly controlled to ensure reproducible separation (Tsao, 1982). This was not possible in the department as the room temperature could vary by 10°C from day to day or even during the course of a day. Controlling the temperature by immersing the tanks in a water bath was tried for a short time but this did not improve consistency of separation. It is apparent from the results of more particularly the saturated fatty acids that PC and PE bands were not discrete (Tables 3.1 and 3.2) but on occasion were merged with other lipids. This resulted in high standard errors (e.g. breast-fed group PC C16:0) compared with those associated with two-dimensional TLC (Tables 3.21 and 3.22).

Two-dimensional chromatography was thus employed to ensure complete separation of the major phospholipids from those present in lesser amounts. Several solvents were assessed to find the combination most suitable for the separation of PC, PE and PS. Two chromatography plates were spotted per sample in order to obtain sufficient material to derivatise PS which is present at a much lower concentration than PC and PE.

In recent years, several groups of workers have chosen HPLC for the separation of the phospholipids (Yeo and Horrocks, 1988; Hundreiser and Clark, 1988). This technique has the advantage of quantifying phospholipid

classes as it is non-destructive. The intact phospholipid fractions can then be subsequently analysed for their fatty acid composition.

4.1.2. Recovery studies

These studies which were carried out by Abbasi (1989) demonstrated that over 80% of lipid was recovered in the first chloroform/methanol extraction (Table 3.3) therefore a single organic extraction without internal standard was performed routinely. Accurate weighing of specimens proved impractical because of their varying degree of hydration on receipt. For this reason all fatty acid data presented in this study were expressed as weight percentage of total fatty acids rather than weight percentage of unit tissue weight although it is appreciated that the latter leads to a clearer picture of overall LCPUFA incorporation in the growing brain.

4.1.3. Precision studies

As a result of the good precision in both GLC and GCMS techniques, it was considered that this minimisation of error would enhance the identification of significant differences in fatty acid compositions between feeding groups.

4.1.4. GCMS

The introduction of the GCMS resulted in slightly lower overall CVs probably largely due to automatic sampling. Capillary columns allowed improved separation of fatty acids with the added benefit of split injection, neither of which were fitted to the GLC system used in the study. In addition mass spectrometry offered identification of unknown compounds. Mead acid was identified by GCMS from its fragmentation patterns, when compared with a commercial standard. On a similar theme, there were occasions when other unidentified peaks appeared on the chromatogram. By use of the GCMS these were rapidly identified as phthalates which are commercial plasticisers and most probably arose due to cross-contamination from pipette tips.

The derivatisation procedure of esterification continued to be employed with the introduction of the GCMS, although other derivatives such as pyrrolidides or picolinyl esters are often recommended as methods of choice. Methyl esters provided adequate sensitivity for the purposes of the study and also gave satisfactory EI fragmentation patterns which were readily compared to a library compiled from commercial standards.

4.1.5. Human and Formula Milks

Breast milk fatty acid concentrations from our local milk bank (Table 3.12) were compared with data published world-wide and quoted in the methods chapter (Tables 2.3). Remarkably there is little apparent demographic effect on breast milk fatty acid compositions (Koletzko *et al.*, 1992) with the exception of populations with a high dietary fish intake such as those of the Penang (Table 2.3) and Inuit mothers (Innis, 1992) in whom breast milk DHA concentrations were at their highest. Prior to the more extensive analyses of human milks (Jensen *et al.*, 1992) which have included identification of positional (cis:trans) isomers, trans fatty acids (Koletzko, 1992) and the array of LCPUFA present in trace amounts, lack of knowledge has led to the formulation of milk substitutes bereft of all LCPUFA. To date, however, no viable human milk sample devoid of the major cerebral LCPUFA, arachidonic, docosatetraenoic and docosahexaenoic acids, has been identified.

4.2. Study results

It is appreciated that ideally there should be equal numbers of subjects in each feeding group and also that the subjects should be age-matched. However, by the very nature of this study using tissue from cot-death infants, this was not possible. The incidence of cot death has also fallen appreciably over the period since the start of the study. The Greater Glasgow Health Board area has very low levels of breast-feeding (27%) when compared to other areas of Scotland (Aberdeen - 53%) (Ferguson *et al.*, 1994) and therefore it has been

difficult to obtain tissues from exclusively breast-fed infants especially in the older ages. The same philosophy applies to sex-matching of groups. Statistical differences were found between groups with a preponderance of females in the breast fed group and in general males in the formula fed groups.

Tissues from premature infants were also analysed. Although insufficient data were available for statistical analysis, it was nevertheless included as observational data in view of the unique neurodevelopmental and vascular disorders associated with prematurity (Crawford *et al.*, 1997).

This discussion section is set out similarly to the results section examining the results for each part of the infant brain separately and then a final section reviewing the results obtained with published research on infant visual and neurodevelopmental outcomes.

4.2.1. Cerebral Cortex

It appears that irrespective of wide variation between infant formula feeds in their content of saturated and mono-unsaturated fatty acids, "genetic" control has a powerful influence on the incorporation of these fatty acids into the cerebral cortex. Direct dietary uptake or de novo synthesis of saturated and mono-unsaturated fatty acids must supply the cerebral cortical tissue, although it is thought that the majority of cerebral saturated fatty acids and cholesterol are synthesised in situ (Edmond *et al.* 1991). If this apparently strictly controlled mechanism also operates for LCPUFA, where synthesis would require the parent essential linoleic and α-linolenic fatty acids to be available, then the mechanism is clearly under severe stress in the postpartum period and may have failed to operate in the case of two preterm singletons and two unrelated 7 week old twin infants.

Before the introduction of the new Cow & Gate (1990) formula, manufacturers' milks (in the UK) did not contain more than 1.5% by weight of α-linolenic acid and, unlike breast milk, were devoid of endogenous DHA. These formula milks also had higher than recommended (Aggett et *al.* 1991) n6:n3 essential fatty acid ratios of between 10:1 - 40:1 and low absolute amounts of α-linolenic acid. As linoleic and α-linolenic acids are metabolised by essentially the same elongase and desaturase enzyme systems, the increased competition provided by linoleic acid may in turn have had an inhibitory effect on the enzymatic formation of DHA from its parent α-linolenic acid. This appears to be reflected in the lower percentages of n-3 series fatty acids found in the cerebral cortex of the CGOST formula fed relative to the SMA fed infants.

Although the numbers are small, infants fed the C&G formulation with an α-linolenic acid concentration of 2.2% of total fatty acids, have cerebral cortex DHA levels greater than the SMA fed infants with no concomitant increase in n-6 series fatty acids (Table 3.16). As neonatal microsomal/peroxisomal synthesis of DHA from its precursor, α-linolenic acid, has now been identified (Carnielli *et al.*, 1997) this may be evidence of a direct correlation between exogenous substrate (α-linolenic) and endogenous product (DHA) concentrations. However dietary provision of such "non-physiological" levels of α-linolenic acid to the infant may have detrimental metabolic consequences by reducing AA synthesis and restricting growth. (Jensen et *al.*, 1997; Carlson, 1993). In addition the linoleic acid concentration in this revised formula at 11% of total fatty acids is lower than previously used (Table 2.2) resulting in a n6:n3 ratio of 5:1.

The ratio of linoleic:α-linolenic acid in breast milk is on average around 10:1 but endogenous DHA and DPA (both n-3 and n-6) in the mothers' milk reduce this ratio to nearer 5:1 and perhaps this is the balance which should be

aimed for by manufacturers of formula milks. In this investigation, preterm infants displayed lower DHA levels than their term counterparts. This could be due in part to lower subcutaneous tissue reserves of DHA associated with preterm delivery along with relative immaturity of fatty acid elongase/desaturase enzyme systems. The 10 week old preterm infant fed exclusively SMA formula also had an "apparent need" to incorporate substantial amounts of n-9 series Mead and dihomo-Mead acids to maintain the balance between PUFA and saturated and monounsaturated fatty acids. Such a finding in animal studies (Galli *et al.*, 1970; Sun, 1972) was associated with feeding of diets deficient in both n-3 and n-6 fatty acids. Together with the possibility of some immaturity in peroxisomal activity and reduced hepatic capacity (Farquharson *et al.*, 1995), this may indicate a need to also supply AA in milk formulae designed for preterm infants. If maintenance of higher DHA concentrations in the cerebral cortex of breast-fed infants is beneficial to neurodevelopment and function then the "deficiency" in formula fed infants may be detrimental. It had always been thought that even trace amounts of parent α-linolenic acid in the diet of infants would provide sufficient amounts of precursor to allow for synthesis of the required amounts of DHA. However at the time of rapid brain growth this may not be true (Fig 3.11).

In addition, animal studies show the incorporation of dietary DHA into brain tissue is much greater than the rate of in-vivo synthesis from the α-linolenic acid precursor (Sinclair *et al.*, 1975). DHA deficiency in rats leads to increased accumulation of n-6 series fatty acids, especially DPA, in brain phospholipids, (Carlson *et al.*, 1986) a finding that is confirmed by the cerebral cortex results. Martinez *et al.* (1992) demonstrated a steady rise in brain DHA during human fetal development, and it seems unlikely that this prenatal rise would be followed by a gradual reduction starting immediately after birth when neurological development is still active. Makrides *et al.* (1994) presented a study analysing cerebral cortex tissue from SIDS infants

comparing breast fed to formula fed infants. In this work the formula fed infants received a milk with α-linolenic acid concentrations in the range 1.0 - 1.6% of total fatty acids, similar to that of the SMA fed group. Their results show differences in cerebral cortex DHA and n-6 fatty acids between breast fed and formula fed groups which are comparable to the present study findings.

Analysis of human brain fatty acid composition necessarily involve analyses of necropsy material which is difficult to obtain. Animal studies to ascertain cerebral fatty acid concentrations resulting from both essential fatty acid deficient and replete diets have invariably demonstrated structural fatty acid differences with "low" α-linolenic acid diets devoid of LCPUFA and some workers (Bourre *et al.*, 1989; Neuringer *et al.*, 1986) have sought to relate these findings to electrophysiological (mainly visual) and direct neurological outcome. With necropsy material this is clearly not possible.

4.2.2. Cerebral Cortex Individual Phospholipids

Cerebral cortex neuronal membrane phospholipids are composed of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol. While the membrane receptor and secondary messenger characteristics of PI and its metabolites have been extensively investigated (Fisher *et al.*, 1987; Araki *et al.*, 1992; Henzi *et al.*, 1992) the functions of other phospholipids are less certain. PC is known to confer a stabilising influence within the neuronal membrane (Cullis and De Kruijff, 1979). The carboxyl groups of PS function as ion-exchange sites (Cook *et al.*, 1972) while both PS and PE have an important influence on the distribution of protein molecules in the membrane (Fenske *et al.*, 1990). Incorporation of proteins into the PS and PE rich areas of membrane is critically dependent on the chain length, degree of unsaturation, and hence configuration of the 2 fatty acids attached to each PE moiety (Salem *et al.*, 1986). It is therefore the presence of

high concentrations of DHA on PE and PS, particularly at the inner aspect of the phospholipid bilayer that allows rapid and repeated complex biochemical activities to take place at the neuronal synaptosomes (Stubbs and Smith 1984; Salem 1988). DHA has a highly specific distribution and is the predominant membrane fatty acid of synaptosomes, retinal photoreceptors, mitochondria and spermatozoa. Membrane thickness, elasticity, porosity and ability to support or transmit other molecules depends on the organic bases such as choline, ethanolamine, serine and inositol which are attached to the phosphoglycerides. The control mechanism that determines the siting and the type of phospholipid in the lipid bilayers of membranes is unknown. Stability of mammalian membranes is crucially dependent upon the presence of long chain polyunsaturated fatty acids. Whereas protein synthesis will cease if essential amino acid supplies are deficient, incorporation of fatty acids into membrane phospholipids will proceed and it appears from the results that the fatty acid closest in molecular structure is substituted thus altering the properties of that membrane. In severe fatty acid deficiency states LCPUFA of the n-3 and n-6 series are replaced by LCPUFA of the n-9 family. Where there is a lesser deficiency of n-3 series, then n-6 fatty acids alone may substitute for them.

The differences in the DHA concentrations in phosphatidylserine and phosphatidylethanolamine between the breast and formula fed infants are of an order of magnitude which, in vitro, would be sufficient to alter membrane function and could therefore critically affect the resultant responses to electrical and chemical stimulation and alter membrane and neurotransmitter function. It can be seen from Figs 3.26 and 3.34 that there is a preferential replacement of DHA in the phospholipid membrane by DPA. This selective substitution has already been reported in animal studies (Bourre *et al.*, 1984; Galli *et al.*, 1971; Mohrhauer and Holman 1963). It appears that it is only from about the fourth month of life (Figs 3.26 & 3.34) that DHA is specifically

replaced by DPA and this is particularly prominent in the CGOST group where the ratio of linoleic to α-linolenic acid is approximately 40:1. Synthesis of both DHA and DPA from the parent essential fatty acids is probably ultimately dependent on a peroxisomal β-oxidation reaction which may not be working maximally in early infancy.

Svennerholm and Vanier (1973) reported fatty acid percentages for the major phospholipids from fetal and infant cerebral cortex tissue. It is difficult to make comparisons with the present study as only 7 of their tissues collected were from infants who died in the first 6 months of life, however, the n-3 fatty acids of PE and PS did appear to fall with age during the first six months and the authors mention in their paper that when DHA was low, DPA n-6 was high. They also state that the infants were fed formula milks after two months.

When comparing breast-fed and SMA-fed infants, it may be that the DHA content of phosphatidylserine in cortical neuronal membranes is "protected" and preferentially incorporated when compared with the degree of substitution of DHA with DPA seen in phosphatidylethanolamine. From the data presented in Table 3.23 it can be seen that the DHA concentrations in the phosphatidylserine of the SMA group are nearer to those of the breast fed infants, although still significantly lower. Subcutaneous fat reserves of the parent essential fatty acid α-linolenic acid are negligible at birth and those reserves of DHA which are present are rapidly exhausted (Farquharson *et al.*, 1993). This increased DHA must have been synthesised from dietary α-linolenic acid and preferentially distributed to phosphatidylserine as no significant differences were found between the formula fed groups in the phosphatidylethanolamine fraction. From Fig 3.27 it can be seen that there is one breast fed infant with the highest DHA content (25.8%). This infant had a very high concentration of α-linolenic acid (0.9%) in subcutaneous tissue triglyceride (Farquharson *et al.*, 1993). It appears that optimal synthesis of

DHA does not occur until an α-linolenic acid substrate concentration in excess of 1% of total fatty acids is present in the diet, although this value was found in only 30% of breast milks analysed. In human milk, however, DHA is provided directly from the milk feed so that the need for synthesis of DHA from α-linolenic acid is not as critical in the breast fed infant. Anderson *et al.* (1990) in animal studies have confirmed the earlier work of Sinclair (1975) that preformed long chain PUFA are incorporated into the developing rat brain with a greater than ten fold efficiency when compared with those synthesised from the parent essential fatty acids. It should also be noted that there are major differences in the DHA content of PE and PS between breast and artificially fed infants even at 40 weeks after birth in term infants. The finding of significant amounts of Mead acid and dihomo Mead acid in the PS and PE fractions of the preterm infant is very disturbing. These n-9 series fatty acids are very unstable and are most unlikely to allow the membranes to function normally.

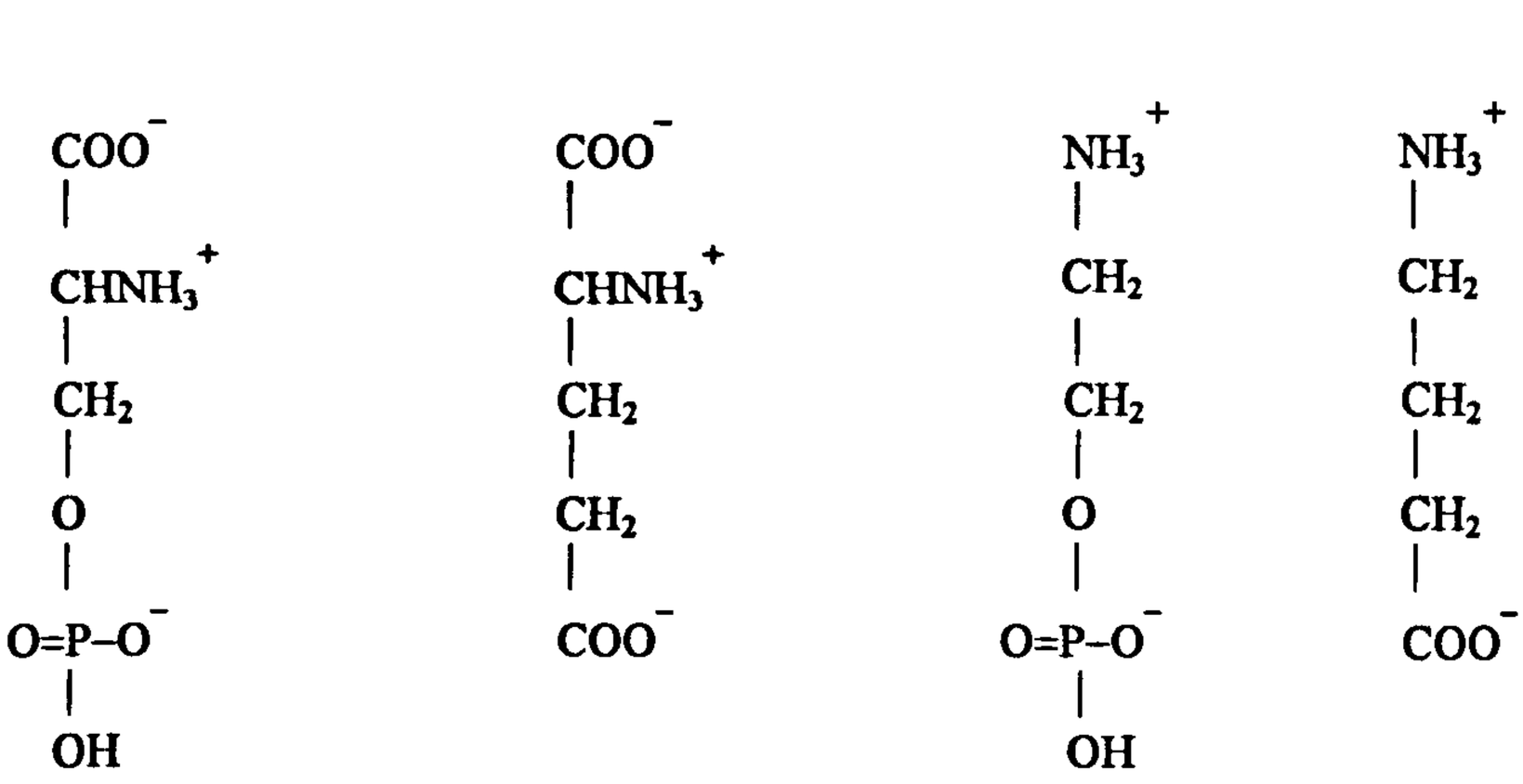
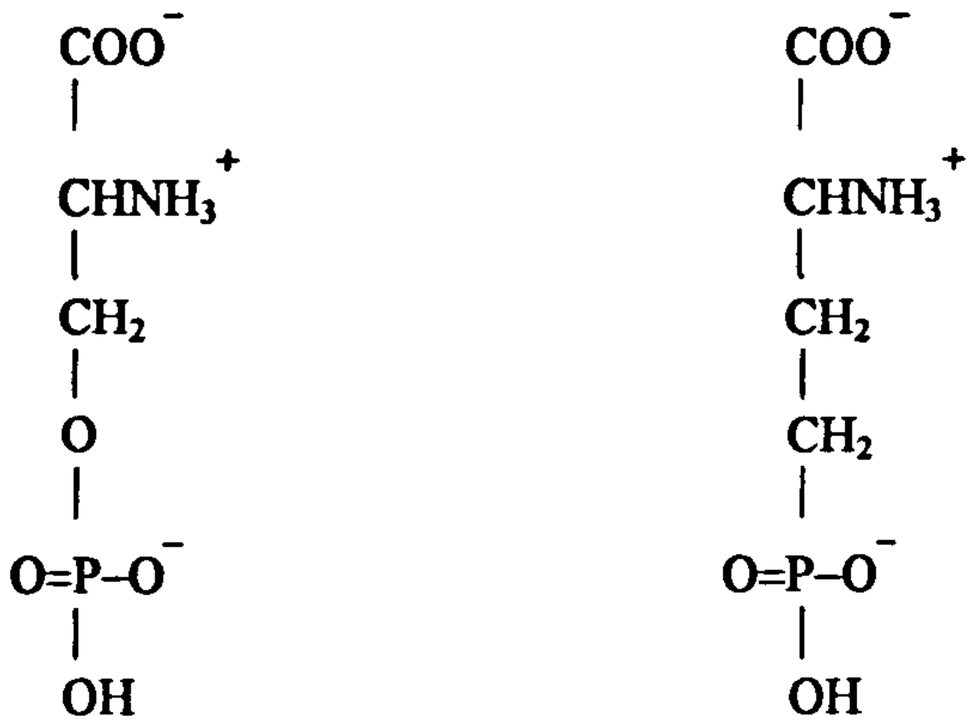


Figure 4.1 - PS and PE polar head groups - structural relationship to glutamate and GABA cerebral neurotransmitters.

There is a clustering of PS, PE and PC head groups into membrane areas known as domains. The PS and PE domains are critically important for neurotransmitter function. It has long been recognised that there are structural similarities between phosphoserine, which is the hydrophilic moiety of PS, and the major central nervous system activatory neurotransmitter glutamate (or aspartate) and the polar phosphoethanolamine of PE, which resembles the inhibitory amino acid neurotransmitter γ-amino butyric acid (GABA) (Fig 4.1). The phospholipids PE and PS are known to exchange polar head groups directly in vivo. PS may also be converted to PE in a simple irreversible decarboxylation reaction similar to that which neutralises the effect of glutamate by its conversion to GABA (Porcellati *et al.*, 1971). Although there is no direct evidence that PS functions in tandem with glutamate it is known that L-AP4 (L-2-amino-4-phosphonobutanoic acid), a phosphorus containing molecule structurally similar to phosphoserine is a proven L-glutamate agonist at presynaptic terminals and retinal bipolar receptors (Forsyth and Clements,1990; Nawy and Jahr, 1990; Slaughter and Miller, 1981). As can be seen (Fig 4.2), PS and L-AP4 differ only in the replacement respectively of an



Phosphoserine L AP4

Figure 4.2 - Structures of L-2-amino-4-phosphonobutanoic acid and phosphoserine.

ester oxygen by a methylene (CH_2) group, both species with very similar charge characteristics and molecular dimensions which would be essential if phosphoserine were to mimic the neuroexitatory role of L-AP4. It may be then that PS and PE have a neurotransmitter function which could be independent or work in parallel with the glutamate-GABA system.

4.2.3. Cerebellar cortex

In evolutionary terms, after the brain-stem, the cerebellum was next to develop in humans from the anterior dorsal part of the primitive mid-brain. The cerebellum is composed of two hemispheres connected by the vermis in the midline. The hemispheres display numerous deep fissures with folia between. Similar to the cerebrum, the cerebellum is also composed of lobes, the oldest in evolutionary terms being the flocculonodular followed by the anterior and posterior. The cerebellum acts to temper and balance the sensory stimuli reaching the cerebral cortex with that of the generated neurological response and it therefore has a "fine-tuning" remit. The cerebellum was thought to specialise predominantly in the co-ordination of movement and balance but it may also undertake some of the higher sensory functions until recently considered to be the preserve of the cerebral cortex (Kolb and Whishaw, 1985; Leiner *et al.*, 1991). In humans it was thought that the increased cerebellar complexity, particularly the lateral expansion of the posterior lobe, was associated with our increased manual dexterity. However positron emission tomography appears to show maximal activity in the cerebellar posterior lobe in response to cognitive and linguistic tasks, generally centred in the cerebral prefrontal cortex (Fox *et al.*, 1985).

From previous studies it has been shown that in the infant, the cerebellum has a faster and shorter growth spurt when compared to the cerebrum, starting later but achieving adult proportions earlier (Dobbing and Sands, 1973) and involves incorporation of specific PUFA (Ballabriga and Martinez, 1978).

Despite missing the growth spurt, formula fed premature infants do not appear to be "disadvantaged" relative to their term born contemporaries in terms of accretion of LCPUFA, specifically DHA, in the first year of life. Indeed cerebellar grey DHA concentrations of the SMA fed group were not significantly higher than those of the SMA-prem group (Table 3.27) and although prenatal accretion may explain these findings it is also worth noting that provision of α-linolenic acid at 1.5% of total fatty acids (SMA group) may have been more than adequate to allow for synthesis of sufficient DHA. The cerebellar cortex constitutes <10% by weight of that of the cerebral cortex and DHA concentrations at birth in both tissues are equivalent (Figs 3.11&3.45). The lowest cerebellar grey DHA compositions were encountered in the infants fed the CGOST formula with low (<0.4%) α-linolenic acid content. Cerebellar grey matter DHA concentrations show a gradual decline with age in breast-fed infants (Fig 3.45) which is in contrast to those of cerebral grey matter (Fig 3.11) but apart from the CGOST group, no overt increase in DPA (C22:5n-6) is apparent in the remaining infants (Fig 3.44), although it was noted that the highest DPA concentration belonged to the 19 week old, CGOST fed, preterm (34 wk gestation) infant. As increased DPA concentrations are a general indication of DHA deficiency it would seem that only by provision of "inadequate" amounts of dietary α-linolenic acid (CGOST group) will a subsequent deficit in cerebellar grey DHA arise. Although, as discussed, premature infants do not appear to be specifically vulnerable to reduced cerebellar DHA incorporation, in two term-born unrelated twins (6.5 and 7 wk old), disproportionately low DHA concentrations were found (Fig 3.45), however this might have been a feature of shared maternal DHA supply in utero.

The study findings with respect to feeding groups must be somewhat tempered because of the lower mean birth weights of the SMA (2.99 kg) and CGOST fed (2.94 kg) than those infants breast fed (3.36 kg). In contrast the small

C&G fed group (n=4), despite a mean birth weight of 2.98 kg similar to the other formula groups, given a diet containing α-linolenic acid at 2.2% of total fatty acids, was able to maintain cerebellar cortex DHA concentrations that were 50% greater than that of the age-equivalent CGOST group.

Whether with the extreme dietary "deprivation" of LCPUFA (as witnessed particularly in the CGOST group) and resultant structural alteration in cerebellar grey matter composition a functional deficit could be induced is unknown. It is interesting to speculate however that inattention in school, the so-called attention deficit hyperactivity disorder has risen remorselessly in particular in American male students in conjunction with an increase in formula feeding of diets both devoid of long chain polyunsaturated fatty acids and very long chain fatty acids both essential to white matter development. Stevens *et al.*(1995) have measured the red cell membrane fatty acids in boys with this disorder and found significantly lower concentrations of both AA and DHA when compared to a group with milder symptoms. Perhaps the first choice treatment Ritalin, which has paradoxically a neuroexcitatory function, is in some way correcting for the dietary induced, biochemical alterations in cerebellar structure. In addition as previously stated, an "epidemic" in uncoordinated, dyspraxic individuals has been identified in upwards of 6% of school-age children (Brenner *et al.*, 1967). However higher incidences of up to 14% were associated with children of low birth weight (<2.5 kg) (Lesny, 1980).

Fine motor co-ordination defects would appear to be at the heart of this problem, and although far from being life-threatening, nevertheless such symptoms can have a destructive effect on quality of life. Animal studies can provide useful information on dietary deprivation and resultant cerebellar-related function. However, because of our ultimate complexity, the human species might be uniquely susceptible to cerebellar membrane structural

alterations, particularly during the neonatal period when there is maximal growth and cell division. It cannot be advisable to introduce mind-altering chemicals to young and developing brains in order to maintain "control" in the early learning environment. Clearly no conclusions can be drawn about neurological outcome from our groups. However, a retrospective study of infants exclusively fed the CGOST formula in infancy with particular reference to subsequent development of mild to moderate dyspraxia may be informative.

4.2.4. HPTLC of Cerebral White and Cerebellar White Matter

HPTLC was used to analyse cerebral and cerebellar white tissues previously assessed for fatty acid content to find a possible explanation for the fatty acid results from those specimens designated *? locus* and considered to be outliers and also to provide more information on cerebral and cerebellar lipid species and their relative variation in infancy.

On visual inspection of the HPTLC plates, it was apparent that bands corresponding to cerebrosides and sulphatides were absent or very faint in parietal cerebral white matter tissue extracts of infants less than 8 weeks old. In contrast the cerebellar white tissue extracts for the same infants had strong bands for both compounds. In spite of their associated infant ages (age range 4 - 11 weeks), the five tissue extracts defined as outliers in the cerebral white matter had well defined bands of both cerebrosides and sulphatides.

Researchers who have examined neonatal brains using Magnetic Resonance Imaging with the purpose of studying white matter maturation have identified significant myelination (grade 3) by birth in cerebellar white matter but not before 3-6 months in cerebral central white matter (Barkovich *et al.*, 1988). Others who studied infants born between 29 and 42 weeks gestation also noted

cerebellar white matter myelination in advance of cerebral myelination (McArdle *et al.*, 1987).

Kinney *et al.* (1994) performed HPTLC in the separation of lipid classes using white matter tissue from various areas of the brain in infants ranging in age from birth to two years. Previous work by the same group (Brody *et al.*, 1987; Kinney *et al.*, 1988) had examined the development of myelination in 62 different white matter sites of the brain. These histological analyses revealed that myelination occurred at different stages of infant development and at different rates that were dependent on the white matter location. Site specific biochemical analyses added further confirmation. The lipids known to be rich in myelin such as cerebrosides, sulphatides and sphingomyelin were present in strong bands from birth in areas histologically identified as "early myelinators" whereas these compounds appeared at a later age in areas such as the frontal pole, defined as a "late myelinator".

In an earlier investigation, Svennerholm and Vanier (1972) quoted values for galactocerebrosides rising from virtually undetectable at birth (0.5) to 20 umol/g wet weight at 8 months, and similarly for sulphatides rising to 5 umol/g. They reported a two-fold increase in cerebral white matter phospholipids in the first eight months of life with the percentages of the individual phospholipids in good agreement with the results of the HPTLC analyses presented in this thesis (Fig 3.46).

Martinez (1982) measured galactolipids in the cerebrum and cerebellum of 14 well-nourished infants from 26 weeks gestation to 15 months of age and included in the study a group of 10 undernourished infants who died between 27 weeks gestation and 4 months of life. She found that the concentration of galactocerebrosides was much higher in the immature cerebellum than in the immature cerebrum. At term galactolipids in the cerebellum were five times

greater than in the cerebrum. She also found that children undernourished postnatally (n=4) had lower cerebral galactolipids than the well nourished population whereas there was no difference in the cerebellar concentrations of the two groups.

Although exclusion of results is not ideal, in view of the data presented in the aforementioned papers, it would seem that the results obtained for these few specimens, if from the parietal central region, would not be compatible with tissue derived from a normally developing brain.

In consideration of the lipid species populated by the rising lignoceric and nervonic acids, the increase in nervonic acid concentrations in cerebral white matter (Fig 3.50) correlated with the rising percentage of both cerebrosides and sulphatides (Fig 3.46). In addition the very low levels of both glycolipids in the first few weeks of life coincided with the absence of both lignoceric and nervonic acids in early life. In contrast, sphingomyelin was present at 5% of total phospholipids from birth (Fig 3.48) and although this ratio increased to approximately 10% by 6 months there was not a dramatic rise in comparison to the other myelin lipids. Cerebellar white matter displayed earlier indication of myelination in relation to cerebral white in terms of sphingomyelin where the ratio to total phospholipids at birth was 10% increasing to 15%.

There are few publications quoting lignoceric and nervonic acid concentrations of myelin lipids in early infancy. O'Brien and Sampson (1965) measured the fatty acid content of the lipids of grey and white matter from the frontal lobes of 5 subjects. They quoted values for an infant aged 10 months for cerebrosides, sulphatides and sphingomyelin. The nervonic acid concentrations were 17.5% and 28.3% for cerebroside and sulphatide respectively as compared to 10.8% for sphingomyelin. Another group of researchers analysed the fatty acid composition of cerebrosides and sulphatides

in neonatal and infant brains (Svennerholm and Ställberg-Stenhagen, 1968). Some of the data was however from whole brain tissue because of the difficulty of separating grey and white in very young infants. Nervonic acid content was in excess of 20% in both cerebrosides and sulphatides in the two white matter tissues which were analysed. Rao (1976) reported the fatty acid composition of cerebrosides from total cerebrum and cerebellum in fetal brains from 20 week gestation to full term. Comparison of data is difficult since the author is quoting tissue not separated into grey and white matter. It seems surprising, however, that the concentration of nervonic acid is lower in the cerebellum when compared to the cerebrum whereas lignoceric acid shows the opposite effect. This observation on lignoceric acid was in general agreement with the results in this thesis. It is also interesting to note that the C22 saturated and monounsaturated fatty acids are considerably higher in cerebellar tissue.

As a consequence of the results reported for this part of the study it would be interesting to analyse the fatty acid composition of these myelin lipids in relation to age and diet as there is a dearth of information of this type in the literature. This would aid in assessment of the importance of nervonic acid which is present in small amounts in breast milk but not in formula milks.

4.2.5. Cerebral Parietal White Matter

Although not exclusively, the deposition of cerebral white matter and myelin in the immediate postnatal period involves phospholipid and cerebroside (including sulphatide) synthesis respectively. Accreted mainly by the oligodendrocytes, these lipids are populated by an array of specific fatty acids, whose incorporation is apparently controlled by a precise series of reactions. These occur mainly in the first year of life and as the fatty acid compositions adopted, thereafter remain unaltered, (Dobbing and Sands, 1973;

Svennerholm,1968; Svennerholm and Ställberg-Stenhagen,1968) any disruption to the process may have far-reaching consequences.

As white matter deposition proceeds, with the exception of docosatetraenoic acid (C22:4n-6), a relative general reduction in LCPUFA is the common feature. From the results (Figs 3.57 & 3.60) it can be seen that the compositions of cerebral parietal white matter AA and DHA nevertheless remain constant for the first two months in the human milk fed infants. In contrast the DHA concentration in the term SMA group is not sustained beyond about five weeks. However, the decline appears to be balanced by an increase in AA, an effect similar to that found in the cerebral cortex. This decline in initial DHA content is more pronounced in the premature SMA fed infants, but again, possibly in compensation, the highest AA concentration is present in the 17 week old infant. What the consequences may be of this decline in DHA in the formula fed groups relative to the breast fed and resultant postponement in the reduction of AA, is not known but it may be reassuring that these between group differences appear to be corrected within the first year of life without recourse to increased insertion of docosapentaenoic acid (C22:5n-6), which usually indicates a DHA deficiency state. The proportion of DHA declined with increasing age in all groups, although more rapidly in the CGOST group. Inspection of the results also revealed that the 7 week old, partially breast fed premature infant could maintain a significantly greater DHA concentration than exclusively formula fed contemporaries and comparable with that of the term born fully breast fed infant (Fig 3.60). This has probably arisen as premature infants will have negligible fatty acids stored at birth (Farquharson *et al.*, 1993) and in spite of the provision of adequate α-linolenic essential fatty acid precursor, we now know that, even in term born infants, hepatic synthesis of DHA in the first postnatal months is minimal (Farquharson *et al.*, 1995). On closer inspection of the divergent DHA compositions of the two 18 week old SMA fed infants

(6.4% and 4.0%) these were found to correlate with their birth weights of 3.63kg and 2.21kg respectively and probably indicate greater subcutaneous tissue reserves of DHA in the former.

In the white matter, metabolism of LCPUFA is predominantly confined to the non-choline containing phospholipids, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol. Possible implications of altered fatty acid compositions in PE and PS have been considered in respect of neurological function in the infant cerebral cortex. Although the between group differences in white matter appear to be eliminated more rapidly than in the cortical grey matter, nevertheless white matter neural structures tend to display a certain permanence and rigidity not present in the plasticity of the grey matter and therefore may be more affected by early deviations from the normal pattern of fatty acid deposition.

In early infancy the initial differentiation between cerebral grey and white matter is further complicated by division of the white matter into myelinated and unmyelinated fibres. The great difficulties experienced in providing an early diagnosis in the leukodystrophies, in spite of the use of the most modern diagnostic imaging technology, may be related to the aforementioned gestational age and dietary dependent variations in the deposition of the component fatty acids. As there appeared to have been "developmental delay" in the processes involved in white matter formation in the formula fed infants, investigation of the very long chain fatty acids (VLCFA) would be expected to indicate whether the effect translated to myelination. As these VLCFA have a high "insulating capacity" and are hydrophobic it was not surprising that their incorporation into myelin was delayed until the cerebral water content reached a minimum (75-80%) an observation supported by animal studies (Uzman and Rumley, 1958). A direct insight into this is provided by the fatty acids, lignoceric and nervonic both of which predominate in the cerebrosides. These

VLCFA increased logarithmically in the total lipid fraction of the breast fed group parietal white matter at the end of the second month, but not until the end of the third month in the CGOST and SMA groups (Figs 3.61 & 3.62). A subsequent further delay may also be evident in the premature SMA-fed infants. This may have been as a consequence of the differences in white matter LCPUFA discussed previously but other explanations are possible. From previous research no evidence of significant hepatic synthesis of these VLCFA has been found (Farquharson *et al.* 1995) and in addition although trace quantities (0.05-0.1%) of lignoceric acid were identified in the formula milk, nervonic acid was undetected (<0.05%). This contrasted with mature human milks which contained similar levels of lignoceric but nervonic acid at <0.05-0.3% (median 0.15%) of total fatty acids. This dietary supply may have enabled the earlier incorporation of nervonic acid into the myelin in those breast fed, circumventing the need for microsomal synthesis by chain elongation of its long-chain precursors. This enzymatic sequence, as it was not evident in the liver, may also have been competitively inhibited by the presence of "excess" long-chain saturated fatty acids, such as arachidic (C20:0) and behenic (C22:0), found in significant quantities (>0.2%) in the SMA formula (Wilson and Sargent, 1993). It may be confirmatory that the partially breast fed, 18 week old term infant seemed capable of maintaining a lignoceric acid concentration similar to that of breast fed infants (Fig 3.61), whereas nervonic acid correlated more closely with the formula fed infants (Fig 3.62). The presence of immature enzyme systems in the premature infants may explain the further delay in their accretion of the VLCFA. This delay, however, may not be inappropriate in the preterm infant in whom myelination may correlate with postconceptual rather than chronological age.

An indication that mechanisms involved in LCPUFA and VLCFA metabolism may be working independently may also be demonstrated in the results of the CGOST group. With the CGOST feeding regimen not only being devoid of

LCPUFA but containing very low levels of α-linolenic acid, cerebral parietal white DHA concentrations are in general very similar to those pertaining in the SMA preterm group (Fig 3.60). This may be confirmed by the resultant higher concentrations of DPA in both the CGOST and SMA preterm groups (Fig 3.59).

However analysis of the VLCFA results appears to confirm a trend whereby infants within the CGOST group have generally lignoceric and nervonic acid concentrations which are intermediary between the breast fed and term SMA group. A possible explanation may be in the formulation of both C&G and Farley's milks which constitute the CGOST group. These contain significantly higher concentrations of both nervonic acids immediate monoenoic precursors, eicosenoic and erucic acid (Table 3.12) and may push the momentum of chain elongation reactions towards nervonic acid synthesis when compared with the SMA group as previously discussed. This effect also appears to translate to the long-chain monounsaturated fatty acids where C16:1 and C18:1 concentrations in the CGOST group are also quite dissimilar from those of the SMA preterm group (Figs 3.53 & 3.55).

As previously stated, because of the dynamics of white matter and myelin deposition in early life it was essential that specimens were taken at necropsy from precise loci. For a period of 4 months results were obtained from 5 specimens in which the fatty acid concentrations (in those that varied with age) were found to be extreme outliers. Further investigation revealed that these probably had been obtained from a more posterior cerebral site. This may have been confirmed by the HPTLC/densitometry of the phospholipids and cerebrosides.

4.2.6. Cerebellar white matter

In contrast to cerebral white matter, cerebellar white matter occupies a relatively restricted area. It is composed of several central isolated nuclei. The largest of these, the dentate nucleus is sited in the neocerebellum, whose efferent impulses alight, via the thalamus, at the cerebral motor cortex. In conjunction with the less prominent white matter fastigial nuclei, their axonal impulses ultimately serve to control both balance and eye movement.

Davison and Dobbing (1966) examined the brain-growth spurt in different species and demonstrated that development of the cerebellum proceeded with a similar sequence of events but at rates that were species specific. The rat is often used as an animal model to study the development of the human cerebellum as both species have an immature cerebellum at birth and morphogenesis mainly occurs during postnatal life. This "growth spurt" includes the period of rapid myelination and it has been suggested that this is a critical period in terms of nutrition (Budowski *et al.*, 1987). In rats post natal growth is retarded by imposing a nutritional deficit exclusively after birth and during the suckling period. Alterations in the brain growth are exacted by either introducing more pups per dam to suckle or by removing the pups from the mother for several hours each day. The cerebellum of animals fed in this manner has disproportionately fewer cells, especially granular neurons and glial cells (Dobbing and Sands, 1971) and the brain lipids, especially those characteristic of myelin, are permanently reduced (Dobbing, 1968).

As a result of the above findings, researchers tested the hypothesis that nutritional deprivation in early postnatal life may affect motor co-ordination. (Lynch *et al.*, 1975; Jordan *et al.*, 1979). They found that growth restricted animals performed worse in specific tests of co-ordination when compared with control animals. Balazs *et al.* (1986) noted retarded development of spontaneous motor activity and a variety of motor reflexes in undernourished

rats. Other groups have shown that cell numbers of neurons and oligodendrocytes are not restored by nutritional rehabilitation (Clos *et al.*, 1982) and that myelin thickness is also not restored (Wiggins, 1982).

There is a dearth of literature on cerebellar white matter lipid fatty acid composition in either animal or human nervous tissue. Martinez (1982) however presented a study comparing cerebral and cerebellar myelin lipids in undernourished infants with those fed a normal diet. She found significantly reduced levels of galactolipids in the cerebrum of the malnourished infants but no difference in the cerebellum of these subjects. In contrast the phospholipids and cholesterol in both forebrain and cerebellum were indistinguishable from normal values. Plasmalogens were decreased in both loci but not so markedly in the cerebellum. These data do not agree with the findings of Chase *et al.* (1972) who examined the cerebellum of a group of small for gestational age (SGA) infants and compared them with a group of appropriate for gestational age (AGA) infants all of whom died shortly after birth. The cerebellar tissue of the SGA infants was found not only to be reduced in cell size, but there was also a significant reduction in cerebrosides and sulphatides but no difference in phospholipids and cholesterol when compared to the AGA group. This study may differ in its findings because it is affected primarily by intrauterine undernutrition whereas that of Martinez involved postnatal undernutrition.

When the results obtained by Martinez (1982) are related to the results of the present study, they can provide an explanation for the lack of differences observed in nervonic acid in relation to diet in cerebellar white matter as compared to the cerebral white matter where there was a deficit in nervonic acid in both the formula fed infants and the preterm infants. Lignoceric acid in the cerebellum showed the opposite effect to that in the cerebrum, SMA-fed infants having significantly higher concentrations than the breast-fed group. A similar trend was also noted in the CGOST-fed infants although no statistical

analysis was performed due to lack of age-matching of individuals. Both lignoceric and nervonic acids are however present at about 1% of total lipids at birth in contrast to the cerebral parietal white matter in which both these fatty acids were undetected. These findings may imply that myelination is further advanced in the cerebellum than cerebrum at birth, which is in agreement with Kinney *et al.* (1994) who showed in their study of autopsied brains that cerebellum matured ahead of cerebral parietal white matter.

The study results for cerebellar white matter DHA show significantly lower concentrations in the SMA-formula fed infants when compared to an age-matched breast-fed group. This finding, similar to that of the cerebellar cortex, is surprising when the cerebellum requires only one tenth of the supply of DHA needed to sustain the cerebrum but might indicate a higher turnover in infant cerebellar polyunsaturated fatty acids. It is however unknown whether the dietary induced differences in DHA are sufficiently large to influence the predominantly neuro-inhibitory characteristics of the cerebellum.

4.3.1. Infant feeding and developmental outcome

This thesis has examined the fatty acid composition of neural membranes with special reference to diet. This section summarises recent publications relating the effects of early feeding to the development of the retina and cerebrum as estimated by visual and developmental studies.

Most of the studies have examined the hypothesis that breast feeding gives infants developmental advantages over formula feeding. Some workers have looked at infants and toddlers (< 2 years) while other groups have concentrated on older children. Studies can be further subdivided into those which have examined preterm children and those which concentrate on term-born infants.

Five studies of term-born infants measured cognitive development by the Bayley's scale of mental developmental index (MDI) up to 24 months. These studies compared breast-fed with formula-fed individuals and also considered the duration of breast-feeding. Temboury *et al.* (1994) examined 229 infants born in Madrid and found bottle fed babies had lower MDI scores between 18 and 29 months when compared to breast-fed infants. Two American groups classified breast-fed infants according to length of breast feeding. Morrow-Tlacak *et al.* (1988) subdivided the breast-fed group into <4 months or >4 months. They examined 229 infants and found a significant difference between bottle-fed children, children breast-fed <4 months and those fed >4 months both at 12 and 24 months. Further regression analysis indicated breast feeding duration was significantly correlated with improved MDI scores at 12 and 24 months allowing for confounding variables. Rogan and Gladen (1993) examined 600 subjects up to 24 months and observed a difference of +6.7 points on MDI when comparing very long (>20 weeks) with short term (<4 weeks) breast-feeding and +5.6 points when comparing very long with formula feeding. Young *et al* (1982) performed a retrospective study on 1000 infants from Tunis birth records and classified the subjects according to length of breast feeding. Artificial feeding was defined as <2 months breast feeding, mixed feeding as breast fed for 2 months followed by cow's milk and breast-fed as exclusive breast feeding for >7 months. They found significantly higher MDI scores for breast feeding over artificial and mixed at 12 and 24 months allowing for confounding variables. The fifth study was performed with 846 first-born singletons from Dundee and measured both motor and mental development at 18 months (Florey *et al.* 1995). Group mean difference for the MDI scores were 8 points higher in the breast-fed group but no mean differences were found for psychomotor development index (PDI) scores.

Studies on older children included one published as early as 1929 by Hoefer and Hardy where their 383 subjects were aged 7-13 years. The groups were

categorised as artificially fed or according to the length of breast feeding and assessed for intelligence quotient (IQ), educational quotient (EQ) and performance quotient (PQ) scores. Of two publications from a group in New Zealand, the first examined a large number (n>1000) of children at 3, 5 and 7 years using IQ and language comprehension (LC) tests and compared bottle feeding, breast feeding <4 months and breast-feeding >4 months. (Fergusson *et al.*, 1982). The follow-on study (Horwood and Fergusson, 1998) reported a range of measures of cognitive and academic outcomes for the same children between the ages of 8 and 18 years. After correction for confounding variables, the authors concluded that breast feeding was associated with small but detectable increases in child cognitive ability and academic achievement. They also noted that these effects were relatively long-lasting extending throughout childhood into young adulthood. Rogan and Gladen (1993) produced a follow-up to their infant study where they used McCarthy Scale scores as a measure of cognitive development at 3, 4 and 5 years. Their results showed a trend for higher scores in the breast feeding groups at all ages. A study in the U.K. compared the performance of exclusively breast-fed with exclusively bottle fed children (>1000 in each group) at 8 and 15 years (Rodgers, 1978). They found significant differences at age 8 on picture intelligence and at age 15 on sentence completion, non-verbal ability and maths tests.

A similar study by Pollock (1994) followed children born in the United Kingdom during seven days in April 1970. The cohort was divided into those mothers who had exclusively breast-fed for at least three months and those who had exclusively bottle-fed over the same period. Children were assessed at five and ten years for several medical, physical and intellectual characteristics. This study differed from the others already discussed in that control for those confounders focused on differences between the two groups and not on factors which necessarily contributed to the outcome being assessed. This was to ensure that results were not attributable to differences between the social and

biological background of infants fed by different methods. No significant differences were observed between infant feeding methods for any medical outcomes or physical characteristics at five or ten years. At five years only one measurement of development achieved significance, namely the English Picture Vocabulary Test in which the breast-fed children had higher scores. By ten years of age, exclusively breast-fed children scored above average on the pictorial language test and also had higher scores on the British Ability Scales for Word Definitions, Similarities and Matrices. The study concluded that intellectual attainment was superior among children that were exclusively breast-fed for at least three months. Pollock hypothesised that it was unlikely that the intellectual difference was due to small differences in the protein, carbohydrate or total fat content of human or formula milk, but was more likely to be due to suboptimal levels of specific micronutrients in formula milk at a critical period of neural tissue growth and differentiation.

All the aforementioned studies have been on term children. Lucas has published several studies on preterm children. His first main study was to follow-up 300 children from 5 different centres who were categorised according to whether they had received their own mother's milk (Lucas *et al.* 1994). Using the Weschler Intelligence Scales for Children (WISC-R) test at 7.5 years the authors found a 10-point higher IQ in the breast fed group. Adjustment for mother's education, social class and sex reduced the difference to 8.3 points. These infants received their mothers' milk via nasogastric tube and therefore any advantage conferred by the act of breast-feeding was cancelled out. The next study involved preterm infants whose mothers had chosen not to breast feed. In one trial infants fed on standard term formula for an average of 4 weeks had a 15 point deficit in PDI at 18 months compared to those fed a nutrient-enriched preterm formula. In another trial a group of infants fed donor breast milk had scores similar to those fed the preterm formula. Lucas suggested that the donor breast milk was similar to the term

formula in that it did not provide adequate nutrition for the needs of the preterm infant. The author also compared the group fed expressed breast milk with those fed standard term formula. The children fed the expressed breast milk had a significant 9 point advantage in PDI at 18 months over those fed standard term formula (Lucas *et al.*, 1990).

The effect of dietary essential fatty acid supply on retinal development in the human infant has been examined by several authors (Carlson *et al.*, 1996; Innis *et al.*, 1994; Jorgensen *et al.*, 1996; Auestad *et al.* 1996). Development of the retina and visual system was assessed by measurement of visual acuity, which is a non-invasive test readily applied to non-verbal individuals. The above studies all concentrated on term born infants. Innis and Jorgensen merely compared breast fed versus standard formula fed infants and their visual acuity. Innis found no difference in visual acuity at either 14 days or 3 months whereas Jorgensen found the visual acuity of the breast fed infants significantly greater than that of the formula fed group. This was paralleled by a decrease in red cell membrane DHA in formula fed infants. This finding was similar to that observed by other groups. Auestad *et al.* (1996) evaluated the addition of DHA and AA or DHA alone to term formula and assessed both growth and visual function. They found no differences in growth or visual function during the 12 month period. This study had involved 197 infants. In 1996, Carlson had performed a similar experiment on 58 infants in USA. She found a transient improvement in visual acuity in the supplemented group over the standard formula but it was not evident after 2 months. Makrides *et al.* 1995) reported a small trial comparing a group of term infants fed standard formula with a group receiving a formula supplemented with fish oil as a source of DHA and gamma-linolenic acid as a precursor of AA. The supplemented group had higher visual acuity, as measured by visual evoked potentials, than the group fed standard formula at 4 and 7 months. A reference breast-fed group performed similarly to the supplemented infants.

Preterm infants have also been given supplemented formula and visual acuity measured comparing with both breast-fed infants and those given standard preterm formula. These studies have differed in the source of LCPUFA and the amount added. Carlson *et al.* (1992) added fish oil to standard preterm formula and found that the supplemented group had significantly lower weight, length and head circumference at 12 months. The supplemented group were also lower at 12 months in both MDI and PDI and there was no difference in visual acuity between groups. The negative findings were presumably due to the high amount of EPA in the fish oil preparation. In a second trial (Carlson *et al.,* 1994) the infants were only given the supplemented formula for two months and the dietary EPA concentration was lower at 0.06%. Visual acuity was higher in the supplemented group at 2 months but no different at subsequent testing up to 12 months. The supplemented group was however still reduced in weight and head circumference at 6 and 9 months. Presumably this is due to the competitive inhibition of the n-6 pathway by the added DHA and EPA.

The above work can be broadly divided into two categories. In the first, researchers have tried to show that breast fed infants have an advantage in neurological outcome as compared to formula fed infants. It is very difficult to compare the publications, although those assessing cognitive function in childhood include a majority which show mild to moderate benefits in the breast fed population. The inconsistency in the outcome between the various studies could relate to population differences and variation in the number of confounding variables applied. Many of the publications do not give details of formula feeding and has been mentioned in this thesis there is a wide variation between formulas in both alpha-linolenic acid concentrations and linoleic:α-linolenic acid ratios.

In the second category, researchers have started to compare standard formulas with supplemented formulas. Here again it is difficult to compare results as there have been large variations in supplementation, ranging from different sources of LCPUFA to the amounts of DHA and/or AA added, not to mention again the difference in the standard formula to which these LCPUFA are being added.

4.3.2. The preterm infant

The preterm infant is defined as having been born at a gestation of less than 37 weeks. Such infants are therefore born with only limited transfer of AA and DHA from the placenta. The amount of subcutaneous tissue will also be less in a premature infant, resulting in lower stores of fatty acids at birth. A baby born several weeks prematurely will be fed parenteral nutrition, expressed breast milk or supplemented formula milk, none of which will match the amount of AA and DHA supplied by the placenta had the infant still been in utero. This deficit in AA and DHA which occurs in the first few weeks of the preterm infant's life coincides with the most rapid period of brain growth.

The incidence of neurodevelopmental disorders is greater in both preterm infants and low birth weight for gestational age infants. A birthweight of 2.5 kg or less in a term-born infant is associated with a greater chance of a central nervous system defect. Of the four infants in this study from twin pregnancies two could be classified as intrauterine growth retarded. Here also the infants are under stress at birth having shared placental supplies of LCPUFA during pregnancy. All four infants however were in the same feeding group and it was not possible to assess the effect of diet in such individuals.

Manufacturers of milks have accepted as a result of research that addition of AA and DHA is recommended for the preterm infant and all specially formulated milks for this group now contain these LCPUFA. Future research

should include where possible analysis of nervous tissue from infants fed these new formula milks. In addition further visual and long term developmental studies should be instigated.

4.4 Conclusions

Controversy still remains in relation to formula milks for term-born infants. This is most probably due to visual and developmental studies producing contradictory results between those who found significant differences between breast feeding and formula feeding and those who found no differences. From the results presented in this thesis, it would appear beneficial to add AA and DHA to term formulae in order to eliminate structural differences in nervous tissue. The other interesting finding of lower nervonic acid in cerebral parietal white matter of formula fed infants leads to the suggestion that this fatty acid, which is present in breast milk, should also be added to infant formula milks.

The ratio of n-6 to n-3 fatty acids in milks is still the subject of much debate. Certainly from the few infants in this study who received the new C&G formulation, it would appear that their levels of LCPUFA in nervous tissue were closer to that of the breast fed infants but further work is necessary to confirm this finding. Evidence exists that the new-born infant is capable of desaturating and elongating linoleic and α-linolenic acids to their longer chain homologues, but whether the rate is sufficient to supply the requirements of the rapidly growing neural network is still not proven. An additional dietary supply of LCPUFA may still be essential.

As stated further dietary related, long-term developmental studies are required in humans. Is it possible that people have become more aware of disorders such as dyslexia, attention deficit hyperactivity disorder and behavioural problems in the last 20 to 30 years or may it be that there is a true increase of these disorders as a result of the decrease in the percentage of mothers breast

feeding? In the adult population, the incidence of multiple sclerosis is much lower in countries such as Japan where the population are predominantly fish eaters and a large proportion of mothers breast feed providing not only dietary AA and DHA but also nervonic acid essential for myelination. Alzheimer's disease, which is showing an increasing prevalence with an ageing population, whilst perhaps not directly caused by deficits of LCPUFA and VLCFA in the brain, disruption of their supply may be a contributory factor.

It would therefore seem prudent to attempt to improve rates of long-term breast feeding and for those mothers who are unable or do not wish to breast-feed, to provide a substitute formula which contains all prominent neural fatty acids in a preformed state, in similar proportions to that found in the milks of well nourished mothers. In conjunction with optimal dietary advice to mothers during pregnancy in an attempt to improve uptake of essential fatty acids by the fetus and possibly reduce the occurrence of both intrauterine growth retardation and premature delivery, it would then be practical to invoke non-invasive longitudinal studies to answer the question, whether introduction of formula milks devoid of the fatty acids discussed, has been detrimental to the neurodevelopment of previous and present generations.

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APPENDIX

Publications

Farquharson, J., Cockburn, F., Patrick, W.A., Jamieson, E.C. & Logan, R.W. (1992) Infant cerebral cortex phospholipid fatty acid composition and diet. *Lancet* **340**, 810-813.

Jamieson, E.C., Abbasi, K.A., Cockburn, F., Farquharson, J., Logan, R.W. & Patrick, W.J.A. (1994) Effect of diet on term infant cerebral cortex fatty acid composition. In *Fatty Acids and Lipids: Biological Aspects.* ed. Galli, C., Simopoulos, A.P. & Tremoli, E. pp. 139-141. Basel, Switzerland: Karger.

Farquharson, J., Jamieson, E.C., Abbasi, K.A., Patrick, W.J.A., Logan, R.W. & Cockburn, F. (1995) Effect of diet on the fatty acid composition of the major phospholipids of infant cerebral cortex. *Archives of Disease in Childhood* **72**, 198-203.

Farquharson, J., Jamieson, E.C., Logan, R.W. Patrick, W.J.A., Howatson, A.G. & Cockburn, F. (1996) Docosahexaenoic acid and nervonic acids in term and preterm infant cerebral white matter. *Prenatal and Neonatal Medicine* **1**, 234-240.

PM No. 402/94

EXTERNAL APPEARANCE

A well nourished well cared for male infant, weighing 6126g, measuring CH 63cm, CR 42.5cm, OFC 42.5cm, foot 8.5cm, with two small superficial abrasions, 1-2mm and 3-4mm lateral to and below the outer angle of the left eye, an oblique curved elliptical pressure mark 2 x 1cm consisting of a central area of pallor and a surrounding hyperaemic zone over the upper outer aspect of the left cheek bone, greenish postmortem discolouration of the anterior abdominal wall, postmortem lividity on the back of the body and head, and no signs of non-accidental injuries.

INTERNAL EXAMINATION

There were no development anomalies.

Head:

The skull and meninges were normal. The brain was soft and showed slight generalised swelling with some flattening of the gyri. The pituitary was normal.

Neck and Thorax:

The pharynx and oesophagus were normal.

The thymus was normal. The pleural sacs were normal, apart from a few fine petechial haemorrhages in the visceral pleurae in the posterior aspect of both lungs. The pericardium was normal.

The epiglottis, larynx, trachea and main bronchi were normal. The lungs showed some lobular collapse of a minor nature. There was no apparent consolidation or evidence of gastric aspirate in the main and peripheral airways.

The heart had a probe patent foramen ovale of no clinical significance. The great vessels were normal. The ductus arteriosus was closed. The carotid bodies were not enlarged.

Abdomen:

The peritoneal sac was normal. The gastrointestinal tract showed some gaseous distension but was otherwise normal. The stomach contained a small amount of milky feed. The mesenteric lymph nodes were not unduly prominent.

The liver, biliary tract, pancreas and spleen were normal.

The adrenal glands, kidneys and urinary tract were normal.

The testes were normal and situated in the scrotum.

The hypogastric ligaments were normal.

WEIGHTS:

Brain 779g, thymus 34.4g, right lung 70.0g, left lung 47.3g, liver 261.5g, spleen 21.0g, adrenals 3.4g, right kidney 27.0g, left kidney 27.2g.

Written 24.10.94
Typed 27.10.94

FEEDING REGIME

34 How did you start with feeding when baby was born?

Breast.....1

Bottle.....2

17 ☐

35 Were you still breastfeeding when baby died? Yes/No/NA

18 ☐

36 If no, when did breast-feeding stop completely? (weeks)

19-21 ☐☐☐

37 Reason for stopping -

You had a problem	Yes/No/NA	22 ☐
The baby had a problem	Yes/No/NA	23 ☐
You went back to work	Yes/No/NA	24 ☐
Baby's age	Yes/No/NA	25 ☐
Other (specify)		26 ☐

38 If you breastfed, did you regularly complement with other milk? Yes/No/NA

39 If yes, at what age (weeks) did you start?

40 If you bottlefed, was the formula you first used one out of Group 1 or Group 2? Group 1

Group 1 - SMA Goldcap ✓
Cow and Gate Premium
Aptamil
Ostermilk

Group 2 - SMA Whitecap
Cow and Gate Plus
Milumil
Ostermilk 2

41 How many times did you change from one group to the other - Never/Once/Twice/More/NA

42 At what age was the first change (weeks)? 6

43 Reason for this change? Very hungry baby

44 Which Group did you change to? Group 2 SMA Whitecap

45 At what age was the second change (weeks)?

46 Reason for this change?

47 Which Group did you change to?

48 When did baby start on solid food? (weeks) 12

49 Was baby ever given other fluids? Yes/No

50 At what age (weeks) did you start baby on -

	Weeks
Full strength cow's milk	
Semi-skimmed cow's milk	
Skimmed cow's milk	
UHT full fat	
UHT semi-skimmed	
Progress	
Other - specify	

27 28-29 30 31 32-33 34 35 36-37 38 39 40-41 42 43-44 45-46 47-48 49-50 51-52 53-54 55-56

Table 3.14 - Infant cerebral cortex phospholipid fatty acid results

Path. No.	Age (Wk)	C16:0	C16:1	C18:0	C18:1	C18:2n-6	C20:3n-6	C20:4n-6	C22:4n-6	C22:5n-6	C22:6n-3
Breast											
382/95	1	30.31	3.01	21.28	14.52	0.11	0.45	11.27	7.11	2.30	9.64
251/95	3	30.74	2.83	21.23	14.59	0.25	0.93	12.10	6.67	1.52	10.27
2/89	5	28.99	2.91	20.88	17.59	0.38	1.13	10.27	5.87	2.77	9.18
472/88	6	27.64	2.47	21.93	16.99	0.32	1.47	10.28	6.39	2.35	10.19
249/86	7	30.53	2.44	22.50	13.41	0.41	1.11	11.10	6.89	2.58	9.03
217/86	8	31.68	2.25	23.68	14.51	0.47	0.99	10.12	5.66	2.26	8.39
581/87	8	29.40	2.01	22.37	17.53	0.33	1.24	10.18	5.48	1.52	9.96
48/95	9	27.88	1.81	23.21	15.07	0.64	1.00	12.50	6.85	1.86	9.08
225/88	11	30.57	2.13	21.65	16.69	0.28	1.17	10.83	5.65	2.49	8.54
81/94	14	29.29	1.69	23.32	16.17	0.31	1.44	11.01	5.72	1.21	9.84
457/89	16	28.33	1.75	21.82	16.93	0.49	1.41	11.45	5.65	1.68	10.45
75/93	19	26.02	1.50	21.74	15.08	0.89	2.07	13.24	7.19	2.37	9.89
19/85	38	25.90	0.75	21.57	18.22	0.85	1.60	12.48	5.98	1.47	11.12
Mix											
110/94	18	29.27	1.66	22.68	15.13	0.53	1.65	12.79	6.21	1.37	8.71
C&G											
275/94	5	31.21	2.45	22.73	14.64	0.40	1.53	10.80	6.05	1.91	8.28
180/94	13	30.06	2.00	22.92	15.31	0.46	1.17	11.61	6.02	1.72	8.72
90/93	21	26.43	0.88	21.07	16.36	0.70	2.04	12.88	6.65	3.07	9.92
164/94	33	25.68	1.66	23.91	20.16	0.50	1.55	10.96	6.25	1.85	7.49

Table 3.14 - Infant cerebral cortex phospholipid fatty acid results (continued)

Path. No.	Age (Wk)	C16:0	C16:1	C18:0	C18:1	C18:2n-6	C20:3n-6	C20:4n-6	C22:4n-6	C22:5n-6	C22:6n-3
SMA											
266/95	0.5	30.61	3.68	21.39	15.44	0.17	0.56	10.22	6.80	2.76	8.35
276/95	2	29.68	3.86	21.38	15.31	0.68	1.07	11.10	6.21	2.29	8.42
50/93	4	29.04	3.20	19.87	15.29	0.72	1.62	10.84	7.18	3.34	8.90
277/94	4	30.85	2.09	23.01	13.41	0.35	0.88	11.73	5.70	1.65	10.32
283/94	7	30.85	2.15	22.20	15.07	0.61	1.95	10.90	6.49	2.06	7.73
163/93	9	30.10	2.85	20.43	14.99	0.75	1.99	12.05	6.59	2.87	7.38
193/94	11	30.68	1.91	22.69	14.73	0.38	1.59	12.21	6.05	2.10	7.64
447/94	15	28.69	1.99	23.05	13.63	0.54	1.72	13.12	7.14	3.13	6.97
268/88	16	28.83	1.49	20.84	16.13	0.59	1.48	13.31	6.64	2.96	7.77
402/94	17	27.54	1.62	22.47	14.16	0.60	1.88	13.75	7.04	2.52	8.42
277/87	18	28.35	1.04	23.13	17.88	0.51	1.70	11.65	6.44	2.89	6.43
5/88	18	26.82	0.55	22.63	17.11	0.55	1.77	12.76	6.89	3.10	7.83
197/93	19	26.05	1.63	21.23	15.96	0.83	2.13	13.13	7.20	3.73	8.10
593/87	22	26.13	0.49	21.73	17.12	0.83	1.91	14.17	6.95	2.62	8.08
279/93	25	27.14	2.02	20.98	16.50	0.61	1.46	12.83	6.91	3.94	7.61
291/87	43	26.11	0.54	23.79	17.33	0.56	1.38	12.40	6.87	3.69	7.33

Table 3.14 - Infant cerebral cortex phospholipid fatty acid results (continued)

Path. No.	Age (Wk)	C16:0	C16:1	C18:0	C18:1	C18:2n-6	C20:3n-6	C20:4n-6	C22:4n-6	C22:5n-6	C22:6n-3
CGOST											
103/93	6.5	30.42	2.78	19.74	16.23	0.67	2.20	10.62	6.88	4.53	5.91
51/94	7	32.55	2.90	22.94	14.58	0.34	1.27	10.82	6.61	2.98	5.01
17/88	9	27.11	2.54	20.60	16.76	0.50	2.13	12.98	7.05	3.29	7.04
249/94	9	28.12	1.92	23.27	16.52	0.45	1.47	12.15	5.93	1.62	8.99
67/88	10	27.21	1.72	21.65	17.98	0.42	1.90	11.45	7.03	3.34	7.34
51/93	11	26.15	1.85	21.22	15.29	0.68	2.38	13.36	7.04	2.94	9.08
263/93	18	26.30	1.69	21.30	15.74	0.74	1.89	13.69	7.35	4.44	6.85
256/93	20	25.53	1.71	21.82	15.84	0.64	2.14	13.80	7.32	4.76	6.44
175/88	21	27.38	0.69	23.18	18.86	0.49	1.37	11.78	6.37	3.32	6.55
195/88	29	25.25	0.50	23.58	16.69	0.55	1.67	13.45	6.62	5.24	6.50
189/88	40	23.97	0.37	22.32	18.18	0.95	1.83	13.21	7.67	5.88	5.65
Mix Prem											
72/95	7	29.56	2.10	21.57	14.46	0.53	1.63	12.03	7.02	2.19	8.91
SMA Prem											
114/95	7	28.87	1.96	21.52	17.42	0.35	1.12	10.81	5.98	1.52	10.45
657/87	10	29.81	3.45	19.96	18.57	0.96	2.09	12.59	5.70	2.61	4.27
419/95	12	29.19	2.52	22.17	14.31	0.57	1.78	13.42	7.19	2.84	6.00
429/95	23	27.19	1.21	23.18	16.41	0.45	1.61	13.62	7.57	4.03	4.73
288/88	25	24.91	0.52	22.16	17.34	0.88	1.41	13.98	7.71	4.45	6.60
CGOST Prem											
333/94	19	28.88	2.03	23.97	14.15	0.38	1.30	13.79	6.74	4.52	4.23

Table 3.18 - Infant cerebral cortex phosphatidylcholine fatty acid results

Path No.	Weeks	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2n-6	C20:3n-6	C20:4n-6
	Breast								
472/88	6.0	2.24	51.53	6.13	10.16	25.53	1.12	0.76	2.51
581/87	8.0	1.89	56.48	5.12	7.97	24.93	0.53	0.69	2.38
225/88	11.0	2.18	53.71	2.84	9.56	26.63	0.67	0.82	3.46
457/89	16.0	1.80	54.01	4.15	8.86	25.46	0.73	0.99	3.96
19/85	38.0	1.28	53.16	3.30	10.55	26.45	0.67	1.08	3.44
	SMA								
133/88	6.0	2.80	57.42	5.12	8.77	23.17	0.69	0.90	1.57
268/88	16.0	1.97	54.65	4.05	9.89	23.21	0.91	1.01	4.29
277/87	17.5	1.80	52.59	4.63	8.76	24.75	0.98	1.41	5.07
5/88	18.5	1.81	53.76	4.26	8.95	24.95	1.00	1.16	4.08
593/87	22.0	1.49	55.07	4.22	10.16	24.19	0.85	0.91	3.12
291/87	43.0	2.25	52.93	3.95	11.92	26.73	0.71	0.77	1.72
	CGOST								
17/88	9.0	2.70	55.64	6.31	7.45	21.70	0.77	1.15	4.24
67/88	10.0	2.04	50.77	5.22	10.19	26.70	0.84	1.11	3.17
175/88	21.0	1.02	56.83	3.12	9.99	23.81	0.85	0.91	3.47
195/88	29.0	1.98	50.67	3.89	11.85	25.11	1.23	1.20	4.06
189/88	40.0	1.36	52.75	3.57	9.92	24.57	1.10	1.21	4.61
	Prem								
657/87	10.0	3.58	56.47	9.11	6.08	23.58	1.23	0.78	2.19

Table 3.19 - Infant cerebral cortex phosphatidylethanolamine fatty acid results

Path. No.	Weeks	C16:0	C18:0	C18:1	C20:3n-6	C20:4n-6	C22:4n-6	C22:5n-6	C22:6n-3	Total n-6	Total n-6+n-3
	Breast										
472/88	6.0	8.57	33.10	9.15	1.36	16.43	11.75	3.07	16.59	32.61	49.20
581/87	8.0	10.43	30.48	8.42	1.35	17.29	11.13	2.68	18.22	32.45	50.67
225/88	11.0	9.83	30.59	6.44	1.46	18.17	13.22	4.19	16.13	37.04	53.17
457/89	16.0	8.21	26.85	10.53	1.47	18.19	12.18	3.31	19.24	35.15	54.39
19/85	38.0	4.95	33.10	9.41	1.72	17.86	11.59	2.84	18.51	34.01	52.52
	SMA										
133/88	6.0	6.97	34.39	9.39	2.80	18.69	10.58	3.62	13.57	35.69	49.26
268/88	16.0	6.05	31.38	10.68	1.86	19.89	11.93	5.42	12.63	39.10	51.73
277/87	17.5	7.79	29.80	8.91	1.95	20.27	13.79	4.81	13.07	40.82	53.89
5/88	18.5	6.29	30.55	8.74	2.19	21.05	14.69	4.85	11.85	42.77	54.62
593/87	22.0	5.26	31.85	9.18	1.67	20.68	12.79	4.73	13.98	39.87	53.85
291/87	43.0	5.83	30.57	8.57	1.80	19.88	12.03	5.59	15.34	39.30	54.64
	CGOST										
17/88	9.0	8.53	27.33	8.14	2.52	20.58	14.47	5.36	13.12	42.93	56.05
67/88	10.0	8.84	27.87	10.14	2.17	17.95	14.91	5.46	12.61	40.49	53.10
175/88	21.0	6.94	28.31	10.16	1.51	19.56	14.79	6.33	12.44	42.19	54.53
195/88	29.0	6.82	31.65	9.44	1.93	18.68	12.58	8.41	10.50	41.60	52.10
189/88	40.0	6.70	28.74	8.12	1.61	21.12	14.84	9.66	9.23	47.23	56.46
	Prem										
657/87	10.0	8.59	30.85	11.58	3.28	19.19	11.65	4.46	7.61	38.58	53.80

Table 3.20 - Infant cerebral cortex phosphatidylserine fatty acid results

Path. No.	Weeks	C16:0	C18:0	C18:1	C20:3n-6	C20:4n-6	C22:4n-6	C22:5n-6	C22:6n-3	Total n-6	Total n-6+n-3
	Breast										
472/88	6.0	1.86	44.49	5.44	1.84	7.78	9.65	6.29	22.69	25.56	48.25
581/87	8.0	2.10	46.81	4.05	1.77	9.71	7.80	4.26	23.52	23.54	47.06
225/88	11.0	1.94	45.82	5.32	1.38	7.82	8.05	7.31	22.35	24.56	46.91
457/89	16.0	3.11	42.55	6.06	1.40	8.84	7.22	5.04	25.79	22.50	47.99
19/85	38.0	4.35	43.53	10.70	2.02	5.34	6.95	3.75	23.39	18.06	41.45
	SMA										
133/88	6.0	2.15	45.69	6.51	2.43	8.96	8.19	6.06	20.05	25.64	45.69
268/88	16.0	1.24	43.75	7.29	1.87	7.44	9.38	8.41	20.66	27.10	47.76
277/87	17.5	3.65	42.22	6.89	2.09	10.98	8.33	7.51	18.39	28.91	47.30
5/88	18.5	2.36	44.11	7.97	2.63	9.53	9.15	8.03	16.26	29.34	45.60
593/87	22.0	1.34	46.28	5.64	1.57	9.72	8.87	7.26	19.31	27.42	46.73
291/87	43.0	2.24	43.54	6.49	1.53	7.44	6.84	8.67	21.25	24.48	45.73
	CGOST										
17/88	9.0	1.64	44.48	5.16	3.18	10.06	11.23	8.39	15.88	32.86	48.74
67/88	10.0	4.63	43.15	11.39	2.33	8.07	8.91	7.15	14.35	26.46	40.81
175/88	21.0	1.67	44.73	8.61	2.06	8.79	9.43	9.31	15.41	29.59	45.00
195/88	29.0	3.37	42.70	9.20	1.87	7.81	8.03	12.66	14.34	30.37	44.71
189/88	40.0	2.48	43.31	6.47	2.04	10.13	8.91	14.58	12.06	35.66	47.72
	Prem										
657/87	10.0	2.32	43.94	5.49	3.28	12.05	9.95	7.35	12.39	32.63	45.02

Table 3.25 - Infant cerebellar cortex phospholipid fatty acid results

Path No.	Age (Wk)	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C20:3	C20:4	C22:4	C22:5	C22:6
Breast												
382/95	1	1.62	28.29	4.44	18.14	18.84	0.37	1.06	11.21	5.71	1.16	9.51
251/95	3	2.12	29.79	3.35	19.01	20.20	0.25	1.00	11.29	4.81	0.26	7.91
2/89	5	2.29	29.64	4.13	18.11	19.87	0.79	1.91	10.40	4.25	0.80	7.81
249/86	7	1.90	27.74	3.12	17.45	20.61	1.05	2.05	11.21	4.96	0.68	9.22
217/86	8	2.08	27.89	3.45	16.40	21.50	0.95	2.11	10.41	4.91	0.75	8.35
48/95	9	1.84	29.35	3.18	17.88	19.31	1.40	2.50	11.53	4.29	0.93	7.79
181/86	10	1.93	27.49	3.39	16.84	20.02	0.96	3.05	10.45	4.25	0.65	9.02
81/94	14	1.52	26.73	2.11	20.29	21.80	0.37	1.57	11.09	5.16	0.40	8.79
457/89	16	1.75	29.60	2.80	18.34	18.82	0.91	2.52	11.88	4.00	0.44	8.95
75/93	19	1.42	28.52	2.17	19.42	19.38	1.67	2.39	12.07	5.10	0.83	7.04
C&G												
275/94	5	2.00	29.72	4.26	17.22	19.50	0.88	2.68	10.50	4.67	1.04	7.52
180/94	13	1.03	29.23	2.99	17.67	20.91	1.05	2.67	11.32	4.34	0.79	7.98
90/93	21	2.03	29.74	2.33	17.79	19.39	1.06	2.63	12.15	4.73	1.41	6.74
164/94	33	0.93	26.27	2.07	20.31	24.41	1.16	2.12	10.47	4.94	0.95	6.36
Mix												
110/94	18	1.78	29.55	2.93	18.39	19.90	1.35	2.61	11.36	4.80	0.70	7.33

Table 3.25 - Infant cerebellar cortex phospholipid fatty acid results (continued)

Path No.	Age (Wk)	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C20:3	C20:4	C22:4	C22:5	C22:6
SMA												
266/95	0.5	1.99	30.68	4.11	17.75	17.65	0.27	1.03	10.45	6.31	0.86	8.87
276/95	2	2.36	30.36	4.12	17.14	18.48	1.09	1.66	10.49	5.47	0.83	8.01
50/93	4	2.27	31.20	3.68	17.76	20.19	1.11	2.35	10.83	4.13	0.75	6.48
283/94	7	2.11	30.20	3.51	17.32	19.17	1.40	2.94	10.66	5.19	1.21	6.28
163/93	9	1.74	28.56	3.13	17.40	21.39	1.20	3.21	10.73	4.84	0.98	6.77
193/94	11	1.98	29.64	3.05	18.00	19.51	0.94	3.09	11.88	4.75	1.14	6.15
447/94	15	1.64	29.72	2.57	18.05	19.25	1.17	2.54	12.45	5.14	1.58	5.89
268/88	16	1.73	30.56	2.77	18.75	18.73	1.31	2.32	11.47	4.80	1.22	6.34
402/94	17	1.73	30.20	2.20	19.46	19.52	1.16	2.73	11.58	4.98	0.90	5.55
197/93	19	1.44	26.58	2.14	20.03	21.33	1.01	2.46	10.50	5.62	1.32	5.06
CGOST												
103/93	6.5	2.35	30.66	4.04	17.70	19.56	0.85	3.06	10.15	4.95	1.79	4.88
51/94	7	2.14	29.85	3.64	17.77	21.40	0.83	2.71	10.87	4.61	1.46	4.73
249/94	9	1.52	29.33	3.94	17.52	20.76	1.03	2.69	10.96	4.07	1.34	6.84
51/93	11	1.72	28.56	2.60	18.34	20.66	0.74	2.83	11.64	5.52	1.63	5.74
263/93	18	1.58	28.10	2.35	20.14	21.38	1.01	2.31	12.16	6.29	2.02	4.15
256/93	20	1.39	28.06	2.63	17.96	20.83	0.89	2.92	12.49	6.12	2.43	4.29
175/88	21	1.62	27.98	2.36	22.58	20.83	0.64	1.83	10.37	5.92	1.68	4.25
195/88	29	1.69	28.65	1.93	19.62	19.78	1.09	2.36	12.17	6.08	2.61	4.02
189/88	40	1.49	29.23	1.50	20.24	18.89	1.21	1.53	13.82	6.72	2.63	3.18

Table 3.25 - Infant cerebellar cortex phospholipid fatty acid results (continued)

Path No.	Age (Wk)	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C20:3	C20:4	C22:4	C22:5	C22:6
Mix Prem												
72/95	7	2.21	28.68	3.03	18.04	22.45	1.29	2.69	10.22	5.07	0.42	6.30
SMA Prem												
114/95	7	2.23	29.58	3.81	17.26	19.59	1.13	2.57	10.68	4.58	0.93	7.71
657/87	10	2.34	28.91	3.89	17.16	20.54	1.33	2.47	10.51	5.21	1.35	6.28
419/95	12	1.76	29.09	3.20	18.58	22.34	1.76	3.57	11.73	5.51	1.33	6.31
429/95	23	1.18	29.05	2.92	18.51	20.83	1.21	2.59	12.38	5.10	2.07	4.15
288/88	25	1.73	30.56	2.77	18.75	18.73	1.31	2.32	11.47	4.80	1.22	6.34
CGOST Prem												
333/94	19	1.57	28.99	2.96	17.55	20.19	0.74	2.09	12.11	5.69	3.06	3.49

Table 3.29 - Infant cerebral parietal white matter total lipid fatty acid results

Path No.	Age (Wk)	C14:0	C16:0	C16:1	C18:0	C18:1	C20:3	C20:4	C22:4	C22:5	C22:6	C24:0	C24:1
Breast													
251/95	3	1.41	29.43	3.22	21.82	14.12	0.95	10.84	6.78	2.49	9.11	<0.10	<0.10
249/86	7	1.13	26.14	2.60	22.98	15.84	1.55	11.90	6.96	2.09	8.81	<0.10	<0.10
217/86	8	1.40	27.83	2.76	23.09	15.36	1.36	10.52	6.11	2.37	9.24	<0.10	<0.10
48/95	9	0.92	26.83	2.35	23.69	16.29	1.43	10.59	6.29	1.98	8.67	0.40	0.54
181/86	10	1.44	24.50	2.27	23.13	19.43	1.66	9.53	6.70	1.62	7.23	1.46	1.01
81/94	14	1.23	21.87	2.19	22.50	22.71	1.40	8.63	7.26	1.10	6.33	1.91	2.92
457/89	16	1.72	21.24	2.38	22.12	23.75	1.27	7.52	7.24	0.71	6.40	2.44	3.26
75/93	19	1.26	20.44	1.96	22.37	23.83	1.54	8.58	8.49	1.24	4.82	2.54	3.01
1983	38	2.48	18.43	2.51	21.15	26.49	1.47	7.02	9.34	0.48	3.67	3.14	3.93
SMA													
266/95	1	1.32	29.66	2.87	21.49	14.86	0.73	10.75	6.78	2.17	8.57	0.41	0.39
39/87	4	1.51	29.30	3.11	22.00	14.79	1.52	10.26	6.20	2.55	8.76	<0.10	<0.10
219/86	7	1.20	28.68	2.57	23.23	15.05	1.60	10.91	6.26	2.95	8.00	<0.10	<0.10
38/87	13	1.13	25.56	2.47	23.84	16.04	1.64	11.37	7.02	3.06	7.05	0.45	0.38
630/86	16	1.12	24.18	2.12	23.48	18.95	1.49	10.87	7.19	2.09	6.08	1.20	1.27
617/85	17	1.87	25.12	2.02	24.29	17.74	1.95	10.30	7.77	2.40	5.30	1.42	1.24
248/86	18	1.97	23.46	2.40	22.97	18.29	1.54	10.71	7.27	2.22	6.39	1.34	1.44
277/87	18	1.41	23.03	2.34	24.71	20.33	1.55	10.21	7.50	1.72	4.04	1.57	1.59
85/86	24	1.90	19.66	2.10	22.79	24.84	1.30	8.66	8.45	1.30	3.70	2.68	2.62
291/87	43	1.15	20.29	1.52	20.91	28.03	1.53	6.95	8.34	1.24	3.24	2.56	4.22
Mix													
110/94	18	1.50	22.02	2.43	23.01	22.04	1.42	9.68	7.08	1.16	5.27	2.40	2.05

Table 3.29 - Infant cerebral parietal white matter total lipid fatty acid results (continued)

Path No.	Age (Wk)	C14:0	C16:0	C16:1	C18:0	C18:1	C20:3	C20:4	C22:4	C22:5	C22:6	C24:0	C24:1
CGOST													
51/94	7	1.72	27.97	2.97	22.12	14.53	1.75	11.42	7.12	3.57	6.81	<0.10	<0.10
642/85	11	1.20	26.50	2.32	24.15	14.07	1.56	11.81	7.41	4.03	6.93	<0.10	<0.10
510/85	12	1.23	24.40	2.15	23.57	17.01	1.69	10.78	7.33	3.64	6.03	1.09	1.10
547/85	14	1.76	24.47	2.37	22.56	17.90	1.60	10.82	8.24	3.06	3.85	1.35	1.53
263/93	18	1.02	21.63	2.19	23.67	20.66	1.43	10.09	8.32	2.93	3.81	2.10	2.06
256/93	20	1.33	21.34	1.88	23.91	22.07	1.46	9.50	8.51	2.48	3.23	2.19	2.17
175/88	21	1.02	21.27	2.18	22.53	25.43	1.11	7.07	8.06	1.84	2.94	2.09	2.79
195/88	29	0.92	19.94	2.44	21.10	25.49	1.60	8.09	9.26	2.54	3.18	2.60	2.83
189/88	40	1.03	20.05	1.26	20.67	28.50	1.47	6.15	8.80	2.21	1.78	2.72	4.30
? Locus													
277/94	4	1.33	22.76	2.45	22.79	21.60	0.92	8.59	7.66	1.38	5.96	1.73	2.83
275/94	5	1.90	23.12	2.55	22.15	21.45	1.56	8.65	7.79	1.32	4.95	1.94	2.60
283/94	7	1.50	19.72	2.41	23.25	27.63	1.89	7.55	6.21	0.50	5.02	2.74	2.92
249/94	9	1.75	22.74	2.34	19.67	26.48	1.35	7.72	7.11	1.45	5.00	1.60	2.78
193/94	11	1.14	21.84	2.58	20.57	23.56	1.97	8.11	8.49	1.85	3.82	1.88	4.19
SMA Prem													
114/95	7	1.48	29.60	2.94	22.41	14.46	1.75	10.55	6.14	3.42	7.25	<0.10	<0.10
657/89	10	1.48	29.33	3.03	21.74	16.18	1.99	10.69	6.80	2.69	5.01	0.36	0.69
7/87	17	1.31	27.95	2.54	22.57	16.33	2.62	12.19	6.75	3.31	4.42	<0.10	<0.10
288/88	25	1.22	22.65	1.73	23.14	21.48	1.28	9.80	9.10	2.21	3.24	1.63	2.47
602/86	34	1.77	21.99	1.96	23.72	20.76	1.54	9.76	8.14	2.18	4.19	2.19	1.92
Mix Prem													
72/95	7	1.44	30.87	2.86	21.31	13.91	2.00	9.87	5.79	3.42	8.53	<0.10	<0.10

Table 3.36 Infant cerebellar white matter total lipid fatty acid results

Path No.	Age (Wk)	C14:0	C16:0	C16:1	C18:0	C18:1	C20:1	C20:2	C20:3	C20:4	C22:4	C22:6	C24:0	C24:1
Breast														
382/95	1	1.19	22.93	3.69	19.38	22.74	0.99	0.49	0.98	9.55	5.93	9.26	1.64	1.24
251/95	3	1.13	21.97	3.49	20.15	24.30	1.09	0.55	1.37	9.49	5.00	10.24	1.29	1.12
2/89	5	2.22	20.72	3.15	21.35	28.20	1.44	0.64	1.12	6.99	5.36	5.05	1.91	1.84
217/86	8	1.45	20.32	2.87	21.42	26.93	1.56	0.70	1.52	7.32	5.45	6.42	1.79	2.03
48/95	9	1.05	20.03	2.41	22.84	27.48	1.84	0.78	1.70	7.57	5.86	5.71	1.98	2.87
181/86	10	1.25	19.05	2.20	22.15	27.03	1.60	0.82	1.80	7.42	5.87	6.09	2.02	2.69
81/94	14	1.14	19.68	2.19	21.97	25.95	1.59	0.74	1.96	7.76	5.58	7.04	1.82	2.57
457/89	16	1.34	16.98	2.29	21.96	29.86	2.08	0.97	1.38	6.46	6.58	4.85	2.48	2.75
75/93	19	1.61	16.74	2.03	22.24	31.41	2.55	0.78	1.63	5.81	5.82	4.32	1.95	3.12
SMA														
266/95	0.5	1.16	22.86	3.36	21.78	22.72	0.85	0.42	1.03	9.04	7.20	6.11	1.92	1.57
276/95	2	1.36	24.12	3.43	22.72	21.36	0.60	0.49	1.22	9.89	5.23	8.16	1.60	0.91
50/93	4	1.14	22.21	2.87	22.37	23.18	0.90	0.59	1.80	8.53	6.53	5.76	2.39	1.75
283/94	7	1.44	21.25	2.95	21.13	27.34	1.42	0.78	0.97	6.99	5.95	5.99	1.76	2.00
193/94	11	1.42	20.29	2.48	22.36	26.97	1.44	0.83	1.64	7.27	6.61	4.00	2.12	2.57
447/94	15	1.24	18.13	2.36	21.65	29.29	2.17	0.95	1.51	6.93	5.82	4.84	2.25	2.86
630/86	16	1.26	17.52	1.78	23.26	28.66	1.73	0.69	1.28	6.77	6.31	3.95	2.43	3.10
617/85	17	1.58	17.87	1.90	23.64	28.52	1.96	1.07	1.62	6.03	7.03	3.00	2.47	3.30
402/94	17	1.41	18.35	2.12	21.33	29.58	2.23	0.96	1.54	5.77	6.97	2.64	2.96	4.14
197/93	19	0.72	16.68	1.94	22.99	30.69	2.31	0.87	1.31	5.94	7.03	3.10	2.42	4.09
C&G														
275/94	5	1.53	22.86	2.96	20.53	25.35	1.17	0.85	1.92	7.17	6.66	4.25	2.47	2.29
180/94	16	1.56	20.30	2.49	21.45	28.48	1.56	0.76	1.23	6.37	6.48	4.11	2.21	3.00
90/93	21	0.83	16.44	2.00	23.61	28.31	3.77	1.59	1.87	6.59	5.77	1.99	3.08	4.63
164/94	33	1.14	20.16	2.12	25.09	31.17	2.72	0.90	1.50	4.68	4.73	2.58	2.61	5.66

Table 3.36 - Infant cerebellar white matter total lipid fatty acid results (continued)

Path No.	Age (Wk)	C14:0	C16:0	C16:1	C18:0	C18:1	C20:1	C20:2	C20:3	C20:4	C22:4	C22:6	C24:0	C24:1
CGOST														
103/93	6	1.28	19.72	2.74	21.47	26.76	1.78	1.09	1.98	7.98	6.55	4.86	1.56	1.83
51/94	7	1.60	23.80	3.36	19.36	24.30	1.11	0.67	2.05	9.86	5.78	5.56	1.18	0.70
642/85	11	0.74	18.75	2.07	24.75	28.86	1.38	0.53	1.23	6.16	5.18	3.66	2.83	2.95
510/85	12	0.66	16.48	1.20	24.24	30.28	2.22	1.03	1.15	5.34	5.77	3.12	3.27	4.65
547/85	14	1.10	21.40	2.28	22.73	31.49	1.25	0.49	1.25	6.39	4.77	3.02	2.05	2.45
263/93	18	0.86	17.40	1.89	25.55	29.93	2.10	0.69	1.11	6.08	4.56	3.37	2.38	4.08
256/93	20	0.93	22.65	1.96	23.11	26.35	1.51	0.66	1.78	7.41	5.36	3.01	1.67	2.27
175/88	21	0.81	17.42	1.94	23.58	30.56	2.13	0.96	1.12	6.40	5.74	2.32	2.94	3.16
195/88	29	0.79	18.07	1.64	23.40	30.66	1.90	0.87	1.43	6.40	6.71	2.06	2.57	3.55
189/88	40	1.06	15.62	1.59	21.45	30.47	2.54	0.92	1.60	7.06	7.54	3.31	1.76	3.43
SMA Prem														
114/95	7	2.51	26.10	3.18	21.12	25.34	0.65	0.51	1.67	8.93	4.88	6.28	0.75	0.70
419/95	12	1.29	18.83	2.61	20.49	26.88	1.99	1.00	2.23	7.33	6.49	4.72	2.60	3.54
429/95	23	0.92	17.26	3.43	20.43	25.50	2.12	1.03	1.98	7.94	7.76	4.13	3.04	4.10
288/88	25	1.82	18.48	2.25	20.94	30.43	2.89	1.11	1.38	5.47	7.23	2.32	1.97	3.69
602/86	34	1.11	15.67	1.77	22.55	31.50	2.37	1.09	1.49	5.86	6.99	2.68	2.77	4.15
Mix Prem														
72/95	7	2.36	28.37	3.12	19.08	21.45	0.55	0.38	2.66	9.71	5.49	5.22	0.67	0.65
Mix														
110/94	18	1.20	16.22	1.86	22.42	29.64	2.30	0.86	1.61	6.70	6.02	6.02	2.26	2.88

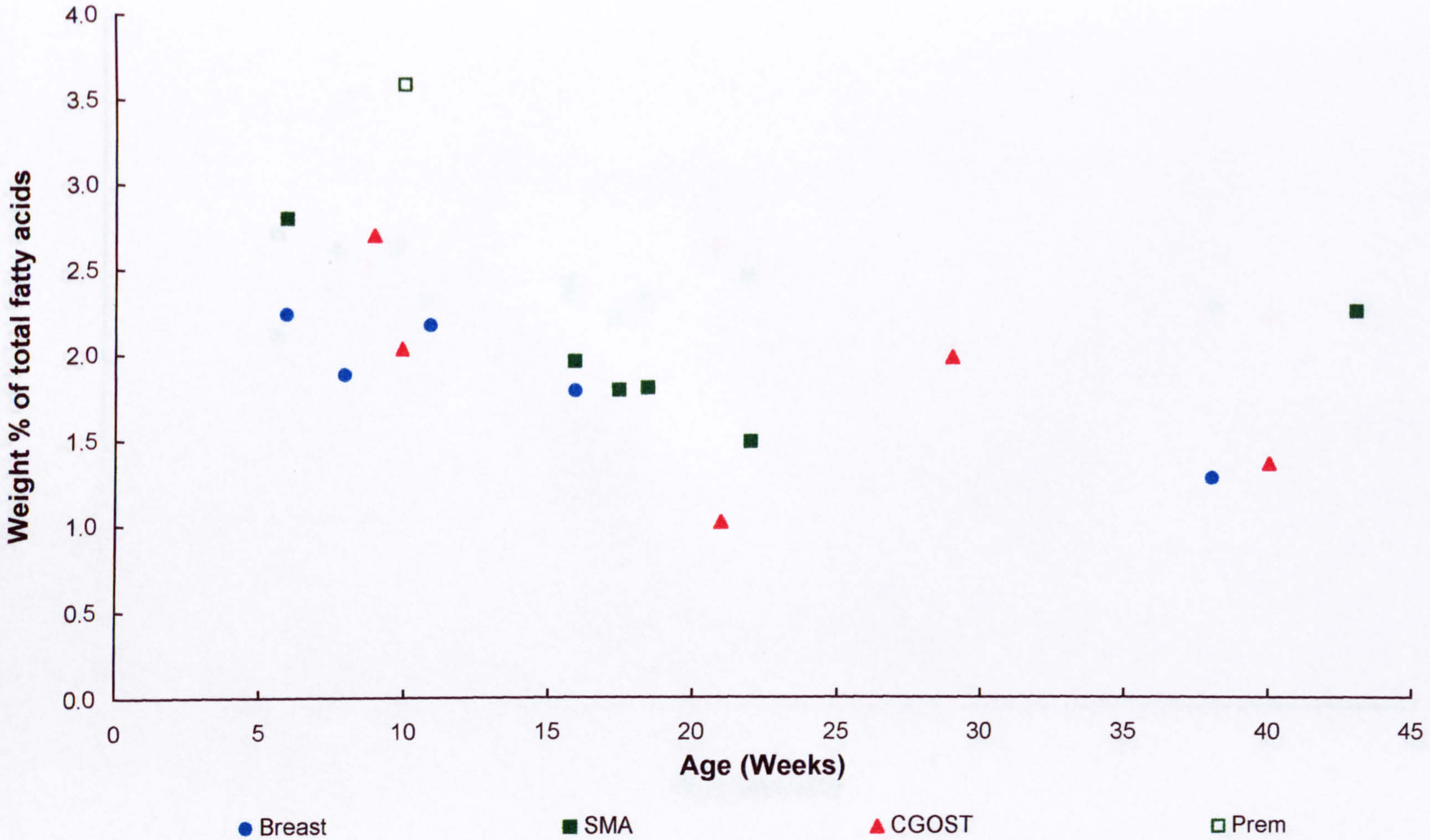

Figure 3.12 - Cerebral cortex phosphatidylcholine myristic acid (C14:0) in relation to infants' diet and age.

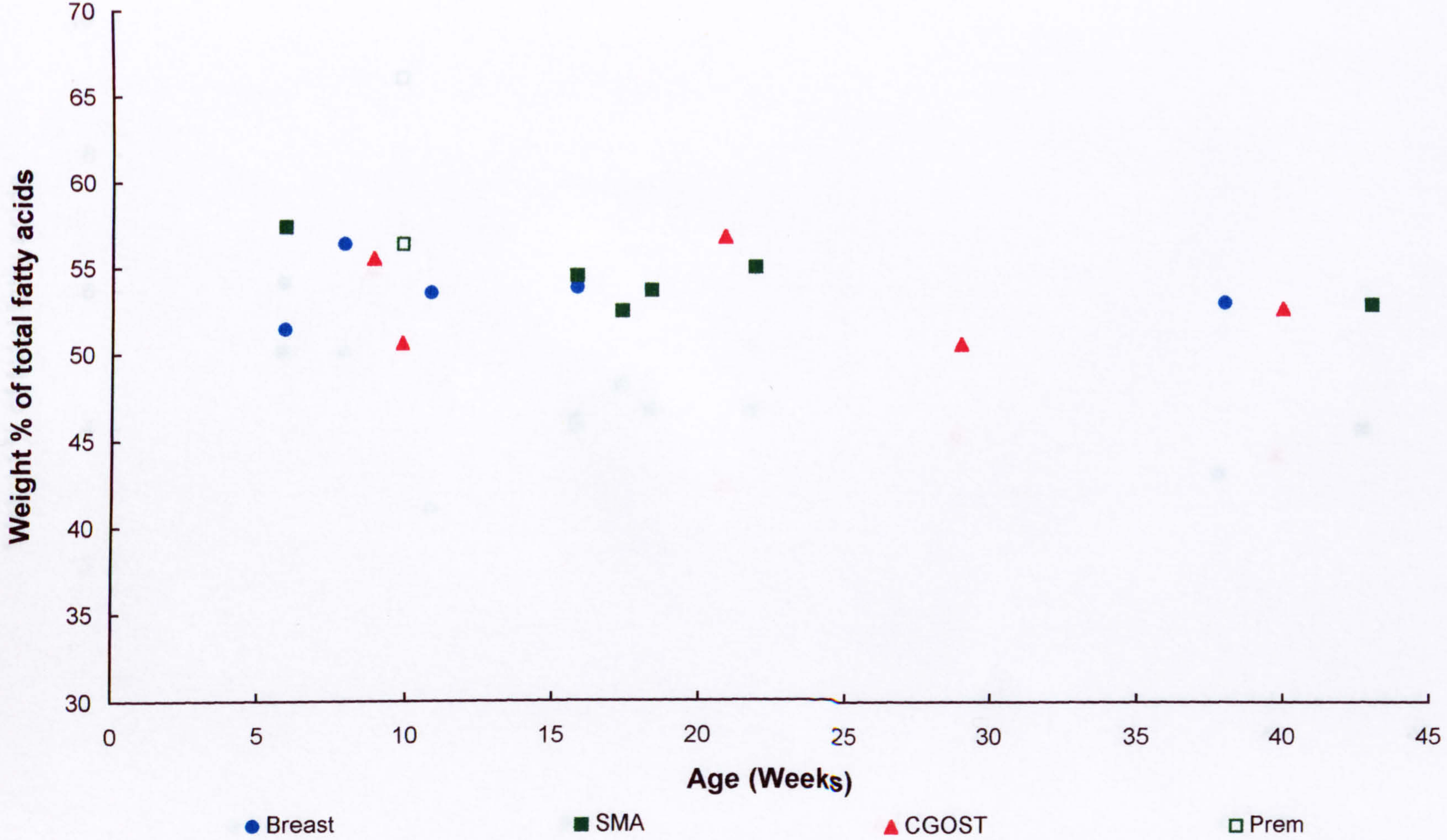

Figure 3.13 - Cerebral cortex phosphatidylcholine palmitic acid (C16:0) in relation to infants' diet and age.

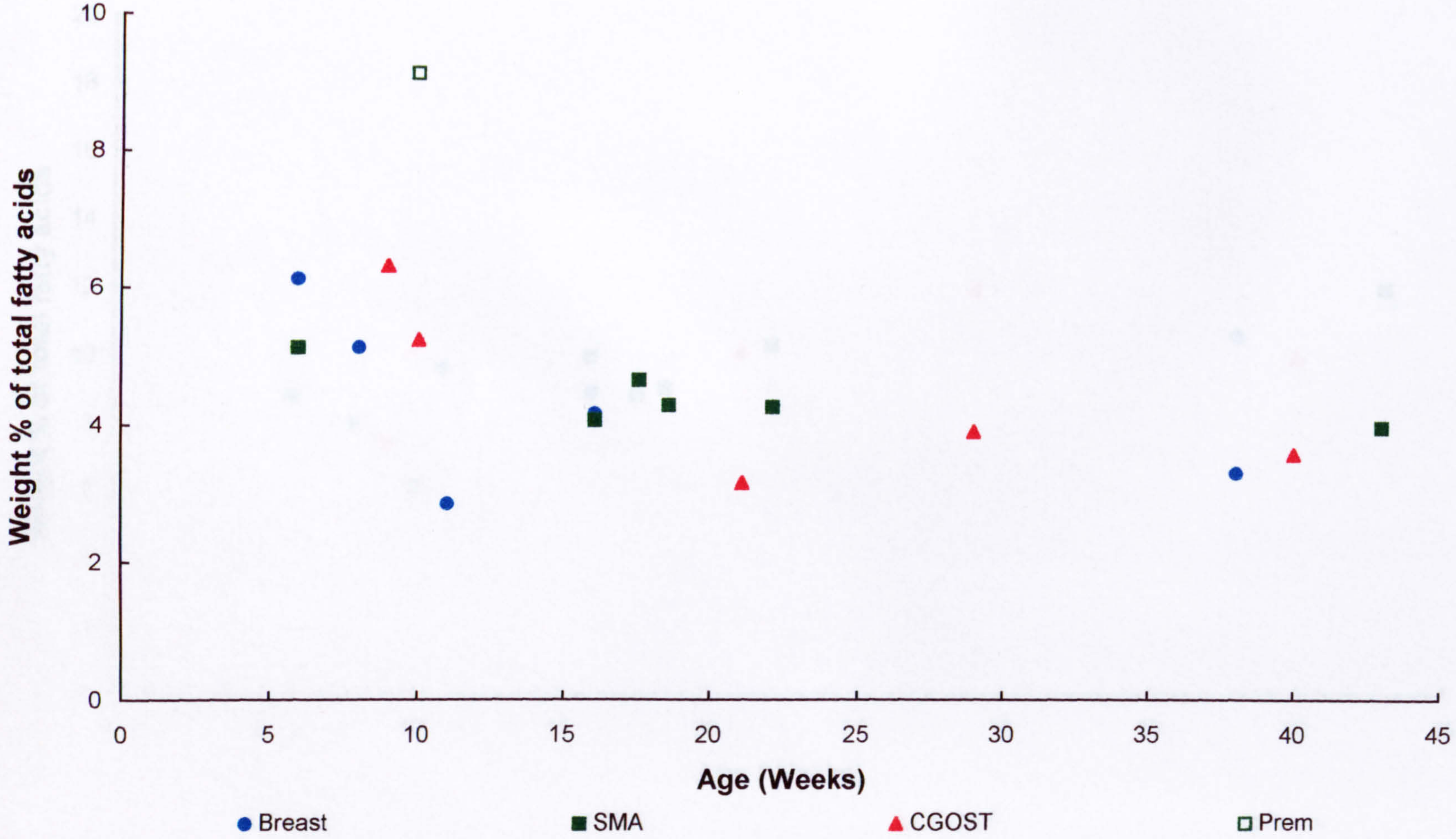

Figure 3.14 - Cerebral cortex phosphatidylcholine palmitoleic acid (C16:1n-7) in relation to infants' diet and age.

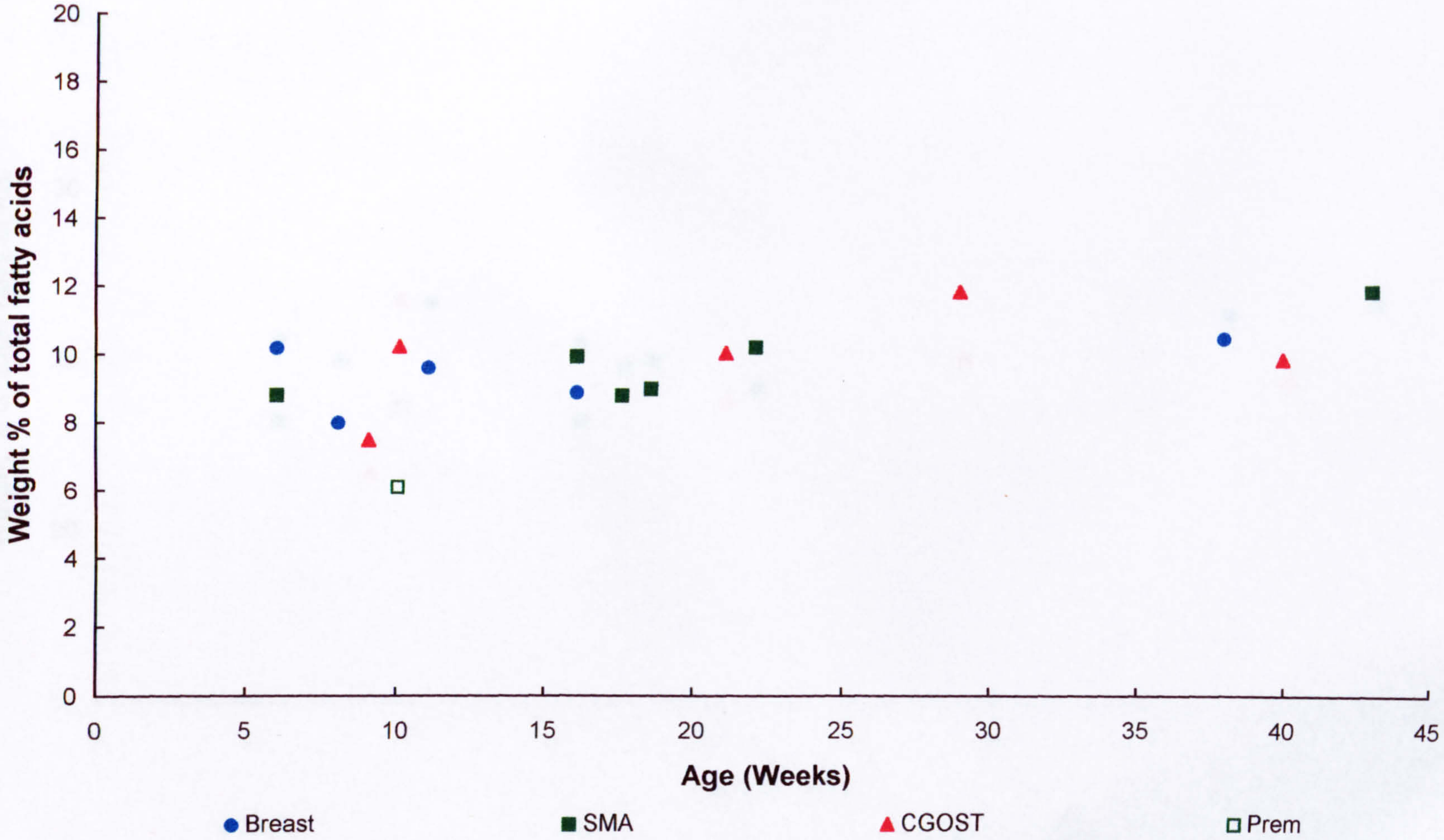

Figure 3.15 - Cerebral cortex phosphatidylcholine stearic acid (C18:0) in relation to infants' diet and age.

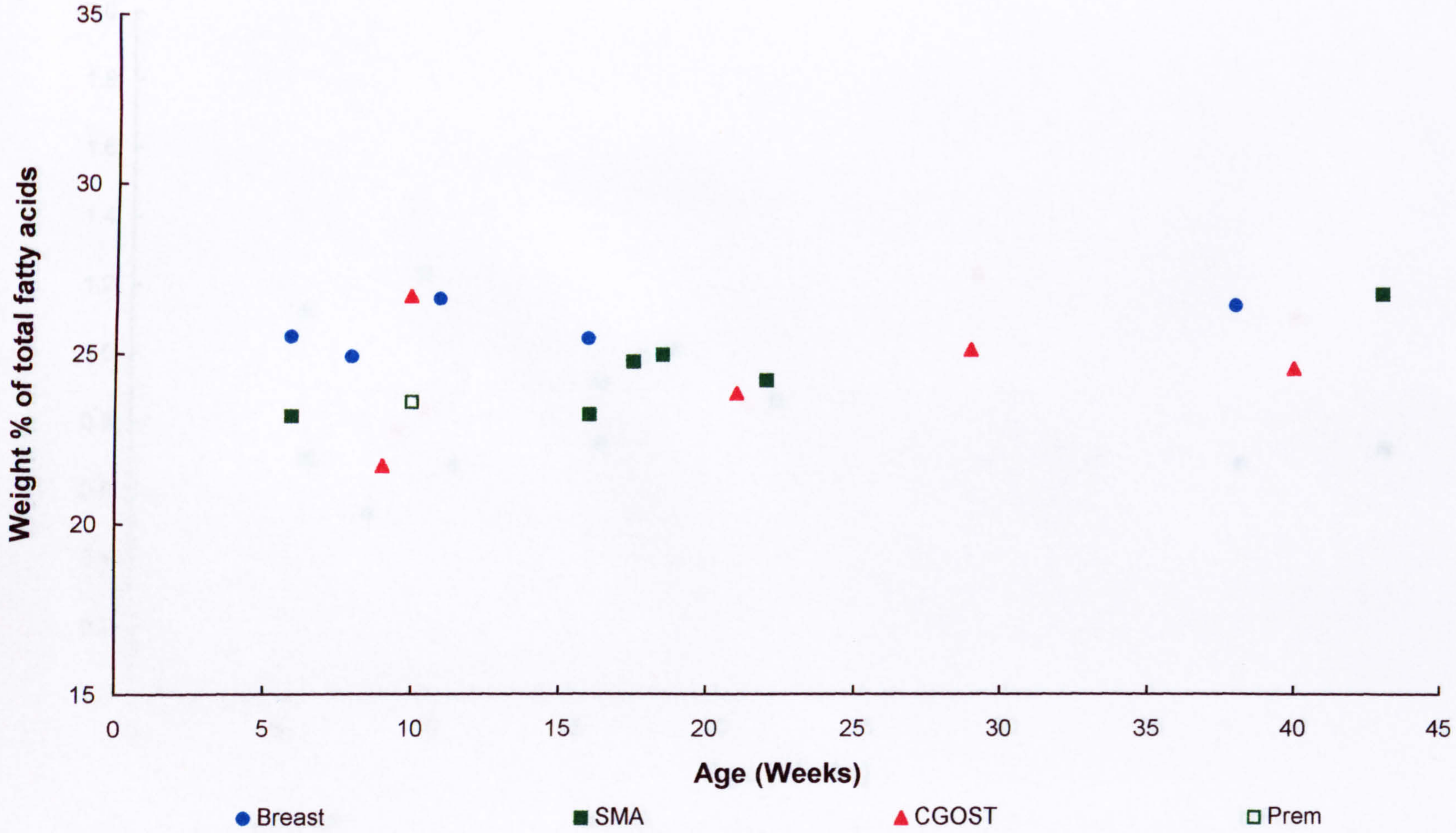

Figure 3.16 - Cerebral cortex phosphatidylcholine oleic acid (C18:1n-7+n-9) in relation to infants' diet and age.

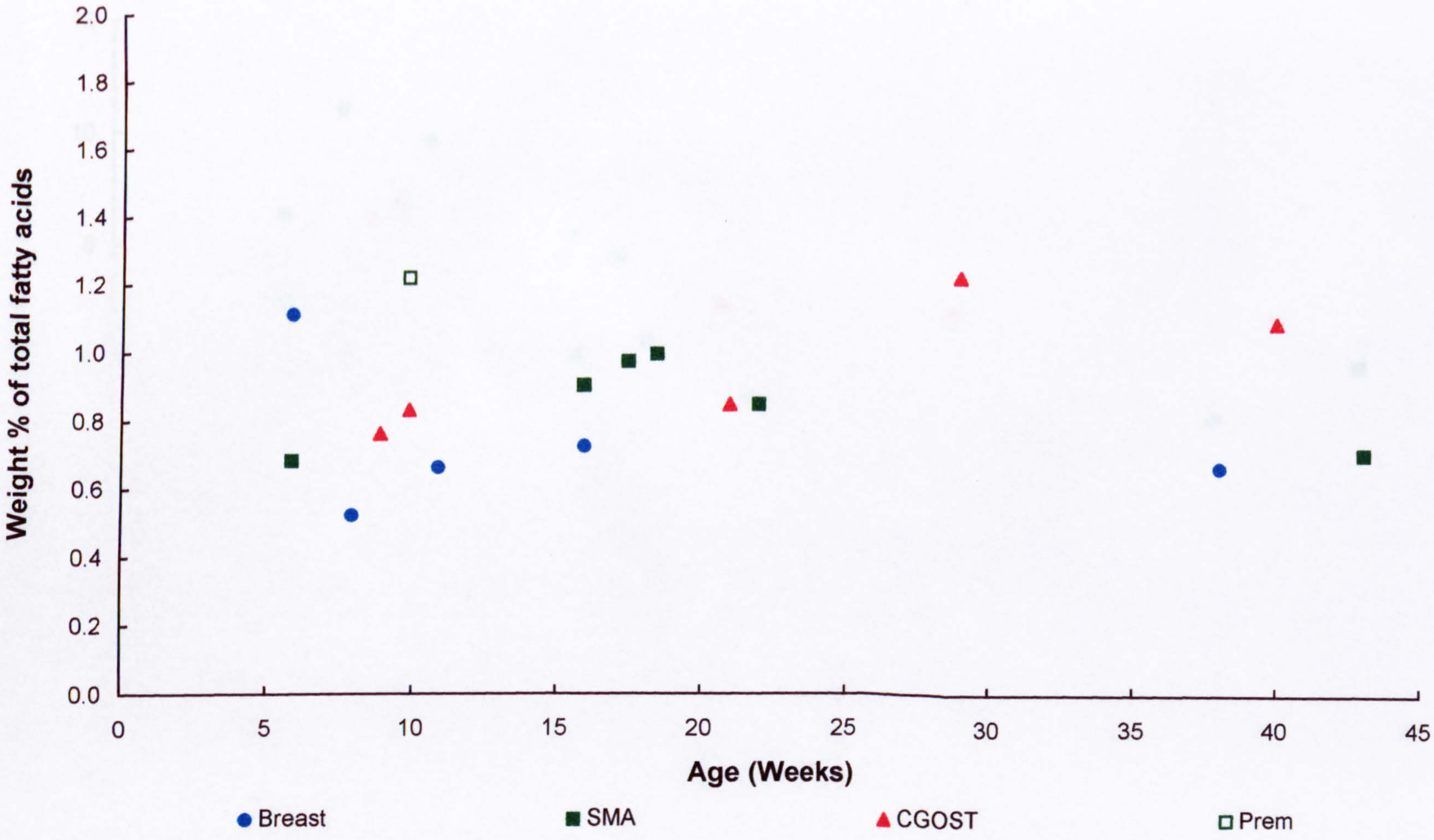

Figure 3.17 - Cerebral cortex phosphatidylcholine linoleic acid (C18:2n-6) in relation to infants' diet and age.

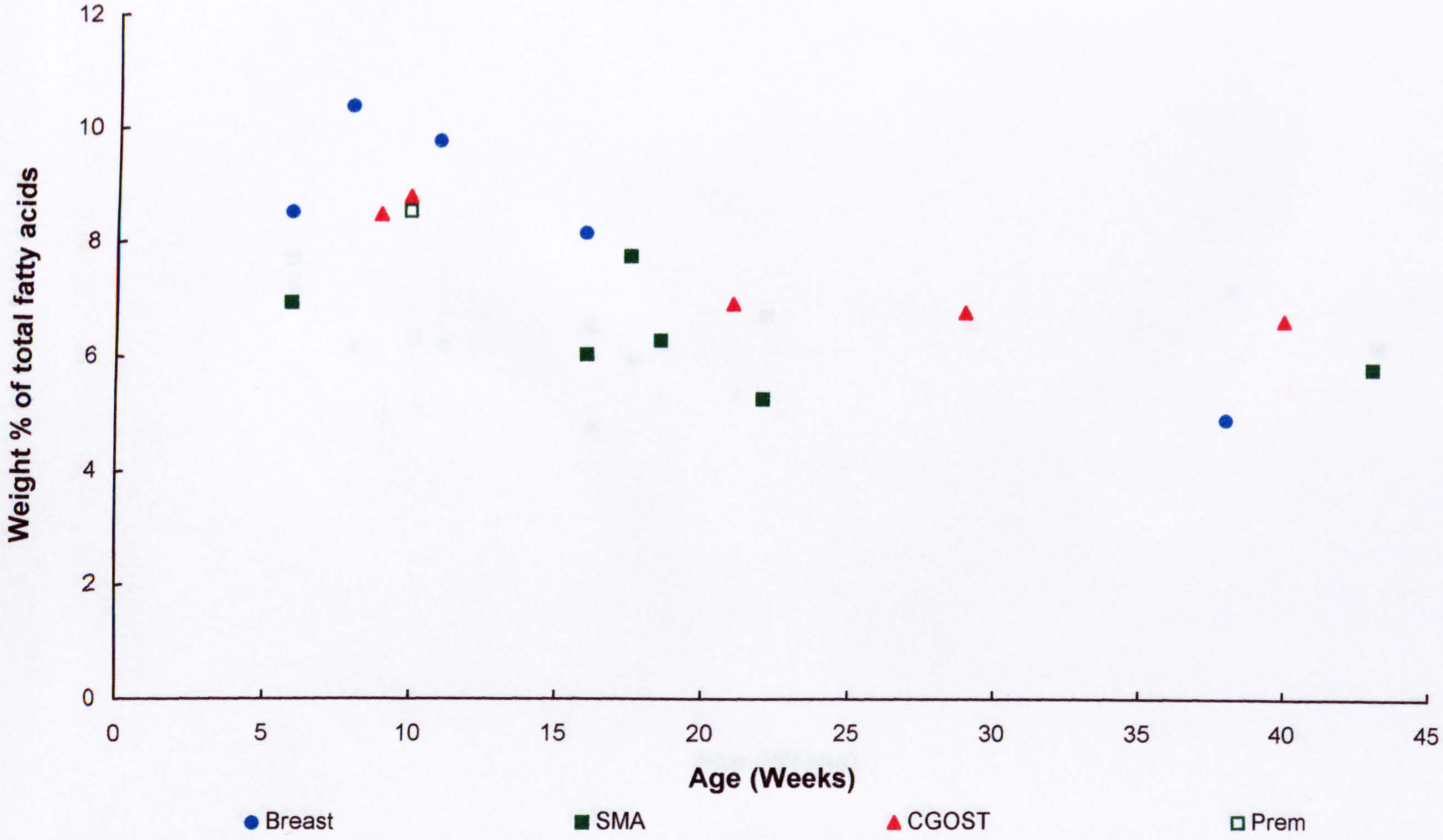

Figure 3.20 - Cerebral cortex phosphatidylethanolamine palmitic acid (C16:0) in relation to infants' diet and age.

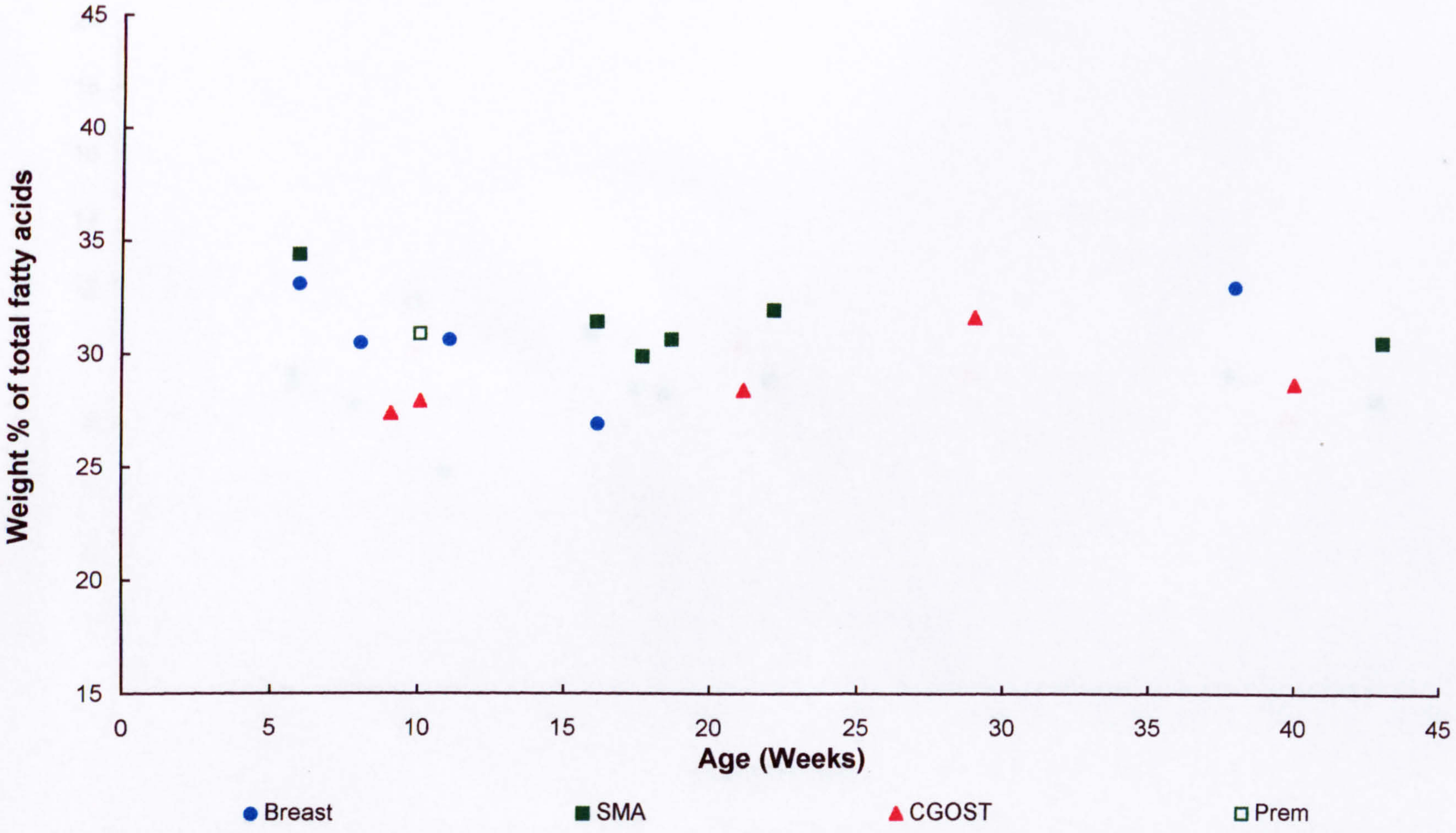

Figure 3.21 - Cerebral cortex phosphatidylethanolamine stearic acid (C18:0) in relation to infants' diet and age.

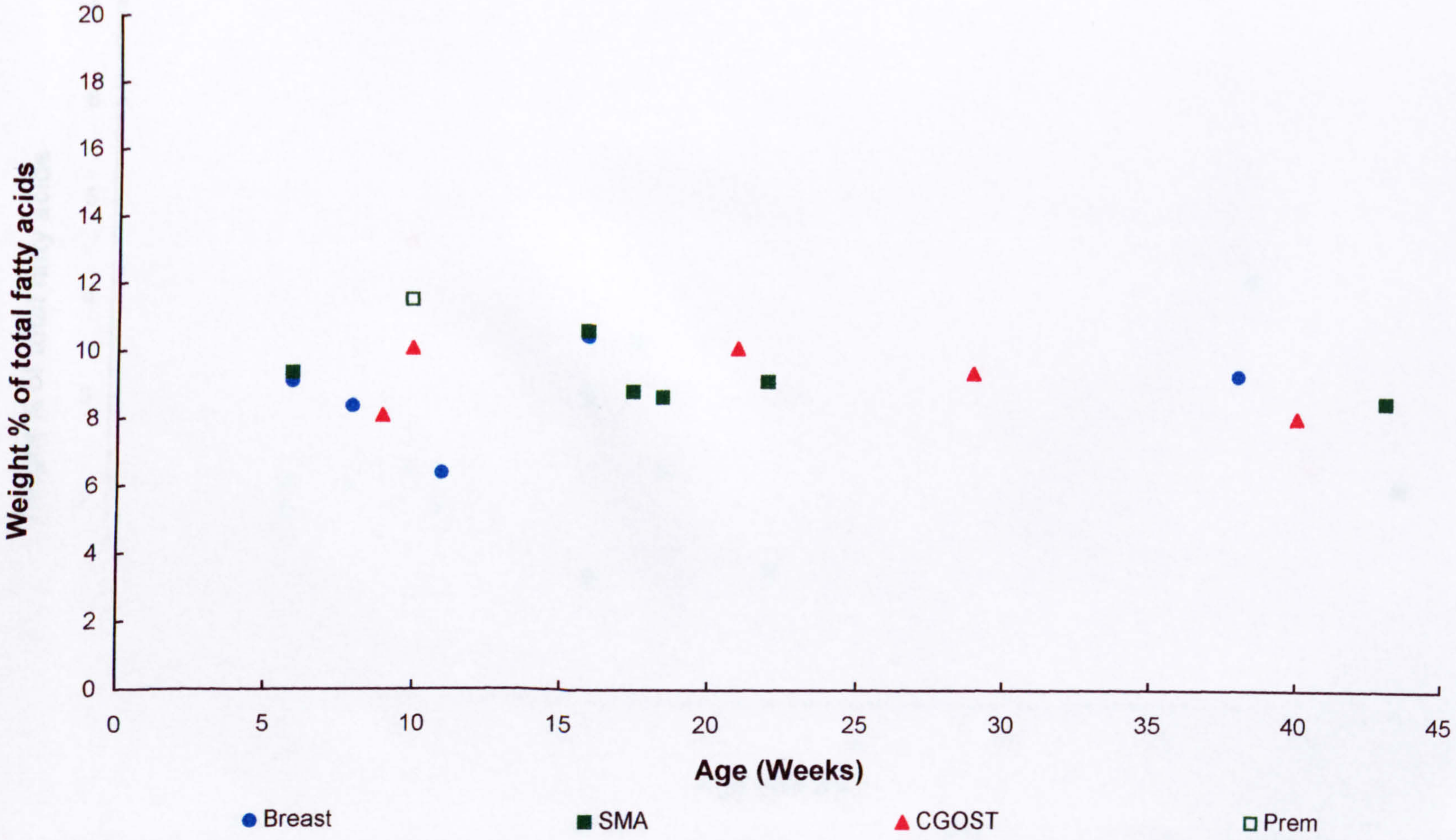

Figure 3.22 - Cerebral cortex phosphatidylethanolamine oleic acid (C18:1n-7+n-9) in relation to infants' diet and age.

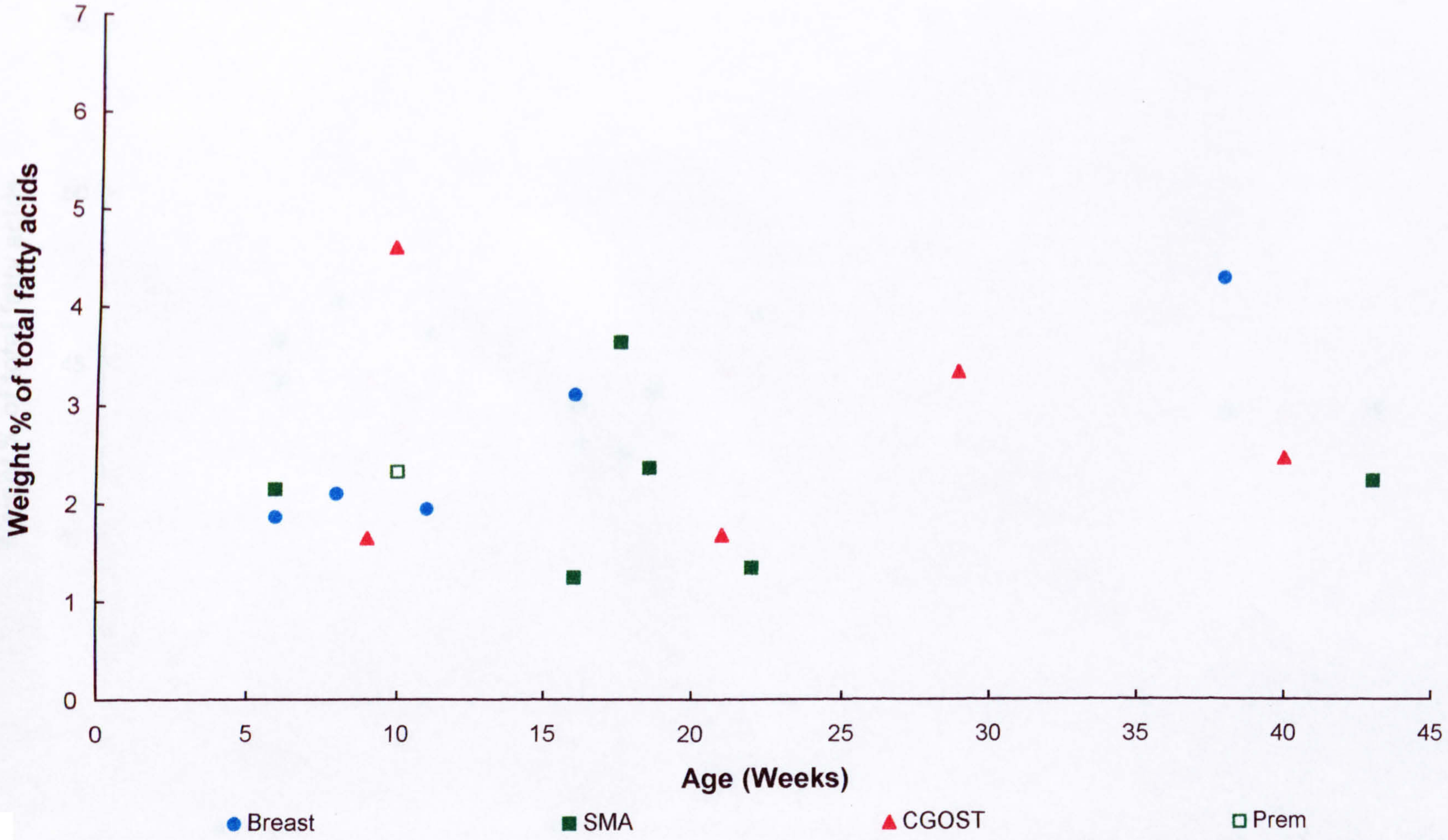

Figure 3.28 - Cerebral cortex phosphatidylserine palmitic acid (C16:0) in relation to infants' diet and age.

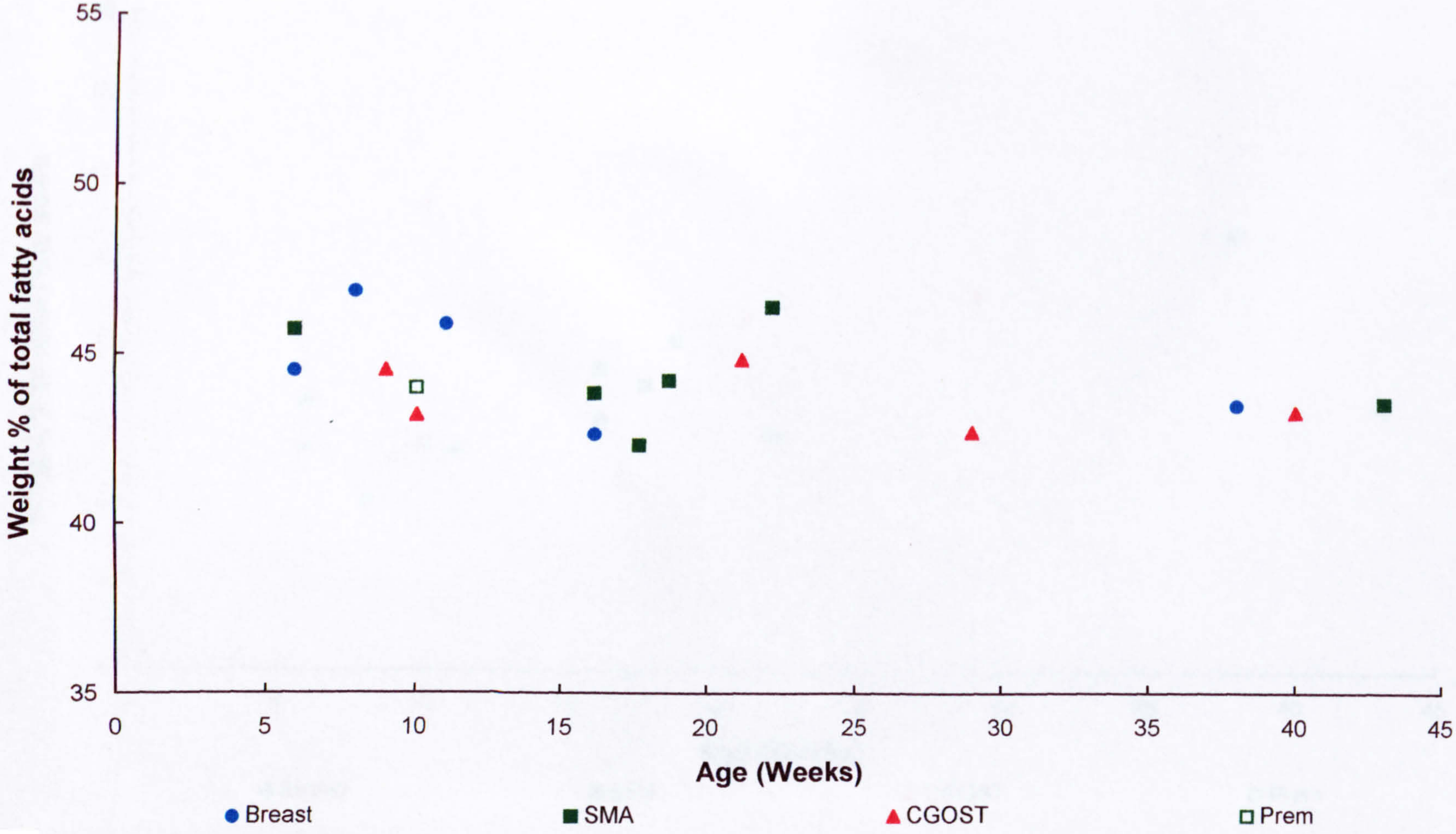

Figure 3.29 - Cerebral cortex phosphatidylserine stearic acid (C18:0) in relation to infants' diet and age.

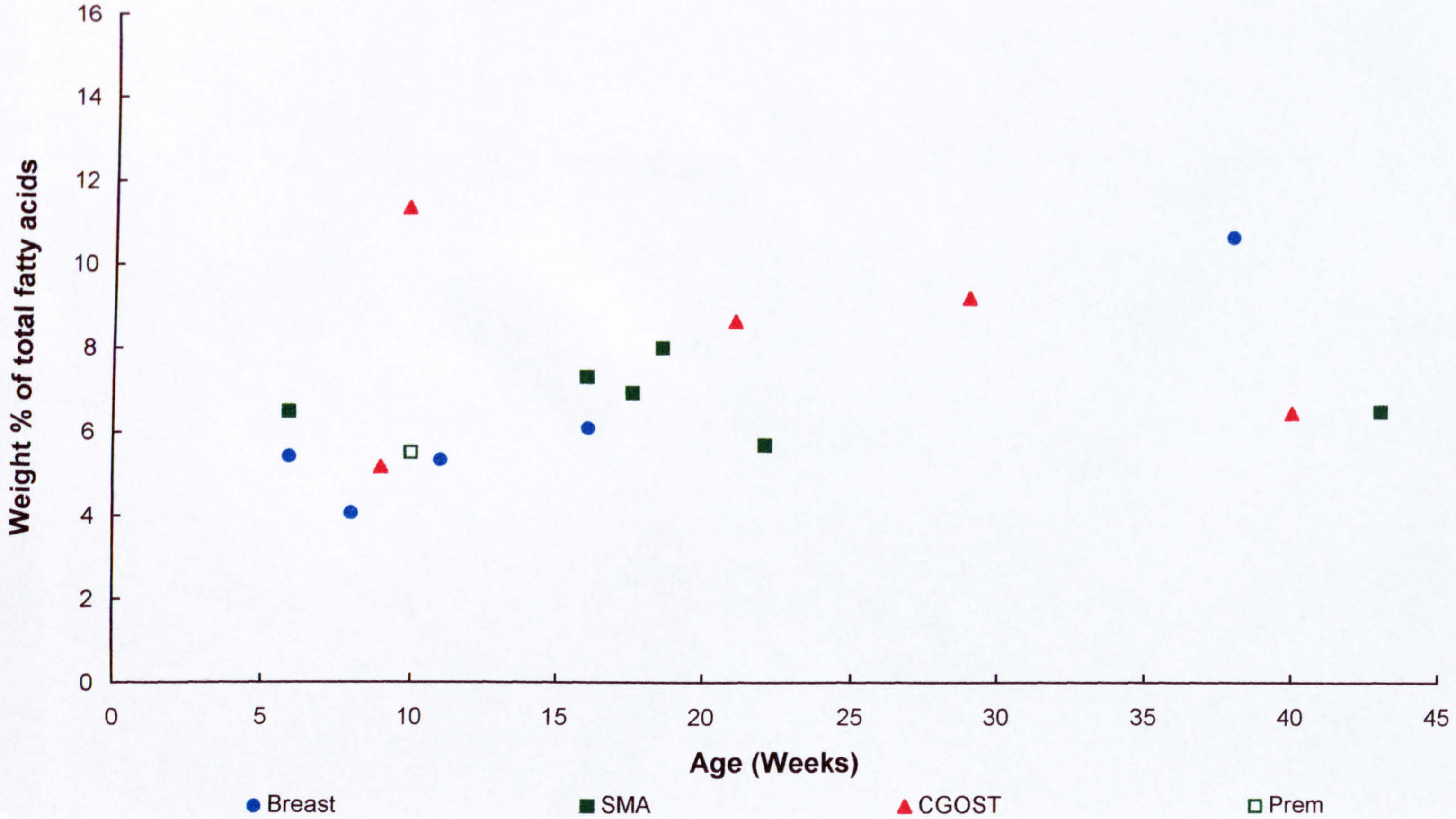

Figure 3.30 - Cerebral cortex phosphatidylserine oleic acid (C18:1n-7+n-9) in relation to infants' diet and age.