NUTRIENTS AND TOBACCO: SHORT AND LONG-TERM EFFECTS ON BRAIN AND COGNITION IN CHILDHOOD AND ADOLESCENCE

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In loving memory of my dearest grandfather, Savvas and
of my precious young cousin, Kyriakos who so suddenly passed away a month ago.

As you set out for Ithaka
hope that your journey is a long one,
full of adventure, full of discovery.
Laistrygonians and Cyclops,
angry Poseidon- do not be afraid of them,
you'll never find things like that on your way
as long as you keep your thoughts raised high,
as long as a rare excitement
touches your spirit and body.
Laistrygonians and Cyclops,
wild Poseidon- you won't encounter them
unless you bring them along inside your soul,
unless your soul sets them up in front of you.

Hope that your journey is a long one.
May there be many summer mornings when,
with what pleasure, what joy,
you come into harbours seen for the first time;
may you stop at Phoenician trading stations
to buy fine things,
mother of pearl and coral, amber and ebony,
sensual perfume of every kind-
as many sensual perfumes as you can;
and may you visit many Egyptian cities
and learn, and learn again from those who know.

Keep Ithaka always in your mind.
Arriving there is what you are destined for.
But do not hurry the journey at all.
Better if it lasts for years,
so you are old by the time you reach the island,
wealthy with all you have gained on the way,
not expecting Ithaka to make you rich.

Ithaka gave you the marvellous journey.
Without her you would not have set out.
She has nothing left to give you now.

And if you find her poor, Ithaka won't have fooled you.
Wise as you will have become, so full of experience,
You will have understood by then what these Ithakas mean.

Ιθάκη (Ithaka)
Κ.Π. Καβάφης (By C.P. Cavafy)

Abstract

The brain undergoes rapid structural and functional changes during gestation and in the first two years of life. But brain development also continues throughout childhood and adolescence with cognitive abilities getting improved. Threats to the vulnerable Central Nervous System can have long-lasting effects throughout the foetal and neonatal periods and beyond infancy, on all aspects of development. This thesis investigates the long-term associations of prenatal tobacco exposure, as well as the long-term associations of breastfeeding with brain and cognitive development of adolescents. Further, it investigates the short-term effects of omega-3 supplementation on brain and cognition of school-aged children. These will be examined in order to identify solutions for optimal development, both at brain and cognitive level, for the new generation.

The first study is entirely based on maternal cigarette smoking during pregnancy association with cognitive development of adolescents. This study found no differences on cognitive development of exposed and non-exposed adolescents when maternal education was held constant in the two groups. The two subsequently studies examined long-term associations of breastfeeding duration with brain and cognition of adolescents. These studies found that breastfeeding duration was positively linked to intelligence and brain structures, such as caudate nucleus, which is vulnerable to environmental influences during critical periods of brain development. Lastly, the omega-3 supplementation study found no differences between active and placebo group on cognition but found associations between omega-3 fatty acids and brain microstructure thus hypothesizing that higher intake of ω-3 fatty acids can alter concentrations of specific ω-6 fatty acids thus influencing membrane fluidity.

As such, our findings suggest that in order to obtain optimal brain and cognitive development, we do not only need to discard toxins or employ nutrients during critical periods of rapid brain development but also take into account other environmental and genetic factors that play vital role in children's development.

List of Publications

Kafouri S., Leonard G., Perron M., Richer L., Seguin J.R., Veillette S., Pausova Z., & Paus T. (2009) Maternal cigarette smoking during pregnancy and cognitive performance in adolescence. *International Journal of Epidemiology* 38, 158–172.

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Contents

Preface

The purpose of this thesis is to investigate the associations between nutrition and tobacco and normal development of brain and cognition in children and adolescents. The General Introduction in Chapter 1 discusses the importance of investigating associations between toxins and brain/cognition during critical periods of development, as well as the efficacy of nutrients in proper brain development. A detailed description of brain and cognitive development during gestation, infancy and adolescence will also be given in this first part of the thesis. Nutrients and toxins regulating brain development during fetal and early postnatal life will also be discussed. As such, the main variables of interest for this thesis are identified, which are prenatal tobacco exposure, breast milk during infancy and omega-3 fatty acids during childhood and how they interact with brain and cognitive development.

In order to examine these associations between nutrients and tobacco and brain/cognitive development, the thesis presents four studies. The first part of this thesis will focus on associations derived from prenatal exposure to tobacco (Chapter 2). The other part of the thesis will concentrate on the associations of nutrients with cognitive and brain development (Chapter 3-5). Chapters 2 to 4 report data derived from a cross-sectional study investigating: (i) the long-term associations of maternal cigarette smoking during pregnancy with offspring's cognitive development, and (ii) the long-term associations of breastfeeding with brain and cognitive development in adolescence. Chapter 5 is a detailed double-blind placebo-controlled investigation on how omega-3 fatty acids supplementation affects brain and cognition of school-aged children. With these studies, the thesis is trying to answer whether we can identify specific nutrients and toxins which alter physiological states of brain development and by either employing (nutrients) or discarding (toxins) them, we can reveal the secret for optimal brain and hence, cognitive development.

Finally the Discussion in Chapter 6 brings together the main findings of the different studies and discusses how these answer the main research questions. Theoretical and methodological contributions to the literature will be emphasized, and further research directions will be considered.

Chapter 1: General Introduction

Effects of nutrients and toxins on brain and cognitive development

This thesis aims to describe associations of nutrition and tobacco with normal development of brain and cognition in children and adolescents. More specifically, long-term associations of prenatal tobacco exposure on adolescents' cognition, as well as long-term associations of breast milk on adolescent's cognition and brain structure will be addressed in the first and mid part of this thesis. The last part will focus on short-term effects of polyunsaturated fatty acids on brain and cognition of healthy school-aged children. These will be examined in order to identify solutions for optimal development, both at brain and cognitive level, for the new generation.

1.1. The main research questions

Nutrients and toxins regulate brain development during the fetal and early postnatal life. But the brain undergoes many structural and functional changes until late adolescence.

A. Is there a relationship between exposure to maternal cigarette smoking and cognitive development of adolescents, when maternal education, the most known predictor of cigarette smoking during pregnancy, is held constant?

Smoking prevalence in early ages and among pregnant women is high even at present. While illegal drugs and alcohol consumption have gained a lot of attention from the media and the medical community as being detrimental for the developing brain, smoking effects have been underestimated and are thought to be of less significance than illicit drugs of abuse. This despite the fact that epidemiological studies have identified that smoking during pregnancy is associated with thousands of spontaneous abortions, perinatal deaths and sudden infant death syndrome.

Hence, the aim of this chapter is to identify long-term associations of maternal cigarette smoking during pregnancy with cognitive development of exposed and non-exposed adolescents who were matched according to level of maternal education and school attended.

Maternal education is a known predictor of cigarette smoking during pregnancy in the general population. Cognition of adolescents will be examined thoroughly, and factors associated with maternal cigarette smoking and adolescents' cognition will be identified.

B. Is there a relationship between breast feeding and adolescents' brain and cognition?

While numerous studies have examined short-term and long-term associations of breast milk with cognition of infants and children (fewer studies on adolescents), long-term associations of breast-feeding with adolescents' brain are largely unknown. The second research question addresses the hypothesis that breast milk is good for optimal brain development. Linking to the previous research question, women who smoke during pregnancy are less likely to breastfeed. As such, the second research question will identify long-term associations of breast milk with brain and cognition of adolescents exposed and non-exposed to maternal cigarette smoking.

C. Does omega-3 fatty acids supplementation facilitates optimal development of school-aged children from less well-off neighbourhoods?

Omega-3 fatty acids are main elements of human milk. Positive effects of breast milk have been linked to omega-3 fatty acids. Most studies have focused on the effects of omega-3 fatty acids on infancy while ignoring school-aged children who are still going through many developmental changes. As the brain undergoes many changes throughout childhood and adolescence, nutrition can still have an impact on it. The third research question aims to identify the effects of omega-3 supplementation on brain and cognition of children from less well-off neighbourhoods.

This general introduction will discuss why and how nutrients and toxins can have a great impact on sensitive periods during brain development. It will outline details on normal brain and cognitive development and how they are related, and how nutrients and toxins affect them. This introduction will then proceed to present the different methodological approaches that have been employed throughout the thesis in order to address the main research questions.

1.2. Brain Development

1.2.1. Stages of brain development

Overview

The formation and development of the human brain arises over a specific period of time, starting soon after conception and continue well through adolescence. Organization of the brain development can be divided in two major periods. The first period begins soon after conception and involves processes such as neurulation, proliferation, migration and differentiation. The second period is characterized by a reorganization of the human cortex with events such as dendritic and axonal growth, synapse production, neuronal and synaptic pruning, and changes in neurotransmitter activity emerging.

The whole Central Nervous System (CNS) originates from the walls of a fluid-filled tube that is shaped at an early stage of the embryonic development. The embryo consists of three different layers of cells called endoderm, mesoderm and ectoderm. Endoderm will give rise to most of the internal organs, whereas from the mesoderm arise all the bones, muscles and connective tissues of the body. The nervous system and skin arise from the ectoderm. Part of the ectoderm, called the neural plate, will transform into the neural tube (a process called *neurulation*) where the entire CNS is being formed. The brain is developed from the three primary vesicles of the neural tube: the forebrain, midbrain and the hindbrain. The forebrain will give rise to the cerebrum (cerebral cortex and basal ganglia) and the diencephalon (thalamus and hypothalamus). The midbrain consists of the tectum and the substantia nigra, whereas the hindbrain differentiates into the cerebellum, the pons and the medulla oblongata.

While the fetal nervous system is being differentiate into the structures we recognize in the adult brain, cell division leads to a vast proliferation of new neurons (*neurogenesis*). The immature neurons, called neuroblasts, migrate from the ventricular zone to the surface of the brain (Bear, Connors & Paradiso, 2001). The earliest migrating cells reside in the deepest layer of the cortex whereas following migrations go through previously formed layers, ending up in the superficial layers (inside-out *cell migration*). Thus, by the 7th prenatal month the neocortex consists of six layers (Nelson, Haan & Thomas, 2006). When the neuroblasts have migrated to their correct location, *cell differentiation* starts with the emergence of neurites developing off the cell body.

A fully grown neuron consists of the *dendrites*, which receive input from other neurons, the *axon*, which transfer information over distance in the nervous system, and in between these two, the *cell body* that contains the nucleus. Within the neuron, information travel through the axon by electrical impulses called *action potentials*; at the end of the axon, the information must cross a junction called *synapse* in order to be sent out to the next neuron by signalling the release of chemical messengers (typically *neurotransmitters*). Spontaneous and environmentally induced neuronal activity leads to the formation and stabilization of synapses (Molliver, Kostovic & Van der Loos, 1973).

Axonal and Dendritic Development

The process of extending axons and dendrites starts once the neuron finished its migratory trip. The axon of a neuron travels through the embryonic environment to its synaptic target. Axons may extend from less than a millimetre to over a meter long; neurons with long axons that extend from one part of the brain to the other are called *Golgi type I neurons*, such as pyramidal cells, whereas neurons with short axons that do not extend beyond the surrounding area of the cell body are called *Golgi type II neurons*, such as the stellate cells. The axon's diameter also plays an important role as the speed of the electrical signal that goes through the axon depends on axonal diameter. The thicker the axon, the faster the impulse travels (Bear, Connors & Paradiso, 2001). The growth cone at the tip of the axon is responsible for the development of the axon itself and in guiding the axon to its target (Webb, Monk & Nelson, 2001).

Dendrites appear as thick processes extending from the cell body with only few spines. As dendrites thicken and increase in number, they offer a better area for synaptic contact (Webb et al., 2001). Aizawa, Hu, Bobb, Balakrishnan, Ince, Gurevich, Cowan and Ghosh (2004) showed that the gene *calcium-regulated transcriptional activator* (CREST) is crucial for the dendrites development. Spine number and density increase as a consequence of dendritic maturation, thus making it easier to connect with the neighbouring axon via synaptic contact.

Dendrites continue to grow and change well beyond birth achieving the growth of dendritic trees and spine in all six layers at the 1st postnatal year; although spines are still undeveloped (Webb et al., 2001). Similar to axonal development, dendritic development also

presents regional and layer-specific differences. For example, in the visual cortex, maximum dendritic arborisation occurs at about 5 months postnatally and then regresses to an adult level by 2 years (Michel & Garey, 1984). To create an efficient synapse, axons must be appropriately connected with dendrites. Thus, an "overproduction" of dendrites, dendritic spines and axons is needed to follow a final "overproduction" of synapses. Excessive production of axons starts during perinatal life and by the process of competitive elimination it may reach the final number during the postnatal period (Webb et al., 2001). For example, in the corpus callosum of the infant rhesus monkey, the number of axons increases between mid-gestation and birth by exceeding at least 3.5 times the number of callosal axons of the adult monkey, and at about the 3rd post-natal month axon elimination is completed (LaMantia & Rakic, 1990). Excessive synaptogenesis during the early postnatal period is strongly associated with the overproduction of cortico-cortical axons (Goldman-Rakic, 1987).

Synaptogenesis

Synapse is the point of contact between two neurons and it comes in two types: electrical and chemical. In the vertebrates nervous system most neurons communicate through chemical synapses; they convert electrical signals (in the form of action potentials) travelling down the axon of one neuron, into chemical signals and then back to electrical impulses within the postsynaptic dendrite of another neuron by releasing neurotransmitters from the presynaptic neuron.

Synapse formation within the cortical plate begins at 23 weeks of gestation and depends on the growth of axons and the dendrites proliferation (Molliver, Kostovic & Van der Loos, 1973). In the 1st year of life there is a peak of synapse production distributed across regions of the brain followed by a gradual reduction, with the timing of the peak overproduction varying by brain area. For example, Huttenlocher and colleagues documented differences in synaptogenesis in visual, auditory and frontal cortex. In the visual cortex (Huttelnocher & de Courten, 1987) greater increases in synaptogenesis was between 2.5 to 8 postnatal months, in the auditory cortex (Huttenlocher & Dabholkar, 1997) highest values in synaptic density was at 3 postnatal months whereas in prefrontal cortex at age 3 months synaptic density was half the maximum. By the age of 3.5 years both cortical regions (auditory

and frontal cortex) have reached the maximum number of synapses (Huttenlocher & Dabholkar, 1997).

Whereas evidence (Bourgeois, Reboff, & Rakic, 1989) document that the overproduction of synapses is largely under genetic control, loss of synapses (synaptic pruning) in the absence of cell death, seems to be environmentally regulated. Synaptic pruning follows the Hebbian principle where an increase in synaptic efficacy and stabilization arises when a presynaptic cell is repeatedly and persistently stimulating a postsynaptic cell; synapses that are less active tend to be eliminated with those which are weaker to be the first to be removed (Chechic, Meilijson & Ruppin, 1998).

While the first evidence for synaptic pruning came from post-mortem studies by Huttenlocher and colleagues, it should be noted that these studies had low number of samples for the different stages of human development. The study by Bourgeois and Rakic (1993) in rhesus monkeys showed that the high synaptic density of the visual cortex between the 2nd and 3rd postnatal month was maintained for the next two years, and that a remarkable loss of synapses became more pronounced during puberty, between 2.7 and 5 years. Their data were expressed as density of synapses per unit volume of neuropil, thus neuronal and glial cell bodies or formation of myelin sheets and enlargement of capillaries could not have affected their results. Their results could not have been due to changes in the overall percentage of neuropil in the cortex or the volume of the cortex because neither of them changes significantly during puberty (Williams, Ryder & Rakic, unpublished observations quoted in Bourgeois & Rakic, 1993).

Myelination

Myelin is a fatty substance that insulates axons thus providing increased conduction velocity; is composed of lipid proteins and glucolipids and, in the CNS, it is generated by oligodendrocytes (Webb, Monk, & Nelson, 2001). Not all axons are myelinated; in general a minimum calibre is required (~ 1μm) before an axon can be myelinated (Sherman & Brophy, 2005). Formerly, myelin was studied in post-mortem tissue using staining methods; these studies evidently established that myelination persists during childhood, adolescence and young adulthood (Benes, Turtle, Khan, & Farol, 1994). It begins prenatally and follows a regional pattern resembling the one of synaptogenesis and dendritic development, with the

prefrontal regions myelinating later than other regions of the cortex. Major tracts of the visual system (optic tract and optic nerve) begin to myelinate prenatally and mature by 9 months of age; whereas during the 1st year regions of the brain stem, cerebellum and corpus callosum myelinate (Brody, Kinney, Kloman, & Gilles, 1987).

A number of studies (Jernigan, Trauner, Hesselink & Tallal, 1991; Giedd, Blumenthal, Jeffries, Castellanos, Liu, Zijdenbos, Paus, Evans and Rapoport, 1999; Paus, Zijdenbos, Worsley, Collins, Blumenthal, Giedd, Rapoport & Evans, 1999; Barnea-Gorally, Menon, Eckert, Tamm, Bammer, Karchemskiy, Dant & Reiss, 2005; Lenroot, Gogtay, Greenstein, Wells, Wallace, Clasen, Blumenthal, Lerch, Zijdenbos, Evans, Thomspon & Giedd,, 2007) have showed: age-related increases in white matter (WM) and non-linear changes in cortical gray matter (GM) following an inverted U shaped trail. The volume of white matter incorporates the number of axons, their calibre and the thickness of myelin sheath; thus increases in white matter during development can be explained by increases in axonal calibre and/or thickness of the myelin sheath (Paus, 2010).

Brain growth

After birth the volume of the human brain undergoes dramatic changes with brain weight reaching 75% of its adult weight at the 2nd year of life (Carmichael, 1990). Brain volume is determined by the number, size and density of neurons and glial as well as dendritic and axonal number and density. Brain tissue is divided into gray and white matter wherein gray matter includes the cell bodies of neurons and their dendrites, glial cells and blood vessels, and white matter consists of myelinated and unmyelinated axons. In the cerebral cortex of an adult mouse, electron-microscopic analyses showed the following relative volumes: axons 29.3%, dendrites 30.2%, dendritic spines 12.06%, glia 9.5%, cell bodies and blood vessels 13.8%, and extracellular space 5.2% (Braitenberg, 2001). Variations in brain volume reflect changes in cerebral GM, WM and cerebrospinal fluid volumes. As already mentioned above, WM volume increases linearly during childhood and adolescence, with boys showing steeper rate of increase during adolescence (Lenroot et al., 2007; Perrin, Hervé, Leonard, Peron, Pike, Pitiot, Richer, Veillette, Pausova & Paus, 2008), whereas GM volume shows a more multifaceted progress. In the frontal and parietal lobes, GM volumes peaks around 10 and 12 years of age and then decreases; in the temporal lobes the peak appears at the age of 16 yr (Giedd et al.,

1999). Exploring GM decreases, researchers have proposed that phylogenetically older brain areas develop earlier than newer ones (e.g. prefrontal cortex), with primary sensory-motor cortices and frontal and occipital poles maturing first, with the rest of the cortex developing in a back-to-front (parietal-to-frontal) direction, and that frontal and occipital poles lose GM early and in the frontal lobe, dorsolateral prefrontal cortex loses GM at the end of adolescence (Gogtay, Giedd, Lusk, Hayashi, Greenstein, Vaituzis, Nugent, Herman, Clasen, Toga, Rapoport & Thompson, 2004).

It has been proposed that GM decreases are attributable to synaptic pruning and normal neuronal elimination during childhood; according to the work of Bourgeois and Rakic (1993) on the visual cortex of the macaque monkey it has been established, however, that a decrease in synaptic density during puberty affects very little the volume of the cortex. On the other hand, WM increases during development can be explained by increases in axonal calibre and myelin thickness. A recent study by Perrin et al. (2008) observed increases in WM volume in adolescent males but a decrease in Magnetization Transfer Ratio (MTR; an indirect index of myelin in WM) values in WM suggesting that increases in WM are not driven by increases in myelin but hypothesized that may be due to changes in axonal calibre.

Taken as a whole, brain development commences within weeks of gestation and persists until young adulthood. Different stages such as neurogenesis, cell differentiation, axonal and dendritic development, synaptogenesis, and myelination, contribute on their own way in generating a perfect circuit, the human brain. While most of these processes are genetically driven, our understanding on how environment and experience can alter these processes is far from complete.

1.3. Sensitive periods in brain development: Environmental effects on prenatal, postnatal and adolescent brain development

The human brain is susceptible to many exogenous influences during different developmental periods. While in utero, infancy, childhood and later on in adolescence, the maturing brain undergoes many structural and functional processes, such as synaptogenesis, axonal growth (longitudinal and radial) and myelination, which can be influenced by factors

such as mother's nutrition, mother's emotional state, family socioeconomic background, and/or other environmental agents. As many neuronal processes of the developing brain are undergoing during foetal life, prenatal influences are the first to be more crucial.

Since foetal nutrition is solely based on maternal diet, agents such as vitamins, iron, folic acid, drugs, illegal substances, alcohol, and cigarettes are those which can severely influence the developing brain. When the mother is undernourished, the placenta fails to develop sufficiently resulting in fewer nutrients available for the foetus.

1.3.1. Deficiencies and Toxins

One of the most sensitive periods of brain development is within four weeks of gestation where the neural tube closes. Maternal malnutrition produces neural tube defects (NTD) in animals. Deficiencies in iron, zinc, and folic acid have been shown to cause NTD in rats (DeLong, 1993). In humans, observational and supplementation studies have shown a somewhat reasonable relationship between maternal nutrition and NTD. In 1991, a large-scaled randomized control trial of folic acid supplementation in 1030 women, who had already had a NTD-affected pregnancy, confirmed 1.0% recurrence rate of NTD in the folic acid supplemented group and 3.5% among those who did not take folic acid (MRC Vitamin Study Research Group, 1991). Diabetes, obesity and epilepsy, more specifically anti-epileptic drugs, are also known risk factors for NTD. Anti-epileptic drugs cross the placenta, raise the drug concentration in the foetus, modify folate metabolism and decrease the plasma folate (Kondo, Kamihira & Ozawa , 2009). It is generally known that folate affects embryogenesis of the brain and it is also important for the later development of the hippocampus.

Neural tube defects illustrate sensitivity of brain development to nutritional state of the mother and how timing is crucial. Early exposure to malnutrition has revealed, from experimental studies with animals, that cerebral cortex shows a reduction in volume with a sparing of the total number of cortical neurons; Golgi staining methods revealed disruption in pyramidal cells, reduction in the density of cortical dendritic spines, a decrease in the width of cortical cells and reduction in the total number of cortical glial cells (Levitsky & Strupp, 1995).

Iodine and iron deficiencies are the most widespread and well-studied causes of impaired brain development. Iodine is required for the synthesis of thyroid hormones that are, in turn, needed for the brain development during foetal and early postnatal life by regulating

the metabolic pattern of most cells of the organism (Delange, 2000). The most severe form of brain damage due to iodine deficiency is endemic cretinism, in which number of neurons is decreased, arrangement of neurons is irregular, and degeneration of neurons has been seen in many areas of the brain (Delong, 1993). Mild and moderate iodine deficiency has been shown abnormalities in the intellectual and psychomotor development of children (Delange, 2000).

Experimental studies in rodents have shown that iron deficiency during gestation/ lactation modifies neurometabolism, neurotransmitters and myelination (Lozoff & Georgieff, 2006). Iron is engaged in tissue oxygenation and energy metabolism during early brain development (Rao & Georgieff, 2002). Iron is also a co-factor for the desaturases involved in the synthesis of long-chain polyunsaturated fatty acids (PUFA), which are vital for brain development (LeBlanc, Surette, Fiset, O'Brien & Rioux, 2009); iron deficiency in rats has been associated with altered tissue fatty acid profiles including reduced docosahexaenoic acid (DHA) content in the brain myelin (Oloyede Folayan & Odutuga, 1992).

Alcohol

Prenatal exposure to alcohol has the most severe consequences known as Fetal Alcohol Syndrome (FAS), with permanent changes in the brain: reduced brain size, variations in shape, tissue density and symmetry, volumetric reductions and abnormalities in the cerebellum, basal ganglia and corpus callosum (Riley & McGee, 2005). Abnormalities of the cerebellar vermis (Sowell, Jernigan, Mattson, Riley, Sobel & Jones, 1996; Archibald, Fennema-Notestine, Gamst, Riley, Mattson & Jernigan, 2001) and white-matter hypoplasia (Archibald et al., 2001) are well documented. Further, there is evidence for volume reduction in basal ganglia and the parietal lobe of children prenatally exposed to alcohol, and those diagnosed with FAS (Archibald et al., 2001). It appears that the timing of exposure determines severity of the brain damage and which structures are mostly affected. During the first trimester, exposure to ethanol interferes with the migration of neurons (Miller, 1993) and the proliferation of cells in the ventricular and subventricular zone in rats (Miller, 1996), whereas the third trimester is the time when hippocampus is especially sensitive to alcohol (Livy, Miller, Maier & West, 2003; West, Goodlett & Kelly, 1986). *In vivo* and *in vitro* studies revealed that ethanol modifies astrogliogenesis in humans and experimental animals, by

affecting key astroglia functions, such as boundary formation during neural morphogenesis, neuronal proliferation, and axon outgrowth (Guerri & Renau-Piqueras, 1997).

Cigarettes

Prenatal exposure to maternal cigarette smoking comes with a variety of severe consequences, such as spontaneous abortions, perinatal deaths, and Sudden Infant Death Syndrome (DiFranza & Lew, 1995). Pharmacological effects of nicotine and other chemicals that are contained in cigarette smoke can harm the foetus in numerous ways: induced constriction of the uteroplacental vessels leading to decreased levels of oxygen and nutrients flow to the foetus, increased levels of carboxyhemoglobin reducing tissue oxygenation of the foetus, and suppressed mother's appetite leading to reduced energy supply to the foetus (Slotkin, 1998). By stimulating nicotinic acetylcholine receptors, nicotine can affect the fetal brain development directly; by providing excessive cholinergic stimulation during the fetal life, nicotine alters events of cell replication, differentiation and synaptic development (Slotkin, 2005).

A study by Roy and Sabherwal (1998) detected reduction in brain weight and cortical thickness in the rat somatosensory cortex. Prenatal exposure to nicotine seems to reduce the number of binding sites for the serotonin transporter in the rat cerebral cortex, an effect that lasted into adulthood (Xu, Seidler, Ali, Slikker & Slotkin, 2001). At a receptor level, prenatal nicotine exposure increases the number of $5HT_{1A}$ (serotonin) receptors in the cerebral cortex of male but not female rats (Slotkin, Tate, Cousins & Seidler, 2006). Further, differences in the expression of nicotinic and muscarinic acetylcholine receptors were found in the brains of 5-to-12 weeks old foetuses exposed and non-exposed to maternal cigarette smoking (Falk, Slotkin, Mencl, Frost & Pugh, 2005). A more recent study by Toro, Leonard, Lerner, Lerner, Perron, Pike, Richer, Veillette, Pausova and Paus (2008) observed 'thinning' of the orbitofrontal cortex in adolescents exposed in utero to maternal cigarette smoking, and in the same sample, Paus, Nawazkhan, Leonard, Perron, Pike, Pitiot, Richer, Veillette & Pausova, (2008) found reductions in the overall size of the corpus callosum in the exposed females. Further, the striatum of the exposed adolescents with a particular variant of a nicotinic-receptor gene was larger than that of exposed adolescents with the other variant of this gene (Lotfipour, Leonard, Perron, Pike, Richer, Séguin, Toro, Veillette, Pausova & Paus, 2010). The above findings

indicate that prenatal nicotine exposure can cause cell damage, reduce cell number, and impair synaptic activity (Slotkin, 1998).

Illegal Drugs

It is quite difficult to estimate the effect of a distinct illicit drug, while women who abuse one substance during pregnancy are also prone to use other drugs, smoke cigarettes and drink alcohol. Cocaine shares several characteristics with nicotine. "Both are vasoconstrictors that converge on adrenergic neurotransmission as their underlying mechanism... cocaine by preventing presynaptic uptake of catecholamines, thus intensifying their actions" (Slotkin, 1998). Observations from epidemiological studies have found that foetal cocaine exposure comes with congenital anomalies, growth retardation, microcephaly, CNS infarction, seizures and cortical atrophy and cysts (Bauer, Langer, Shankaran, Bada, Lester, Wright, Krause-Steinrauf, Smeriglio, Finnegan, Maza & Verter, 2005).

The intoxicating chemical in marijuana, THC, changes fundamental developmental processes, and in particular impairs the establishment of connectivity between brain regions that play role in motivation, mood and cognition (Jutras-Aswad, DiNieri, Harkany & Hurd, 2009).

As aforementioned, one of the most critical periods of brain development is during prenatal life when neural processes such neurogenesis, cell migration, axonal and dendritic growth, and synaptogenesis take place. Given that brain growth continues well into adolescence, compared with the "helpless" foetus depending entirely on its mother's state, the adolescent can consciously improve or harm his brain development by either choosing a healthier lifestyle or becoming a prey of substances.

During adolescence, there is an increase in myelination, fiber density of amygdalocortical and corticoaccumbens connections continues to increase, and maturation of mesocorticolimbic dopamine system is marked (Dwyer, McQuown & Leslie, 2009). Normal development of neuronal pathways can be disturbed by agents such as nicotine, alcohol and illicit drugs. Nicotine administered during adolescence has been shown to induce greater increases in extracellular levels of dopamine and serotonin in the nucleus accumbens of the adolescent rat (Shearman, Fallon, Sershen & Lajtha, 2008). Moreover, alcohol consumption in adolescence, in particular ethanol, increases dopamine in the nucleus accumbens (Maldonado-

Devincii, Badanich & Kirstein, 2010). Cannabis exposure is associated with discrete alterations of the endogenous opioid system in limbic-related neuronal pathways known to facilitate reward behaviour (Ellgren, Spano & Hurd, 2007). Several agents act differently in numerous neuronal processes and have discrete effects on critical developing periods.

1.3.2. Nutrition and the developing brain

As described above, nutrition is essential for brain development during foetal and early postnatal life, and in adolescent years. Certain nutrients have bigger effects on brain development than others. In addition, the timing, dose and duration of any nutrient deficiency or excess, plays different role in brain development. In early development, stem cells that give rise to neurons, are rapidly dividing and then they differentiate and grow. These processes need optimal nutrition. In order for the brain to have an ideal development, axonal outgrowth is necessary, which requires production of trophic agents that are influenced by nutrition. Axonal and synaptic development as well as myelination, is vulnerable to malnutrition (Prohaska, 2000).

While I have discussed the effects of several nutrient deficiencies as well as of substance abuse on the foetal and adolescent brain, neonatal brain development is also influenced by nutrition via breastfeeding (or formula-feeding).

Human milk provides different lipids that supply energy; between these lipids, essential ω-6 and ω-3 polyunsaturated fatty acids (PUFA) are among them, establishing the vital growth of the breastfed infant. Prior to birth, all of the omega-3 (ω-3) fatty acids that are essential for fetal development are provided through placental transfer from the mother's circulation (Innis, 2005). "Transfer of DHA across the placenta involves fatty acid binding proteins, with a release of DHA to the fetal circulation, followed by transport to liver where it is esterified and resecreted in lipoproteins" (Innis, 2008). After birth and almost until the 1st year of life, breast milk is the only source of ω-3 fatty acids to the infant's brain.

DHA, the most essential ω-3 fatty acid, is enriched especially in membrane lipids; also is vital for protection from oxidative damage, for neurogenesis and neurotransmitter metabolism, function of membrane proteins, and for myelination (Innis, 2007). Reduced levels of DHA in the brain have been associated with impairment of neurogenesis and neurite

outgrowth and altered metabolism of several neurotransmitters such as dopamine, serotonin and acetylocholine (Bazaan, 2006). A recent study by Isaacs, Fischl, Quinn, Chong, Gadian & Lucas (2009) found that the percentage of breast milk in the infant diet of 50 adolescents (born prematurely) was significantly correlated with intelligence and white-matter volume assessed with MRI at the age of 15 years 9 months old thus supporting the notion that breast-milk, and in particular, ω-3 fatty acids promote brain development.

In conclusion, evidence from experimental studies with humans and animals on how nutrition affects brain development will facilitate our understanding on how environment can alter neuronal processes in the foetal, infant and adolescent developing brain, and can also provide us with details on how to promote our children's brain growth.

1.4. Cognitive Development

1.4.1. What is the relation of brain development to cognitive development?

Cognitive processes mature concurrently with brain development. Paediatric neuroimaging and developmental neurobiology have taught us a great deal about the ways the brain grows: neurons migrating, axons and dendrites growing, synapse overproducing, and myelin covering up the axons until young adulthood. While the human brain undergoes all these momentous changes in its structure, it also goes through a functional organization leading to the formation of cognition. A direct relationship between neural and cognitive development has been under researcher's microscopes for decades using different methodologies: lesion studies and imaging studies. The most common method of assessing, *in vivo*, the developmental physiological path of behaviour, is the functional MRI. Haemoglobin (the iron-containing oxygen-transport metalloprotein in the red blood cells) becomes strongly paramagnetic in its deoxygenated state; and thus "it can be used as a naturally occurring contrast agent, with highly oxygenated brain regions producing a larger MR signal than less oxygenated areas" (Casey, Giedd & Thomas, 2000).

Brain-behaviour relations revealed by structural MRI

As aforementioned, MRI-based studies have mapped the anatomical course of normal brain development. In summary, MRI studies designate that gray matter follows an inverted U-

shape pattern, whereas white matter increases until young adulthood. In general, cortical regions subserving primary functions mature first while higher-order association areas mature later (Gogtay et al., 2004). Developmental changes in gray matter have been found to correlate with cognitive processes. Frangou, Chitins & Williams (2004) found that, in a sample of 40 young people aged between 12 and 20 years, intelligence quotient (IQ) was positively correlated with prefrontal gray matter (in particular orbitofrontal regions), and gray matter density in the anterior cingulate and medial frontal gyrus. In a longitudinal study of 45 children scanned between the ages of 5 and 11 years old, Sowell, Thompson, Leonard, Welcome, Kan & Toga (2004) found that a greater thinning of cortical gray matter was associated with improved performance on the vocabulary subtest of Wechsler Intelligence Scale for Children (WISC). A more comprehensive study by Shaw, Greenstein, Lerch, Clasen, Lenroot, Gogtay, Evans, Rapoport & Giedd (2006), using a longitudinal design with 307 participants with age range covering from early childhood to early adulthood, illustrated a developmental shift from a negative correlation between intelligence and cortical thickness in early childhood to a positive correlation in late childhood and beyond.

Brain-behaviour relations revealed by functional MRI

It has been noted that, through structural MRI data, prefrontal cortex follows a delayed maturation and its development is believed to play an important role in the maturation of higher cognitive skills, named 'executive functions', including inhibition, working memory, attention and cognitive control. Thus, functional MRI studies assessing the relationship between neural and cognitive development have focused mainly in prefrontal cortex.

In a study of prefrontal activation during performance on a Go-No-Go task (inhibition task), Casey, Trainor, Orendi, Schubert, Nystrom, Giedd, Castellanos, Haxby, Noll, Cohen, Forman, Dahl & Rapoport (1997) found that the location of activation in the prefrontal cortex was the same for both children and adults but the volume of activation was significantly greater for children compare with adults. Same findings were reported by Tamm, Menon & Reiss (2002) with younger participants activate more extensively than older participants, whereas older participants showed focal activation in specific areas critical for response inhibition. Adleman, Menon, Blasey, White, Warsofksy, Glover & Reiss (2002) provided evidence, using a Stroop task (interference task), that increases in prefrontal activation persist into adulthood.

The ability to suppress voluntarily context-inappropriate behaviour was examined by Luna, Thulborn, Munoz, Merriam, Garver, Minshew, Keshavan, Genovese, Eddy & Sweeney (2001) and fMRI results denoted that prefrontal cortex activation was greater in adolescents than children. Taken together, data from functional MRI studies evidenced that cognitive performance increases with brain maturation from childhood to early adulthood, and that particular brain regions become increasingly specialized, for particular cognitive processes, with development. A popular albeit unproven neurobiological explanation of these MR-based observations is that pruning of synapses and elimination of connections result in more efficient processing, strengthening of connections and in turn increased cognitive performance.

1.4.2. Effects of nutrients and toxins on cognitive development: from infancy to adolescence

While several nutrients can affect the developing brain of a foetus on several ways, there is also evidence that toxins have both short-term and long term effects on cognition and behaviour. As stated above, basic nutrients such as iron, iodine, and fatty acids are essential for foetal and neonatal neuronal development. Toxins, such as nicotine and alcohol, can alter axonal and dendritic growth, synaptogenesis, and myelination. They can cause reductions in cerebral cortex volume, and can interact with neurotransmitters by either decreasing or increasing their concentrations. Consequently, brain development alterations will modify cognitive development.

Research has mostly focused on associations between different types of substances use during pregnancy on the developing brain and cognition of children and adolescents. Illegal substances are extensively used by young people and in particular young women in pregnancy. Two longitudinal cohort studies have focused on the short-term and long-term association between prenatal marijuana and offspring's development (Fried & Smith, 2001). The researchers have noticed that there was no association of prenatal marijuana use and infant mental development at 1 year of age. At the age of 48 months, however, verbal and memory outcomes were negatively associated with prenatal daily marijuana use and this association remained even after controlling for confounders, including family income, mother's weight, age, education, nutrition and other drugs use, as well as sex, parity, gestation and birth weight (Fried & Watkinson, 1990). Fried and colleagues hypothesized that the absence of any

association of IQ and executive functioning impairment at early ages and prenatal marijuana use is due to the fact that executive functioning is still undeveloped at these early ages (Fried &Smith, 2001). At the ages of 9 and 12 years, prenatal marijuana use was negatively associated with abstract and visual reasoning (Fried, Watkinson & Gray, 1998). The same observation was made in the 10 years old children in another longitudinal study where marijuana was a significant predictor of poorer abstract/visual reasoning (Richardson & Gray, 1997; abstract). Deficits in executive functioning provoked by prenatal marijuana use are persistent and long-lasting since 18-22 years old adults from one of the two longitudinal studies showed altered neuronal functioning during visuospatial working memory processing (Smith, Fried, Hogan & Cameron, 2006). Taken together, these two longitudinal studies emphasize the importance of higher order cognitive processes impairments due to prenatal marijuana use.

Associations between prenatal alcohol and offspring's cognitive development are perhaps the most frequently studied. In general, heavy prenatal alcohol exposure is responsible for a range of neuropsychological deficits, including impairments in intelligence, memory, language, attention, processing speed, executive functioning and fine motor skills (Riley & McGee, 2005). Prenatal exposure to alcohol can permanently alter brain structure and function, with consequences in cognition and behaviour.

A fairly large literature focuses on the long-term association between prenatal cigarette smoking and offspring's development. Impairments have been found stable even after controlling for possible confounding variables. Prenatal maternal cigarette smoking has been found to associate negatively with different aspects of cognition, including visuoperceptual functioning, reading achievement, verbal comprehension, learning and memory, even after controlling for various confounding variables such as sociodemographics, other substances use, and current tobacco use (Davie, Butler & Goldstein, 1972; Fried & Watkinson, 1990; Fried and Watkinson, 2000; Cornelius, Ryan, Day, Glodschmidt & Willford, 2001).

It is worth mentioning that studies examining the association of prenatal exposure of different substances with the child's cognitive development should always take into account that environment and genes play an important role in the development of cognition. Thus, one should always co-vary for parental education when measuring child's intelligence as well as

family income, as these two variables (mother education and income) can have vital significance. Few studies examining the association of prenatal exposure to cigarette smoking with cognition found no differences between exposed and non-exposed children and adolescents after controlling for maternal education and maternal IQ (Breslau, Paneth, Lucia & Paneth-ollak, 2005; Batty, Der & Deary, 2006; Kafouri, Leonard, Perron, Richer, Séguin, Veillette, Pausova & Paus, 2009).

Apart from prenatal nutrition, the child's cognitive development is also affected by nutrition during infancy via breastfeeding or formula-feeding. A review of 24 published studies on the association between breastfeeding and intelligence by Drane and Logeman (2000) revealed that infants who were breastfed had advantages in cognitive development. Advantages in IQ were present in breastfed infants at 5 years, 7.5 to 8 years, 10 years, 13 years and 18 years (Drane & Logeman, 2000). In another study by Helland, Smith, Saarem, Saugstad & Drevon (2003), children who were born to mothers who were supplemented with cod liver oil (PUFAs) during pregnancy and lactation, were advantaged in intelligence scores at 4 years of age compare to children whose mothers were supplemented with corn oil. Altogether, these results highlight the importance of long-chain n-3 PUFAs on the infant's mental development both through breastfeeding and pregnancy.

1.5. Summary of the findings in the literature

The human brain develops rapidly during the first two years of life with cell proliferation, cell differentiation, axonal and dendritic grow, synaptogenesis and myelination taking place. But the brain development also continues throughout childhood and adolescence with cognitive abilities getting improved. Threats to the vulnerable CNS can have long-lasting effects throughout the foetal and neonatal periods and beyond infancy, on all aspects of development- neuronal growth and organization, synaptogenesis, synaptic organization, myelination, gliogenesis- as well as alterations in cognition and behaviour not detectable at birth, such as intelligence, visuospatial, attention, memory and language. By investigating long-term associations of prenatal exposures with cognition we can identify the consequences behind them and perhaps prevent future detrimental events for next generations.

On the other hand, nutrients such as omega-3 fatty acids can enhance brain development. Breast milk, which contains omega-3 fatty acids, has been shown to have positive effects on cognitive and brain development of pre-term infants. Indeed, exploring long-lasting associations of breast-feeding with brain and cognitive development of adolescents born at term might unravel noteworthy mechanisms that underlie omega-3 fatty acids. Plus, short-term effects of omega-3 fatty acids on brain and cognition of healthy children can help us identify nutrients for optimal development.

1.6. Methodological approaches of the thesis

To answer the research questions stated in the beginning of this Introduction, this thesis describes four studies. The first study is entirely based on maternal cigarette smoking during pregnancy association with cognitive development of adolescents. While numerous studies have reported associations between maternal cigarette smoking during pregnancy and offspring's cognition, this study has the benefit of matching the exposed and non-exposed adolescents according to maternal education, the best known predictor of cigarette smoking during pregnancy, as well as using an extended neuropsychological battery consisting of 33 cognitive tasks.

The second and third studies focus on the long-term associations of breast milk with cognitive development (2nd study) and brain structure (3rd study) of adolescents. Associations between breast milk and brain structure are largely unknown. Thus, the 3rd study aims to identify a vital gap in the methodological literature of breastfeeding long-lasting associations with brain development, and relate effects on brain with effects on cognition.

Last, the fourth study is a detailed double-blind placebo-controlled investigation on how omega-3 fatty acids supplementation affects brain and cognition of school-aged children. Studies on omega-3 fatty acids supplementation have focused their interest on infants by leaving an important gap on the effects of omega-3 fatty acids in childhood. Few studies have examined the omega-3 supplementation effects on cognition of school-aged healthy children, as well as children with Attention Deficit Hyperactivity Disorder. This study is the first to be investigating fatty acids supplementation effects on brain structure of children, as well as uses a wide range of cognitive and behavioural tests to identify any momentous omega-3 effects.

Taking these methodological issues into account, this thesis is trying to answer the question as to whether we can identify specific nutrients and toxins which alter physiological states of brain development and by either employing (nutrients) or discarding (toxins) them, we can reveal the secret for optimal brain and hence, cognitive development.

Chapter 2: Study 1 - Maternal Cigarette Smoking During Pregnancy and Cognitive Performance in Adolescence

2.1. Introduction

The incidence of cigarette smoking during pregnancy remains high. In the United Kingdom, 17% of women smoked throughout pregnancy in 2005 (ONS, 2006). In the United States, 6 to 26% of pregnant women smoked (depending on the State) during this same period (Martin, Hamilton, Sutton, Ventura, Menacker, & Munson, 2005). In Canada, the National Longitudinal Study of Children and Youth reported that 23.7% of mothers had smoked during pregnancy (Connor and McIntyre, 2002). In the Saguenay–Lac-Saint-Jean (SLSJ) region of Quebec, Canada, i.e. in the area in which the study was conducted, a similar frequency of cigarette smoking during pregnancy was observed with the expected differences between women of low vs. high socioeconomic status (SES): 39.0% and 23.6% respectively, for low vs. high levels of education and 47.2% vs. 27.4% for low vs. high income (Institut de la statistique du Québec, 2001).

Maternal smoking during pregnancy is known to be associated with increased risk of complications during pregnancy and early infancy, as well as with behavioural and cognitive sequelae in childhood and adolescence (Pausova, Paus, Abrahamowicz, Almerigi, Arbour, Bernard, Gaudet, Hanzalek, Hamet, Evans, Kramer, Laberge, Leal, Leonard, Lerner, Lerner, Mathieu, Perron, Pike, Pitiot, Richer, Séguin, Syme, Toro, Tremblay, Veillette, & Watkins, 2007). Inhaled cigarette smoke can influence the fetus in many ways, including a reduction of tissue oxygenation and flow of nutrients to the foetus, lower intake of nutrients related to the suppressed appetite of the mother (Lambers & Clark, 1996), as well as altered cellular growth and activity of the central and peripheral nervous system (Slotkin, 1998). Several epidemiological studies have reported an association between maternal smoking during pregnancy and psychopathology during childhood, including hyperactivity, aggressiveness, depression and anxiety (Falk, Nordberg, Seiger, Kjaeldgaard, & Hellstrom-Lindahl, 2005).

The effect of maternal cigarette smoking during pregnancy on the development of the human brain is not well documented. Indirect evidence suggests disadvantageous effects on brain growth; using the Swedish Medical Birth Registry (1,362,169 infants born during 1983-

1996), Källén (2000) showed a negative correlation between maternal cigarette smoking during pregnancy and head circumference at birth. Falk, Nordberg, Seiger, Kjaeldgaard & Hellstrom-Lindahl (2005) demonstrated changes in the expression of nicotinic (nAChRs) and muscarinic (mAChRs) acetylcholine receptors in brainstem and cerebellum of 5-to-12 weeks foetuses exposed to maternal cigarette smoking during the first trimester of pregnancy. A more recent study measured brain activity, using functional MRI (fMRI), during the performance of auditory and visual attention tasks by adolescent smokers with and without *in utero* exposure to maternal cigarette smoking. This study found that exposed female smokers performed less accurately than non-exposed female non-smokers; in contrast, among male adolescents adverse effects of tobacco smoking were most evident among smokers with prenatal exposure during auditory attention tasks (Falk, Slotkin, Mencl, Frost & Pugh, 2005). Recent studies from our laboratory showed that orbitofrontal, middle frontal and parahippocampal cortices were thinner in exposed, as compared with non-exposed adolescents (Toro et al.,2008); corpus callosum of exposed females was smaller than that of the non-exposed female adolescents (Paus et al., 2008); and the striatum of the exposed adolescent with a particular variant of a nicotinic-receptor gene (associated with higher drug experimentation and smoking during adolescence) was larger than that of exposed adolescents with the other variant of this gene (Lotfipour et al., 2010).

The possible associations of maternal smoking during pregnancy with children's cognitive and neuropsychological development have been examined in a number of longitudinal studies. Fifteen cohorts will be described in the following section: The American National Collaborative Perinatal Project (Hardy & Mellits, 1972), The British National Child Development Study (NCDS; Butler & Goldstein, 1973), The Ottawa Perinatal Prospective Study (Fried, 1995), The Finnish Cohort Study (Rantakallio, 1983), The Maryland Cohort Study (Sexton, Fox & Hebel, 1990), The Port Pirie Prospective Study (Baghurst, Tong, Woodward, & McMichael, 1992), The Dunedin Multidisciplinary Health and Development Study (McGee & Stanton, 1994), The Christchurch Child Development Study (Fergusson & Lloyd, 1991) and, more recently, the Scandinavian Prospective Multicenter Study (Trasti, Jacobsen, & Bakketeig, 1999), The Maternal Health and Child Development project (Cornelius, Ryan, Day, Goldschmidt, & Willford, 2001), The Birmingham Anti-smoking

During Pregnancy Experimental Study (MacArthur, Knox, & Lancashire, 2001), The Michigan Low Birth-weight Study (Breslau, Paneth, Lucia, & Paneth-Pollak, 2005), The US National Longitudinal Survey of Youth (Batty, Der, & Deary 2006), The Swedish Medical Registry Study (Lambe, Hultman, Torrang, MacCabe, & Cnattingius, 2006) and the Menorca Birth Cohort Study (Julvez, Ribas-Fitó, Torrent, Forns, Garcia-Esteban & Sunyer, 2007). A summary of the studies is obtained in Table 1. Overall, the results of these studies have been equivocal.

The British NCDS study included 16,000 children born in Britain in 1958 and evaluated the associations of maternal cigarette smoking with their cognition; these children were followed from birth to ages 7, 11 and 16 years old (Lassen & Oei, 1998). At the age of 7 years, Davie, Butler, & Goldstein (1972) reported significant differences in reading and social adjustment between exposed and non-exposed children; these differences were present after adjusting for several confounders such as maternal age, sex, birth weight, paternal occupation status and having younger siblings. At 11 years, exposed children were still delayed on measures of general ability, reading and mathematical achievement (Butler & Goldstein, 1973). At 16 years, Fogelman (1980) noted a slight association between maternal smoking during pregnancy and reading and mathematical skills.

Similar results were obtained from the Ottawa Perinatal Prospective Study. In a sample of 84 children aged 13 months, Gusella and Fried (1983) found that verbal comprehension was negatively associated with maternal smoking during pregnancy, but the association was significant only after adjusting for paternal education. In a later publication, after adjusting for potential confounders, Fried and Watkinson (1990) reported poorer language development and lower cognitive scores of exposed vs. non-exposed children at the age of 3 and 4 years (n=133 and n=130, respectively). At 5 (n=135) and 6 years (n=137), maternal smoking during pregnancy was still associated with lower language and cognitive scores (Fried, O'Connell, Watkinson, 1992). Between the age of 9 and 12 (n=146), maternal smoking during pregnancy was associated with overall lower scores on visuo-perceptual functioning as assessed by the four subtests comprising the Perceptual Organization Index of WISC-III (Picture Completion, Picture Arrangement, Object Assembly, and Block Design).

Table 1: Summary of 15 studies on maternal cigarette smoking associations with offspring's cognitive development

Study	N	Age (yrs)	Covariates	Cognitive Tests	Results
The American National Collaborative Perinatal Project					
Hardy & Mellits (1972)	88	4 & 7	Nonsmoking controls	Tests of IQ, concept formation (4 yrs), academic achievement.	No differences.
National child Development Study (NCDS)					
Davie, Butler & Goldstein (1972)	16,000	7	Birth weight & gestation, age, sex, maternal height & parity, paternal occupational status, number of younger siblings	Tests of general ability, mathematics, & reading.	Differences in reading achievement & social adjustment.
Butler & Goldstein (1973)	16,000	11	Birth weight & gestation, age, sex, maternal height & parity, paternal occupational status, number of younger siblings	Tests of general ability, mathematics, & reading.	Delay in general ability, reading achievement, and mathematical achievements.
Fogelman (1980)	16,000	15	Birth weight & gestation, age, sex, maternal height & parity, paternal occupational status, number of younger siblings	Tests of general ability, mathematics, & reading.	Differences in reading and mathematical skills.
Ottawa Perinatal Prospective Study					
Gusella & Fried (1983)	84	13 (mon)	Father's education	Bayley MDI	Differences in verbal comprehension

Fried & Watkinson (1990)	263	3 & 4	Demographic, obstetric information	McCarthy Scales of Children's Abilities, Reynelle Developmental Language scales, tactile form recognition test, Pegboard.	Differences in language development and cognition
Fried, O'Connell, & Watkinson (1992)	272	5 & 6	Mother's age at delivery, parental education, home environment	McCarthy Scales of Children's Abilities, Reynelle Developmental Language scales, tactile form recognition test, Pegboard.	Differences in cognitive and receptive language scores
Fried & Watkinson (2000)	146	9- 12	Parental education, maternal drug use, prenatal passive smoke exposure, gender of offspring, home environment, socioeconomic status, environmental tobacco smoke exposure of the child.	Perceptual Organization Index of WISC-III	Differences in visuo-perceptual functioning
Finnish Cohort Study					
Rantakallio (1983)	3544	14	Maternal education, age, social class, sex of child, number of siblings, & paternal smoking.	School Achievement Mental Retardation (MR)	Differences in academic performance
Maryland Cohort					
Sexton, Fox & Hebel (1990)	364	3	Maternal age, height, parity, race, education, source of obstetric care, household income, alcohol, caffeine consumption, number of adults & children in the household, pre-pregnancy weight, & maternal	McCarthy Scales of Children's Abilities & the Minnesota Child Development Inventory.	Differences in the General Cognitive Index (GCI)

The Christchurch Child Development Study					
Fergusson & Lloyd (1991)	1,265	8- 12	Environmental covariates of smoking	WISC-R at 8; Burt reading test at 8, 10, 12; Progressive Tests of math (age 11) & reading comprehension (age 12)	No differences
The Post Pirie Prospective Study					
Baghurst, Tong, Woodward, & McMichael (1992)	548	0- 4	Socioeconomic status, quality of home environment, & mother's intelligence.	Bayley Scales (2 yrs) McCarthy Scales (4 yrs).	No differences
Dunedin Multidisciplinary Health and Development Study – New Zealand					
McGee & Stanton (1994)	765	3-9	SES, marital status, maternal age, maternal mental ability, maternal neuroticism, perinatal complications, maternal childrearing attitudes, maternal rejection of child, maternal over protectiveness, early maternal-child separations, birth weight, head circumference, length, neurological signs in newborn period, & other newborn complications; child health status	PPVT & Reynell Developmental Language Scales (RDSL)—age 3; Stanford Binet & RDSL comprehension & Expressive language (age 5); WISC-R & BurtWord Recognitions Test, age 9	No differences

The Scandinavian prospective multicenter study					
Trasti, Jacobsen & Bakketeig (1999)	745	13 (months) & 5	Maternal education, Raven score, breastfeeding.	Bayley Scales of Infant Development (13 mths); Wechsler Preschool & Primary Scales of Intelligence Revised (WPPSI-R) (5 yrs); Peabody Developmental Motor Scales (PDMS) (5yrs).	No differences
The Maternal Health and Child Development project					
Cornelius, Ryan, Day, Goldschmidt, Willford (2001)	593	10	Maternal socio-demographic & psychosocial characteristics, prenatal & current maternal substance use, & child characteristics (age, gender, IQ, illnesses, birth weight etc.)	Wide Range Assessment of Memory & Learning-Screening (WRAML-S); Wisconsin Card Sorting; Stroop Interference Test; Trail Making Test; Pediatric Assessment of Cognitive Efficiency (PACE); Grooved Pegboard	Differences in learning and memory.
The Birmingham anti-smoking during pregnancy experimental study					
MacArthur, Knox, & Lancashire (2001)	1,218	9	Sex, Mother height,Total IQ, CSE, GCSE, Higher qual., Parity Mother age, Child's height at 9.4 years, Birth weight, Chronic child illness.	British Ability Scales	No differences
The Michigan low-birthweight study					
Breslau, Paneth, Lucia & Paneth-Pollak (2005)	713	6, 11, 17	Maternal IQ & education.	WISC-R (6 & 11 yrs); WAIS-III (17 yrs).	No differences.

The US National Longitudinal Survey of Youth					
Batty, Der & Deary (2006)	5578	14 – 21	Mothers IQ, race, infant feeding, gestational age, birth weight; alcohol consumption, drug use during pregnancy, live with spouse or partner, quality of care-giving of the parent(s), maternal education.	Peabody Individual Achievement Test (PIAT)	No differences
A Swedish medical registry study					
Lambe, Hultman, Torrang, MacCabe, & Cnattingius, 2006	400.000	15	Maternal characteristics, maternal smoking compared with no tobacco use during pregnancy, sex, birth weight, birth length, head circumference, gestational age, & Apgar score at 5 minutes.	School performance was assessed by a grade-point summary score	Associations between maternal smoking and poor cognitive function in the offspring may not be causal due to unmeasured characteristics.
Menorca Birth Cohort Study					
Julvez, Ribas-Fitó, Torrent, Forns, Garcia-Esteban & Sunyer (2007)	420	4	home location, maternal alcohol consumption, mother's social class and level of education, parity, marital status, father's education, child's gender, birth weight & height, breastfeeding duration, passive smoking, school season, age during test administration and evaluator (psychologist).	McCarthy Scales of Children's Abilities (MCSA)	Lower McCarthy's global cognitive scores in the 4-year-old offspring of mothers who reported smoking at least one

The Finnish cohort study and the Maryland cohort study found significant negative associations of maternal smoking during pregnancy with academic achievement and General Cognitive Index (Rantakallio, 1983, n=1,819 14-year-olds; Sexton, Fox, & Hebel, 1990, n=364, three-year-olds). A more recent study by Cornelius, Ryan, Day, Goldschmidt, and Willford (2001; n=593, 10 years old) found that after controlling statistically for other prenatal substance use, current tobacco, other substance use variables, and multiple socio-demographic covariates, prenatal tobacco exposure was significantly associated with deficits in learning and memory. In 2006, Lambe, Hultman, Torrang, MacCabe, and Cnattingius reported that in a model adjusted for maternal characteristics, maternal smoking compared with no tobacco use during pregnancy was associated with an increased risk of poor scholastic achievement (n>400.000, 15 yrs old). These risks remained unchanged when they also adjusted for smoking-related pregnancy outcomes such as foetal growth restriction and preterm birth. Nonetheless, these authors concluded that the association between maternal smoking during pregnancy and school achievement might reflect the influence of unmeasured characteristics that differ between smokers and non-smokers.

On the other hand, in the New Zealand Christchurch Child Development Study of maternal smoking during pregnancy in children from birth to 12 years (n=1,265), Fergusson and Lloyd (1991) found no significant association between maternal smoking during pregnancy and children's cognitive abilities after adjusting for potential confounders. Similar results were reported from the Port Pirie Prospective study of 548 children followed from birth until 4 years of age. After adjusting for confounders, differences between exposed and non-exposed children were no longer statistically significant (Baghurst, Tong, Woodward, & McMichael, 1992). MacArthur, Knox, & Lancashire (2001) compared long-term outcomes of maternal smoking during pregnancy on Intelligence Quotients (IQ) of 1,218 9-year old children. They concluded that any cognitive associations demonstrated in early life seem to be overcome in later childhood, with no evidence of direct long-term associations on growth or cognitive functioning.

Differences in social and environmental factors between smokers and non-smokers are key potential confounders when examining the relationship between maternal smoking and neuropsychological outcomes in childhood and adolescence (Baghurst, Tong, Woodward, &

McMichael, 1992). Inconsistencies reported in the literature may in part be attributable to these confounders. Furthermore, one cannot discard the possibility that cognitive differences between children who were and were not exposed to maternal cigarette smoking during pregnancy may be associated in part or whole with genetic/familial factors that were not measured in the studies (Sexton, Fox, & Hebel, 1990). Although it is important to take potential confounding variables into account (e.g., maternal and paternal education, maternal age at birth, nutrition, or parenting skills), this may not be sufficient because children's physical and cognitive development are determined by a largely unknown combination of environmental factors, genes, and the interactions between these two.

The present chapter describes a rich neuropsychological dataset obtained in the Saguenay Youth Study (SYS). The SYS is a retrospective cross-sectional study of the effects of maternal cigarette smoking during pregnancy on brain and cognition, and cardiovascular and metabolic health during adolescence. The SYS is carried out in the Saguenay Lac-Saint-Jean region in Quebec, Canada, in a French-Canadian population. The non-exposed adolescents (controls) were matched to the exposed ones (cases) according to the level of maternal education and school attended, thus minimizing differences between cases and controls in the SES of the families. The fact that the sample comes for a relatively geographically isolated population increases its genetic and environmental/cultural homogeneity and, therefore, decreases the likelihood of major systematic differences between the exposed and non-exposed individuals in these two domains.

2.2. Methodology

2.2.1. Design

The Saguenay Youth Study (SYS) was designed to appraise long-term outcomes of prenatal exposure to maternal cigarette smoking in adolescence and to evaluate how such an adverse intrauterine environment relates with the individual's development. The design of the SYS has several features: (i) family-based (sib-ship) design where children with only one or more siblings and with both biological parents are included; (ii) cross-sectional design with participants ranging between the ages of 12 and 18 years, and equal proportion of exposed (*n=500*) and non-exposed (*n=500*) adolescents of both sexes; (iii) retrospective-cohort design

where in utero exposure is assessed retrospectively; and (iv) quantitative assessment of brain and behaviour as well as cardiovascular and metabolic phenotypes.

2.2.2. Setting and Participants

Participants were recruited from a relatively geographically isolated population living in the Saguenay-Lac-Saint-Jean region of Quebec, Canada. Because of the relatively high-birth rates and low immigration into the SLSJ region in the past two centuries, the population represents one of the largest population isolates in North America with almost 300,000 inhabitants. As a consequence, the prevalence of several recessive disorders is higher in the SLSJ region than in other populations indicating that genetic heterogeneity is reduced in the SLSJ population (Pausova, Paus, Abrahamowicz, Almerigi, Arbour, Bernard et al., 2007).

Selection Criteria

Participants were 503 adolescents between the ages of 12 to 18 years. Selection criteria for the exposed participants are: (i) age 12-18 years; (ii) one or more siblings in the same age group; (iii) maternal and paternal grandparents of French-Canadian ancestry; (iv) positive history of maternal cigarette smoking (>1 cigarette/day in the 2^{nd} trimester of pregnancy). The main exclusion criteria for all participants, exposed and non-exposed, are: (i) positive history of alcohol abuse during pregnancy; (ii) positive medical history for meningitis, malignancy, and heart disease requiring heart surgery; (iii) severe mental illness (e.g. autism, schizophrenia) or mental retardation (IQ<70); (iv) premature birth (<35 weeks); and (v) MRI contraindications. The non-exposed adolescents were matched to the exposed ones based on the level of maternal education and school attended. Mothers of non-exposed adolescents should have had negative history of maternal cigarette smoking during pregnancy and during the 12-month period preceding the pregnancy.

Recruitment

Adolescents were recruited through regional high schools. The research team visited individual classrooms and presented the project, after briefing of the teachers. Before the visit, a letter was sent to all parents of students attending the visited school, containing an information brochure, a letter from the principal, a consent form for a telephone interview and a self-addressed and stamped response card. The parents were asked to send the response card

back to the team, indicating if they want to participate in the project or not, how many children between the ages of 12-18 years they have and to provide a home phone number for a follow-up call by a research nurse. The follow-up interview covers the demographics of the parents, pregnancy (smoking, drinking, drug use, medical complications) and medical history of the children and parents. If the family is eligible for participating, a home visit is being set up. Written consent is given from both parents and adolescents (assent form).

2.2.3. Measurements

Data collection started with telephone interview, continued with a home visit, neuropsychological testing during a laboratory visit, and a hospital visit for the magnetic resonance imaging (MRI).

2.2.3.1. Cognitive Measures

All testing was done in French.

Intelligence

The Wechsler Intelligence Scale for Children III (WISC-III; Wechsler, 1991) was used to measure intelligence (IQ). The WISC-III consists of 12 subtests, 6 measuring verbal IQ and 6 measuring performance IQ. The scores obtained on the verbal and performance tests provide, respectively, Verbal and Performance IQ, with the combined score indicating Full Scale IQ. The WISC-III has four indices: Verbal Comprehension, Perceptual Organization, Freedom from Distractibility and Processing Speed.

Academic achievement

Woodcock-Johnson III (WJ-III) provides a comprehensive assessment of academic achievement (Woodcock, McGrew, & Mather, 2001). We used the following three subtests: Math Calculation Skills, Math Fluency and Reading Comprehension.

Spelling was measured using Orthographe d'usage (premie`re a` sixie`me anne´e; Poulin, 1982).

Short-term and verbal working memory

The Digit Span subtest of WISC-III was analysed separately; it consists of two tasks, digits forward and digits backward (Wechsler, 1991). Digits forward measures auditory attention and short-term memory whereas digits backwards can be used as a measure of verbal working memory.

Long-term and immediate memory for auditory/verbal material

The Stories Immediate, Stories Delayed and Stories Recognition subtests of Children's Memory Scale (CMS; Cohen, 1997) were used to measure immediate and long-term memory for auditory/verbal material.

Long-term and immediate memory for visual/non-verbal material

The Dot Locations Learning, Total Score and Long Delay subtests from CMS were used to assess immediate and delayed visual memory (Cohen, 1997). For this test, a pattern with blue dots placed in eight locations is shown to the adolescent for 5 s and then the adolescent is given eight chips to place on a blank 4"4 grid in the same locations as had been shown. This procedure is repeated three times (Dot Location Learning). Immediately after this, the examiner presents the adolescent with a different pattern with red dots again for 5 s and the adolescent must then places the chips in the same locations. Following this interference trial, the adolescent is asked to recall the first pattern, i.e. the one they did three times. A Total Raw Score is computed from the sum of the correct responses on the first three trials and the short delay score. After a period of almost 40 min, the adolescent is again presented the blank 4"4 grid and asked to recall the location of the first pattern (long delay).

Executive function

Working memory

Self Ordered Pointing Task (Petrides, & Milner, 1982) was used to measure participants' ability to manipulate items in working memory. The task consists of a set of 12 stimulus items (abstract pictures), which are presented on 12 different pages, their order changing quasi randomly each time the subject turns a page. Participants have to point to a different item on each page without pointing to the same picture twice. Successful performance

involves working memory and challenges the participants to organize, maintain and monitor their responses.

Selective attention

Ruff 2-&-7 Selective Attention Test was used to measure two types of attention: sustained and selective (Ruff & Allen, 1996). The task provides information on the power of automatic vs controlled information processing. In the automatic condition, capital letters are intermixed with the digits 2 and 7. In the controlled condition, the digits 2 and 7 are intermixed with other digits. In both conditions (10 blocks per condition) the adolescent has to read through each line and cross out 2 s and 7 s within a time limit of 15 s per block (each block consist of three lines with 50 items per line).

Interference

Stroop Color-Word Test (Stroop, 1935) consists of three tasks: (i) reading colour words printed in black ink (in a time limit of 45 s); (ii) naming as many colours of rectangular blots printed in green, red and blue (45 s) and (iii) naming the colour of the ink in which the word is printed (45 s). The Stroop interference score is calculated using the following formula: $(W*C)/(W+C)=CW^1$; $CW-CW^1$= Interference, where C is the number of colours named, W is the number of words read and CW is the number of correct colour-word targets named (Golden, 1978; Adleman Menon, Blassey, White, Warsofsky, Glover, & Reiss, 2002).

Cognitive flexibility—verbal fluency

This task is based on the NEPSY Verbal Fluency test (Korkman, Kirk, & Kemp, 1998). In the semantic condition, adolescents have to name animals and food/drinks each within 1-min time limit. In the phonemic part, adolescents have to name as many words as possible starting with letters F, A and S, each within 1 min time limit.

Fine motor skills

The Grooved Pegboard Test was used to measure finger dexterity. Participants are required to fit key shaped pegs into similarly shaped holes on a 4"4 inch board beginning at the left side with the right hand and at the right side with the left hand. Participants are urged to

complete the task as rapidly as possible. Two trials for each hand were utilized. The score obtained was the mean time required to complete the task with each hand.

Puberty Development Scale

All participants filled out the Puberty Development Scale (PDS; Petersen, Crockett, Richards, & Boxer, 1988), which is an eight-item self-report measure of physical development based on the Tanner stages with separate forms for males and females (Marshall & Tanner, 1969, 1970). For this scale, there are five categories of pubertal status: (1) prepubertal, (2) beginning pubertal, (3) midpubertal, (4) advanced pubertal, (5) postpubertal. Participants answer questions about their growth in stature and pubic hair, as well as menarche in females and voice changes in males.

2.2.3.2. Other Measures

A number of pregnancy/birth-related and SES measures, such as birth weight, maternal age at delivery, alcohol during pregnancy, number of pregnancies, breastfeeding and in utero exposure to second-hand smoking, as well as household income and parental education, were assessed using a set of questionnaires and by a structured telephone interview with the biological mother in the majority of participants (Pausova et al., 2007). A number of self-attributes (e.g. parental monitoring and warmth) and behaviours (e.g. parental antisocial behaviour) were evaluated using the following two instruments answered, respectively, by the adolescents and their biological parents, namely the Positive Youth Development (based on the 5C's model: Competence, Confidence, Character, social Connection, and Caring; Lerner, Lerner, Almerigi, Theokas, Phelps, Gestdotir et al., 2005) and a Mental Health & Anti-social Behaviour questionnaire (Huijbregts, Séguin, Zoccolillo, Boivin & Tremblay, 2008).

2.2.4. Statistical Methods

In all analyses, we used raw scores instead of standard scores; the latter are used to express the performance of an individual among his/her peers of the same age. Outliers, defined as values three standard deviations from the mean, were excluded. Outliers can have adverse effects on statistical analyses; they increase error variance and reduce power of statistical tests, they can decrease normality if non-randomly distributed thus altering the odds of making both Type I and Type II errors, and they can influence estimates that may be of

substantive interest (Osborne & Overbay, 2004). For identifying an outlier, simple rules of thumb, such as data points three or more standard deviations from the mean, are usually good starting points (Osborne & Overbay, 2004). Instead of removing an outlier, transformation was selected, if possible, in order to keep the individual in the dataset and to minimize consequence of his/her removal to statistical inference.

Statistical analyses were carried out using JMP (version 1.5.2) and SPSS 13 (for Mac). To obtain a global view of our data, a factor analysis was conducted that included raw scores from the 33 subtests comprising our neuropsychological battery. Due to the exploratory nature of the neuropsychological dataset a confirmatory factor analysis could not be performed. The data were analyzed by means of a principal component analysis with varimax rotation. Varimax rotation is an orthogonal type of rotation that tries to load smaller number of variables highly onto each factor resulting in more interpretable clusters of factors (Field, 2005). Varimax rotation was used as this is the most common rotation used in psychological research and mostly recommended by text books. The various indicators of factorability were good and the residuals indicate that the solution was a good one. Kaiser's criterion was used to identify the number of derived components. Kaiser's criterion for factor selection recommends retaining all factors with eigenvalues greater that 1 (Kaiser, 1960). The criterion is based on the fact that eigenvalues represent the amount of variation explained by a factor and that an eigenvalue of 1 represents a substantial amount of variation. Kaiser's criterion is accurate when the number of variables is less than 30 and the resulting communalities (after extraction) are all greater than .7; Kaiser's criterion is also accurate when the sample size exceeds 250 and the average communality is greater or equal to .6 (Field, 2005). Here, the sample size is 503 and the average communality is .643. Another criterion for selecting factors or components is by examining the *scree* plot. Seven components with an eigenvalue greater than 1.0 were found; the scree plot also indicated seven components. The seven components can be thought of as representing the following cognitive abilities: Component 1- verbal abilities, Component 2 - processing speed, Component 3 - verbal memory, Component 4 - visuo-spatial skills, Component 5 - visual memory, Component 6 - resistance to interference and Component 7 - motor dexterity (Table 2).

Table 2: Seven components as revealed by Principal Component Analysis: Classification of subtests loadings

Components	Subtests Included
FA1: Verbal Abilities	WISC-III Information WISC-III Vocabulary WISC-III Comprehension WISC-III Arithmetic WISC-III Digit Span WISC-III Similarities WJ Math WJ Reading Comprehension Spelling Fluency Phonemic Fluency Semantic
FA2: Processing Speed	Ruff Total Letters Ruff Total Digits Stroop Colors Named Stroop Words Reading WISC-III Coding WISC-III Symbol Search WJ Math Fluency
FA3: Verbal Memory	CMS Stories Immediate CMS Stories Delayed CMS Stories Recognition
FA4: Visuo-Spatial Skills	WISC-III Picture Completion WISC-III Block Design WISC-III Object Assembly
FA5: Visual Memory	CMS Dot Locations Learning CMS Dot Locations Total Score CMS Dot Locations Long Delay
FA6: Resistance to Interference	Stroop Color-Words Named Stroop Interference
FA7: Motor Dexterity	Pegboard Mean Time Left Pegboard Mean Time Right

Before extracting factor scores for each component, we chose those subtests that had scores higher than .5 (31 subtests, Table 2). Factor scores were extracted using the *Regression* method to allow correlations between factor scores (Field, 2005). To evaluate the association of exposure status with cognitive abilities, statistical analyses began by assessing the association between each potential confounder and maternal cigarette smoking during

pregnancy. Afterwards, the relationship between offspring's cognitive abilities and maternal smoking during pregnancy in an unadjusted analysis was performed. Finally, this relationship was examined again after adjusting for a number of confounders. Potential confounders were grouped into three models: Model A - SES; Model B - pregnancy and postnatal environment; and Model C - parenting. By multiple regressions, Model A was adjusted first, then more covariates were added in the regression analyses and adjusted for Model B and finally added and adjusted for Model C (Table 3).

To evaluate the effect of Age, linear regressions with Age as the predictor were performed. The Age by Sex interaction was tested by performing linear regressions with Age separately for males and females and then comparing the beta confidence intervals of the slopes of the (male vs. female) regression lines; if the confidence intervals did not overlap, the slopes were deemed to be significantly different from one another thus indicating significant Age by Sex interaction. To examine the main effect of Sex, residuals from the first set of regressions were used (i.e. after removing the effect of Age) and one-way ANOVAs with Sex as the independent variable and the age-adjusted residuals of a given factor score as the dependent variable were carried out.

Table 3: Potential Confounders separated by exposure status for Model A, B and C

		Exposed	Non Exposed	p
MODEL A – SES				
Household Income	Mean ± SD (n)	53050.8 ± 24320.1 (118)	56794.9 ± 21779.3 (117)	.2152
Mother Education	Mean ± SD (n)	4.87 ± 1.58 (116)	4.69 ± 1.39 (118)	.3459
Father Education	Mean ± SD (n)	**4.80 ± 1.63 (105)**	**5.25 ±1.70 (115)**	**.0512**
MODEL B - Pregnancy & Postnatal				
Alcohol during Pregnancy	Frequency of Yes (n)	**76 (237)**	**47 (263)**	**.0002**
Number of pregnancies	Mean ± SD (n)	3.25 ± 1.45 (119)	3.33 ± 1.16 (119)	.6237
2ndHandSmokingDuringPregnancy	Frequency of Father (n)	**154 (237)**	**79 (262)**	**<.0001**
Maternal Age at Delivery	Mean ± SD (n)	25.50 ± 3.59 (118)	26.22 ± 3.60 (117)	.1303
Birth Weight	Mean ± SD (n)	**3233.04 ± 462.512 (238)**	**3529.47 ± 469.549 (263)**	**<.0001**
Breast Feeding	Frequency of Yes (n)	**86 (236)**	**150 (265)**	**<.0001**
Birth Order	Mean ± SD (n)	**1.74 ± 0.74 (236)**	**2.02 ± 0.88 (261)**	**.0002**
MODELC - Mother/Father - Parenting				
Father Warmth	Mean ± SD (n)	27.44 ± 6.80 (113)	27.50 ± 7.62 (115)	.9486
Mother Warmth	Mean ± SD (n)	30.01 ± 6.80 (118)	29.45 ± 7.40 (117)	.5439
Parent Monitoring	Mean ± SD (n)	2.87 ± 0.73 (119)	2.87 ± 0.84 (119)	.9968
Mother's Antisocial Behaviour	Mean ± SD (n)	**0.30 ± 0.54 (119)**	**0.16 ± 0.45 (119)**	**.0404**
Mother's Antisocial Behaviour Adult	Mean ± SD (n)	0.03 ± 0.25 (119)	0.03 ± 0.22 (119)	.5892
Father's Antisocial Behaviour	Mean ± SD (n)	**0.69 ± 0.91 (115)**	**0.40 ± 0.81 (117)**	**.0104**
Father's Antisocial Behaviour Adult	Mean ± SD (n)	0.24 ± 0.57 (115)	0.17 ± 0.44 (117)	.2976

2.3. Results

2.3.1. Sample Characteristics

Table 4, separately for each sex, describes the adolescents' age and Tanner stage of pubertal development, as well as the family SES, as indicated by household income and parental education. Parental education ranged from the lowest level, which is 8th grade or less, up to a doctoral degree. Male and female adolescents did not differ in their age or in their family's SES. Male and female adolescents differed significantly in Tanner stage ($p<.0001$). The number of participants in each Tanner stage were: a) Stage 1: 6 males and 2 females, b) Stage 2: 24 males and 4 females, c) Stage 3: 84 males and 39 females, d) Stage 4: 106 males and 138 females and e) Stage 5: 19 males and 78 females. Generally, Tanner stage increased with age ($r = .59$, $p <.0001$) and both males ($r=.61$, $p<.0001$) and females ($r=.65$, $p<.0001$) have shown this effect.

Table 4: Demographics

	Age (Years)	Tanner Stage	Father Education	Mother Education	Household Income
Males					
N	240	239	222	234	239
Mean ± SD	14.53 ± 1.85	3.45 ± 0.87	5.05 ± 1.62	4.69 ± 1.47	54916.3 ± 22935.6
Min-Max	12-18	1-5	3-10	3-10	15.000-85.000
Females					
N	263	261	246	261	257
Mean ± SD	14.62 ± 1.90	4.09 ± 0.75	5.008 ± 1.71	4.86 ± 1.48	54630.3 ± 23225.2
Min-Max	12-18	1-5	3-9	3-9	15.000-85.000

Descriptive statistics for the standardized neuropsychological measures are displayed in Table 5. Means and standard deviations for measures for which there are no norms for ages above 12 years (Verbal Fluency, Stroop Test and Self Order Pointing Test) are presented by age (Table 6, Table 7, and Table 8).

Table 5: Mean ± SD and range of standardized scores for tests with published norms for exposed and non-exposed adolescents (SS: Standardized score[1])

Test	Exposed			Non-Exposed		
	N	Mean ± SD	Min-Max	N	Mean ± SD	Min-Max
WISC III Verbal IQ (SS)	235	103.98 ± 13.38	74-142	265	103.49 ± 12.17	67-138
WISC III Performance IQ (SS)	235	106.35 ± 14.09	60-146	265	105.58 ± 12.99	73-139
WISC III Full Scale IQ SS)	235	105.46 ± 13.39	65-138	265	104.72 ± 11.81	71-135
WISC III Verbal Comprehension Index (SS)	235	103.92 ± 12.92	73-140	265	103.71 ± 11.77	67-137
WISC III Perceptual Organization Index (SS)	235	106.82 ± 13.75	56-142	265	106.27 ± 12.98	73-138
WISC III Freedom of Distractibility (SS)	235	100.17 ± 14.47	61-142	265	98.34 ± 12.95	67-137
WISC III Processing Speed (SS)	235	110.71 ± 14.22	75-143	265	108.78 ± 14.59	64-143
CMS Dot Locations Learning (Scaled Score)	235	10.6 ± 2.97	1-14	264	10.43 ± 2.86	1-14
CMS Dot Location Total Score (Scaled Score)	235	10.74 ± 2.84	1-14	264	10.68 ± 2.61	1-14
CMS Dot Location Long Delay (Scaled Score)	235	10.65 ± 2.82	1-13	264	11.07 ± 2.47	3-13
CMS Stories Immediate (Scaled Score)	235	10.43 ± 3.27	3-19	264	10.25 ± 3.21	2-19
CMS Stories Delayed (Scaled Score)	235	10.37 ± 3.21	3-19	264	10.04 ± 3.23	1-18
CMS Stories Recognition (Scaled Score)	235	8.87 ± 3.16	0-18	264	8.93 ± 2.93	1-17
WJ Math (Raw Score)	235	26.59 ± 4.57	14-42	265	26.53 ± 4.31	14-41
WJ Math Fluency (Raw Score)	235	90.37 ± 20.37	47-127	265	88.28 ± 21.2	37-127
WJ Reading Comprehension (Raw Score)	235	33.6 ± 3.52	22-42	265	33.53 ± 3.69	21-43
Spelling (Raw Score)	235	41.8 ± 4.15	0-45	265	41.3 ± 3.76	27-45
Ruff 2 & 7 Selective Attention Test- Letters (Hits)	235	151.91 ± 28.03	91-226	265	146.71 ± 28.91	73-217
Ruff 2 & 7 Selective Attention Test- Digits (Hits)	235	116.15 ± 20.08	68-178	265	113.65 ± 20.59	66-182
Semantic Fluency (Total Number)	235	40.37 ± 10.47	14-96	265	39.46 ± 9.41	17-67
Phonemic Fluency (Total Number)	235	25.12 ± 8.82	6-50	265	23.27 ± 7.87	5-46
Overall Fluency (Ttotal Number)	235	65.5 ± 16.8	26-133	265	62.73 ± 15.36	24-106
Pegboard Mean Time Left	234	13.37 ± 2.29	9-26	265	13.35 ± 2.16	8.5-20
Pegboard Mean Time Right	234	11.89 ± 1.91	8-20	265	12.2 ± 1.69	9-18.5
Pegboard Dropped All	235	1.77 ± 1.69	0-8	265	1.83 ± 1.77	0-12
Stroop Naming Colors (Total Number)	234	71.54 ± 12.07	34-119	265	69.11 ± 12.21	39-109
Stroop Words Reading (Total Number)	235	95.72 ± 14.26	44-136	265	93 ± 14.18	55-147
Stroop Color-Word Naming (Total Number)	234	42.26 ± 9.65	22-74	265	40.65 ± 9.74	14-73
Stroop Interference	234	1.53 ± 6.92	(-14.1)-31.8	265	1.23 ± 6.52	(-14.4)-21.5
Sum Self Order Pointing Task	231	6.05 ± 3.26	0-23	259	5.93 ± 3.59	0-33

[1] Standardized scores of tests with published norms were used only for comparison purposes when illustrating demographics; Raw scores were used when running statistical tests or when published norms of a test are not given thus extraction of standardized scores is not possible. For Tables 6-8 raw scores are used.

Table 6: Means ± SD by age in years for correct number of words on Verbal Fluency task

Age	N	Semantic	Phonemic	Total
12	89	35.28 (8.51)	20.55 (6.91)	55.83 (13.77)
13	77	37.32 (8.71)	20.79 (7.21)	58.11 (14.04)
14	97	40.02 (8.37)	23.14 (6.89)	63.16 (13.14)
15	65	41.18 (9.55)	24.18 (8.16)	65.36 (14.84)
16	78	42.61 (10.48)	27.30 (8.46)	69.92 (16.52)
17	54	43.14 (11.80)	28 (8.44)	71.14 (17.39)
18	40	42.97 (10.78)	29.55 (9.92)	72.52 (18.26)

Table 7: Means±SD by age in years for Stroop Test

Age	N	Naming Colors	Reading Words	Color-Word Naming	Interference
12	89	61.59 (8.82)	84.70 (10.47)	34.28 (7.08)	-1.15 (5.79)
13	77	66.40 (11.79)	89.06(14.09)	38.71 (9.96)	0.93 (8.04)
14	97	69.54 (11.37)	93.23 (12.90)	41.25 (9.17)	1.59 (6.52)
15	65	73 (11.69)	98.23 (11.93)	44.13 (9.33)	2.44 (6.33)
16	78	75.76 (12.07)	98.5 (13.67)	43.83 (8.09)	1.32 (5.56)
17	53	76.11 (11.59)	102.28 (13.83)	46.66 (9.96)	3.26 (7.35)
18	40	75.67 (9.54)	103.07 (14.45)	46.7 (8.25)	3.16 (6.60)

Table 8: Means ± SD by age in years for errors on Self Ordered Pointing Test (SOPT)

Age	N	SOPT Trial 1 (Errors)	SOPT Trial 2 (Errors)	SOPT Trial 3 (Errors)	Sum SOPT (Errors)
12	84	2.35 (1.89)	2.23 (1.91)	2.32 (1.82)	7 (4.82)
13	75	2.21 (1.33)	2.29 (1.41)	2.2 (1.55)	6.61 (3.49)
14	95	1.90 (0.97)	2 (1.35)	1.84 (1.16)	5.68 (2.79)
15	65	1.81 (1.08)	2.12 (1.23)	1.93 (1.29)	5.96 (2.70)
16	77	1.94 (1.15)	1.79 (1.20)	1.87 (1.54)	5.53 (3.25)
17	55	1.85 (1.22)	1.74 (1.22)	1.81 (1.37)	5.62 (2.83)
18	40	1.75 (1.31)	1.62 (1.14)	1.5 (1.17)	4.87 (2.97)

2.3.2. Factor Analysis of Cognitive Abilities

As forementioned, indicators of factorability and residuals indicate that the solution was a good one. Kaiser-Meyer-Olkin Measure of Sampling Adequacy was .843 showing the amount of variance within the data that could be explained by factors and Bartlett's Test of Sphericity had a p-value <.05 meaning that the data is probably factorable. According to the total variance explained, seven components have eigenvalues larger than 1.0 and all together explain 67.1% of the variance. Table 2 shows which tests loaded on each component.

2.3.3. Exposure Status

The relationships between prenatal maternal cigarette smoking and potential confounders are presented in Table 3. For covariates that are continuous, the mean score (±SD) is listed, while for categorical covariates, the frequency is shown. Eight out of 17 potential confounders were found to be significantly different between exposed and non-exposed adolescents. Socioeconomic status did not differ among exposed and non-exposed families, thus validating the case-control matching procedure (maternal education, school attended) used at enrolment. In comparison with the mothers of non-exposed adolescents, mothers who smoked during pregnancy were also more likely to consume alcohol and be exposed to second-hand smoking at home (especially from the father). The exposed adolescents were more likely to be first born and to weigh less at birth than non-exposed adolescents. Postnatally, exposed adolescents were less likely to be breast-fed (Table 3). It is also worthwhile mentioning that both mothers and fathers of exposed adolescents were more likely to exhibit antisocial behaviour in their adolescence as determined by a mental health and anti-social behaviour questionnaire.

In the unadjusted analysis (only age effect was removed; see below for age adjustment), two-way ANOVAs showed no main effect of Exposure or Exposure by Sex interaction on any of the seven factors. In the adjusted analysis, only Model A revealed a significant Exposure effect on Processing Speed [$F(1,454)=3.79$, $p=.0519$; exposed adolescents performing *better* than non-exposed ones]. This effect disappeared after adjusting for Model B [$F(1,441)=0.66$, $p=.4149$], and Model C [($F(1,417)=1.60$, $p=.2056$]. One-way ANOVAs revealed no significant main effect of Exposure in any of the other six factors, when adjusting for all three models. These results are presented in Table 9, together with the effect of

Exposure on Verbal, Performance and Full-scale IQ (for comparison with other studies only; note that different WISC-III subtests were included in the factor analysis) (Table 2).

2.3.4. Age Effect

Factor scores were analyzed using linear regression with age (in months) as the predictor. Significant age effects were found on verbal abilities (r^2=.21, p<.0001), processing speed (r^2=.32, p<.0001), visuo-spatial skills (r^2=.15, p<.0001), resistance to interference (r^2=.107, p<.0001) and motor dexterity (r^2=.039, p=<.0001) but not on verbal (r^2=.006, p=.078) and visual (r^2=.003, p=.19) memory (Figure 1). Performance on these five components was increasing with age, whereas there was a plateau on both verbal and visual memory.

2.3.5. Sex Effect

After removing the effect of age, one-way ANOVAs showed that adolescent girls were better than boys in Processing Speed ($F_{(1,497)}$=19.63, p<.0001), resistance to interference ($F_{(1,495)}$=5.13, p=.02) and motor dexterity ($F_{(1,498)}$=30.73, p<.0001). Boys were better than girls on verbal ($F_{(1,498)}$=6.92, p=.0008) and visual ($F_{(1,498)}$=16.32, p<.0001) memory (Fig.1).

2.3.6. Age by Sex Interaction

As seen in Figure 1, motor dexterity was the only component with a significant Age by Sex interaction, with girls improving their performance with age (r^2=.11, p<.0001); this was not the case for boys (r^2=.001, p=.51).

Table 9: Results of adjusted analysis with potential confounders [Mean ± SD (n)]

Factors	Model A			Model B			Model C		
	Exposed	Non-Exposed	p	Exposed	Non-Exposed	p	Exposed	Non-Exposed	p
Verbal Abilities	0.01 ± 0.81(210)	0.006 ± 0.75(245)	.8680	-0.01 ± 0.79(206)	0.03 ± 0.74(236)	.4414	-0.02 ± 0.77(197)	0.04 ± 0.73(221)	.3163
Processing Sp.	**0.08 ± 0.76(209)**	**-0.06 ± 0.8(246)**	**.0519**	0.03 ± 0.76(205)	-0.02 ± 0.78(237)	.4149	0.05 ± 0.75(196)	-0.03 ± 0.77(222)	.2132
Verbal Memory	0.02 ± 0.95(209)	0.006 ± 0.92(245)	.8297	0.007 ± 0.94(205)	0.02 ± 0.906(236)	.8821	-0.01 ± 0.92(196)	0.04 ± 0.87(221)	.5025
VisuoSpatial Skil.	0.05 ± 0.83(207)	0.006 ± 0.86(245)	.5699	0.06 ± 0.81(203)	0.001 ± 0.85(236)	.4356	0.05 ± 0.81(194)	0.003 ± 0.83(221)	.5341
Visual Memory	0.08 ± 0.88(206)	0.03 ± 0.9(243)	.5917	0.09 ± 0.88(202)	0.02 ± 0.91(234)	.4738	0.086 ± 0.92(194)	0.032 ± 0.91(219)	.5516
Resistance Interf.	0.004 ± 0.86(206)	-0.05 ± 0.89(246)	.5258	0.008 ± 0.86(203)	-0.05 ± 0.89(237)	.5761	0.003 ± 0.84(194)	-0.06 ± 0.88(222)	.5060
Motor Dexterity	-0.04 ± 0.49(208)	-0.05 ± 0.46(246)	.7702	-0.03 ± 0.49(204)	-0.06 ± 0.46(237)	.4733	-0.02 ± 0.49(195)	-0.06 ± 0.46(222)	.4377
Full Scale IQ	105.6 ± 11.3(209)	104.6 ± 11.18(247)	.3588	105.3 ± 10.9(239)	104.9 ± 10.8(239)	.6660	105.1 ± 10.8(196)	105.1 ± 10.8(223)	.9999
Performance IQ	106.5 ± 12.2(209)	105.6 ± 12.8(247)	.4591	106.3 ± 12.8(205)	105.7 ± 12.4(238)	.5925	106.3 ± 11.7(196)	105.7 ± 12.4(223)	.6316
Verbal IQ	103.9 ± 11.7(209)	103.5 ± 11.09(246)	.6818	103.5 ± 11.4(205)	103.8 ± 10.9(237)	.7929	103.3 ± 11.2(196)	104.1 ± 10.7(222)	.4926

Model A - SES; Model B – SES, pregnancy and postnatal environment; and Model C – SES, pregnancy and postnatal environment, parenting.
Education was measured starting from the lowest level, which is 8th grade or less, and going up to a doctoral degree.

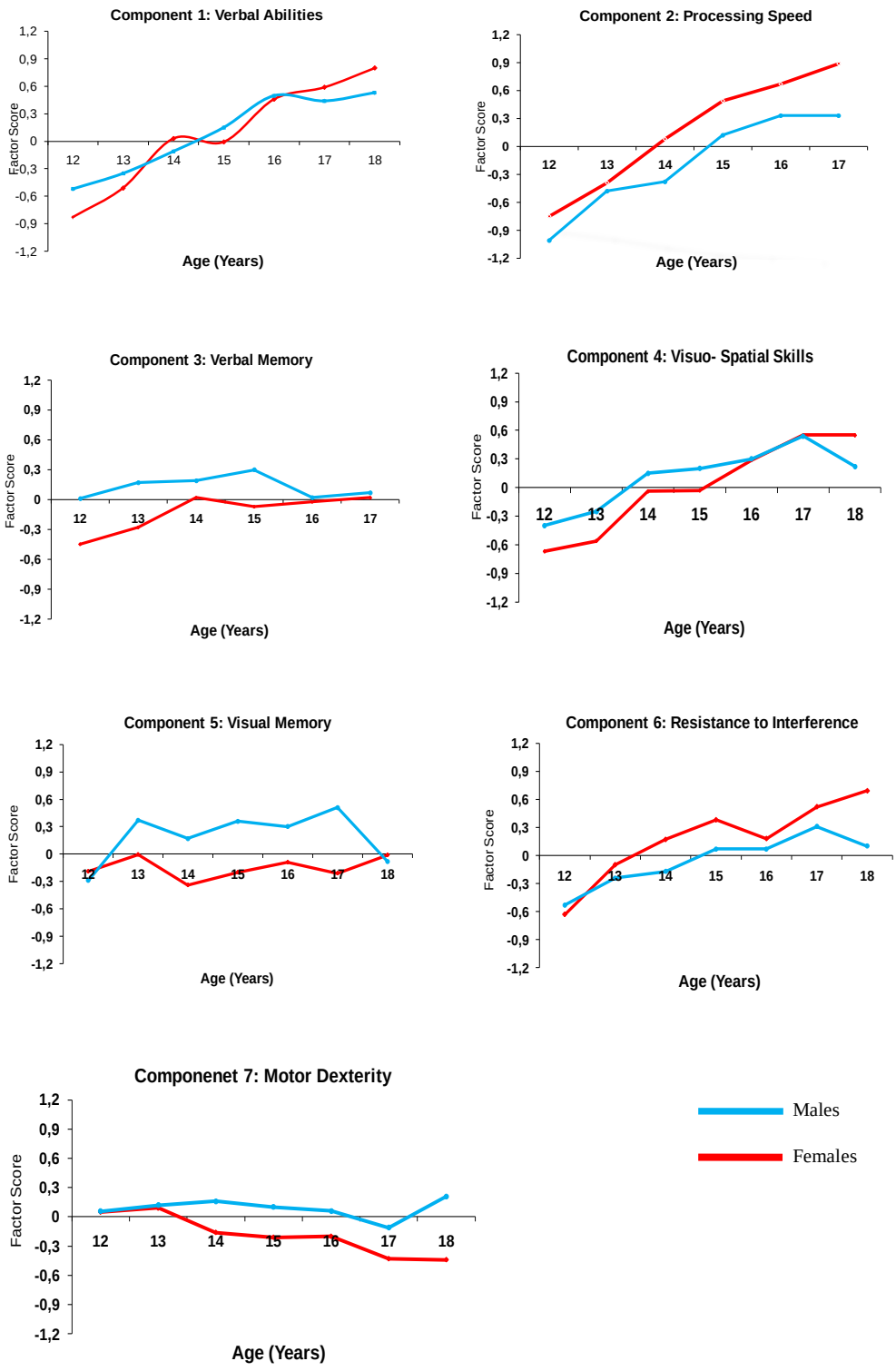


Figure 1: Age and Sex effects on the seven components. Note that, for Component 7 only, lower scores indicate better performance

2.4. Discussion

In the present study, a large sample of adolescents exposed and non-exposed to maternal cigarette smoking during pregnancy were tested in order to examine the effects of exposure, age, and sex on their cognitive abilities. To obtain a comprehensive view of the data, factor analysis was used and grouped the different neuropsychological tests into seven components: verbal and visuo-spatial skills, verbal and visual memory, processing speed, resistance to interference and motor dexterity.

2.4.1. Maternal Cigarette Smoking during Pregnancy

No associations of maternal smoking during pregnancy were found with any of the seven cognitive components or, when tested separately, on the IQ scores. Whether or not adjusting for a number of potential confounders, maternal smoking during pregnancy had no association with cognitive abilities. The one exception was the Processing Speed component that appeared to be higher in the exposed vs. non-exposed adolescents after adjusting for SES. This association disappeared after adjusting also for prenatal and postnatal environment and parenting. Further analysis revealed that the confounder that eliminated the association of exposure status with Processing Speed was Second Hand Smoking At Home. The study had matched the non-exposed to the exposed adolescents according to the level of maternal education and school attended, thus minimizing differences in parental education between the two groups. The findings suggest that controlling carefully for maternal education, a known predictor of cigarette smoking during pregnancy in the general population (Cornelius, Leech, Goldschmidt, & Day, 2000), is sufficient to eliminate possible differences between exposed and non-exposed adolescents in their cognitive abilities.

A recent study by Batty, Der, & Deary, (2006) found that maternal smoking during pregnancy was associated with lower IQ of the offspring (n=5,578; 14 to 21 years old) in unadjusted analysis, but this association was not observed when controlling for maternal education and maternal IQ. Similar results were obtained by Breslau, Paneth, Lucia, & Paneth-Pollak (2005) who concluded that offspring IQ is independent of maternal smoking when controlling for maternal characteristics such as the mother's cognitive abilities measured by IQ and education. The New Zealand cohort study (Fergusson & Lloyd, 1991), a study by McGee & Stanton (1994), the Scandinavian prospective multicenter study (Trasti, Jacobsen and

Bakketeig, 1999) and a study by McArthur, Knox, & Lancashire, (2001) obtained similar results indicating that, after statistical adjustment for confounders, no associations of maternal smoking during pregnancy with offspring's cognitive abilities remained. Fergusson and Lloyd (1991) suggest that children whose mothers smoked during pregnancy perform worse not due to possible causal effects of smoking but rather because these children tend to come from a relatively disadvantaged home environment. But, in our sample, there were no differences between the exposed and non-exposed adolescents in their (perceived) experience of parenting. In the future, a hypothesis whether individuals who differ in their genetic background, for example, in genes coding nicotinic receptors, may differ in their cognitive abilities as a function of maternal cigarette smoking during pregnancy should be taken into account.

2.4.2. Age and Sex Effects on Task Performance

The observed effects of age and sex on cognitive abilities of the 12-to-18 year old adolescents support previous findings of age-related increases in children's cognitive abilities during adolescence. Five out of seven components were found to change significantly with age: Verbal abilities, Processing Speed, Visuo-spatial skills, Motor Dexterity and Resistance to Interference. Similar results were obtained in the study of Waber, De Moor, Forbes, Almli, Botteron, Leonard, Milovan, Paus, & Rumsey (2007) where children (n=385, 5 to 17 years of age) were assessed on different neuropsychological measures including WJ-III Calculation and Passage Comprehension, WISC-III/ WASI, verbal fluency and motor dexterity. These results are further supported by previous cognitive studies of verbal fluency (Sauzèon, Lestage, Raboutet, N'Kaoua, & Claverie, 2004; *n*=140, 6 to 17 years old) and verbal working memory (Conklin, Luciana, Hooper, & Yarger, 2007; *n*=117, 9 to 17 years old). Note that the above studies included not only adolescents but also younger children; our sample is the largest to date to evaluate in a comprehensive manner age-related changes between 12 and 18 years of age. Two of the seven components, namely Verbal and Visual Memory, did not change significantly with age. Schneider (2002) reports findings from earlier studies in that a linear increase in memory performance was found from 6 to 11 years of age and that there was a plateau in performance during early adolescence. This suggests that either memory is fully developed by age 12 years and/or that high-school educational experience does not contribute to further development of this ability.

By age 12, adolescents are already members of a specialized society, namely a secondary school, where they interact with their peers and adults. Adolescents participating in this study spent four periods a day (75 minutes each) in class, starting high school at 12 years old until 17 years of age. Thus, they spend 5 hours per day in class having compulsory lessons in mathematics, French, English (as second language), history, arts and physics; and optional lessons in science and technology. Note, however, that they are examined only in mathematics, French (speaking, comprehension, writing and reading), English and physical science. It might be that these courses, especially languages, facilitate the progress of verbal abilities at a higher level than memory capacity.

A number of sex differences in cognitive abilities were also observed. Female adolescents performed better than males on Processing Speed, Resistance to Interference and Motor Dexterity whereas male adolescents were better than females on Verbal and Visual Memory components. Prior studies (Waber, De Moor, Forbes, Almli, Botteron, Leonard, Milovan, Paus, & Rumsey, 2007; Weiss, Kemmler, Deisenhammer, Fleischhacker, & Delazer, 2003; Kimura & Hampson, 1994) have shown similar results in the case of females outperforming males on processing speed, as well as on verbal fluency and spelling measures. The finding of males scoring better than females on verbal and visuospatial memory and recognition tasks are inconsistent with previous studies carried out in adults (Bleecker, Bolla-Wilson, Agnew, & Meyers, 1988; Kimura, 1996; 2002; Stumpf and Jackson, 1994) and children/adolescents (Geffen, Moar, O'hanlon, Clark, & Geffen, 1990, Kramer, Delis, Kaplan, O'Donnell, & Prifitera, 1997; Mann, Sasanuma, Sakuma, N., & Masaki, 1990). According to some authors, better memory in females may be related to their verbal abilities, indicating that females are verbalizing the material to be recalled (see Herlitz, Nilsson & Bäckman, 1997, for an overview). However, when examining sex differences on the Verbal Abilities component, we found no effect of sex. On the contrary, males had a slightly higher Verbal IQ than females ($F_{(1,498)}$=5.00, p=.02). The Verbal Abilities component consists of all verbal tests from WISC-III, as well as two other fluency tasks and three tests from WJ, which measure reading comprehension and spelling. The Verbal IQ is the index derived from WISC-III that measures a person's ability to work with abstract symbols, verbal fluency, verbal memory skills and generally the degree to which they have benefited from education (Groth-Marnat, 2000). The difference between Verbal Abilities component and Verbal IQ may lay on the fact that Verbal

Abilities component consists of a wider range of tests that have to do with verbal skill as well as more tests that have to do with education level (reading comprehension, spelling, fluency) whereas the Verbal IQ index is more centred, consists of less tests, which are question-based ("How are an apple and a banana alike?" "What does ancient mean?"). The Verbal IQ index lacks the "reading" part that perhaps points to another, but not distinct, issue of cognition. After adjusting for Verbal IQ, the effect of sex on the Verbal Memory component was no longer significant ($F_{(1,496)}$=3,44, p=.06). These results suggest that males in this sample might have excelled on episodic verbal memory because of their ability to verbalize the recalled material. It is important to note that the distinction between verbal, nonverbal and visuospatial episodic memory does not suggest that a task is either verbal or visuospatial in nature, but rather requires more verbal or visuospatial processing (Lewin, Wolgers & Herlitz, 2001). Visual Memory component consists of a visuospatial task, namely Dot Locations from Children's Memory Scale. There are not many studies on visuospatial episodic memory. Nonetheless, the study by Lewin, Wolgers & Herlitz (2001) reported that men (n=91, 20-40 years old) were superior on a number of visuospatial tasks, whereas women (n=94, 20-40 years old) did not perform at a higher level than men on the verbal production tasks. This is consistent with the findings in female and male adolescents. It has been suggested that females will excel in episodic memory in which a verbalization of the material is possible, whereas no difference between females and males will be found in material in which verbalization is not possible; and males will outperform females in episodic memory tasks with nonverbal material requiring visuospatial processing (Lewin, Wolgers & Herlitz, 2001). Because males in our sample had higher Verbal IQ than their female counterparts, it could be argued that it is not surprising that they excelled on the Verbal Memory component as well.

Finally, as already mentioned, we found no significant sex differences on Verbal Abilities component; this is consistent with results obtained by several studies in adults and children suggesting that males and females do not differ on general intelligence (vocabulary tests) and verbal and non-verbal reasoning tests (Halpern, 2000; Maccoby & Jacklin, 1975). The Verbal Abilities component, as mentioned above, includes the verbal tests from WISC-III but also fluency tasks from the NEPSY, reading comprehension from WJ, and a spelling task. Thus, in contrast with Verbal IQ, it adds the reading (and spelling) components.

Motor Dexterity was the only component with a significant Age by Sex interaction, with girls improving their performance with age; this was not the case in boys. Increases with age on this component demonstrate development of fine motor skills that can be defined as coordination of small muscle movements.

2.5. Study Limitations and Strengths

One of the limitations of our study is the fact that pre-natal exposure to maternal cigarette smoking was determined by maternal reports, which may not be free of recall bias. As detailed elsewhere (Pausova et al., 2007) we were able to verify most of the mothers' reports using medical charts completed during pregnancy. In order to assess the overall agreement between the medical records and the maternal reports, we calculated Kappa statistics and found a value of 0.69±0.04, indicating a 'good' strength of agreement (good agreement: >0.6 to ≤0.8; Landis & Koch, 1977). The observed differences between exposed and non-exposed adolescents in their birth weight further support validity of the retrospective assessment of maternal smoking during pregnancy in our sample. Finally, it has been suggested by others (Huizink & Mulder, 2006) that underreporting of smoking during pregnancy may be higher at the time of pregnancy, as compared with later recalls, due to the immediate influence of the stigma associated with smoking at the time of pregnancy. We have observed a similar tendency when comparing medical records and current maternal reports (Pausova et al. 2007).

Most of the studies that observed the association of maternal cigarette smoking during pregnancy with cognitive abilities are longitudinal and have large sample sizes. The limitations of the Saguenay Youth Study lie in a relatively small sample size (n=503) and the cross-sectional design. One of the main strengths of this study is the comprehensive nature of the cognitive assessment. The neuropsychological battery consists of 33 cognitive tasks, thus providing a wealth of information along a broad spectrum of cognitive abilities. The large epidemiological studies, such as the National Child Development Study, used more limited tools, often focusing on tests of academic achievement such as math and reading. Another strength of the study is that exposed and non-exposed adolescents were matched according to maternal education and school attended at enrolment, thus eliminating the effect of maternal education that other studies found to be a significant covariate in their analyses. Last but not least, the fact that the sample comes for a relatively geographically isolated population

increases its genetic and environmental/cultural homogeneity and, therefore, decreases the likelihood of major differences between the exposed and non-exposed individuals in these two domains. This genetic and environmental/cultural homogeneity will allow us to investigate whether maternal smoking during pregnancy has an association with adolescent cognition in individuals with specific variants of genes, which may increase the impact of different constituents of the cigarette smoke on the developing brain.

2.6. Conclusion

There was no association of maternal cigarette smoking during pregnancy with cognitive abilities of the adolescent offspring. Unlike the majority of previous studies, exposed and non-exposed adolescents were matched on the level of maternal education at ascertainment. This matching procedure effectively eliminated any differences between the two groups in family socio-economic status, the most common confounder of maternal cigarette smoking during pregnancy. It should be noted, however, that significant differences between exposed and non-exposed adolescents in other domains were observed, including questionnaire-based indicators of positive youth development (Toro et al., 2008), cortical thickness (Toro et al., 2008) and the size of the corpus callosum (Paus et al., 2008). Therefore, the negative findings reported in this report should not be taken as indicating a lack of long-term consequences of maternal smoking during pregnancy on brain and behaviour in general.

Chapter 3: Study 2 – Nutrition at birth: Long-term effects of breastfeeding examined in adolescence: Cognition

3.1. Introduction

After birth, the infant's exclusive source of nutrients is breast milk or formula milk. The latter is the case if the mother chooses not to breastfeed or cannot breastfeed for medical reasons (e.g. due to maternal exposure to radioactive materials; maternal use of antimetabolites or chemotherapeutic agents, medications or drugs of abuse; infected with human immunodeficiency virus [HIV]), cannot produce enough milk or infant suffers from classic galactosemia (American Academy of Pediatrics, 2005). The prevalence of breastfeeding differs across countries and it seems to have been increasing throughout the years. Thus, the incidence of breastfeeding, defined as the percentage of infants who were breastfed initially (including one occasion only), increased from 2000 to 2005 in the United Kingdom (from 71% to 77% in England and Wales, 63% to 70% in Scotland, and 54% to 63% in Northern Ireland). When standardised for two factors strongly associated with breastfeeding, namely age and education level, the standardised rates were 62% in 2000 and 67% in 2005 suggesting that the observed variations in breastfeeding rates between 2000 and 2005 are due to a real increase and do not represent changes in the sample composition (Infant Feeding Survey, 2005). Incidence of breastfeeding does not, however, inform us about the duration of breastfeeding. Therefore, no conclusions can be inferred about the benefits of breastfeeding on infant's cognitive development and health. A recent study carried out in Canada (Al-Sahab, Lanes, Feldman & Tamim, 2010) has assessed the prevalence of exclusive breastfeeding, defined by the World Health Organization (WHO) "as [the infant] being exclusively breastfed for six months, initiated within the 1st hour of life, the infant only receives breast-milk without any additional food or drink, not even water, it is as often as the child wants, day and night, and no bottles, teats or pacifiers should be used". The study by Al-Sahab *et al* (2010) states that, in Canada, "ever" breastfeeding was high at 90.3% but, after three months, only half of the mothers continued to breastfeed exclusively, and only 13.8% were exclusively breastfeeding for 6 months. In the Canadian province of Quebec (1999/2000), exclusive breastfeeding was 62% and 10% for the 1 and 5 months respectively (Haiek, Gauthier, Brosseau, Rocheleau, 2007). In

the United States, 59.8% mothers ever breastfeed, 25.4% breastfeed for at least 6 months, and 17.5% breastfeed for at least 12 months (CDC's Pediatric and Pregnancy Nutrition Surveillance System, 2008). In summary, it appears that, in developed countries, the rates of exclusive breastfeeding are decreasing, with more mothers initiating exclusive breastfeeding within 24 hours of birth but then withdrawing by either adding other liquids (such as water) or shifting to formula feeding.

3.1.1. Composition of human milk

Human milk is the only source of nutrition for an infant. At no other stage of life is a single source of food sufficient for nourishment. According to the WHO, human milk should be the only nutrient source for an infant for the first four to six months of life. After six months, complementary foods are needed. The exceptionality of human milk is being recognized as promoting brain growth and development in infants, as well as protecting against illness in infancy and later in life (Rodriguez-Palmero, Koletzko, Kunz, et al., 1999).

During lactation, high nutritional requirements are needed; production of 750 to 1,000 mL/d of human milk corresponds to 2100 to 2500 kJ/d transferred as energy macronutrients to nursing infants. Human milk is a composite biological fluid that consists of thousands of elements: true solutions (87%), colloidal dispersions of casein molecules (0.3%), emulsions of fat globules (4%), fat-globule membranes, and live cells (Jensen, 1999; Picciano, 2001). Human milk elements are categorized in Table 1 according to their physical properties (Picciano, 2001).

Lipids are the major energy-yielding ingredient of human milk. About 97-98% of lipids are triacylglycerols. Fatty acids (FAs) correspond to 88% of milk fat (Koletzko, Rodriguez-Palmero, 1999) and they are undoubtedly the most variable element of human milk because they are highly dependent on maternal diet (Innis, 2007).

Table 1: Human milk composition elements

Proteins	Nonprotein nitrogen	Carbohydrates	Lipids	Water-soluble Vitamins	Mineral & ionic elements	Trace Minerals	Cells
a-Lactalbumin P-Lactoglobulin Caseins Enzymes Growth factors Hormones Lactoferrin Lysozyme Secretory IgA & other immunoglobulins	a-amino nitrogen Creatine Creatinine Glucosamine Nucleic acids Nucleotides Polyamines Urea Uric acid	Lactose Oligosaccharides Glycopeptides Bifidus factors	Fat-soluble vitamins **(A,** D, E, and K) Carotenoids Fatty acids Phospholipids Sterols and hydrocarbons Triglycerides	Biotin Choline Folate Inositol Niacin Pantothenic acid Riboflavin Thiamin Vitamin B, Vitamin B, Vitamin C	Bicarbonate Calcium Chloride Citrate Magnesium Phosphate Potassium Sodium Sulfate	Chromium Cobalt Copper Fluoride Iodine Iron Manganese Molybdenum Nickel Selenium zinc	Epithelial cells Leukocytes Lymphocytes Macrophages Neutrophils

Breast milk is rich in Essential Fatty Acids (EFAs), linoleic acid (LA; 18:2ω-6) and a-linolenic acid (ALA; 18:3ω-3), their long-chain polyunsaturated fatty acids (LC-PUFA) derivatives, arachidonic acid (AA; 20:4ω-6), dihomo-gamma-linolenic acid (DGLA; 20:3ω-6), and eicosadienoic acid (20:2ω-6) for the ω-6 series, docosahexaenoic acid (DHA; 22:6ω-3), and docosapentaenoic acid (DPA; 22:5ω-3) of the ω-3 series. Nonetheless, the highest proportions of LC-PUFA in human milk are AA (0.4-0.6%) and DHA (0.2-0.4%; Koletzko, Rodriguez-Palmero, 1999).

The fatty-acid composition in human milk is influenced by a variety of factors such as stage of lactation (from colostrum to mature milk, content of EFAs is increasing whereas the percentages of LC-PUFAs are decreasing; Luukkainen, Salo, Nikkari, 1994) and gestational age [(some studies have found that milk samples of mothers delivering preterm had higher fat contents (Guerrini, Bosi, Chierici & Fabbri, 1981; Bitman, Wood, Hamosh, Hamosh & MEHTA, 1983); but other studies report that term and preterm milk do not differ in LC-PUFA percentages; Genzel-Boroviczény, Wahle, Koletzko, 1997)]. Maternal diet is also a factor influencing fatty-acid composition of the milk; milk fat produced by women following vegan diets contains <0.1g DHA/100g total fatty acids (Sanders & Reddy, 1992), whereas higher amounts are found in the milk of women consuming diets high in fish and other marine animals (Innis, 2004). Levels of EPA and DHA in breast milk of Inuit women were approximately 5.5 and 3.5 fold higher, respectively, compared with Canadian or North American women [(Inuit's major portion of marine lipid come from marine mammal flesh rather than fish (Innis, 1989)]. Last, metabolic disorders can also be a factor influencing composition of fatty acids in the human milk [insulin-dependent diabetes mellitus can alter fatty-acid metabolism resulting in changes in the fatty-acid composition of human milk (Koletzko, Rodriguez-Palmero, 1999)].

Linoleic acid and a-linolenic acid in the human milk are obtained from maternal diet or by mobilization of body stores. The source of LC-PUFA in human milk is threefold: they may be produced in the liver (or the mammary gland) by synthesis from their precursors LA and ALA (Figure 1), be provided as preformed LC-PUFA by diet, or be mobilized from body stores (Koletzko, Rodriguez-Palmero, 1999; Francois, Connor, Wander & Connor, 1998). Preformed AA and DHA can be found in foods such as fatty fish and eggs. After absorption of preformed AA and DHA, dietary long-chain fatty acids are re-esterified into triacylglycerols,

which enter the circulation in the form of chylomicrons (chylomicrons are large lipoprotein particles that consist of triglycerides, phospholipids, cholesterol and proteins) and are transferred into human milk. When preformed, AA and DHA are not taken through diet, triacylglycerols are transported from the liver as VLDL (very-low-density lipoprotein is a type of lipoprotein made by the liver) into the mammary gland. Fatty acids from adipose tissue are transported as unesterified fatty acids bound to albumin (albumin refers to any protein with water solubility) into the mammary alveolar cells (Koletzko, Rodriguez-Palmero, 1999). Before penetrating into the mammary alveolar cells, fatty acids containing triacylglycerols are released by the action of lipoprotein lipase (lipoprotein lipase is an enzyme that hydrolyses lipids in lipoproteins, two free fatty acids and one monoacylglycerol molecule).

Several studies have shown that direct transfer of PUFA from diet provides only a small percentage of milk PUFA. Using ^{13}C labelled 18:2n-6, Demmelmair, Baumheuer, Koletzko, Dokoupil and Kratl (1998) showed that only 30% of 18:2n-6 (LA) in milk was transferred from diet, while only 11% of AA and 1.2% of DGLA were derived from endogenous conversion of LA. These results indicated that maternal adipose tissue contributed mainly to the lipid composition of human milk (Demmelmair et al., 1998).

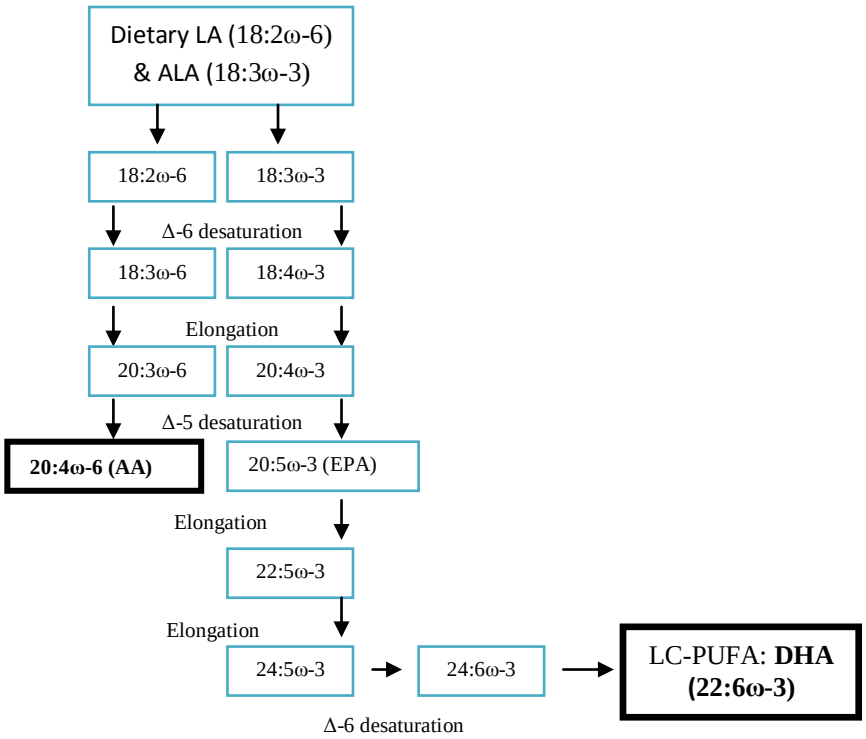


Figure 1: Conversion of Linoleic Acid (18:2ω-6) and A-Linoleic Acid (18:3ω-3) to the long-chain polyunsaturated fatty acids Arachidonic Acid (20:4ω-6) and Docosahexaenoic Acid (22:6ω-3).

3.1.2. Associations of breastfeeding with cognitive development

Since the 1929 study by Hoefer and Hardy, many studies have investigated the possible associations of breastfeeding with cognitive abilities. Overall, the results of these studies demonstrate a consistent pattern of positive but small association of breastfeeding with "better" cognition. But the association becomes smaller after taking into account relevant confounders such as maternal intelligence, parenting skills and socioeconomic status; in fact, many researchers argue that the remaining association could be due to residual confounding (Michaelsen, Lauritzen, Mortensen, 2009).

Most of the available research is based on observational studies. Four reviews published between 1999 and 2002 have included almost all observational studies examining at the association of breastfeeding with cognitive abilities. Only one out of the four reviews is a meta-analysis and will be discussed in more detail later on.

Grantham-McGregor, Fernald, and Sethuraman (1999) discussed the effects of breastfeeding in terms of short-term and long-term associations. In their short-term associations review, they included eight studies from 1982 to 1998 on children 24 months old and younger. Most of these studies used the Bayley Scale of Infant Development as the main outcome measure; some of the studies controlled for socio-economic status and maternal characteristics. In summary, at 24 months, breastfed infants showed a small but consistent benefit over non-breastfed infants in mental development even after adjusting for confounders.

For the longitudinal associations of breastfeeding with cognition, Grantham-McGregor *et al* (1999) identified 14 studies from 1929 to 1998 with assessments on children between the ages of 4 to 18 years old. They concluded that, in some studies, the benefit of breastfeeding remained robust with the inclusion of confounders such as socio-economic status, whereas others covered up the benefit of breastfeeding with the inclusion of confounders.

The main limitation of the Grantham-McGregor's *et al* review is that while they state sample characteristics for each study, they do not report any inclusion or exclusion criteria for the included studies thus making any observations less reliable. It seems that researchers, after understanding the mechanism of breastfeeding, have made a clear distinction between term and preterm infants, and covariates that should be controlled for when evaluating effects of

breastfeeding; thus any review study of breastfeeding associations should make clear which studies are including and for what reason.

Twenty four investigations on breastfeeding associations with cognition were included in a 'critical evaluation' by Drane and Logemann (2001). Studies reviewed in their paper were published between 1966 and 1998, and had to meet three methodological standards: definition of cognitive outcome, correct classification of type of infant feeding (duration of exclusive breastfeeding, exclusive formula), and control for potential confounders. From 24 studies, only 6 met all three methodological standards. Only four out of these six studies suggested that breastfeeding was positively associated with cognitive development. Specifically, they report an advantage of the order of 2-to-5 IQ points for term infants and 8 points for low birth-weight infants. They conclude, however, that the phenomenon of breastfeeding associated with cognitive ability is not entirely understood and has not yet been comprehensively answered.

In 2002, Jain, Concato and Leventhal, published a 3rd review in which they identified 40 studies published between 1929 and February 2001. For each study, they examined the overall design and seven methodological aspects: target population (full-term *Vs* preterm infants), sample size, collection of feeding data, control of susceptibility bias (control for socio-economic background and stimulation of the child), blinding (observers of the outcome were blind to feeding status), outcome (standardized test of general intelligence), and format of results (reported effect size). Jain *et al* (2002) detected 27 (68%) studies with beneficial effects of breastfeeding on cognitive outcome but many of these, according to Jain's *et al* methodological standards, had methodological flaws. Only two studies that included full-term infants met all their quality criteria. One of these showed a beneficial effect of breastfeeding on cognitive outcome with an effect of 5 IQ points, which was reduced to 4.6 after adjustment of confounders, whereas the other study showed an improvement of 3.8 IQ points reduced to 0.8 IQ point after adjustment. Jain *et al* concluded that results from higher quality studies are inconsistent and do not favour the beneficial effects of breastfeeding.

The only formal meta-analysis was published by Anderson, Johnstone and Remley in 1999. They included 20 studies published between 1966 and June 1996 that met their initial inclusion criteria: studies comparing predominantly breast-fed infants with infants who were predominantly formula-fed, the primary outcome measure had to be a widely applied test of

cognitive development, and participants were tested between infancy and adolescence. From those 20 studies only 11 controlled for ≥ 5 covariates and presented unadjusted and adjusted results. Before adjusting for covariates, the benefit in cognitive development was 5.32 points, whereas after adjustment the increase in cognitive ability between breast-fed and formula-fed children was 3.16 points. Three more important outcomes derived from this meta-analysis are: higher levels of cognitive ability were obvious in breast-fed than formula-fed children at 6 to 23 months of age and these differences were stable across consecutive ages; low birth-weight infants showed larger benefits than did normal birth-weight infants; and duration of breastfeeding was a significant factor for advantageous cognitive development.

In 2007, Horta, Bahl, Martines and Victora, working on behalf of the WHO, performed their own meta-analysis using the studies from Jain *et al*, and three more that were identified from their own systematic review. Their results indicated that eight studies observed a positive association of breastfeeding with cognition but only six of them were statistically significant.

Since the publication of the above reviews and meta-analyses, few other studies have investigated the association of breastfeeding with cognitive development. In a prospective population birth cohort study of term infants in Australia, Oddy, Kendall, Blair, de Klerk, Stanley, Landau, Silburn and Zubrick (2003) examined the association between duration of exclusive breastfeeding and cognitive outcome measured at 6 (Verbal IQ) and 8 (Performance subtest) years of age, after taking into account numerous perinatal, social and family factors. In 1,450 children tested at six years of age, they observed a significant relation between verbal cognitive ability and duration of breastfeeding after adjustment; in the 1,375 eight-year old children, there was no relation with the non verbal subtest (WISC-III Block Design). Gustafsson, Duchén, Birberg and Karlsson (2004) reported that length of breastfeeding, used in a multiple regression with relevant confounders, contributed significantly to total IQ, verbal and performance IQ. In 2006, Clark, Castillo, Calatroni, Walter, Cayazzo, Pino and Lozoff, followed a cohort of 784 healthy full-term children born in Chile, who were enrolled in a study on prevention of iron deficiency, to investigate the association between breastfeeding and cognitive outcome at 5½ years in a socioeconomically homogeneous population where breastfeeding is universal. They observed a non-linear relationship where children breastfed for 2 to 8 months had higher scores for language, motor and cognition tests than those breastfed

for <2 months or >8 months, after adjustment for socioeconomic factors and home stimulation. In 2008, Kramer, Aboud, Mironova, Vanilovich, Platt *et al* published new evidence on breastfeeding and child cognitive development from a large randomized trial known as Promotion of Breastfeeding Intervention Trial (PROBIT) in the Republic of Belarus. The purpose of this randomized trial was to promote exclusivity and duration of breastfeeding among mothers who have already decided to initiate breastfeeding. Mothers were randomly assigned into the experimental or control group. Experimental intervention was based on the Baby-Friendly Hospital Initiative, which was developed by the WHO and UNICEF to promote and support breastfeeding (World Health Organization; UNICEF. *Protecting, Promoting and Supporting Breastfeeding: The Special Role of Maternity Services.* Geneva, Switzerland: World Health Organization; 1989), whereas control group was assigned to standard care (Kramer, Chalmers, Hodnett, Sevkovskaya, Dzikovich, et al., 2001). A total of 13,889 breastfed infants were followed up at the age of 6½ years. The experimental intervention was found to have higher means on all of the intelligence measures than the control group, and also teacher's academic ratings were significantly higher in the experimental group for both reading and writing (Kramer et al., 2008). Last but not least, an important study by Caspi, Williams, Kim-Cohen, Craig, Milne, Poulton, Schalkwyk, Taylor, Werts, Moffitt (2007) showed that the association between breastfeeding and IQ is moderated by a genetic variant in *FADS2*, a gene involved in the genetic control of fatty acid pathways, even when ruling out potential confounding of this gene-environment interaction.

Association of breastfeeding with cognitive abilities assessed in adulthood

In the above reviews only one study was included in which intelligence in adults was evaluated. Gale and Martyn (1996) followed up 994 men and women, born between 1920 and 1930, with the AH4 IQ test that measures logical, verbal and arithmetic reasoning. To assess effects of breastfeeding, adults were classified as breast-fed, bottle-fed and "combined"; they found a positive association between breastfeeding in infancy and intelligence in adulthood, which disappeared after controlling for family and perinatal factors. At the ages of participants tested (around 60 to 70 years old), test scores may be affected by individual differences in age-related decline in cognitive ability thus weakening the association between breastfeeding and

cognition (Michaelsen et al., 2009). Also, researchers did not control for maternal education, intelligence or parental skills, which are strongly associated with offspring's intelligence.

Since 1996, three more studies have examined the breastfeeding association with adult's intelligence. Mortensen, Michaelsen, Sanders *et al* (2002) conducted a prospective longitudinal birth cohort study in a sample of 973 men and women, and a sample of 2,280 men, all born in Copenhagen between 1959 and 1961. Assessment of breastfeeding was divided into five categories according to duration. Intelligence was assessed with the Wechsler Adult Intelligence Scale (WAIS) for the mixed group at the mean age of 27.2 years, and with the Børge Priens Prøve (BPP) test in the male sample at the mean age of 18.7 years. Their results demonstrated a robust association of duration of breastfeeding with adult intelligence on both samples with larger effects associated with breastfeeding for the WAIS sample, perhaps because it is a more sensitive measure of intelligence than the BPP. Richards, Hardy and Wadsworth (2002) included 1,739 men and women from the British 1946 birth cohort, and assessed them at the age of 53 years using three cognitive tests: reading ability (using the National Adult Reading Test; NART), verbal memory measured by a word-list learning task, and mental speed and concentration measured by a timed letter search task. The possibility of achieving advanced educational qualifications at the age of 26 years was also assessed and found to be positively associated with breastfeeding. For the three cognitive tests, only reading ability was positively associated with breastfeeding even after adjustment for early social background, education, social class. But when cognitive ability at 15 years was included in the adjustment model, there was no association between breastfeeding and reading ability. According to the researchers, reading ability is stable over time whereas other cognitive abilities may be more vulnerable to age-related decline (Richards et al., 2002). In a more recent study by Elwood, Pickering, Gallacher, Hughes and Davies (2005), 779 men in UK were tested when they were 60 to 74 years old with the NART, the AH4 test for verbal and mathematical reasoning, and the choice reaction time (CRT) that measures hand-brain reaction speed. Researchers concluded that men having low birth-weight and being artificially fed, after adjustment for possible confounders, had poorer cognitive outcome in late adult life.

3.2. Summary, Aims and Hypothesis

Over the past 50 years, research on breastfeeding has shown some consistent findings. On the whole, it seems that the rates of (initial) breastfeeding are increasing over time. At the same time, however, duration of exclusive breastfeeding is decreasing. As a general rule, researchers agree that there is a robust association between breastfeeding and maternal education, social class and parenting skills. Women who choose to breastfeed tend to be older, more educated, have higher incomes, and provide a more stimulating home environment, and are less likely to smoke cigarettes (Reynolds, 2001). Another important conclusion from the above reviews is that most of the studies were observational in design and included children who were born healthy and at term. Results of these studies indicate that there is a modest positive association between breastfeeding and intelligence of term infants; an advantage of the order of 2-to-5 IQ points for term infants was observed by Drane and Logeman (2001). In preterm infants, larger effects of breastfeeding are found; e.g. 8-point IQ advantage reported by Lucas, Morley, Cole, Lister, and Leeson-Payne (1992). Consequently, special notice should be drawn in differences among term and preterm breastfed children. In addition, several studies demonstrated a clear-cut relationship between duration of breastfeeding and advances in cognitive ability. Overall, studies examining the associations of breastfeeding with cognitive ability in infants, children, adolescents and adults show a consistency in their results favouring breastfeeding.

The main purpose of this chapter is to examine association between exclusive breastfeeding and cognitive abilities in 599 adolescents tested with a rich neuropsychological dataset between 12 and 18 years of age. The participants were drawn from the Saguenay Youth Study (SYS) in Quebec, Canada, which is a retrospective cross-sectional study of the effects of maternal cigarette smoking during pregnancy on brain and cognition, and cardiovascular and metabolic health during adolescence. The non-exposed adolescents (controls) were matched to the exposed ones (cases) according to the level of maternal education and school attended, thus minimizing differences between the two groups in the socioeconomic status (SES) of the families at ascertainment. Due to the nature of the study, exposure status will always be controlled for even if it's not a significant predictor for the cognitive measures. In an unadjusted analysis, breastfeeding and exposure to maternal cigarette smoking will be examined in combination in order to evaluate possible interaction between the hypothesized

adverse (smoking during pregnancy) and beneficial (breastfeeding) environments on cognitive development. Previous research has shown that maternal cigarette smoking is negatively associated with breastfeeding. In most studies, exposure to maternal cigarette smoking is considered a potential confounder. Two similar types of analysis will be performed: in the first set, breastfeeding will be examined as a categorical variable with two levels (breastfed *Vs* non-breastfed) and in the second one, breastfeeding will be four-level variable measuring exclusive breastfeeding duration. The reason for using two different variables measuring breastfeeding is to examine the effect size on the outcomes of each variable and to establish the significance of using duration of breastfeeding when examining effects of breastfeeding.

3.3. Methodology

3.3.1. Design

As detailed described in Chapter 2, the design of the SYS is family-based, with children with one or more siblings and both parents being included, cross-sectional with participants' age ranging from 12 to 18 years old, and retrospective where in utero exposure is evaluated retrospectively. Further, the design includes quantitative assessment of brain and behaviour, as well as cardiovascular and metabolic phenotypes, and also acquisition of DNA sample from the adolescents and their biological parents.

3.3.2. Setting and Participants

Details about the population of SLSJ, selection criteria, recruitment and measurements are detailed described in Chapter 2.

Briefly, participants were 599 adolescents between the ages of 12 to 18 years, recruited from the SLSJ region of Quebec, Canada. As the SYS is designed for evaluating long-term consequences of exposure to maternal cigarette smoking, selection criteria for exposed and non-exposed adolescents are explained in Chapter 2. Overall, main exclusion criteria for all 599 participants are: (i) alcohol abuse during pregnancy; (ii) premature birth (<35 weeks); (iii) positive history of heart disease, brain trauma, brain tumour, meningitis, or epilepsy; and (iv) severe mental illness (e.g. autism) or mental retardation (IQ<70).

3.3.3. Measurements

3.3.3.1. Breastfeeding status

Breastfeeding was measured through a medical questionnaire in which mothers were asked if they breastfed their child, and if yes, for how long (weeks or months) did they breastfeed exclusively. They were also asked at what age they introduced water or unsweetened liquids, at what age did they first introduced formula or milk of any kind, at what age did they introduced cereals, fruits and other solid foods, and lastly at what age did they completely stop breastfeeding. These data were summarized and, in order to create a new variable with exclusive duration of breastfeeding, categorized as: (i) never breastfed, (ii) breastfed for less than 4 weeks, (iii) breastfed for 4 to 16 weeks, and (iv) breastfed for more than 16 weeks. The use of such a three-level (when breastfeeding) categorical variable prevents the loss of statistical power and misclassification bias.

3.3.3.2. Cognitive measures

Cognitive measures were detailed described in Chapter 2.

3.3.3.3. Pre-natal, peri-natal and family environment measures

A number of pregnancy and birth-related variables, such as birth weight, maternal age at delivery, alcohol during pregnancy, number of pregnancies, breastfeeding and *in utero* exposure to second-hand smoking were assessed using a set of questionnaires and by a structured telephone interview with the biological mother in the majority of participants. A number of socioeconomic indicators, such as household income and parental education, were also obtained. In addition, a number of parental characteristics, including antisocial behaviour, anxiety and depression, were evaluated with a Mental Health & Anti-social Behaviour questionnaire answered by the biological parents.

3.3.4. Statistical Approaches

In all analyses, raw scores of cognitive tests were used rather than standard scores. Standard scores express the performance of an individual relative to peers of the same age. While they capture individual differences, they are insensitive to developmental differences, which will be best correlated with total performance on the task (Waber, Moor, Forbes, Almli,

Botteron, Leonard, Milovan, Paus, Rumsey et al., 2007). Outliers, defined as values three standard deviations from the mean, were excluded.

Statistical analyses were carried out using JMP (version 8), SPSS 14 (for Windows) and hierarchical linear modelling (HLM statistical software, version 6.0; Scientific Software International, Lincolnwood, Illinois). Variables that were negatively skewed were reflected and then transformed using LG10, which produces a variable that contains the logarithmic (to base 10) values of the variable, in order to obtain normally distributed data.

Using HLM, we accounted for the clustering of sibling pairs within families. This procedure allows us to resolve the relationship of the offspring and family predictors (such as socioeconomic factors) with the main outcome variables, namely, the cognitive measures. Within HLM, parental data are specified as a level 2 variable, which estimates between family effects, and the sibling data as a level 1 variable, which estimates within family variability. This procedure allows one to model how family-based variables interact with sibling-based variables and to calculate the correct error terms, as well as to examine the influence of the single-parent estimates of socioeconomic status on each of the sibling scores without double counting (Lotfipour, Ferguson, Leonard, Perron, Pike, Richer, Seguin, Toro, Veillette, Pausova & Paus, 2009). Further details on HLM analysis will be described later on.

In Chapter 2, we performed a principal component analysis with varimax rotation, in order to obtain a more global view of our neuropsychological data. Here, we performed instead a principal component analysis with oblique (direct oblimin) rotation. Oblique rotation was chosen to allow correlation between the derived components; whereas varimax (orthogonal) rotation gives factors (components) that are not correlated with one another. The reason for choosing oblique instead of orthogonal rotation at this point is because we believe that our cognitive components should be related to each other (for example visual and verbal memory). Due to the exploratory nature of the neuropsychological dataset a confirmatory factor analysis could not be performed. When principal component analysis is performed in one dataset, conclusions are restricted to the sample collected and generalization of the results can be achieved only if analysis using different samples reveals the same factor structure (Field, 2005). Thus, the seven cognitive components derived from the exploratory factor analysis in Chapter 2 were not used for this study because the dataset increased (599 adolescents *Vs* 503 adolescents in Chapter 2). A second exploratory factor analysis was performed on the 599

adolescents in order to evaluate if the same components from Chapter 2 could have been derived.

Raw scores from all 33 cognitive tests were included in the principal component analysis with direct oblimin rotation. While various indicators of factorability were good, anti-image correlation matrix revealed that two of the 33 cognitive tests (Self-Ordered Pointing Sum of Errors & Stroop Interference) had Kaiser-Meyer-Olkin (KMO) below .5; the KMO can be calculated for individual and multiple variables and represents the ratio of squared correlation between variables to the squared partial correlation between variables and varies between zero and one. Individual variables with KMO below .5 should be considered removed from the analysis (Field, 2005). After removing these two cognitive tests, principal component analysis with direct oblimin rotation was performed again with 31 variables.

The 2nd principal component analysis with direct oblimin rotation revealed the various indicators of factorability to be good and the residuals indicated that the solution was a good one. Kaiser's criterion was used to identify the number of derived components. The *scree* plot of the 2nd principal component analysis revealed six components. Also the sample size exceeds 250 (599 participants) and average communality was .642 thus Kaiser's criterion for extracting components can definitely be used. The six components can be thought of representing the following cognitive abilities: Component 1: Verbal Conceptualization and Sequential Processing; Component 2: Processing Speed; Component 3: Visual Memory; Component 4: Perceptual Organization; Component 5: Verbal Memory; and Component 6: Motor Dexterity. Components are quite similar to components derived in Chapter 2, with only one component missing (Component 6: Resistance to Interference), which reflects the deletion of the Stroop interference cognitive test from the analysis.

For further analysis, instead of extracting the factor scores with options offered from SPSS (Anderson-Rubin, Bartlett, Regression methods), items loaded above .40 (Fergusson & Cox, 2007) on a component were selected and for each component the sum of included items was calculated for each individual.

Further statistical analyses began by assessing the association between each potential confounder and breastfeeding status. Potential confounders were separated into two levels: Level 1: Age and exposure status; Pregnancy: maternal age at delivery, pregnancy duration, and alcohol during pregnancy; Post-natal: Sex, birth-weight, and birth order; Parenting:

Parental monitoring, and Level 2: SES: household income and parental education (these variables are thought to be strongly associated with breastfeeding and cognitive outcome of the child) and parental antisocial behaviour in adolescence. Confounders were selected after examining for multicollinearity. For variables that were highly correlated (e.g. number of pregnancies and maternal age at delivery) only one was included for further analysis.

Firstly, the relationship between breastfeeding, exposure status and cognitive abilities was examined in an unadjusted analysis (only age effect was removed) in order to examine if there was a significant interaction between breastfeeding and exposure status. The effect of breastfeeding and exposure status on age-adjusted cognitive abilities was assessed by carrying out two-way ANOVAs with breastfeeding and exposure status as the independent variables and the age-adjusted residuals for a given component as the dependent variable. Main effects of breastfeeding and exposure status, as well as an interaction effect of breastfeeding and exposure status were reported.

As already mentioned, hierarchical linear modelling was used in order to resolve the issue of sibling pairs and thus, to assess if breastfeeding is a significant predictor of cognitive abilities when other important predictors are also controlled for. The possible offspring-based confounders, as well as the parent-based predictors chosen for further analysis, are stated above. A 'step-up' strategy of building up from a univariate to a multivariate model was used (Raudenbush & Bryk, 2002). To establish which predictors should remain in the model and which should be omitted, variables were added individually and linearity of the predictor variable with that of the outcome variable was assessed. Predictor variables that were significantly ($p<.05$) associated with the outcome variables were retained in the final model. Once the offspring-based model was determined (without addition of breastfeeding variable), we added parental variables to the model. Similarly, parental variables were added individually and were assessed for linearity with the outcome variables. Maternal education was the first parental predictor variable to be added in the model. At the end of the analysis, when the significant predictor variables were already added to the models, breastfeeding status was also added as the last important predictor variable. Exposure status was also added (significant or not) due to the nature of our study design. Since we have six different cognitive components and three IQ indexes, the final models were different from each other. As an example, the final model for Full Scale IQ is given below:

$$FIQ = \gamma_{00}+\gamma_{01}*(\textbf{\textit{Maternal\_education}}) + \gamma_{02}*(\textbf{\textit{Paternal\_Education}}) +$$

$$\gamma_{10}*(\text{Breastfeeding}) + \gamma_{20}(\text{Exposure}) + \gamma_{30}*(\textbf{Age}) + u_0 + r,$$

in which γ_{01} & γ_{02} are the level-2 variables, γ_{10} to γ_{30} are the level-1 variables. Predictors in boldface type represent group-mean centered variables, whereas predictors in bold-italic type represent grand-mean centered variables.

Last but not least, breastfeeding effects can be assumed to vary in proportion to the length of time to weaning and the daily dose of breastfeeding; therefore breastfeeding is more accurately measured as a continuous variable or at least a three-category variable. In order to evaluate effects of duration of exclusive breastfeeding on cognitive abilities, duration of breastfeeding was split into three categories according to interquartiles. Thus, a new variable was prepared having four categories: non-breastfed, breastfed for less than four weeks, breastfed for 4 to 16 weeks, and breastfed for more than 16 weeks.

The second type of analysis was performed again, as stated above, by substituting breastfeeding status with breastfeeding duration. Thus we could compare effect sizes from first set of analysis using breastfeeding status (breastfed/non-breastfed) and with the 2nd set of analysis using breastfeeding duration. In order to evaluate effects of breastfeeding duration on cognitive abilities, the hierarchical linear modelling was used again for all cognitive measures and IQ indexes. As an example, the final model for FIQ would be as follows:

$$FIQ = \gamma_{00}+\gamma_{01}*(\textbf{\textit{Maternal\_education}}) + \gamma_{02}*(\textbf{\textit{Paternal\_Education}}) +$$

$$\gamma_{10}*(\text{Exclusive Breastfeeding Duration}) + \gamma_{20}(\text{Exposure}) + \gamma_{30}*(\textbf{Age}) + u_0 + r$$

3.4. Results

3.4.1. Sample Characteristics

Table 2, separately for breastfed and non-breastfed adolescents, describes the adolescents' age and exposure status, as well as the different confounders. Breastfed and non-breastfed adolescents have the same mean age but differ in exposure status. There are significantly more exposed adolescents in the non-breastfed than in the breastfed group [x^2(1,

N=593) = 31.61, *p*<.0001] thus indicating that women who smoke during pregnancy usually choose not to breastfed their offspring. From Level 2, maternal education is significantly different in the two groups; mothers with higher level of education have chosen to breastfeed more than mothers with lower education level [maternal education: *Welch F* (1,278 =7.17, *p*=007; paternal education: *Welch F* (1,264)=2.36, *p*=.125][2]. Parental education ranged from the lowest level, which is 8th grade or less, up to a doctoral degree. No differences were apparent between the two groups in household Income, and in the various confounders in *Pregnancy* and *Parenting*. In *Post-natal* variables, there was a significant difference in birth weight between the two groups [F (1,587) =11.31, *p*=.0008], but no difference in sex and birth order. Descriptive statistics for the neuropsychological measures are displayed in Table 3.

[2]In parental education, Levene's test of Homogeneity of Variances was significant (p<.0001) indicating that the variances in the two groups are significantly different thus violating one of the assumptions of ANOVA. In this case Welch's F is reported (Field, 2005).

Table 2: Sample characteristics separated by breastfeeding status

		Breastfed (*N*)	**Non Breastfed** (*N*)	***P***
Level 1:				
Age	Mean ± SEM	14.7 ± 0.1 (285)	14.5 ± 0.1 (308)	.094
Exposure Status	Percentage of Exp.	16.7% (285)	30% (308)	<.0001
2nd Hand Smoking (num. of cigar.)	Mean ± SEM	5.9 ± 0.6 (280)	7.4 ± 0.6 (304)	.072
Pregnancy				
Maternal Age at Delivery	Mean ± SEM	27.1 ± 0.2 (285)	26.9 ± 0.2 (308)	.781
Pregnancy Duration	Mean ± SEM	39.2 ± 0.1 (285)	39 ± 0.1 (307)	.100
Alcohol During Pregnancy	Percentage of Yes	11,7% (283)	12,4% (307)	.864
Post-natal				
Sex	Males (*n*)	148 (285)	139 (308)	.097
Birth Weight	Mean ± SEM	3464.3 ± 27.9 (283)	3327.2 ± 29.5 (306)	.0008
Birth Order	Mean ± SEM	1.9 ± 0.05 (285)	1.8 ± 0.04 (308)	.298
Parenting				
Parental Monitoring	Mean ± SEM (*n*)	2.9 ± 0.04 (285)	2.9 ± 0.04 (306)	.412
Level 2: SES and paternal antisocial beh.				
Income	Mean ± SEM	56000 ± 1907 (145)	51250 ± 2018.9 (136)	.088
Mother Education	Mean ± SEM	5.02 ± 0.1 (146)	4.5 ± 0.107 (133)	.007
Father Education	Mean ± SEM	5.1 ± 0.1 (139)	4.8 ± 0.1 (126)	.125
Maternal Antisocial Behaviour (Adolescence)	Mean ± SEM (*n*)	0.28 ± 0.04 (144)	0.2 ± 0.03 (135)	.171
Paternal Antisocial Behaviour (Adolescence)	Mean ± SEM (*n*)	0.5 ± 0.07 (141)	0.6 ± 0.07 (136)	.296

Table 3: Mean±SD and range of scores for breastfed and non-breastfed adolescents

Test	Breastfed			Non-Breastfed		
	N	Mean ± SD	Min-Max	N	Mean ± SD	Min-Max
WISC III Verbal IQ (SS[3])	285	105.1 ± 12.3	67-142	305	101.7 ± 12.5	72-137
WISC III Performance IQ (SS)	285	107.7 ± 13.5	70-142	305	103.1 ± 12.9	60-146
WISC III Full Scale IQ (SS)	285	106.7 ± 12.3	71-136	304	102.6 ± 11.8	73-138
WISC III Verbal Com. Index (SS)	285	105.0 ± 11.8	67-140	305	101.8 ± 12.1	72-140
WISC III Perceptual Org. Index (SS)	285	108.6 ± 12.8	70-142	305	103.5 ± 13.2	56-141
WISC III Freedom of Distractibility (SS)	285	100.2 ± 14.1	69-142	305	97.7 ± 13.1	61-140
WISC III Processing Speed (SS)	285	109.2 ± 14.5	67-143	305	108.9 ± 14.3	64-143
CMS Dot Locations Learning (SS[4])	282	10.7 ± 2.8	2-14	298	10.2 ± 3.1	1-14
CMS Dot Location Total Score (SS)	282	10.8 ± 2.5	2-14	298	10.4 ± 2.8	1-14
CMS Dot Location Long Delay (SS)	282	10.9 ± 2.5	3-13	298	10.7 ± 2.7	1-13
CMS Stories Immediate (SS)	282	10.7 ± 3.3	2-19	298	10 ± 3.1	2-19
CMS Stories Delayed (SS)	282	10.4 ± 3.2	1-19	298	9.8 ± 3.1	1-19
CMS Stories Recognition (SS)	282	9.1 ± 2.8	1-18	298	8.5 ± 3.1	0-16
WJ Math (Raw Score)	282	26.8 ± 4.7	14-41	299	26.1 ± 4.2	15-42
WJ Math Fluency (Raw Score)	282	88.1 ± 19.3	47-127	299	89.1 ± 22.3	37-127
WJ Reading Comprehension (Raw Score)	282	33.8 ± 3.4	21-43	299	33.1 ± 3.7	22-42
Spelling (Raw Score)	282	41.6 ± 3.4	27-45	298	41.4 ± 3.5	26-45
Ruff 2&7 Sel. Att. Test-Letters (Hits)	282	147.1 ± 28.4	73-226	299	147.8 ± 28.6	83-217
Ruff 2&7 Sel. Att. Test Digits (Hits)	282	114.5 ± 19.9	60-182	299	112.9 ± 20.5	68-178
Semantic Fluency (Total N.)	282	40.1 ± 9.6	14-70	299	39.7 ± 10.2	17-96
Phonemic Fluency (Total N.)	282	24.2 ± 8.5	6-51	299	24.2 ± 8.6	4-49

[3] SS: Standard Scores
[4] SS: Scaled Scores

Overall Fluency (Total N.)	282	64.4 ± 15.8	28-111	299	64.1 ± 16.5	24-133
Pegboard Mean Time Left	282	13.2 ± 2.3	8.5-26	298	13.5 ± 2.2	9-21
Pegboard Mean Time Right	282	11.9 ± 1.7	8-18	298	12.1 ± 1.8	8-20
Pegboard Dropped All	282	1.87 ± 1.8	0-11	299	1.86 ± 1.7	0-12
Stroop Naming Colors (Total N.)	282	69.8 ± 11.8	34-100	298	70.2 ± 12.2	39-119
Stroop Words Reading (Total N.)	282	93.9 ± 13.9	55-135	298	94.1 ± 14.6	44-147
Stroop Color-Word Naming (Total N.)	282	41.7 ± 9.6	14-74	298	41.2 ± 9.8	18-73
Stroop Interference	282	1.7 ± 6.6	-14.1 to 31.8	298	1.2 ± 6.8	-22.4 to 22.5
Sum Self Order Pointing Task	283	5.8 ± 3.1	0-19	298	6.7 ± 3.8	0-33

3.4.2. Factor Analysis of cognitive abilities

As aforementioned, indicators of factorability and residuals indicate that the solution was a good one. Kaiser–Meyer–Olkin Measure of Sampling Adequacy was 0.911 showing the amount of variance within the data that could be explained by factors and Bartlett's Test of Sphericity had a P-value <.05 meaning that the data is probably factorable. According to the total variance explained, six components have eigenvalues >1.0 and all together explain 64.2% of the variance. Table 4 shows which tests loaded on each component.

Table 4: Six Components revealed by principal component analysis: classification of subtest loadings

Components Extracted	Subtests Included
FA1: Verbal Conceptualization and Sequential Processing	WISC-III Vocabulary WISC-III Comprehension WISC-III Similarities WISC-III Arithmetic WISC-III Digit Span WISC-III Information WJ Math WJ Reading Comprehension Spelling Fluency Phonemic Fluency Semantic
FA2: Processing Speed	Ruff Total Letters Ruff Total Digits Stroop Colors Named Stroop Words Reading Stroop Color-Words Named WISC-III Coding WISC-III Symbol Search WJ Math Fluency
FA3: Visual Memory	CMS Dot Locations Learning CMS Dot Locations Total Score CMS Dot Locations Long Delay
FA4: Perceptual Organization	WISC-III Picture Completion WISC-III Block Design WISC-III Object Assembly WISC-III Picture Arrangement
FA5: Verbal Memory	CMS Stories Immediate CMS Stories Delayed CMS Stories Recognition
FA7_MD: Motor Dexterity	Pegboard Mean Time Left Pegboard Mean Time Right

3.4.3 Breastfeeding status

The relationships between breastfeeding status and potential confounders are presented in Table 2. For variables that are continuous the mean score (±SD) is listed, while for categorical variables, the percentage is stated. Out of 12 confounders, 3 were different between breastfed and non-breastfed adolescents. While income and paternal education were not different among the two groups, maternal education was higher among breastfed adolescents. Also, in agreement with previous studies, adolescents with low birth weight were more likely not to be breastfed. Results showed that adolescents who were exposed to maternal cigarette smoking during pregnancy were less likely to be breastfed. Same results about relationship of breastfeeding and exposure status were revealed by the study in Chapter 2. It is also worthwhile mentioning that parental monitoring was not different among breastfed and non-breastfed adolescents.

In the unadjusted analysis (only age effect was removed), two-way ANOVAs revealed a main effect of breastfeeding on *Visual Memory* [F (1,577) =4.96, p=.026], *Perceptual Organization* [F (1,585) =20.6, p<.0001], *Verbal Memory* [F (1,576) =6.66, p=.01], *Verbal IQ* [F (1,586) =11.97, p=.0006], *Performance IQ* [F (1,586) =22.22, p<.0001], and *Full Scale IQ* [F (1,585) =22.41, p<.0001], a main effect of exposure status on *Perceptual Organization* [F (1,585) =5.83, p=.016], *Performance IQ* [F (1,586) =5.40, p=.020], and *Full Scale IQ* [F (1,585) =6.25, p=.012], and no significant interaction of exposure and breastfeeding status. Results on mean differences on cognitive abilities between groups of breastfeeding and exposure status, and interaction effects are shown in Table 5.

Table 5: Unadjusted analysis of breastfeeding and exposure status effects on cognitive abilities. T tests (p-value) from 2-way ANOVA are reported; minus sign indicates lower score for non-exposed/non-breastfed adolescents.

Cognitive Factors	Exposure	Breastfeeding	Interaction
Fa1: Verbal Consep.	-1.7 (.075)	-1.8 (.061)	1.4 (.164)
Fa2:Processing Speed	-1.8 (.072)	.76 (.446)	.62 (.535)
Fa3: Visual Memory	-0.4 (.638)	**-2.2 (.026)**	-0.4 (.683)
Fa4: Perceptual Org.	**-2.4 (.016)**	**-4.5 (<.0001)**	.30 (.764)
Fa5: Verbal Memory	-1.4 (.152)	**-2.6 (.010)**	.76 (.449)
Fa6: Motor Dexterity	1.7 (.087)	1.7 (.093)	-0.5 (.588)
Verbal IQ	-1.6 (.115)	**-3.5 (.0006)**	0.7 (.482)
Performance IQ	**-2.3 (.020)**	**-4.7(<.0001)**	1.2 (.237)
Full Scale IQ	**-2.5 (.012)**	**-4.7 (<.0001)**	.96 (.339)

3.4.4. Duration of exclusive breastfeeding status

Table 6 shows breastfeeding characteristics and separation of the exclusive breastfeeding duration into three categories.

Table 6: Breastfeeding Characteristics

Breastfeeding Characteristics	
Median number of weeks breastfed	8
Min-Max weeks of breastfed	1 – 28
Interquartile range (25% - 75%)	4 – 16
Distribution of Breastfeeding duration	
< 4 weeks	19%
4-16 weeks	67%
≥ 16 weeks	14%

3.4.5. Hierarchical Linear Modelling

For the HLM analysis, potential confounders were grouped into two levels: offspring-based Level 1: age, exposure status, pregnancy and post-natal environment, and parenting; and parent-based Level 2: SES and parental antisocial behaviour. Table 7 provides information on which predictor loaded on each of the different cognitive models. After addition of all Level 1

and Level 2 predictors, breastfeeding status was added into the model as the last important predictor. In the 1st set of analysis breastfeeding status was used as the final predictor added to the model. In the 2nd set of analysis exclusive breastfeeding duration, instead of breastfeeding status, was used as the final predictor that was added to the models. Both set of analyses revealed the same results (Table 8). Breastfeeding status was significantly predicting performance on perceptual organization component, Performance and Full Scale IQ. Similarly, exclusive breastfeeding duration was significantly predicting performance on perceptual organization and motor dexterity components, as well as Performance and Full Scale IQ. Table 8 also presents data on effect sizes from the two type of analysis.

Table 7: Significant (except exposure status) Level 1 and Level 2 predictors that were added to the different models. (Breastfeeding variable was added to the end of the analysis.)

Models	**Level 1 Predictors Total Sample (n=597)**			**Level 2 Predictors Total Sample (n=239)**		
		T-ratio	***p***		**T-ratio**	***p***
Fa1: Verbal Consep.	Age Exposure	14.708 1.129	<.0001 .260	Maternal educ. Paternal educ.	5.48 3.87	<.0001 <.0001
Fa2:Processing Speed	Age Sex Exposure	15.278 4.68 1.26	<.0001 <.0001 .206	Paterna edu.	2.66	.009
Fa3: Visual Memory	Sex Birth Order Exposure	-3.78 -1.95 0.67	<.0001 .050 .499	Maternal edu.	2.37	.018
Fa4: Perceptual Org.	Age Exposure	12.23 0.57	<.0001 .056	Maternal edu. Paternal edu.	3.93 2.60	<.0001 .010
Fa5: Verbal Memory	Age Sex Exposure	4.07 -2.19 0.52	<.0001 .028 .599	Maternal edu. Paternal edu.	4.26 3.00	<.0001 .003
Fa6: Motor Dexterity[5]	Age Sex Maternal age at delivery Exposure	-5.41 -6.22 -2.97 0.31	<.0001 <.0001 .006 .752			
Verbal IQ	Age Alcohol during pregnancy Exposure	4.33 2.13 -0.40	<.0001 .033 .686	Maternal edu. Paternal edu.	5.86 5.50	<.0001 <.0001
Performance IQ	Age Exposure	2.14 0.58	.032 .562	Maternal edu. Paternal edu.	4.35 2.98	<.0001 .004
Full Scale IQ	Age Exposure	3.79 0.46	<.0001 .640	Maternal edu. Paternal edu.	6.10 4.80	<.0001 <.0001

[5] Minus sign in Motor dexterity indicates better performance over time

Table 8: Two sets of analyses (HLM) revealing breastfeeding (ever and exclusive) associations with cognitive measures and IQ indexes. Effect sizes are also reported.

Models	Breastfeeding status		Exclusive breastfeeding duration		Effect size[6]	Effect size
	T-Ratio	p_1	T-Ratio	p_2	r_1	r_2
Fa1: Verbal Consep.	0.55	.577	1.02	.305	.024	.045
Fa2:Processing Speed	-0.78	.431	-0.67	.503	.034	.029
Fa3: Visual Memory	0.78	.431	1.68	.092	.034	.074
Fa4: Perceptual Org.	**2.14**	**.032**	**3.11**	**.002**	**.095**	**.137**
Fa5: Verbal Memory	0.87	.381	1.00	.315	.038	.044
Fa6: Motor Dexterity	-1.87	.061	**-2.60**	**.010**	**.083**	**.115**
Verbal IQ	1.25	.211	1.53	.126	.055	.068
Performance IQ	**2.22**	**.026**	**2.87**	**.005**	**.098**	**.127**
Full Scale IQ	**2.12**	**.034**	**2.68**	**.008**	**.094**	**.118**

[6] r is indicative of effect size, $\mathbf{r=\sqrt{[(t)^2/(t)^2+df]}}$

3.5. Discussion

The main purpose of the present study was to evaluate long-term associations of exclusive breastfeeding duration with cognitive abilities in a large sample of adolescents half of whom were exposed to maternal cigarette smoking during pregnancy and the other half were non-exposed but matched according to maternal education to the exposed adolescents. Firstly, we examined both the main effects of breastfeeding and exposure, as well as the interaction between the two independent variables without adjusting for any possible confounder. Next, using hierarchical linear modelling, we performed two similar types of analysis: the first set of analysis used breastfeeding status as a categorical variable with two levels (breastfed *Vs* non-breastfed), whereas the second type of analysis used exclusive breastfeeding duration as a categorical variable with four levels (non-breastfed/breastfeed for less than 4 weeks/ breastfed for 4 to 16 weeks/ and breastfed for more than 16 weeks). Breastfeeding effects can be assumed to vary in proportion to the length of time to weaning and the daily dose of breastfeeding; therefore breastfeeding is more accurately measured as a three-category variable.

To obtain a comprehensive view of the cognitive measures, factor analysis was used and grouped the different cognitive tests in six components: verbal comprehension and sequential processing, processing speed, visual and verbal memory, perceptual organization skills, and motor dexterity.

3.5.1. Breastfeeding and exposure to maternal cigarette smoking

We found that women who smoked during pregnancy were less likely to breastfeed their children. Mills (1950) was the first to describe the association between maternal cigarette smoking and breastfeeding duration; he found that duration of lactation was shorter among smoking mothers. Our finding is consistent with several other reports: mothers who smoke during pregnancy are less likely to breastfeed their infants (Horta, Kramer & Platt, 2001; Amir, 2001; Letson, Rosenberg & Wu, 2002). The explanation may lay in several factors. Women who smoke tend to be younger, less educated, and have lower income than non-smokers and women who breastfeed (Scott & Binnis, 1998). A review provided evidence that women who smoke have less motivation to breastfeed: they are less likely to intend breastfeeding and less

likely to initiate breastfeeding (Amir, 2002; Donath et al., 2004). Furthermore, it is believed that the two most important hormones involved in lactation, prolactin and oxytocin, are decreased by nicotine levels thus affecting milk synthesis and production, respectively. But despite the lowered levels of prolactin in milk of smoking women, no relationship was identified between levels of prolactin and rate of milk synthesis (Amir, 2001; Donath, Amir & the ALSPAC team, 2004).

Last but not least, there is substantial evidence to show that smoking mothers who breastfeed are exposing their children to nicotine and its metabolite cotinine, with possible effects on the infant's behaviour. Using gas liquid chromatography, Luck and Nau (1984) analyzed 44 milk samples from 23 nursing smokers and found that nicotine accumulates rapidly in breast milk and that nicotine concentrations in milk were higher than the corresponding (maternal) serum concentrations. Nicotine concentrations in milk of smoking mothers depends on the number of cigarettes smoked prior to nursing, as well as the time interval between the last cigarette consumed and the next nursing period (Luck & Nau, 1987; Dahlström, Ebersjö & Lundell, 2004). Also, breastfed infants of mothers who smoke have median urinary cotinine (nicotine's metabolite) levels 10 fold higher than those artificially fed infants of smoking mothers (Mascola, Vunakis, Tager, Speizer & Hanrahan, 1998). Thus, infants of smoking mothers are exposed to nicotine during early neonatal life, if breastfed. This "milk-mediated" exposure is likely combined with an "air-mediated" exposure to the second-hand smoke produced by the mother and/or her partner; Dahlström *et al* (2004) found nicotine in breast milk of non-smoking women exposed to second-hand smoke at home. The major question concerning nicotine and cotinine in breast milk is whether this has a detrimental effect on the child. According to the American Academy of Pediatrics (1994), nicotine (smoking) can cause shock, vomiting, diarrhoea, rapid heart rate and restlessness to the breastfed infant. These were based on two of the earliest publications relating case history of a six-week old infant (Bisdom, 1937) and three-to-four days old infants (Majewski, 1979) whose mothers smoked during breastfeeding. Symptoms of restlessness, insomnia, vomiting, diarrhoea and rapid pulse disappeared once the mothers stopped breastfeeding their infants. But a revised section of the American Academy of Pediatrics (Committee on Drugs, 2001) states that there is no substantial evidence vis-à-vis a health risk of nicotine in breast milk to the nursed infant.

3.5.2. Breastfeeding status

Results from the two-way ANOVAs (unadjusted analysis) showed significant main effects of breastfeeding status on cognitive abilities. There was no significant interaction between exposure status and breastfeeding status. This unadjusted analysis showed that breastfed adolescents had higher Full-scale (by 4 IQ points), Verbal (by 4 IQ points) and Performance (by 4 IQ points) IQ, and better performance on visual and verbal memory, as well as better perceptual-organization skills. However, subsequent analysis revealed that breastfed children tended to be born to socially advantaged families characterized by having better educated mothers and who did not smoke during pregnancy. But even after controlling for these and other potential confounders (by HLM), breastfeeding was still positively associated with Full-scale and Performance IQ, as well as with better perceptual-organization skills. Second set of analysis showed that longer duration of exclusive breastfeeding was positively associated with Full-scale and Performance IQ, as well as higher scores on perceptual organization skills and motor dexterity.

These results are consistent with previous studies indicating that breastfeeding is positively related with later cognitive outcome, even after adjusting for potential confounders. Several studies have analyzed duration of breastfeeding in a manner similar to this study. Clark *et al* (2006) found that children breastfed for 2 to 8 months had higher scores for language, motor and cognition tests than children breastfed for less than 2 months or more than 8 months; these findings were present after adjusting for socioeconomic factors. Similar results were obtained by Horwood and Fergusson (1998) who showed, in a birth cohort study of >1000 New Zealand children, that those who were breastfed for more than 8 months had mean test scores (reading comprehension, mathematical ability, and scholastic ability assessed during the period from 10 to13 years old) higher by 0.11 to 0.30 SD than those not breastfed; again, this was the case even after controlling for socioeconomic and other factors. Another recent study on breastfeeding duration reported a positive dose-response relationship for Full Scale, Verbal and Performance IQ among adult males who were breastfed as infants up to 9 months but lower scores for those breastfed for more than 9 months (Mortensen et al., 2002).

Further, Oddy *et al* (2003) found a positive association between Verbal IQ and exclusive breastfeeding duration at 6 years old children, after adjusting for perinatal, social and

family factors. Finally, Gustafsson *et al* (2004) showed that duration of breastfeeding contributed significantly to Full scale IQ, Verbal and Performance IQ. Overall, there is universal agreement that breastfeeding duration is associated with small but significantly positive outcomes on cognition. In our study, we observed an effect size of .118 and .127 for Full scale and Performance IQ, respectively, indicating that adolescents who had been breastfed for more than 16 weeks have IQ higher by 0.14 and 0.16 SD units than those who had not been breastfed. Small effects can be very important; effect size for many life-saving medical treatments is less than 0.2 standardized units (Brace et al., 2006). Even a small change in mean IQ can affect the number of children falling below any given cut-off of concern, as well as the number considered high functioning (Rogan and Gladen, 1993).

Motor-dexterity component reflects the time that a person needs to perform a task with his fingers, thus reflecting fine motor skills. Perceptual organization component is the same as the perceptual organization index derived from WISC-III. It measures non-verbal, fluid reasoning, attention to detail, and visual-motor integration. The difference between perceptual organization component and Performance IQ is that processing speed is off less importance for the former.

It should be also noted that when evaluating breastfeeding associations with cognitive ability, the main confounder is maternal intelligence. One of the reasons for this confounding effect is the relatively high heritability of intelligence (Mackintosh, 1998; Bouchard & McGue, 2003). By removing the effect of intelligence, one can examine the association between breastfeeding and child's intelligence in a manner less confounded by genetic factors. In a study using data from a national database, adjustment for maternal intelligence removed the breastfeeding effect (Der, Batty & Deary, 2006). Here, maternal intelligence is not included in the data, but maternal education is. It is often assumed that educational attainment has lower heritability than intelligence; yet, a large number of twin and adoption studies have challenged this view, supporting the notion that intelligence and educational attainment have nearly similar heritability (Mackintosh, 1998). There is also a noteworthy correlation between intelligence and total number of years of education (McCall, 1977), and thus between intelligence and educational qualifications. Furthermore, even when controlling for parental socioeconomic status, intelligence scores are still strongly related with educational attainment

(Herrnstein & Murray, 1994). Thus, when examining the effects of any variable, such as breastfeeding status, on child's cognitive ability, maternal education is a reasonable proxy of maternal intelligence.

3.5.2.1. Potential Mechanisms

There are several potential mechanisms that could explain the beneficial associations of breastfeeding with cognitive abilities. Many factors that are considered confounders when estimating the presence and/or strength of an association between breastfeeding and cognitive abilities are highly correlated with both breastfeeding and cognition (residual confounding). As pointed out above, an example of this is the most significant predictor of both breastfeeding and cognitive development, namely maternal intelligence. Other possible mechanisms could be the physical contact and psychological interactions between the mother and the child during breastfeeding, which may enhance the bonding process (Newton, 1971). Also, as Drane and Logemann stated, lactating women have higher circulating levels of prolactin and oxytocin than non-lactating women, thus activating feelings of calmness and nurturing behaviour and facilitating positive mother-child interactions. Finally, extensive research has focused its interest on components of human milk, with the most plausible explanation given by the content of long-chain polyunsaturated fatty acids. We will now turn our attention to this potential mechanism.

3.5.2.1.1. The LC-PUFA Hypothesis

As already mentioned in the Introduction, human milk is rich in essential fatty acids, but also in their long-chain polyunsaturated fatty acids derivatives, arachidonic acid and docosahexaenoic acid (DHA). Infants born at term and fed with mother's milk had approximately twice as much DHA in red blood-cell phospholipids as infants receiving formula with ALA but not DHA (Putnam, Carlson, DeVoe & Barness, 1982). Byard, Makrides, Need, Neumann and Gibson (1995) studied DHA levels, measured by gas chromatography, in samples of frontal lobe and brainstem taken from 28 and 26 infants, respectively, who had died of sudden infant death syndrome. They found significantly higher DHA levels within the frontal lobes of breastfed infants compared with those who were formula fed. Similar results were obtained by Makrides, Neumann, Byard, Simmer and Gibson (1994) who studied total lipids, measured with capillary gas chromatography, in erythrocytes,

retina and brain cortex from 35 term infants who had died of sudden death. They found higher proportion of DHA in the erythrocytes and brain cortex of breastfed infants compared with formula fed infants. They also showed that cortex DHA increased in breast-fed (but not formula-fed) infants with age, presumably related to the feeding duration.

Taking these studies together, Cunnane, Francescutti, Brenna, and Crawford (2000) concluded that, over the first six months of life, DHA accumulates at about 10mg/d in the whole body of breastfed infants, with 48% of it appearing in the brain. They also report that, despite the fact that term infants have a store of about 1,050mg of DHA in body fat and an intake of about 390mg/d of ALA, the brain of formula fed infants not consuming DHA accumulates at a rate half of the DHA of the brain of breastfed infants. Formulas before 1990 did not contain any LC-PUFAs, hence these differences among breastfed and formula fed infants led to the explanation that LC-PUFAs in the human milk, and especially DHA, are driving these positive effects on infant's development.

High levels of LC-PUFA, especially DHA, are contained in the cellular membranes of the CNS. More specifically, high concentrations of DHA are present in phosphatidylserine and the ethanolamine phosphoacyl-glycerols of the brain gray-matter and the outer segments of rod and cone photoreceptors in the retina (Innis, 2003). In the brain, DHA is enriched in synaptic terminal membranes (Innis, 2007). From 26 prenatal weeks to 8 postnatal years, DHA increases in phosphatidylethanolamine (PE) and phosphatidylcholine, while AA remains constant (Martinez & Mougan, 1998). The brain seems to be selective in the absorption of LC-PUFA, preferring those with 20 and 22 carbon chain fatty acids rather than their 18 carbon polyunsaturated fatty acid (PUFA) precursors (Innis, 2003). Reduced levels of DHA in the brain impair neurogenesis, and neurite outgrowth and modify the metabolism of several neurotransmitters such as dopamine and serotonin (Innis, 2007). The rate of brain growth is very high during the last three months of gestation and the first 12 months after birth, thus DHA requirements for the infant may be high during these stages. Before birth, DHA is extracted from the maternal circulation through placental transfer; after birth DHA should originate from mother's milk or formula diet.

Supplementation during pregnancy and lactation

In a recent meta-analysis, worldwide mean DHA and AA concentrations in human milk were 0.32±0.22% and 0.47±0.13% (Brenna, Varamini, Jensen, Diersen-Schade, Boettcher and Arterburn, 2007). DHA content in breast milk increases with DHA supplementation of lactating women (Makrides, Neumann & Gibson, 1996; Henderson, Jensen, Lammi-Keefe, Ferris & Dardick, 1992; Jensen, Maude, Anderson & Heird, 2000), while supplementation with ALA increases milk ALA but has modest effect on DHA content in breast milk (Francois, Connor, Bolewicz & Connor, 2003).

In the study by Helland, Smith, Saarem, Saugstad and Drevon (2003), pregnant women received 10mL of cod liver oil (1183mg/10 mL DHA & 803mg/10Ml EPA) or 10mL corn oil (4747mg/10mL LA & 92mg/10mL ALA) from the 18th week of gestation until three months after delivery. Infant's cognitive development was assessed at six and nine months after birth and at four years of age with the Kaufman Assessment Battery for Children (K-ABC). Children of mothers supplemented with cod oil during pregnancy and lactation scored higher on the Mental Processing composite score of K-ABC than those whose mothers had taken corn oil. Further, children's K-ABC score at four years of age correlated with maternal intake of DHA and EPA during pregnancy. Given the design of this study, it is unclear whether benefits on cognitive development are due to supplementation during pregnancy, lactation or both.

Few randomized controlled trials have assessed the effect of DHA supplementation during lactation on infant's visual and cognitive development. Even though these studies found higher DHA level in the content of human milk and in the infant's blood status, as compared with placebo, they observed no significant effects of supplementation on any of the indicators of visual development (Gibson, Neumann & Makrides, 1997; Lauritzen, Jorgensen, Mikkelsen et al., 2004; Lauritzen, Jorgensen, Olsen, Straarup & Michaelsen, 2005; Jensen, Voigt, Prager et al., 2005). Effects on cognitive development are somewhat inconsistent. In Gibson's *et al* study (1997) mothers were supplemented with DHA for the first 12 weeks post partum; DHA levels in breast milk correlated with DHA levels in infant plasma and erythrocytes. Furthermore, DHA levels in infant erythrocytes were associated with Bayley Mental Development Index at 12 weeks but not at 2 years of age. There was a positive effect of

supplementation in girls' performance on a problem-solving test (Lauritzen et al., 2005) and a positive association of psychomotor development at 30 months of age and supplementation in Jensen's study (Jensen et al., 2005).

Overall, these interventional studies illustrate that DHA supplementation during lactation has no effect on infant's visual development, whereas there is some evidence that cognitive development is somewhat affected by DHA supplementation. Mixed results are probably due to different methodological designs, use of different cognitive tests and use of different dosage of DHA supplementation.

Supplementation of term infants with LC-PUFA containing formula

Varied results were also obtained in infant formula studies. A recent Cochrane review included 14 randomized controlled trials (RCTs) with 1,719 children fed with formula enriched with DHA and AA or DHA alone, as compared with standard milk formula (Simmer, Patole & Rao, 2008). The main outcomes assessed in these studies were: visual acuity, neurodevelopmental and physical growth. Only three out of nine studies showed a beneficial effect of LC-PUFA supplementation on visual acuity throughout the three first years of life. Eleven studies assessed neurodevelopmental outcome at different ages until the age of two years; 8 out of 11 studies used the Bayley Scales for Infant Development (BSID), with only 1 study finding a positive effect of LC-PUFA supplementation. Pooled meta-analysis of the data from the eight studies did not show any benefit of LC-PUFA supplementation on either mental or psychomotor developmental index of BSID. Other studies, in which different developmental measures were used, yielded inconsistent results. Taken together, the authors concluded that the results of most well-conducted RCTs have not shown any advantageous effect of LC-PUFA containing formula milk on visual or cognitive development of infants born at term, thus supplementation of milk formula with LC-PUFA to improve infant's development cannot be recommended unless more evidence is provided in the future.

Preterm vs Term Infants

Preterm infants are usually considered separately since they have lower LC-PUFA concentrations than infants born at term; this is probably due to the fact that preterm infants miss the major period of in utero DHA and AA accretion in the brain, which occurs during the last trimester of gestation (Clandinin, 1980). Studies investigating the association between

breastfeeding and cognitive development of premature infants report greater effects than term infants (Reynolds, 2001). The most prominent study is the one by Lucas *et al* (1992) in which higher IQ was documented at the ages of 7½-8 years in premature breastfed infants than infants who received no maternal milk. Fifteen randomized trials were included in a Cochrane review (Simmer, Schulzke & Patole, 2008) examining the effects of LC-PUFA containing formula milk on visual acuity, development and growth of preterm infants. Most studies found no valuable effect of LC-PUFA supplementation on any visual assessment; meta-analysis of BSID of four studies at 12 months (n=364) and three studies at 18 months (n=494) showed no effect of supplementation on neurodevelopment.

Taken together, supplementation studies during lactation, at term and preterm infants revealed no beneficial effect of LC-PUFA on visual or mental development. Studies of the association between breastfeeding status and visual development and cognitive ability in childhood and adolescence continue to demonstrate a positive, even though weak, association of breastfeeding with cognitive ability. Could it be that the beneficial effects of breastfeeding or LC-PUFA supplementation are revealed later on in life? Does DHA, even though it is highly accumulated by the brain during the last trimester of gestation and during the first year of life, need time to build up in the brain and mostly support brain areas that are fully developed later on in adolescence? Genetic factors should also be considered. After eating a meal with added DHA, lactating women with the 347S variant of ApoA-IV had 40% more DHA in their breast milk than women with the 347T variant (Weinberg, Greenwood & Chacon-Angobaldo, 2005). Additionally, lactating women with the E4 variant of ApoE had lower breast milk fat content than women who did not have this variant. And, interestingly, the study by Caspi *et al* (2007) showed that the association between breastfeeding and IQ is moderated by a genetic variant in *FADS2;* C-carrying breastfed children showed an advantage on IQ than C-carriers non breastfed, whereas GG homozygotes neither gained an advantage from breastfeeding nor suffered a disadvantage from not being breastfed.

The LC-PUFA hypothesis has been proposed as a key explanation for neurodevelopmental benefits of breast milk. However, breast milk consists of many ingredients besides fatty acids, thus fatty acids can not be causative in the relationship between breastfeeding and cognition. Isaacs *et al* (2009) suggested that dietary cholesterol intake may

explain an impact of breast milk on white matter development and cognition through enhanced myelination/glial production, due to reports stating that breast milk contains noteworthy quantities of cholesterol whereas infant formulas contain little (Uauy, Mize, Castillo-Duran; 2000), and that cholesterol is an essential component of myelin membranes in mice (Saher, Brugger, Lappe-Siefke, Mobius, Tozawa et al., 2005).

3.6. Study Limitations and Strengths

One of the limitations of this study is the fact that breastfeeding status was determined by maternal reports, which may not be free of recall bias. The limitation of SLSJ study, comparing at the studies included in the reviews of Anderson, Jain, and Drane and Logemann, is the relatively small sample size (n=599) and the cross-sectional design. One of the main strengths of this study is the comprehensive nature of the cognitive assessment. The neuropsychological battery consists of 33 cognitive tasks, from which 31 were included in further analysis, thus providing a wealth of information along a broad spectrum of cognitive abilities. Most of the studies included in the above reviews used only measures of intelligence or general ability. Also the age range of the study is well defined comprising all ages during the adolescence period, whereas other studies have focused on specific ages during childhood and adolescence. Last but not least, the fact that breastfeeding was measured both as a 2-level variable (yes/no) and a 4-level variable (providing information on duration and exclusivity of breastfeeding) had the advantage of comparing results from both sets of analysis with previous studies using either of the two approaches.

3.7. Conclusion

A longer duration of exclusive breastfeeding was associated with higher intelligence in a group of adolescents exposed and non-exposed to maternal cigarette smoking during pregnancy, even after adjusting for potential confounders. Potential confounders were grouped into two levels; maternal education and birth-weight were the most noteworthy ones. Similarly with other studies, duration of exclusive breastfeeding appeared to be a superior way of measuring association between breastfeeding and cognitive abilities. Breastfeeding is thought to enhance cognitive development through different mechanisms. Different elements of breast

milk, such as polyunsaturated fatty acids and cholesterol, can enhance cognitive development. By breastfeeding, prolactin and oxytocin can activate feelings of calmness thus making the mother more affectionate to her child. According to the above, is still unknown if breastfeeding on its own is beneficial for infant's cognitive development. Probably, both maternal behaviour and breast milk are positively influencing infant's cognitive development.

Chapter 4: Study 3- Nutrition at birth: Breastfeeding and brain structure in adolescence

4.1. Introduction

4.1.1. Brain Development

The human brain begins to grow during the first trimester of gestation, with whole brain volume, cortical gray matter and white matter volume increasing with age (Hüppi, Warfield, Kikinis, Barnes, Zientara, Jolesz, Tsuji, and Volpe, 1998). The third trimester is also the time period during which cortical folding and gyrification begins (Dubois, Benders, Cachia, Lazeyras, Ha-Vinh, Leuchter, Sizonenko, Borradori-Tolsa, Mangin and Hüppi, 2008). A recent study showed that total brain volume measured with MRI in 98 children increased by 101% in the first year of life, with another 15% increase in the second year; gray matter increased by 149% in the first year while white matter increased by only 11% (Knickmeyer, Gouttard, Kang, Evans, Wilber, Smith, Hamer, Lin, Gerig & Gilmore, 2008). The large increase in total brain volume in the first year of life suggests that this is a critical period in which any environmental factor can have long-lasting, if not permanent, effects on brain structure and function (Knickmeyer et al., 2008).

The progression of key events that underlie brain development is detailed in Chapter 1; nonetheless, a brief description follows. The neural tube, which is formed in the first four weeks of gestation, will give rise to the central nervous system (CNS). While the nervous system is being differentiated, cell proliferation and differentiation into neurons and glia begins. At the same time neurons migrate from the ventricular zone to their final position. Migration is essential as it establishes the identity of neurons and defines their functional properties and their future connections (Wainwright, 2002). Cell differentiation follows the migration stage with axons and dendrites development. During the last trimester of gestation, synapse formation begins, followed by a series of events including programmed cell death (apoptosis), and axonal and synaptic pruning. Myelination starts prenatally and extends well beyond adolescence. All these processes are influenced by the environment.

Among a number of environmental factors, diet plays a significant role in brain development and its maturation; some examples are the effect of folate deficiency on neural

tube development during early gestation, as well as the influence of essential fatty acids deficiency during gestation and postnatal life on visual function of infants (Fernstrom, 2000). Clandinin and Jumpsen (1997) report that "... Even under conditions of a nutritionally adequate diet, brain lipid metabolism and the functions of integral membrane proteins may be influenced by variations in quantitative and qualitative aspects of food consumed." (pg.17). Hence, the amount of lipids in the post-natal diet can play crucial role on the structural and functional aspects of the developing brain, such as the cell membrane. The following section addresses the role of dietary lipids on brain development.

4.1.2. Dietary long-chain polyunsaturated fatty acids and brain development

The dry weight of the adult brain consists of 50% lipids, of which approximately 35% are in the form of LC-PUFA, primarily arachidonic acid (20:4 ω-6; AA) and docosahexaenoic acid [(or otherwise cervonic acid, 22:6 ω-3; DHA) Wainwright, 2002]. These LC-PUFA are obtained by biosynthesis from their respective dietary essential fatty acids (EFA) precursors, namely the linoleic acid (18:2 ω-6; LA) and α-linolenic acid (18:3 ω-3; ALA), or they are gained directly from diet. The latter fatty acids are called essential because they can only be obtained from diet; they are formed in plants and the human body cannot synthesize them because of the absence of the Δ12 and Δ15 enzymes necessary to insert a double bond at the ω-6 or ω-3 position of a fatty acid carbon chain (Innis, 2003).

Among different tissues, the nervous system has the highest concentrations of PUFAs, with DHA as the major acid in the outer segments of the retina rods and cones where it constitutes almost 50% of the fatty acids in phospatidylethanolamine (PE)[7] and phosphatidylserine (PS)[8]. These membranes are responsible for the rapid transmission of light and contain 90% to 95% of the lipid as phospholipid (Innis, 2003). The phospholipids of brain gray-matter consists of high amounts of DHA in PE and PS and high proportions of AA in phosphatidylinositol (PI)[9]; unlike other organs, EFAs concentrations in the brain are quite low with LA representing <1% of brain and retina fatty acids, and ALA concentration are even lower (Sastry, 1985).

[7] (PE) is a lipid found in biological membranes.
[8] (PS) is a phospholipid component usually kept on the inner-leaflet of cell membranes.
[9] (PI) is a negatively charged phospholipid and a minor component in the cytosolic side of cell membranes.

In neurons, LC-PUFAs internalized from extracellular fluids are incorporated into phospholipids (PLs; Rapoport, 2001) where they influence membrane fluidity and modify the function of many integral and membrane-associated proteins (Marszalek & Lodish, 2005; Innis, 2003). Because neurotransmission depends on membrane receptors, which interact with G protein and other second-messenger systems, variations in membrane phospholipid-fatty acid composition may affect the nature of these interactions (Wainwright, 2002). Chronic ω-3 fatty acid deficiency decreased dopamine-receptor binding in the frontal cortex of rats, while altering the dopamine metabolism (Zimmer, Hembert, Dward, Breton, Guilloteau, Besnard & Chalon, 1998; Zimmer, Vancassel, Cantagrel, Breton, Delamanche, Guilloteau, Durand & Chalon, 2002). PUFAs can be released from the PLs by phospholipases and serve as signaling molecules or be converted into prostaglandins, leukotrienes and thromboxanes (also known as eicosanoids; Marszalek & Lodish, 2005). Plus, LC-PUFAs can have direct effect on gene expression through binding directly with transcription factors (Jump & Clarke, 1999).

Before birth, the foetus obtains all the EFAs and LC-PUFAs from maternal circulation through placental transfer. "Transfer of DHA across the placenta involves fatty acid binding proteins, with release of DHA to the fetal circulation, followed by transport to liver where it is esterified and resecreted in lipoproteins" (Innis, 2008). Plasma DHA varies among newborn infants thus indicating that DHA and AA proportions are highly influenced by maternal diet. After birth, all ω-6 and ω-3 fatty acids are derived from breast or formula milk. The vital difference, however, between breast and formula milk is that the latter contains only the essential fatty acids and not the long-chain polyunsaturated fatty acids derivatives. Hence, the question that arises is to what extent the developing foetus and infant is able to convert LA and ALA into AA and DHA respectively. Term and preterm infants are capable of converting LA to AA and ALA to DHA as it was shown by several tracer studies using stable isotopes of LA and ALA (Carnielli, Wattimena, Luijendijk, Boerlage, Degenhost & Sauer, 1996; Demmelaier, Schenck, Behrendt, Sauerwalk & Koletzko, 1995; Salem, Wegher, Mena & Uauy, 1996; Sauerwald, Hachey, Jensen & Heird, 1997; Uauy, Mena, Wegher, Nietro & Salem, 2000). A more recent study using a whole-body natural isotope-tracer approach showed that in one month preterm infants consuming formulas with 0.64% w/w DHA, an average of 42% of DHA were biosynthesized from ALA which dropped in 7% in seven months (Carnielli, Simonato,

Verlato, Luijendijk, De Curtis, Sauer & Cogo, 2007). The amount of ALA in infant formulas may be, however, inadequate to support the proportion of DHA in the human milk.

In an analysis of Cunnane, Francescutti, Brenna and Crawford (2000) it was estimated that DHA accumulates at about 10mg/d in the whole body of breast-fed infants, with 48% of that amount appearing in the brain. They also estimated that despite 1,050 mg of DHA in body fat at term birth and an intake of approximately 390mg/d ALA (from formula), formula-fed infants not consuming DHA would accumulate only half the DHA (in the brain) of breast-fed infants (Cunnane et al., 2000).

Inclusion of DHA in infant formulas has been shown to increase DHA in the blood lipid of the infant (Makrides, Neumann, Simmer & Gibson, 1995; Carlson, Ford, Werkman, Peeples & Koo, 1996), as well as to increase visual acuity, cognitive and motor development of full term supplemented with DHA and DHA+AA formula infants (Birch, Hoffman, Uauy, Birch & Prestidge, 1998; Birch, Garfield, Hoffman, Uauy & Birch, 2000).

4.1.3. Deficiency of ω-3 fatty acids and brain development in animals

Numerous animal studies have examined the effect of ω-3 fatty acids deficiency on brain structure and function. Wainwright, Bulman-Fleming, Lévesque, Mutsaers and McCutcheon (1998) showed that mice fed a saturated-fat diet deficient in both ω-3 and ω-6 fatty acids (and raised in an enriched environment[10]) had less dendritic branching in pyramidal cells of occipital cortex than mice fed the control diet with ω-6:ω-3 fatty acids of 4:1. Plus, mice fed the saturated-fat diet showed retarded growth and impaired ability to learn the location of a platform, and had lower levels of DHA in the brain (Morris water maze; Wainwright, Huang, Bulman-Fleming, Lévesque & McCutcheon, 1994).

Ahmad, Moriguchi and Salem (2002) measured the neuronal size in hippocampus, hypothalamus, piriform cortex and parietal cortex in rats that were either under a DHA-deficient diet or in a supplemented diet with flaxseed oil (as a source of ALA) and with DHASCO[11] (as a source of DHA). Size of neurons in the hippocampus, hypothalamus, and parietal cortex was lower at weaning (21 days) in rats raised on the DHA-deficient diet, as

[10] Enriched environment involved housing mouse in groups of 12 in large cages with food and bedding, as well as opportunities for exploration and physical activity, such as digging trays and rotating series of toys.
[11] Docosahexaenoic acid single cell oil

compared with rats in the ω-3 supplemented diet; these rats exhibited a decrease of 90% of brain DHA, as compared with rats in the ω-3 supplemented diet.

A study by Coti-Bertrand, Kusky and Innis (2006) showed that DHA was 55-65% lower and DPA[12] was 150-225% higher in brain phospholipids at embryonic day 19 of the ω-3 deficient rats, as compared with ω-3 control rats. From 2 weeks prior to mating and throughout pregnancy, female rats were fed either a ω-3 deficient diet (0.03% energy from ALA from safflower oil) or a diet with 1.2% energy from ALA from canola oil. Their diets also differed in ω-6 fatty acid intake, with the ω-3 deficient diet having a high dietary ω-6 fatty acid intake and a high ω-6/ω-3 ratio. The main purpose of this study was to evaluate neurogenesis in the embryonic rat brain. The results showed that the mean thickness of the cortical plate and mean sectional area of the primordial dentate gyrus were 26 and 48% lower, and the mean thicknesses of the cortical ventricular zone[13] and the primary dentate neuroepithelium[14] were 110 and 70% higher in the ω-3 deficient embryos. As such, these results suggest that ω-3 fatty acid deficiency alters neurogenesis in the embryonic rat brain, perhaps by delaying or inhibiting normal development.

Moreover, DHA deficiency results in decreased neurite growth in rats' hippocampal neurons; in hippocampal slices/cultures, DHA supplementation results in increased individual neurite length, as well as number of branches (Calderon & Kim, 2004). The uniqueness of DHA effects on neurite growth was apparent in the same study of Calderon and Kim, when *in vitro* supplementation of hippocampal neurons with arachidonic, oleic and docosapentaenoic acid had no effect on neurite growth. DHA also promotes the differentiation of neural stem cells into neurons both *in vitro* and *in vivo*, by supporting cell-cycle exit and by suppressing cell death (Kawakita, Hashimoto & Shido, 2006). Lastly, a more recent study by Suganuma, Arai, Kitamura, Hayashi, Okumura and Shimizu (2010) suggested that maternal DHA supplementation during pregnancy in rats offer neuroprotection by inhibiting oxidative stress and apoptotic neural death.

[12] Docosapentaenoic acid (22:5 ω-6)
[13] Contains progenitor cells that give rise to both intermediate progenitor cells and post-mitotic neurons and glia destined for the cerebral cortex
[14] Contains progenitor cells that give rise to proliferative cells of the secondary dentate matrix and post-mitotic neurons

4.1.4. Deficiency of ω-3 fatty acids and brain development in humans

As already mentioned, LC-PUFAs are accumulated by the infant's brain; through placental transfer during pregnancy and from breast or formula milk after birth. The animal literature suggests that ω-3 deficiency has crucial consequences on the animal's brain development; hence, predicting that if the infant's brain DHA is lower than the proportion of DHA needed for the brain to develop properly, then there would be functional consequences especially in those infants that are formula fed, since formulas do not include DHA in their composition. Levels of DHA in the human milk can also vary within and among populations, since mothers following vegetarian diets will lack DHA and mothers consuming diets rich in fish will have higher amounts of DHA (Innis, 2007).

The associations of breastfeeding with cognitive development, thus potentially indicating functional consequences of LC-PUFA deficiency or adequacy, are described in detail in Chapter 3. Therefore, only studies concentrating on the content and composition of fatty acids in the human brain will be described here.

Farquharson, Jamieson, Logan, Cockburn and Patrick (1992) measured phospholipid fatty acids in the cortical gray-matter in term and preterm infants who have died of sudden infant death syndrome (SIDS). Tissues were analyzed by gas chromatography and showed that the mean weight percentage of DHA in the cortical phospholipids was greater in breast-fed than formula-fed infants. Specifically, Farquharson, Jamieson, Abbasi, Patrick, Logan and Cockburn (1995) showed that DHA content in cerebral cortex Phosphatidylethanolamine (PE) and Phosphatidylserine (PS) of breast-fed infants was greater than formula-fed infants by 9.1% (in PS). Phosphatidylserine functions as ion exchange, while both PE and PS can influence the distribution of protein molecules in the membrane (Fenske, Jarrell, Guo & Hui, 1990). Makrides *et al.* (1994) went a step further by illustrating that term infants who died from SIDS and were breast-fed had higher amounts of DHA in their erythrocytes and brain cortex than formula-fed infants, and that cortex DHA was increasing with age in the breast-fed group whereas cortex DHA in formula-fed infants did not increase with age. This study is vital as it determines the fact that breast-feeding duration has a clear effect on cortex DHA. This is verified by studies examining the association of breastfeeding duration with cognition (Anderson, Johnstone & Remley, 1999). Similar results were obtained by Byard, Makrides,

Need, Neummann, and Gibson (1995) who demonstrated that higher levels of DHA were reached in the frontal lobes of 13 breastfed infants compared with the 15 formula-fed infants; the 28 infants had died of SIDS and approximately 2 g of anterior frontal cortex and 1g of brain-stem were removed for the fatty acid analysis using gas chromatography. There were no differences between breast and formula fed group on DHA concentration in brain stem. Lastly, Jamieson, Farquharson, Logan, Howatson, Patrick, Weaver and Cockburn (1999) showed that DHA concentration in the cerebellar cortex and cerebellar white matter was significantly lower in the formula-fed than breast-fed infants.

4.1.5. Summary, Aims and Hypothesis

The human brain grows the fastest during the 2nd and 3rd trimester of pregnancy and then in the two post-natal years. The large increase of total brain volume (101%) in the first year of life implies that this is a crucial time in which environmental factors, such as breastfeeding or formula feeding, can have long-lasting effects on brain structure and function.

Breast milk (or formula milk) is the only source of nutrient for the infant. Breast milk is rich in polyunsaturated fatty acids DHA and AA, and thus provides the infant with the amounts needed for proper brain development. Long-chain polyunsaturated fatty acids are shown to influence brain development in both animals and humans by modifying the physical properties of membranes and thus influencing a variety of membrane functions. Both rodent and human studies have shown that brain DHA accumulation is higher in breast-fed than formula-fed groups, and that DHA supplemented pregnant mothers can support DHA accumulation of their offspring by promoting neurogenesis in animals, and later cognitive development and visual acuity in humans.

Associations of breastfeeding with brain structure are largely unknown. A recent study by Isaacs, Fischl, Quinn, Chong, Gadian and Lucas (2010) showed that percentage of expressed maternal breast milk correlated significantly with verbal intelligence, as well as, white matter in the brain of adolescent boys who were born prematurely. Another recent work by Isaacs Gadian, Sabatini, Chong, Quinn, Fischl and Lucas (2008) studying a group of 76 pre-term adolescents who were randomly assigned to a Standard-nutrient diet (term formula or un-supplemented banked donor breast-milk) *versus* a High-nutrient diet (formulated to meet the increased macronutrient and micronutrient needs of this population) as infants, found that the

High-nutrient diet group had significantly higher left and right caudate nuclei, only in boys, even when correcting for total brain volume. As such, this chapter has two main purposes: a) to examine the association of breastfeeding duration with the size of the basal ganglia (the caudate nucleus, the putamen, and the globus pallidus) and the brain stem, and b) to examine the associations of breastfeeding duration with the cortical thickness of 36 'IQ-related' regions. We will do so in a sample of 599 adolescents aged between 12 and 18 years old. These 'IQ-related' regions were chosen due to results from the previous Chapter associating breastfeeding duration with total and fluid intelligence, and due to evidence from other research suggesting that breastfeeding is beneficial for later cognitive development. Basal ganglia were chosen because of the above recent evidence which suggests that caudate nucleus may be particularly sensitive to environmental influences such as diet (Isaacs et al., 2008).

Since IQ is positively associated with breastfeeding duration (see Chapter 3), instead of performing multiple correlations with brain regions and IQ measures, and then investigating the associations of breast-feeding duration with the correlated regions, we first carried out a meta-analysis in order to identify brain regions consistently engaged (or activated), in functional neuroimaging studies, when participants performed tasks similar to traditional IQ tests, such as Wechsler Intelligence Scales. After identifying the most frequently activated "IQ-related" regions in this manner, the cortical thickness of these regions was calculated using the sample of 599 adolescents. The sample of 599 adolescents is described in Chapter 3; half of these adolescents were exposed to maternal cigarette smoking during pregnancy. Here, exposure status will be used as a confounder thus taking into account the effect of smoking on adolescent's brain structure.

4.2. Methodology

4.2.1. Design, Setting and Participants

The 599 participants, aged between 12 and 18 years old, were drawn from the Saguenay Youth Study (SYS) in Quebec, Canada, which is a retrospective cross-sectional study of the effects of maternal cigarette smoking during pregnancy on brain and cognition, and on cardiovascular and metabolic health during adolescence. Details about the design, setting, selection criteria and recruitment of participants are thoroughly described in Chapters 2 and 3.

4.2.2. Measurements

Breastfeeding status

Breastfeeding was measured through a medical questionnaire in which mothers were asked if they breastfed their child, and if yes, for how long (weeks or months) did they exclusively breastfed for; they were asked at what age they introduced water or unsweetened liquids, at what age did they first introduced formula or milk of any kind, at what age did they introduced cereals, fruits and other solid foods, and lastly at what age did they completely stop breastfeeding. For this chapter, total breastfeeding duration was used and not exclusive breastfeeding duration. When performing the analysis with "exclusive breastfeeding duration" there was no significant associations between breastfeeding and brain structures/cortical thickness. Exclusive breastfeeding duration had a median of 8 weeks whereas total breastfeeding duration had 14 weeks as a median. Using total breastfeeding duration will give us an advantage over the length of time that breastfeeding was used and thus any small but significant associations might be more straightforward. Total breastfeeding duration was used as a categorical variable having four levels: (i) never breastfed, (ii) breastfed for less than 6 weeks, (iii) breastfed for 6 to 24 weeks, and (iv) breastfed for more than 24 weeks.

Pre-natal, peri-natal and family environment measures

A number of pregnancy and birth-related variables, such as birth weight, maternal age at delivery, alcohol during pregnancy, number of pregnancies, and *in utero* exposure to second-hand smoking were assessed using a set of questionnaires and by a structured telephone interview with the biological mother in the majority of participants. A number of socioeconomic indicators, such as household income and parental education, were also obtained.

4.2.3. MRI Acquisition

For each participant, high resolution magnetic resonance (MR) images of the brain were collected on a Phillips 1.0-T superconducting magnet. These structural images were obtained in a session that lasted around 45 to 60 min, and were acquired using the three following sequences: a) T1-weighted images [T1W: Three dimensional (3D) ratio frequency (RF)-spoiled gradient echo scan with 140–160 slices, an 1-mm isotropic resolution, a repetition

time of (TR) 25 ms, an echo time of (TE) 5 ms and a flip angle of 30˚]; b) T2-weighted images (T2W; 2D multi-slice fast spin echo scan with 70–80 2-mm contiguous slices with a 1 mm in-plane resolution, TR=3,300 ms, TE effective=11 ms); and c) proton-density weighted images (PDW; same as for T2 scan, but with TE effective of 105 ms).

4.2.4. MRI Image Analysis

Using Freesurfer, a set of automated tools for reconstruction of the brain cortical surface (Fischl and Dale, 2000), the total volume of the left and right caudate, putamen, globus pallidum and brain stem were measured. Structural labelling of the above structures was achieved using Freesurfer's subcortical segmentation procedure (Fischl, Salat, Busa, Albert, Dieterich, Haselgrove, van der Kouwe, Killiany, Kennedy, Klaveness, Montillo, Makris, Rosen & Dale, 2002). Freesurfer's segmentation processes integrate information about the image intensity of different tissue classes with probabilistic information about the relative location of different brain regions, such that each voxel in a participant's structural image is given a neuroanatomical label (Pizzagalli, Holmes, Dillon, Goetz, Birk, Bogdan, Dougherty, Iosifescu, Rauch & Maurizio, 2010). FreeSurfer generates 37 labels of the brain (Fischl et al., 2002) including 18 labels of subcortical structures and cerebrospinal fluid (CSF). The steps for creating subcortical segmentations were detailed described by Fischl *et al* (2002) and are the following: a) pre-processing: non-uniform intensity normalization is carried out on the MRI image (T1-weighted), b) registration: affine transformation to Talairach space, c) intensity normalization: variations in scan intensity are corrected and adjusted, d) skull stripping: after removal of the skull and meningeal surfaces, only the brain and the pial surface remains, e) registration: calculation of a transform matrix to align participant's volume with the Freesurfer atlas, in order to be used for when applying segmentation labels, and f) labelling: final volume labels are utilized to subcortical structures based on the prior probabilities of voxel identity assigned by the atlas in addition to the probability of voxel identity based on the tissue class assignment of surrounding voxels.

4.2.5. ALE Meta-Analysis

4.2.5.1. Meta-Analysis Methodology

Meta-analysis in this Chapter has been carried out using the activation-likelihood estimation (ALE) method that was developed by Turkeltaub, Eden, Jones and Zeffiro (2002). Brain-imaging experiments that report 3-D coordinates in stereotactic space are used to perform an ALE meta-analysis. These coordinates are normally published under Montreal Neurological Institute (MNI) or Talairach (Talairach and Tournoux, 1988) space, and must be spatially renormalized to a single template using a proper transformation. In the past, the Brett transform (Brett, 1999) was utilized to convert MNI coordinates to Talairach space. At present, the *icbm2tal* is used instead to convert MNI coordinates to Talairach space; it has been validated and shown to provide better fit over the Brett *mni2tal* transform (Lancaster, Tordesillas-Gutiérrez, Martinez, Salinas, Evans, Zilles, Mazziotta & Fox, 2007). Once all coordinates are set in a single stereotactic space (i.e. the "Talairach" space), the ALE analysis begins.

An experiment, either with Positron Emission Tomography (PET) or with functional MRI (fMRI), refers to a contrast between one condition of interest and a control condition. Activation refers to the difference in brain activity between the two conditions, either indicated by cerebral blood-flow (CBF, with PET) or blood oxygenation level-dependent (BOLD) signal (with fMRI). The coordinates where the difference in CBF or BOLD was statistically significant are the "focus" of activation (e.g. Grosbras, Laird & Paus, 2005). Each focus, from multiple studies, is then combined to generate a whole-brain map that indicates statistically consistent results across multiple studies that may not be obvious by simple visual comparison of the reported findings.

As such, the ALE method can identify the pattern of brain activity for a given task while estimating the concordance across different experiments.

4.2.5.2. Selection of studies for the Meta-Analysis

Functional brain-imaging and PET studies related to intelligence were selected for this meta-analysis. Studies published from 1997 to 2009 were selected from ISI Web of Knowledge

and Sleuth (BrainMap[15] database) by using the terms: intelligence, reasoning, Raven's matrices, WISC/WAIS, processing speed, and cognitive control. A study had to describe clearly the methodology and to report 3-D stereotaxic coordinates to be included in the meta-analysis. Studies that did not report results based on whole-brain analysis, because a region of interest approach was chosen, were excluded. In this meta-analysis, 23 PET and fMRI studies reporting a total of 30 experiments were included. Table 1 (pg.102-103) presents a summary of these studies.

4.2.5.3. Coordinate Transformation and Map Generation

Studies included in the meta-analysis published coordinates either derived from normalization to the MNI-305 template (Collins, Neelin, Peters, Evans, 1994) or to the Talairach atlas template. To standardize the coordinates input into the analysis, all coordinates derived from normalization to the Talairach template were transformed using *tal2icbm_spm* transformation from the Talairach template to the MNI template. One of the studies reported Talairach coordinates after Brett transformation (*mni2tal)* thus the published coordinates were then 'un-Brett' using *tal2mni*. GingerALE[16] software was used to apply appropriate transformations.

As described in Turkeltaub *et al* (2002), the ALE maps were created using a 10-mm full-width-half-maximum (FWHM) Gaussian function to model each coordinate. The likelihood of each voxel in standard space representing each primary locus of activation was combined to generate a map of the ALE score at each voxel. ALE maps were thresholded by a permutation test controlling the false discovery rate (FDR) at $P < 0.05$ and clusters of supra-threshold voxels superior to 200 mm^3 in volume were defined as loci of brain activation across all studies included in the meta-analysis. From 23 studies and 30 experiments, 328 foci were included in the meta-analysis (Figure 1). ALE meta-analysis was carried out using a Java version of GingerALE that was developed at the Research Imaging Institute of the University of Texas Health Science Centre San Antonio. The thresholded maps were overlaid onto an

[15] BrainMap is an online database of published functional neuroimaging (fMRI and PET) experiments with coordinate-based (x,y,z) activation locations in Talairach space. BrainMap was created and developed at the Research Imaging Institute of the University of Texas Health Science Centre San Antonio (UTHSCSA).
[16] GingerALE is the BrainMap application that is used to perform an ALE meta-analysis on a group of coordinates in Talairach or MNI space. GingerALE can also be used to convert coordinates between MNI and Talairach spaces.

anatomical (MNI) template that was obtained from Simon Eickhoff through BrainMap website. Mango multi-image software was used to visualize ALE maps.

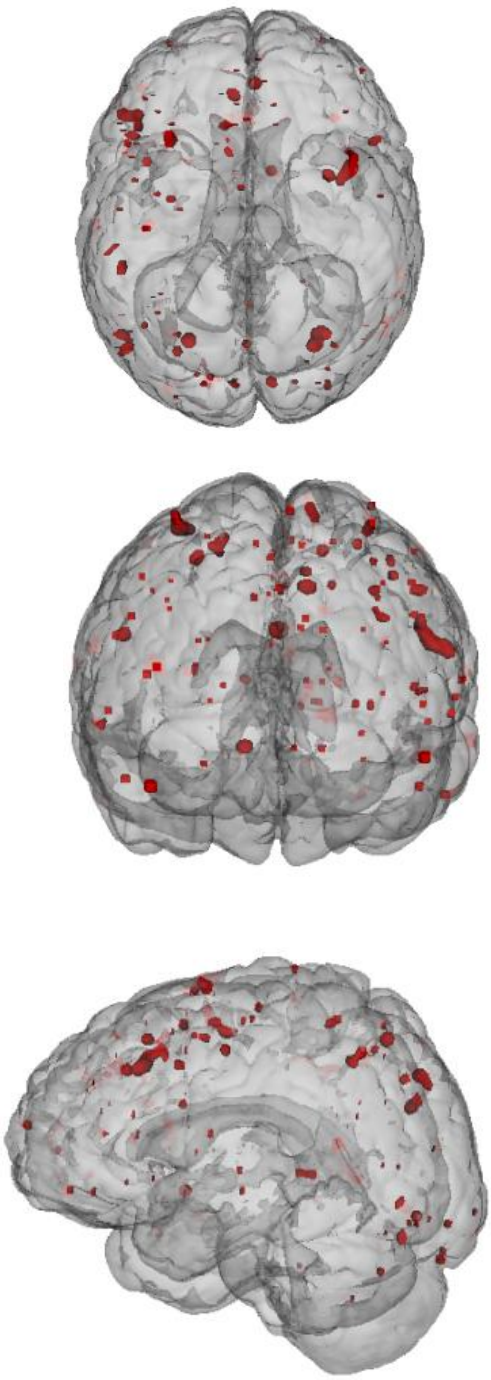


Figure 1: 328 foci included in the ALE meta-analysis on Intelligence: a) Dorsal view, b) Anterior view and c) Lateral view

Table 1: Summary of the articles selected for the meta-analysis on intelligence

Reference	Modality	Key words	n	Age	Control	Comments
Duncan et al., 2000	PET	High *g* problem solving task	13	21-34 yrs	Low *g*	Tasks designed after measuring correlations between candidate tasks for PET and standard measures of *g*.
Kroger et al., 2002	fMRI 3T	Reasoning problems: Ravens Matrices	8	19-32 yrs	Control problems	Control problems complexity held constant & difficulty manipulated by adding distractor forms.
Christoff et al., 2003	fMRI 3T	Reasoning and working memory	12	18-25 yrs	Externally generated	Contrast between internal & external trials designed to identify brain regions involved in evaluating internally generated information.
Gray et al., 2003	fMRI 1.5T	General fluid intelligence	60	18-37 yrs	Non-target trials	Control trials were non-target trials for example, items never seen previously in the task, thus lower interference, less demand.
Knauff et al., 2003	fMRI 1.5T	Reasoning	12	21-35 yrs	Rest	
Luo et al., 2003	fMRI 2T	Analogical reasoning	10	20-25 yrs	A semantic judgment task	Control for activation caused by purely semantic access.
Goel et al., 2004	fMRI 2T	Inductive and Deductive Reasoning	16	27.5 mean	Baseline	Baseline: sentences did not constitute arguments
Canessa et al., 2005	fMRI 1.5T	Deductive reasoning	55	19-26 yrs	Baseline	Baseline was to control for visuoperceptual and linguistic processing & motor response requirements.

Geake & Hansen, 2005	fMRI 3T	Fluid analogical reasoning	12	18-54 yrs	Fixation	
Fangmeier et al., 2006	fMRI 1.5T	Deductive reasoning	12	22.4 mean	Maintenance problems	
Hon et al., 2006	fMRI 3T	Cognitive control	15	23 mean	Unattended change events	
Lee et al., 2006	fMRI 3T	Intelligence (*g*)	36	16.5 mean	Simple *g* task	
Kalbfleisch et al., 2007	fMRI 3T	Fluid reasoning: Matrix reasoning	14	18-47	Easy Task	
Melrose et al., 2007	fMRI 3T	Abstract reasoning	22	21 mean	Matching task	
Schmithorst & Holland, 2007	fMRI 3T	Intelligence & language processing	303	5-18 yrs	Sublexical auditory processing	Bayesian connectivity analysis
Masunaga et al., 2008	fMRI 1.5T	Fluid reasoning	18	21-35 yrs	Dot location matching test	
Song et al., 2008	fMRI 3T	Intelligence	59	18-33 yrs	Rest	Functional Connectivity maps: seed regions
Wendelken et al., 2008	fMRI 1.5T	reasoning	20	18-28 yrs	Fixation	
Eslinger et al., 2009	fMRI 3T	Reasoning problems: Ravens Matrices	16	8-19 yrs	Baseline	Baseline consisted of geometric designs of a single dimension requiring simple perceptual matching response choice.
Perfetti et al., 2009	fMRI 1.5T	Reasoning: Ravens Matrices	20	19-30	Control problems	Control problems devoid of any logical relations.
Rodriguez et al., 2009	fMRI 1.5T	Deductive reasoning	12	26.6 mean	Unrelated sentences	
Waiter et al., 2009	fMRI 1.5T	Intelligence, Ravens Matrices & working memory	47	60-70	Fixation	Correlation between IQ and behavioural tasks performed.
Wartenburger et al., 2009	fMRI 1.5T	Analogical thinking, fluid intelligence	15	18.7 mean	Easy task	

4.2.6. Cortical Thickness of the 36 'IQ-related' regions derived from the ALE meta-analysis.

Freesurfer was used to calculate cortical thickness of 36 'IQ-related' regions that resulted from the ALE meta-analysis. Freesurfer is a set of automated tools for reconstruction of the brain cortical surface (Fischl and Dale, 2000). MRI structural data on all 599 adolescents from the Saguenay Youth Study were utilized to calculate cortical thickness. "For every participant, FreeSurfer segments the cerebral cortex, the white matter, and other subcortical structures, and then computes triangular meshes that recover the geometry and the topology of the pial surface and the gray/white interface of the left and right hemispheres. The local cortical thickness is measured based on the difference between the position of equivalent vertices in the pial and gray/white surfaces. A correspondence between the cortical surfaces across the subjects is established using a nonlinear alignment of the principal sulci in each subject's brain with an average brain." (Toro, Leonard, Lerner, Lerner, Perron, Pike, Richer, Veillette, Pausova & Paus, 2008).

4.3. Statistical Approaches

In all analyses, normal distribution was checked and any outliers, defined as values three standard deviations from the mean, were excluded. Statistical analyses were carried out using JMP (version 8) and SPSS 14 (for Windows).

4.3.1. Basal ganglia and Brain stem: Linear Regressions and two-way ANOVAs

Before any further analysis, putamen, caudate, globus pallidum and brain stem were controlled for brain size using linear regression. We also examined effects of total breastfeeding duration on brain size and found no significant associations. Next, to evaluate the effects of Age and Exposure status, linear regressions with Age and Exposure as predictors were performed. To examine the main effects of Breastfeeding duration and Sex, as well as the Breastfeeding by Sex interaction, residuals from the two sets of regressions (e.g. after removing the effect of brain size, age and exposure) were used and two-way ANOVAs were carried out with total Breastfeeding duration and Sex as the independent variables and the adjusted volumes of the four structures as the dependent variables.

4.3.2. Cortical Thickness: Factor Analysis and two-way ANOVAs

ALE meta-analyses resulted in 36 ‘IQ-related’ regions; because 36 variables would lead to multiple comparisons and thus to rejecting incorrectly the null hypothesis, a reduction method was used in order to obtain a more comprehensive view of the data. As already mentioned, ALE resulted in 36 regions. These regions were grouped by the ALE algorithm into 22 clusters (Table 2).

Table 2: ALE meta-analysis results

Cluster	Region	Side	Coordinates			ALE	Extent mm^3
			x	y	z		
1	Mid Dorsolateral Frontal cortex	L	-44	18	32	.004	4,504
	Mid Dorsolateral Frontal cortex	L	-48	24	26	.003	
	Mid Dorsolateral Frontal cortex	L	-42	14	42	.002	
	Mid Dorsolateral Frontal cortex	L	-38	6	46	.002	
2	Medial PreFrontal cortex	L	-4	28	44	.003	4,136
	Medial PreFrontal cortex	L	2	24	46	.003	
	Medial PreFrontal cortex	L	2	36	32	.002	
	Medial PreFrontal cortex	L	-6	14	66	.002	
	Medial PreFrontal cortex	L	-6	10	48	.002	
	Medial PreFrontal cortex	L	-6	34	32	.002	
3	Superior Parietal Lobule	R	26	-58	56	.003	1,880
	Superior Parietal Lobule	R	12	-64	56	.001	
	Superior Parietal Lobule	R	28	-62	42	.001	
4	Frontal Eye-Field	R	34	2	60	.003	1,744
	Frontal Eye-Field	R	30	-4	54	.003	
5	Angular Gyrus	L	-46	-66	38	.003	864
	Angular Gyrus	L	-48	-64	30	.002	
6	PreMotor Cortex	L	-26	8	62	.003	576
7	Mid Lateral Frontal Cortex	R	50	14	26	.002	568
8	Superior Parietal Lobule	L	-22	-60	56	.002	568
	Superior Parietal Lobule	L	-14	-68	58	.001	
9	Lateral Occipital cortex	R	36	-92	-4	.002	448

	Lateral Occipital cortex	R	34	-84	0	.001	
10	Mid Dorsolateral Frontal cortex	L	-28	20	44	.002	392
11	Medial Occipital cortex	R	12	-90	-10	.002	384
12	Frontal Eye-Field	L	-30	-8	54	.002	312
13	Inferior Parietal Lobule	R	52	-38	54	.002	296
14	Lateral Occipital/ Posterior Temporal cortex	L	-50	-66	-10	.002	288
15	Mid Ventrolateral Prefrontal cortex	L	-48	32	-8	.002	248
16	Mid Ventrolateral Prefrontal cortex	R	48	46	10	.002	240
17	Mid Ventrolateral Prefrontal cortex	L	-50	24	6	.002	216
18	Superior Parietal Lobule	L	-26	-66	42	.002	208
19	Ventrolateral Prefrontal cortex	L	-50	46	2	.002	192
20	Inferior Parietal Lobule	L	-34	-50	46	.002	168
21	Face fusiform area	L	-32	-62	-12	.002	136
22	Mid Dorsolateral Prefrontal cortex	R	56	32	24	.002	136

To obtain a global view of our data, a principal component analysis was conducted that included the 36 "IQ-related" regions that were derived from the ALE meta-analysis (Table 2). The data were analysed by means of a principal component analysis with direct oblimin rotation. Due to the exploratory nature of the dataset a confirmatory factor analysis could not be performed. The 1st principal component analysis with direct oblimin rotation revealed the various indicators of factorability to be good and the residuals indicated that the solution was a good one. Direct oblimin rotation (oblique rotation) was chosen to allow correlation between the derived components. Four out of the 36 regions [(*x=56, y=32, z*=24), (*x=-32, y=-62, z=-12*), (*x*=-50, *y*=-66, *z*=-10) & (*x*=12, *y*=-90, *z*=-10)] were excluded due to low extracted communalities (.2; Brace, Kemp & Snelgar, 2006). The 1st PCA was re-performed after exclusion of the above four regions. Kaiser's criterion was used to identify the number of derived components. Kaiser's criterion for factor selection recommends retaining all factors with eigenvalues greater that 1.0 (Kaiser, 1960). By Kaiser's criterion seven components should be extracted. This criterion is accurate only when there are less than 30 variables and communalities after extraction are greater than .7 or when the sample size exceeds 250 and the average communality is greater than .6 (Field, 2005). Indeed our sample size is much larger than 250, and the average of the communalities is .644 (20.626/32). Thus, Kaiser's rule is accurate in this case. The seven components can also be viewed by scree plot. The *scree* plot revealed seven components (Figure 2). The seven components can be thought of representing the following brain areas: Component A: Bilateral Frontal Eye Field (FEF); Component B: Left Medial Prefrontal Cortex (MPC); Component C: Right Lateral Occipital Cortex (LOC); Component D: Left and Right Superior and Inferior Parietal Lobule (SPL/SIP); Component E: Bilateral Ventrolateral Prefrontal Cortex (VPC); Component F: Left Angular Gyrus (AG); and Component G: Left Mid Dorsolateral Frontal Cortex (MDFC; Table 3).

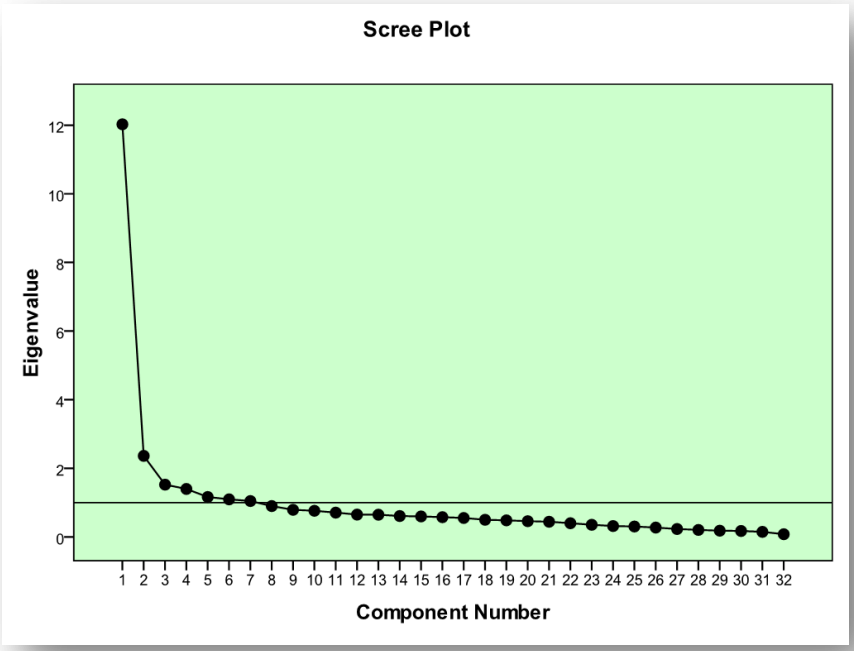


Figure 2: *Scree* plot of PCA revealing seven components

For further analysis, instead of extracting the factor scores with options offered from SPSS (Anderson-Rubin, Bartlett, Regression methods), items loaded above .40 (Fergusson & Cox, 1993) on a component were selected and for each component the weighted sum of included items was calculated for each individual.

Further statistical analysis began by assessing the effect of Age on the seven cortical thickness components by linear regressions. Residuals from first set of regressions were used to assess the Exposure effect on age-adjusted components using one-way ANOVAs. To examine the main effects of total Breastfeeding duration and Sex, two-way ANOVAs were carried out with adjusted components as the dependent variables and total Breastfeeding duration and Sex as the independent variables.

Table 3: Seven Components revealed by principal component analysis: classification of region loadings

Components	Coordinates			Region	Side	Lobe	Factor Loadings
	x	y	z				
A: Left and Right Frontal Eye-Field (bilateral FEF)	34	2	60	Frontal Eye-Field	R	Frontal	.820
	30	-4	54	Frontal Eye-Field	R	Frontal	.801
	-26	8	62	Premotor cortex	L	Frontal	.478
	-30	-8	54	Frontal Eye-Field	L	Frontal	.464
B: Left Medial Prefrontal Cortex (MPFC)	-4	28	44	Medial PreFrontal cortex	L	Frontal	-.764
	-6	34	32	Medial PreFrontal cortex	L	Frontal	-.743
	-6	10	48	Medial PreFrontal cortex	L	Frontal	-.743
	2	24	46	Medial PreFrontal cortex	L	Frontal	-.704
	2	36	32	Medial PreFrontal cortex	L	Frontal	-.687
	-6	14	66	Medial PreFrontal cortex	L	Frontal	-.472
C: Right Lateral Occipital Cortex (LOC)	36	-92	-4	Lateral Occipital Cortex	R	Occipital	.957
	34	-84	0	Lateral Occipital Cortex	R	Occipital	.912
D: Left & Right Superior & Inferior Parietal Lobule (SPL/IPL)	28	-62	42	Superior Parietal Lobule	R	Parietal	.734
	26	-58	56	Superior Parietal Lobule	R	Parietal	.690
	-26	-66	42	Superior Parietal Lobule	L	Parietal	.649
	-22	-60	56	Superior Parietal Lobule	L	Parietal	.628
	-14	-68	58	Superior Parietal Lobule	L	Parietal	.579
	-34	-50	46	Inferior Parietal Lobule	L	Parietal	.532
	12	-64	56	Superior Parietal Lobule	R	Parietal	.432
E: Left & Right Ventrolateral Prefrontal Cortex (VLPFC)	-50	46	2	Ventrolateral Prefrontal Cortex	L	Frontal	.765
	-48	32	-8	Ventrolateral Prefrontal Cortex	L	Frontal	.732
	50	14	26	Ventrolateral Prefrontal Cortex	R	Frontal	.553
	-50	24	6	Ventrolateral Prefrontal Cortex	L	Frontal	.552
	48	46	10	Ventrolateral Prefrontal Cortex	R	Frontal	.514
F: Left Angular Gyrus (AG)	-48	-64	30	Angular Gyrus	L	Temporal	.911
	-46	-66	38	Angular Gyrus	L	Temporal	.907
G: Left Mid Dorso-lateral Frontal Cortex (MDLFC)	-44	18	32	Mid Dorsolateral Frontal Cortex	L	Frontal	.786
	-48	24	26	Mid Dorsolateral Frontal Cortex	L	Frontal	.711
	-42	14	42	Mid Dorsolateral Frontal Cortex	L	Frontal	.640
	-38	6	46	Mid Dorsolateral Frontal Cortex	L	Frontal	.437

4.4. Results

4.5.1. Sample Characteristics

From 599 adolescents, 20 adolescents were excluded due to registration failure during MRI analysis. Table 4, separately for breastfed and non-breastfed adolescents, describes the adolescents' age and exposure status, as well as the family SES, as indicated by household income and parental education. Parental education ranged from the lowest level, which is eighth grade or less, up to a doctoral degree. Sample characteristics are similar as in Chapter 3; there is only a small difference in sample size (599 in Chapter 3 *Vs* 579 in Chapter 4). Breastfed and non-breastfed adolescents have the same mean age but differ in exposure status. There are significantly more exposed adolescents in the non-breastfed than in the breastfed group [$x^2(1, N=576) = 34.94, p<.0001$] thus indicating that women who smoke during pregnancy are less likely to breastfed their child.

Maternal education was significantly different between breastfed and non-breastfed adolescents. Mothers with higher level of education have chosen to breastfeed more than mothers with lower education level [maternal education: *Welch F* (1,270) =6.44, *p*=.011][17].

There was also a significant difference between the two groups on Birth weight and 2nd hand smoking exposure [F (1,571) =12.58, p=.0004; *Welch* F (1,566) =4.86, p=.027 respectively]. Non-breastfed adolescents were more likely to be exposed to 2nd hand smoking as well as weight less at birth than breastfed adolescents. It should be noted, however, that these comparisons are confounded by differences of breastfed and non-breastfed adolescents in their exposure status. Exposed adolescents are more likely to weigh less at birth and to be exposed to second hand smoking (Chapter 2).

[17]Levene's test of Homogeneity of Variances was significant (p<.0001) indicating that the variances in the two groups are significantly different thus violating one of the assumptions of ANOVA. In this case Welch's F is reported (Field, 2005).

Table 4: Sample Characteristics separated by breastfeeding status

Sample Characteristics		Breastfed (*N*)	Non Breastfed (*N*)	*P*
Age	Mean ± SEM	14.7 ± 0.1 (277)	14.5 ± 0.1 (299)	.116
Exposure Status	Exp. Percentage	16,3% (94)	30,3% (175)	**<.0001**
Income	Mean ± SEM	56276 ± 1927.9 (141)	51742 ± 2052.5 (132)	.108
Mother Education	Mean ± SEM	5 ± 0.14 (141)	4.5 ± .10 (129)	**.011**
Father Education	Mean ± SEM	5.1 ± 0.1 (134)	4.7 ± .14 (122)	.123
Sex	Males (*n*)	144 (277)	135 (299)	.101
Birth Weight	Mean ± SEM	3471.9 ± 28.1 (275)	3321.3 ± 30 (297)	**.0004**
Birth Order	Mean ± SEM	1.9 ± .05 (277)	1.8 ± .04 (299)	.216
Maternal Age at Delivery	Mean ± SEM	27.1 ± 0.2 (277)	26.9 ± 0.2 (299)	.749
Pregnancy Duration	Mean ± SEM	39.2 ± .09 (277)	39.1 ± .08 (298)	.179
Alcohol During Pregnancy	Percentage of Yes	11.7% (275)	12.2% (298)	.806
2nd Hand Smoking (num. of cigar.)	Mean ± SEM	5.7 ± 0.5 (272)	7.5 ± 0.5 (295)	**.028**

4.4.2. Duration of breastfeeding status

Table 5 shows breastfeeding characteristics and separation of breastfeeding duration on three categories.

Table 5: Breastfeeding Characteristics

Breastfeeding Characteristics	
Median number of weeks breastfed	14
Min-Max weeks of breastfed	1 - 88
Interquartile range (25% - 75%)	6 - 24
Distribution of Breastfeeding duration	
< 6 weeks	21%
6-24 weeks	58%
≥ 24 weeks	21%

4.4.3. Basal ganglia and Brain Stem

Caudate, Putamen, Globus Pallidum and Brain stem were all corrected for brain size using linear regressions. Next, residuals from the 1st set of regressions were used in a 2nd set of regressions to evaluate the effect of Age (in months) and Exposure status on the four structures. Caudate and Putamen volumes were not significantly affected by Age or Exposure status. Pallidum and Brain stem were adjusted for Age (R^2adj.=.011, df=578, p=.005; R^2adj.=.08, df=578, p<.0001 respectively), and Exposure status (R^2adj.=.021, df=578, p=.0003; R^2adj.=.006, df=578, p=.028 respectively). After correcting for Age and Exposure, two-way ANOVAs showed that males had larger pallidum volume than females [F (1,562) = 5.49, t=2.34, p=.019]. There were no other main effects of sex on any of the other three structures.

Since breastfed and non-breastfed adolescents differed on means of birth weight and maternal education we also assessed the relationship of the basal ganglia structures with the above covariates. We found no significant associations of any of the basal ganglia structures with birth weight or maternal education.

Total breastfeeding duration was significantly and positively affecting caudate volume [F (3,562)=3.89, p=.009] but none of the other parts of basal ganglia. Tukey post-hoc analysis showed that level 1 (breastfeeding for less than 6 weeks) had significantly lower mean from level 3 (breastfeeding for more than 24 weeks; p=.004).

In addition, there was a breastfeeding duration by sex interaction on brain stem [F (3,562)=3.39, p=.017] thus indicating that males and females are affected differently by breastfeeding duration. Tukey post hoc comparisons showed that females breastfed for more than 24 weeks had significantly larger brain stem volume than females breastfed for less than 6 weeks (p=.023) and females who were never breastfed (p=.022; Figure 3). While breastfeeding duration significantly affects brain stem volume, the interaction effects suggests that this is true only for females (males appear unaffected by breastfeeding duration).

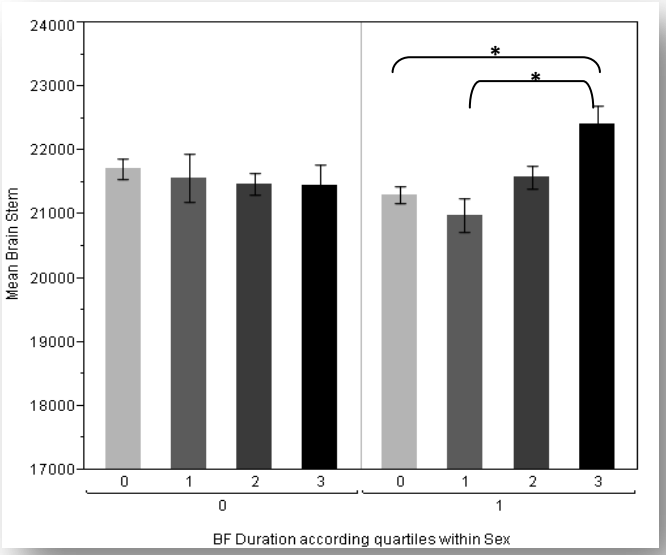


Figure 3: Breastfeeding by Sex interaction on mean Brain stem volume. To visualize better the results on breastfeeding duration, the variable was used as a categorical one with four levels: (0) never breastfed, (1) breastfed for less than 6 weeks, (2) breastfed for 6 to 24 weeks, and (3) breastfed for more than 24 weeks. Left hand side is indicative of males (0), and right hand side is indicative of females (1). Error bars are constructed using 1 standard error from the mean.

4.4.4. ALE meta-analysis

Selected studies of intelligence yielded a total of 328 foci. These foci are viewed in MNI space using Mango Multi-Image software (Figure 1). Pooling the results of 30 experiments onto a single brain resulted in a number of activations in all four lobes, especially the frontal and parietal lobes. Twenty-two clusters were seen in bilateral frontal lobe (11 clusters; four in the right hemisphere, seven in the left hemisphere), superior parietal lobule (3 clusters; two in the left hemisphere, and one in the right), left angular gyrus, right occipital lobe (3 clusters), left fusiform gyrus, bilateral inferior parietal lobule (2 clusters), and left cerebellum (Table 2, Figure 4). Extremely high ALE values were observed in the middle frontal gyrus (*x=-44, y= 18, z= 32*) with a cluster volume of 4,504 mm^3, and in the medial frontal gyrus (*x=-4, y=28, z=44)* with a cluster volume of 4,136 mm^3.

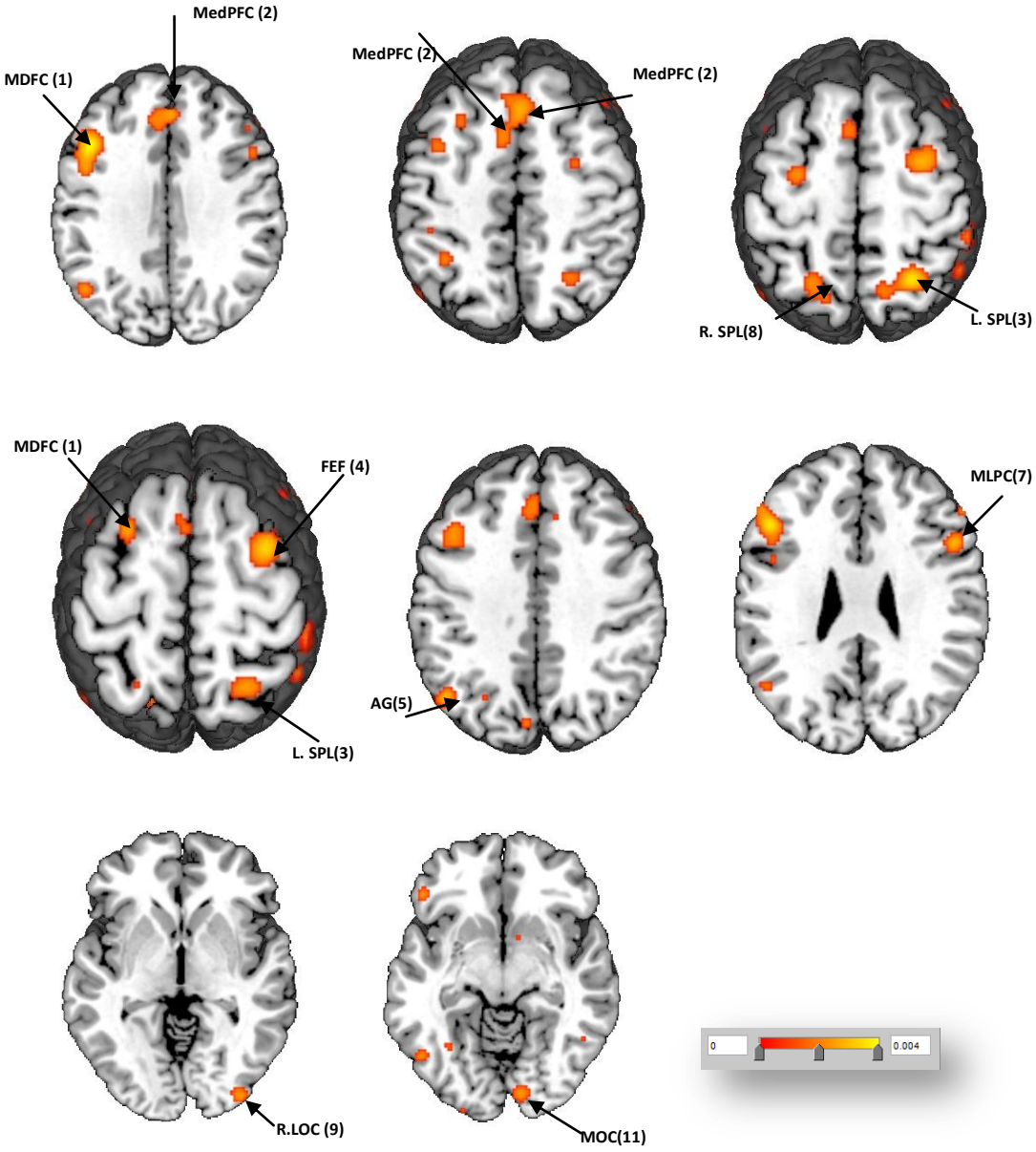


Figure 4: Results illustrating the locations of most of the ALE clusters on axial view. The colour scale represents the ALE score (from 0 to 0.004), significant at p<.05. Numbers in parenthesis represents the number of the cluster. MDFC: Mid Dorsolateral Frontal Cortex; MedPFC: Medial Prefrontal Cortex; L.SPL & R.SPL: Left & Right Superior Parietal Lobule; FEF: Frontal Eye-Field; AG: Angular Gyrus; MLPC: Mid Lateral Frontal Cortex; R.LOG: Right Lateral Occipital Cortex; MOC: Medial Occipital Cortex.

4.4.5. Factor Analysis of 36 'IQ-related' regions

As aforementioned, indicators of factorability and residuals indicate that the solution was a good one. Kaiser–Meyer–Olkin Measure of Sampling Adequacy was 0.922 showing the amount of variance within the data that could be explained by factors and Bartlett's Test of Sphericity had a P-value <0.05 meaning that the data are probably factorable. According to the total variance explained, seven components have eigenvalues >1.0 and all together explain 64.4% of the variance. Table 3 shows which regions load on each component.

4.4.6. Cortical Thickness, Sex and Breastfeeding duration

The seven components were all adjusted for Age. Only the first Component (bilateral FEF) was affected by exposure status [F(1,576)=6.88, p=.008] and thus this was the only component adjusted also for exposure status. Before carrying on with the two-way ANOVAs, we assessed the relationship between cortical thickness components and maternal education as well as birth weight. We found no significant associations between the components and the covariates. Two-way ANOVAs showed that, compared with males, females have thicker bilateral FEF [F(1,561)=20.35, p<.0001], right LOC [F(1,561)=4.82, p=.028], bilateral SPL/IFL [F(1,561)=14.15, p=.0002], bilateral VLPFC [(F(1,561)=7.85, p=.005], Left AG [F(1,561)=7.89, p=.005]and Left MDLFC [F(1,561)=23.13, p<.0001]. There was a main effect of breastfeeding duration on left MPFC [F(3,561)=2.48, p=.05] and on bilateral SPL/IPL [F(3,561)=3.61, p=.013]. Tukey post-hoc comparisons revealed that those who were breastfed more than 24 weeks had significantly thicker bilateral superior/inferior parietal lobule and left medial prefrontal cortex than those who were never breastfed (p=.027; p=.05 respectively). There was no significant interaction of total breastfeeding duration with sex for any of the seven components.

Figure 5 (from L to R): left medial prefrontal cortex; left superior & inferior parietal lobule;

4.5. Discussion

The main purpose of this Chapter was to evaluate the association of total breastfeeding duration with brain structure of adolescents. In order to achieve this goal, two types of analyses were employed. In the first type of analysis, four subcortical structures were selected to examine if they are considerably affected by breastfeeding. These four structures were parts of the basal ganglia (caudate, putamen, and globus pallidum) and the brain stem. The basal ganglia were selected because there is evidence that caudate volume is affected by nutrition (Isaacs et al., 2008), and brain stem development seems to be influenced by breastfeeding (Ünay, Sarici, Ulaş, Akin, Alpay & Gökçay, 2004). From this investigation it has been illustrated that indeed caudate and brain stem volumes (after adjusting for appropriate covariates) of 12-to-18 year old adolescents are considerably affected by total breastfeeding duration during infancy. More specifically, adolescents breastfed for more than 24 weeks have larger caudate and brain stem volumes than adolescents breastfed for less than 6 weeks or never breastfed adolescents (respectively). Subcortical structures were controlled for total brain volume, age and exposure status. Birth weight, as well as maternal education, was found to differ significantly between breastfed and non-breastfed adolescents. For this reason, we assessed the relationship of birth weight and maternal education with all the outcome variables and found no significant associations. Second hand smoking, even significantly different between the two groups, was not used as a covariate as it is highly correlated with exposure status.

In the second analysis, a more complicated method was utilized. Firstly, regions that are significantly engaged during the performance of tasks similar to intelligence tests were selected using an ALE meta-analysis; they were then used in further analysis to extract their cortical thickness and investigate the association of breastfeeding duration with cortical thickness in these "IQ-related" regions. Analyses outcome demonstrated that medial prefrontal cortex (bilaterally) and superior and inferior parietal lobule are considerably influenced by total breastfeeding duration with those breastfed for longer period having thicker cortex in these regions than those who were never breastfed. The seven cortical thickness components were also controlled for age and exposure status. Relationships between maternal education and birth weight with the thickness components were assessed as well.

In the following parts of the Discussion, all outcomes from the Results section will be discussed accordingly by emphasis on breastfeeding conclusions.

4.5.1. Intelligence meta-analysis

Twenty-three studies and 30 experiments with 328 foci were selected for the IQ meta-analysis. ALE meta-analysis revealed 36 regions that are significantly engaged during intelligence tasks, according to fMRI and PET studies. These 36 regions involve medial prefrontal cortex, frontal eye-field and pre-motor cortex, ventro-lateral frontal cortex, mid dorsolateral frontal cortex, lateral occipital cortex, angular gyrus, superior and inferior parietal lobule, temporal cortex and face fusiform area (Figure 6). For most of these brain regions, the left hemisphere appears to be more involved in intelligence tasks than the right hemisphere (from 36 ALE regions, 24 are from the left hemisphere).

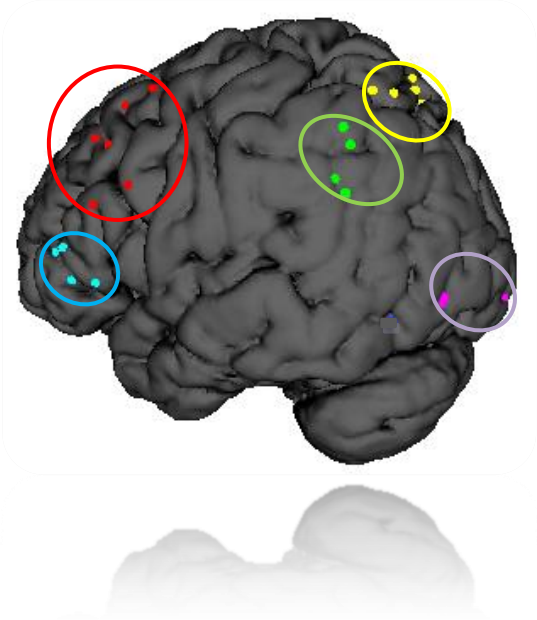


Figure 6: Brain regions involved in intelligence tasks as revealed by fMRI and PET studies. Red: Mid dorsolateral frontal cortex and medial prefrontal cortex; Cyan: ventro-lateral prefrontal cortex; Green: angular gyrus and inferior parietal lobule; Yellow: superior parietal lobule and precuneus; Purple: lateral and medial occipital cortex.

Interestingly, results from this ALE meta-analysis verify the parieto-frontal integration theory of intelligence (P-FIT) put forward by Jung and Haier (2007). The P-FIT was suggested after a review of 37 neuroimaging studies (including voxel-based morphometry studies, diffusion tensor imaging -white matter- studies, functional imaging studies using PET and fMRI) of intelligence and reasoning. According to the P-FIT, humans process sensory information with the extrastriate cortex (temporal and occipital lobes) and fusiform gyrus involving recognition and following ellaboration of visual input, and Wernicke's area involving analysis of auditory information. Thereafter, this perceptual processing is passed to the parietal cortex, mainly the supramarginal, superior parietal and angular gyrus in which structural symbolism, abstraction and elaboration are thought to emerge. The P-FIT assumes that the parietal cortex interacts with frontal regions that serve in testing a range of solutions to a given problem. Once the best solution has been chosen, the anterior cingulate cortex supports response engagement and inhibits alternative responses. All these connections, among different brain regions, are dependent on white matter fibres, such as the arcuate fasciculus (Jung & Haier, 2007; Figure 7).

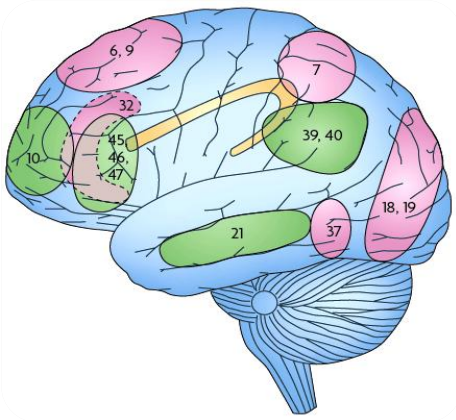


Figure 7: The figure illustrates the P-FIT theory that Jung & Haier (2007) suggested. The figure shows Brodmann Areas (BAs) involved in intelligence, as well as the arcuate fasciculus (shown in yellow). BAs shown in green indicate predominantly left-hemisphere and BAs shown in pink indicate predominantly right-hemisphere regions related with intelligence [Figure adopted from Deary, Penke & Johnson (2010)].

A major weakness of the ALE meta-analysis and of the P-FIT theory is that they are mostly focused on reasoning studies, that is, on *fluid* intelligence. Fluid intelligence generally reflects reasoning and novel problem-solving ability, to be able to search for rules, understand relationships, independently from prior experience and knowledge. Tests involving fluid intelligence are the Raven's Matrices, and letter-series problems. All fMRI and PET studies involved in this ALE meta-analysis, and in the P-FIT theory, are measuring fluid intelligence. Verbal intelligence, or else crystallized intelligence, is more complicated to be measured through functional MRI studies. Some structural MRI studies have used verbal IQ tests to correlate it with brain structures but again correlation studies, as well as functional studies, are not straightforward on the exact relation between intelligence and structure and do not explain causality.

In summary, this ALE meta-analysis, revealed a circuit of brain regions throughout the brain, mostly involving the fronto-parietal cortex. This fronto-parietal circuit is mostly involved during fluid and not crystallized intelligence. In other words, when humans try to solve a problem, by understanding rules and relationships, and select a response that better suits the problem then this fronto-parietal circuit is usually activated.

4.5.2. Basal ganglia and Brain Stem

4.5.2.1. Age and Exposure effects

Results from this analysis pointed up that globus pallidus and brain stem were the structures affected by age and exposure status. The globus pallidus is part of the basal ganglia and is the source of the striatum output to the thalamus. Striatum consists of the caudate and putamen together, and is the target of the cortical input to the basal ganglia. In a recent study by our group (Lotfipour, Leonard, Perron, Pike, Richer, Séguin, Toro, Veillette, Pausova and Paus, 2010), using the same sample of adolescents (423 adolescents 12-to-18 years old), demonstrated a significant interaction between the presence of a single nucleotide variant in the gene coding an a6 nicotinic acetylcholine receptor (nAChR) and the adverse environment of *in utero* exposure to maternal cigarette smoking, in influencing striatum volume. By genotyping the a6 nAChR SNP (rs2304297, C/G), with the minor C allele, they found a significant interaction between *in utero* exposure and the a6 nAChR SNP on striatum volume (striatum defined as a sum of the caudate nucleus, putamen and nucleus accumbens); specifically,

exposed adolescents with the GG having significantly larger striatum volume than exposed C-carriers (Lotfipour et al., 2010). This increase of the size of the striatum maybe explained by possible functional alterations in these nicotinic acetylcholine receptors that, in turn, decrease dopaminergic neurotransmission in the striatum (Lotfipour et al., 2010). Here, results demonstrate that in the same sample of adolescents (599 12-to-18 years old), the volume of the globus pallidus is significantly affected by exposure status with those been exposed having larger pallidum volume than the non-exposed adolescents.

Brain stem has been also found to be influenced by maternal cigarette smoking; brain stem is the site where essential functions are regulated, such as breathing, sleep/waking cycle, and the control of body temperature. Previous studies have demonstrated a marked decline in [^{3}H] nicotine binding in the developing brain stem in foetuses and infants exposed to maternal cigarette smoking during pregnancy (Kinney, O'Donnell, Kriger & White, 1993; Duncan, Randall, Belliveau, Trachtenberg, Randall, Habbe, Mandell, Welty, Iyasu & Kinney, 2008). Franco, Groswasser, Hassid, Lanquart, Scaillet and Kahn (1999) demonstrated that intrauterine exposure to cigarette smoking was associated with a decrease in infant's arousability. Brain stem is associated with the regulation of arousal, attention and somatic motor control (Kinney et al., 1993) thus results on arousal by Franco *et al* (1998) may be explained by the association of maternal cigarette smoking with brain stem function. In this Chapter, brain stem volume was significantly larger in the exposed adolescents than the non-exposed. In contrast, Ekblad, Korkeila, Parkkola, Lapinleimu, Haataja, Lehtonen, and the PIPARI Study Group (2010) found no significant differences in brain stem volume of a group of very low birth weight/ very low gestational age infants exposed to maternal cigarette smoking during pregnancy. The PIPARI study consists of very low birth weight/very low gestational age infants (n=232) born in 2001 through 2006. For volume measurements MRI was performed at term of corrected age (Ekblad et al., 2010).

Age-related differences were only apparent in pallidum and brain stem. Giedd, Snell, Lange, Rajapakse, Casey, Kozuch, Vaituzis, Vauss, Hamburger, Kaysen and Rapoport (1996) found significant decreases in both caudate and putamen volume in male but not female children and adolescents (n=104, 4-to-18 years old), whereas there was no difference in pallidum volume across these ages. The age of our sample is between 12 to 18 years old;

between these ages, there was a significant increase in pallidum and brain stem volume. This may be explained by the fact that the size of brain structures is established by the number, size and density of neurons and glia cells. As some neurons are lost due to apoptosis or cell death, the remaining neurons produce more dendrites, axons become thicker and the number of synaptic boutons increases (Giedd et al., 1996).

4.5.2.2. Breastfeeding Associations with Basal ganglia and Brain Stem

The volumes of caudate nucleus and brain stem were the only structures affected by breastfeeding duration. Caudate volume was significantly increasing with total breastfeeding duration; the more an adolescent was breastfed as an infant, the larger the caudate nucleus during his/her adolescence. To our knowledge, no other study has examined the associations of breastfeeding duration with basal ganglia of human adolescents. A recent work by Isaacs *et al* (2008) studied a group of 76 pre-term adolescents who were randomly assigned to a Standard-nutrient diet (term formula or unsupplemented banked donor breast-milk) *versus* a High-nutrient diet (formulated to meet the increased macronutrient and micronutrient needs of this population) as infants. They found that the High-nutrient diet group had significantly higher left and right caudate nuclei, only in boys, even when correcting for total brain volume (Isaacs et al., 2008). Results from this Chapter, as well as from Isaacs *et al* study, suggest that caudate volume is influenced by nutrition during early post-natal life. The caudate nucleus is known to be one of the brain regions having high DHA content in the neonatal rats (Xiao, Huang & Chen, 2005). Experimental evidence from baboon neonates also indicates that DHA is most concentrated in basal ganglia and limbic regions of the CNS (Diau, Hsieh, Sarkadi-Nagy, Wijendran, Nathanielsz, & Brenna, 2005; Hsieh, Anthony, Diersen-Schade, Rumsey, Lawrence, Li, Nathanielsz & Brenna, 2007). As already mentioned, DHA is transported to the foetus through the placenta *via* pathways involving fatty acids binding proteins; and high dietary intake of the mother facilitates maternal-to-foetal transfer. After birth, DHA is provided to the infant through the mother's milk and again level of DHA depends on the amount of the latter in the mother's diet. Also, a PET study found that basal ganglia showed relatively high glucose metabolism (high metabolic activity) in the newborn period (Chugani, 1996). Lastly, Knickmeyer *et al* (2008) showed that caudate nucleus (after correction for total brain volume) increased by 19% from age 1 to age 2 years in a group of 98 healthy children who received

structural MRI scans from birth to 2: 84 children at 2-4 weeks, 35 at 1 year and 26 at 2 years of age. All together, these studies indicate that there is critical period of development for basal ganglia, and especially caudate nucleus, during early post-natal life and that during this critical period, normal development of the caudate nucleus can be influenced by environmental factors such as diet, and more precisely, by the major constituent of breast milk, DHA.

Brain stem volume was significantly affected by total breastfeeding duration only in females and not males; females who were breastfed for more than 24 weeks had significantly larger brain stem than females who were breastfed for less than 6 weeks or never breastfed. These results indicate that breastfeeding has a different influence on this structure in the two sexes. This may suggest that the female and male brain may be differently vulnerable to environmental influences, such as nutrition, during the period of the fast brain growth. Brainstem is a structure that develops early during gestation (Chugani, Phelps, Mazziotta, 1987). Thus, it could be suggested that it is extremely susceptible to environmental influences. Studies on auditory brain stem maturation have shown that both preterm and term infants exclusively breastfed or supplemented with LCPUFA formulas, respectively, had significantly increased maturation rate of brain stem auditory evoked potentials than non-breastfed or non-LCPUFA supplemented preterm and term infants (Amin, Merle, Orlando, Dalzell & Guillet, 2000; Ünay et al., 2004). Auditory brain stem evoked responses is a process that reflects the first encoding of auditory stimuli and starts with an action potential conducted along the eighth nerve, moving to the brainstem and finally to the auditory cortex (Kable, Coles, Lynch & Carroll, 2009).

4.5.3. Cortical thickness and breastfeeding duration

All seven cortical thickness components showed age-related decreases in 12-to-18 year old adolescents, similarly with other studies on children and adolescents (Shaw et al., 2006; Sowell et al., 2004). Also, results demonstrated sex differences on the six out of seven cortical thickness components. Bilateral frontal eye-field, right lateral occipital cortex, bilateral superior/inferior parietal lobule, bilateral ventro-lateral prefrontal cortex, left angular gyrus and left mid dorso-lateral frontal cortex were thicker in female than male adolescents. These six regions consist of a circuit involving frontal and parietal lobes. These results are also consistent with other studies of cortical thickness in children, adolescents and adults (Sowell, Peterson,

Kan, Woods, Yoshii, Bansal, Xu, Zhu, Thompson & Toga, 2007; Im, Lee, Lee, Shin, Kim, Kwon & Kim, 2006).

Breastfeeding duration was significantly affecting bilateral superior and inferior parietal lobules, as well as left medial prefrontal cortex, with those adolescents breastfed for more than 24 weeks having thicker regions than those who were never breastfed. No other study has shown association of breastfeeding duration with cortical thickness of human adolescents. Animal studies have illustrated that ω-3 (DHA) deficient diet caused decreased neuronal size in hippocampus, hypothalamus and parietal cortex in weaning rats, and in piriform cortex in mature rats (Ahmad et al., 2002); the mean thickness of cortical plate and mean sectional area of the primordial dentate gyrus were lower in an ω-3 deficiency group of embryonic rats, whereas the mean thickness of the cortical ventricular zone and the primary dentate neuroepithelium were higher thus indicating a delay or inhibition of normal brain development in rats (Coti-Bertrand et al., 2006). On the other hand, DHA supplementation has been found to improve/restore DHA levels in the brain of baboon neonates (Diau et al., 2005), as well as increase the population of neurons with longer neurite length and higher number of branches in hippocampus in culture (Calderon & Kim, 2004), and promote the differentiation of neural stem cells into neurons (Kawakita et al., 2006). Taken together, animal studies suggest that DHA improves neurogenesis. DHA addition to infant formulas showed that DHA increased in the blood lipids of infants (Makrides et al., 1995; Carlson et al., 1996), as well as increased visual and neural system maturation in term infants (Birch et al., 1998, 2000). Further, there is an association between infant DHA status during breastfeeding and scores of cognitive tests when measured later in early childhood (Helland, Smith, Saarem, Saugstad & Drevon, 2003). A recent study by Isaacs *et al* (2010) showed a dose-response relationship between early breast milk intake and later intelligence and whole brain volume at adolescence. The effects of breast milk were more strongly presented in white than gray matter in the brain of adolescent boys who were born prematurely. Results from this chapter and chapter 3, verify all the above outcomes by demonstrating associations of breastfeeding with cognitive function (Chapter 3) as well as with brain structure on a relatively large sample of adolescents. Thus, DHA which is present in human milk is vital in the central nervous system and early deficiency has the potential to result in long-term associations with cognition and brain's structure.

4.5.3.1. Biological explanations

MR-based studies showed that cortical thickness appears to decrease during adolescence but the nature of underlying cellular events is largely unknown. Our results of breastfed adolescents having thicker parietal regions than non-breastfed adolescents do not necessarily suggest that breastfeeding is "delaying" normal brain development in adolescence. The thickness of the cortex reflects characteristics of the neuropil including the density and arrangement of neurons, glial cells and nerve fibres (Narr, Woods, Thompson, Szeszko, Robinson, Dimtcheva, Gurbani, Toga & Bilder, 2007). In addition, loss of cortical gray matter volume and thickness may reflect increases in myelination (Paus, Keshavan & Giedd, 2008). In the first year of life, in which breast or formula feeding must occur, gray matter increases by 149% (Knickmeyer et al., 2008). DHA, through human milk during the first year of life may promote cellular processes in gray matter having long-lasting effects on brain structure. As such, at the time of adolescence where gray matter volume and thickness are normally decreasing, adolescents who were breastfed for longer periods (over 24 weeks) have thicker fronto-parietal regions than those who were not breastfed perhaps because continuous DHA during the first year of life supported cellular processes giving rise to thicker cortex. Animal studies have illustrated that DHA supplementation increase the population of neurons with longer neurite length and higher number of branches in hippocampus in culture (Calderon & Kim, 2004). As such, thicker parietal cortex in adolescents who were breastfed for longer periods may suggest that DHA during the first year of life increased the population of neurons and the number of dendrites.

Last but not least, in the brain, astrocytes are responsible for DHA synthesis and delivery to neurons (Innis & Dyer, 2002). A study by Politi, Rotstein & Carri (2001) investigated whether glia could provide DHA to neurons in co-culture, and they found that not only glial cells transfer [^{14}C] DHA to neurons but they also incorporated it and esterified it in their lipids as well. Also, Politi *et al* (2001) found that DHA is necessary for protecting rat retinal photoreceptors *in vitro*, by delaying the onset of apoptosis. They also showed that glial cells protected photoreceptors by delaying cell death in a similar fashion to DHA. Thus, we could suggest that provision of DHA early in life through human milk, not only increases the

population of neurons with longer neurite length but also reduces apoptosis by being involved in the protective role played by glial cells.

Fronto-parietal regions found to be affected by breastfeeding duration are thought to be activated during fluid intelligence tasks, as revealed by ALE meta-analysis. This may suggest that since breastfeeding is promoting cortical thickness on these 'IQ-related' regions, then an advantage in *fluid* intelligence will be noticed in those who were breastfed. This is verified by results from Chapter 3, were associations of breastfeeding were only apparent with Performance IQ (*fluid* intelligence) and Full Scale IQ, and not Verbal IQ (*crystallized* intelligence). Cortical thickness has been related to intelligence in few studies so far (Shaw et al., 2006; Karama, Ad-Dab'bagh, Haier, Deary, Lyttelton, Lepage, Evans and the Brain Development Cooperative Group, 2009; Choi, Shamosh, Cho, DeYoung, Lee, Lee, Kim, Cho, Kim, Gray & Lee, 2008; Narr et al., 2007). Narr *et al* (2007) found positive associations of intelligence (measured with Wechsler Adult Intelligence Scale) with cortical thickness in bilateral prefrontal cortices. Karama *et al* (2009) found significant positive associations between cognitive ability factor (measured with Wechsler Abbreviated Scale of Intelligence) and cortical thickness in most multimodal association areas in a large representative sample of the US population between 6 and 18 years of age. Shaw *et al* (2006) observed that superior intelligent group (of children and adolescents) had thinner cortex in superior prefrontal gyri, but then showed a rapid increase in cortical thickness; by 11 years old, regions of thicker cortex became apparent in the superior intelligence group (compare with the high and average intelligence group) and concluded that intelligence has an important role in dynamic properties of cortical maturation. Taking into account that gray matter increases the fastest during the first year of life and it is possibly associated with intelligence later in life, and DHA accumulates at about 10mg/d in the whole body of breast-fed infants, with 48% of that amount appearing in the brain and high concentrations of DHA are present in brain gray matter, we could suggest that systematic breastfeeding for over a period of 24 weeks in the first year of life interacts with normal brain development and can have long-term associations with brain structure (cortical thickness) and thus function (intelligence).

4.6. Conclusion

Human milk provides the only source of DHA to support the growth and development of the breast-fed infant. High concentrations of DHA are present in phosphatidylserine of brain gray matter and the outer segments of rod and cone photoreceptors in the retina. Animal studies have shown that DHA deficiency can cause decrease in neuronal size, alterations in neurogenesis and neurotransmission including altered cortical thickness. Studies with breastfed and formula fed infants have illustrated that breastfeeding or DHA formula supplementation can increase visual acuity, cognitive function and alter DHA level in brain cortex. Here, results suggest that indeed breastfeeding is facilitating cortical thickness of a number of fronto-parietal regions, as well as increasing caudate and brain stem volume in a sample of 12-to-18 year old adolescents. Brain regions rich in DHA, such as the striatum and portions of the frontal cortex, are involved in development of attention. Neurodevelopmental disorders, such as Attention-Deficit Hyperactivity disorder (ADHD) are thought to have decreased striatum volumes (Ellison-Wright, Ellison-Wright & Bullmore, 2008), as well as lower DHA in plasma phospholipids and erythrocytes (Antalis, Stevens, Campell, Pazdro, Ericson & Burgess, 2006; Burgess, Stevens, Zhang & Peck, 2000). It can be suggested that breastfeeding during early infancy, as well as DHA supplementation, can have an encouraging impact on brain development of ADHD children. Further research should investigate if a link between the DHA deficiency and slower brain maturation in ADHD children exists, and how breastfeeding or DHA supplementation can improve ADHD-related symptoms.

Chapter 5: effects of omega-3 fatty acids supplementation on brain and cognitive development of school-aged children from less well-off neighbourhoods

5.1. Introduction

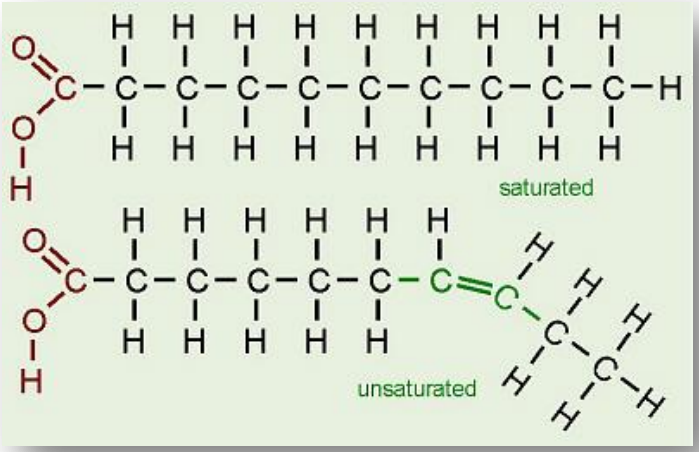
The final part of this thesis describes my work on the effects of ω-3 fatty acids supplementation on brain and cognitive development of 9-to-12 year old children. Most research exploring the impact of nutrition on cognitive development has focused on its effects in children under the age of two years. Although the brain grows the fastest during this period, the brain maturation continues throughout childhood and adolescence. Volumes of cortical gray-matter appear to increase during childhood, reaching peak levels around the time of the puberty onset, after which they gradually decline; this is the case mostly for the frontal and parietal but not temporal lobes (Giedd, Blumenthal, Jeffries, Castellanos, Liu, Zijbendos, Paus, Evans, Rapoport, 1999). Volumes of white matter show a linear increase throughout childhood and adolescence, with the maximum volumes reached often as late as in the third decade of life (Pfefferbaum, Mathalon, Sullivan, Rawles, Zipursky & Lim, 1994). The above evidence supports the idea that the brain continues to mature during childhood and adolescence and, as such, nutrition is likely to continue play an important role in this process.

At term birth, docosahexaenoic acid (DHA) represents 9% of total cortical fatty acid composition, and increases by an additional 6% between birth and age 20 years to contribute a total of 15% of the cortical fatty acids in post-mortem brain tissue (Carver, Benford, Han & Cantor, 2001). Several findings suggest that DHA continues to build up throughout postnatal brain maturation. The linear increase in DHA accumulation in human frontal cortex between birth and 20 years of age parallels the linear increases in white matter of the frontal lobe during this period (McNamara & Carlson, 2006). Thus, there is evidence supporting the possibility of a positive effect of DHA supplementation on brain development even between the ages of 9 and 12 years old.

5.1.1. Chemistry and origin of ω-3 fatty acids

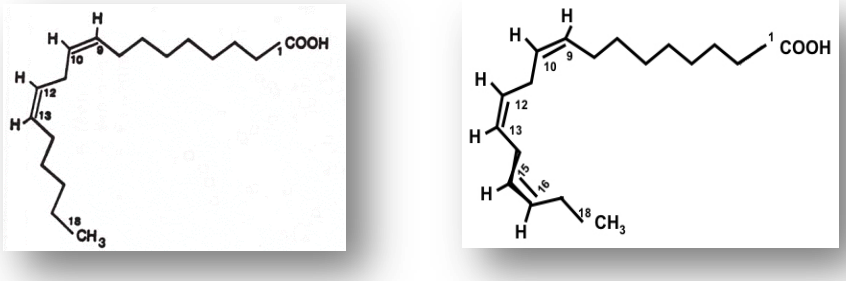
Fatty acids are the building blocks from which other lipids in the body are made [British Nutrition Foundation (BNF), 1999]. Fatty acids are hydrocarbon chains (chains of

carbon atoms to which are attached hydrogen atoms) with a carboxyl group in one end and a methyl group in the other end. Fatty acids are characterized as saturated or unsaturated based on the presence of double bonds in its structure (Figure 1).



<u>Figure 1</u>: Fatty acids can be saturated or unsaturated depending on the presence of double bonds[18]

Polyunsaturated fatty acids (PUFAs) have two or more double bonds in the carbon chain and the ω-3 fatty acids have the first double bond in the third carbon atom from the methyl end of the molecule, whereas ω-6 fatty acids have the first double bond in the 6 position from the methyl group (Drevon, 1993; Figure 2).



<u>Figure 2</u> (from left to right): Structure of ω-6 fatty acid Linoleic acid (LA) and ω-3 fatty acid a-Linolenic acid (ALA) where for the ω-6 fatty acid the first double bond is on the 6 position of the methyl group and for the ω-3 fatty acid the first double bond is in the 3rd carbon atom from the methyl end.[19]

[18] Source: http://biology.clc.uc.edu/graphics/bio104/fatty%20acid.jpg
[19] Source: http://www.thepaleodiet.com/nutritional_tools/fats.shtml

The two distinct families of polyunsaturated fatty acids, the ω-3 and the ω-6, cannot be inter-converted. The ω-6 family is derived from the linoleic acid (LA) and the ω-3 family from the a-linolenic acid (ALA). The parent fatty acids in each family, LA and ALA respectively, cannot be synthesized by the human body because of the absence of the Δ12 and 15 enzymes necessary to insert a double bond, and must be provided by the diet (Innis, 2003; BNF, 1999); for this reason these fatty acids are called essential fatty acids.

The long chain polyunsaturated fatty acids (LC-PUFA) arachidonic acid (AA), eisosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are formed from LA and ALA, respectively, in the liver by a series of desaturation (addition of double bond) and elongation (addition of a 2-carbon unit) reactions (Innis, 2003). The parent fatty acids LA and ALA are mostly derived from plant sources, e.g. seeds and vegetable oil whereas the LCPUFAs are mainly found in animal products. ω-6 AA is mostly derived from eggs and meat (Calder, 2007), while polyunsaturated ω-3 fatty acids DHA and EPA are predominantly found in marine products. Long chain ω-3 fatty acids are made by phytoplankton and transferred via the nutrition chain in the ocean ending up in fatty fish, seal and whale. Herring, mackerel, salmon, trout, fish oil, and cod liver oil are the main sources of the ω-3 products in our diet (BNF, 1999).

5.1.2. Neurobiology of polyunsaturated fatty acids

5.1.2.1. DHA and membrane fluidity

The brain has the highest concentrations of PUFAs, especially DHA. The dry weight of the adult brain consists of 50% lipids, of which approximately 35% are in the form of long chain PUFAs (LC-PUFA), primarily AA and DHA (Wainwright, 2002). In the nervous system, the photoreceptor outer segment is the tissue most enriched in DHA (Fliesler & Anderson, 1983). ω-3 and ω-6 fatty acids are employed in the creation of the lipid bilayer in cell membranes and thus, dietary intake is reflected in membrane levels (Marteinsdottir, Horrobin, Stenfors, Theodorsson & Mathé, 1998). The kinked (introduction of a double bond into the fatty acid causes a ‘kink’ to occur in the FA) chains of PUFA occupy more space in the membrane thus making it more fluid; whereas the straight chains of saturated fatty acids make the membrane more rigid (Heinrichs, 2010). Neurotransmission depends on ion channels embedded in membranes; neurons composed of membranes with inadequate amounts of

PUFAs could possibly alter neurotransmission (Zimmer, Hembert, Durand, Breton, Guilloteau, Besnard & Chalon, 1998). It is also noteworthy to report that the phospholipids of mitochondria are also rich in DHA. High DHA amounts in mitochondrial membranes can increase the efficiency of electron transport by increasing the lateral movement of proteins within bilayer, thus facilitating protein-protein interactions (Valentine & Valentine, 2004).

5.1.2.2. DHA and neurite outgrowth

Several reports establish that DHA supplementation in culture can increase neurite outgrowth (Calderon & Kim, 2004; Kawakita, Hashimoto & Shido, 2006). Animal studies have illustrated that a diet deficient in ω-3 DHA caused decreased neuronal size in hippocampus, hypothalamus and parietal cortex in weaning rats, and in piriform cortex in mature rats (Ahmad et al., 2002); the mean thickness of cortical plate and mean sectional area of the primordial dentate gyrus were lower in an ω-3 deficiency group of the rat embryos, whereas the mean thickness of the cortical ventricular zone and the primary dentate neuroepithelium were higher thus indicating a delay or inhibition of normal brain development in rats (Coti-Bertrand, Kusky and Innis, 2006). Taken together, all these studies support the notion that DHA facilitates neurogenesis. PUFAs seem to improve neurite outgrowth by several mechanisms that include increasing the synthesis and levels of phospholipids (Marszalek & Lodish, 2005). In differentiated PC12 (PC: phosphatidylcholine), a higher percentage of AA and DHA than of Oleic acid (OA), relative to triacylglyceride, was incorporated into phospholipids (Marszalek, Kitidis, Dirusso & Lodish, 2005). Likewise, in rats infused with palmitic acid (PA), AA, or DHA for 15 min, more AA and DHA was incorporated into brain phospholipids relative to PA (Rapoport, 2001). These data suggest that in differentiating and mature neurons, AA and DHA are preferentially incorporated into phospholipids rather than into triacyglycerides, the other major class of cellular molecules that contain fatty acids (Marszalek & Lodish, 2005).

5.1.2.3. DHA and inhibition of apoptosis in neurons

Prevention of apoptosis by the incorporation of DHA into phospholipids has been described in rat retinal photoreceptors (Rotstein, Aveldano, Barrantes, Roccamo & Politi, 1997). Additionally, increased dietary intake of DHA prevents apoptosis in mouse photoreceptors subjected to a strong inducer of apoptosis, N-methyl-N-nitrosourea (Moriguchi,

Yuri, Yoshizawa, Kiuchi, Takada, et al., 2003). The antiapoptotic effects of DHA in neurons take place only after supplementation with DHA, thus indicating that these effects are possibly due to DHA being metabolized into phospholipids (Marszalek & Lodish, 2005). Interestingly, in other non-neuronal cell types, DHA promotes apoptosis; in CaCo-2 cells, a colon cancer cell line, DHA stimulates apoptosis by down-regulating the expression of antiapoptotic genes and increasing the level of several pro-apoptotic genes (Narayanan, Narayanan & Reddy, 2001).

5.1.3. Polyunsaturated fatty acids in human development

Before birth, all of the ω-3 and ω-6 fatty acids of the foetus are derived from the maternal circulation through placental transfer. After birth, all must be provided from the breast milk or formula diet. The human placenta permits free fatty acids to cross it but does not allow phosphoglycerides or triacylglycerols to pass through (Wainwright & Martin, 2005). Levels of DHA in the human brain are low at the beginning of the third trimester of gestation, but they increase rapidly during the third trimester as well as after birth (Clandinin, Chappell, Leong, Heim, Swyer & Chance, 1980a; Clandinin, Chappell, Leong, Heim, Swyer & Chance, 1980b). For this reason, preterm and small-for-gestational age infants may be at risk for fatty acid deficiency. The mechanisms that have been proposed to explain the increasing concentrations of DHA in the foetus with gestational age include an increase in fetal or placental capacity to form LC-PUFAs from precursors or a preferential transfer of these fatty acids across the placenta from maternal to fetal circulation (Wainwright & Martin, 2005).

Lipids are the major energy-yielding ingredient of human milk. Fatty acids (FAs) correspond to 88% of milk fat with the highest proportions of LC-PUFA in human milk being the AA (0.4-0.6%) and the DHA (0.2-0.4%; Koletzko, Rodriguez-Palmero, 1999). Studies comparing breast-fed with formula-fed infants who died from sudden infant death syndrome found lower red blood-cell phospholipid DHA in formula-fed infants (Farquharson et al., 1992; Makrides, Neumann, Byard, Simmer, Gibson, 1994). Based on these studies, and studies on ω-3 deficient animal models, most research has focused on the effects of DHA on infants' development. A recent review by Eilander, Hundscheid, Osendarp, Transler and Zock (2007) examined the effects of ω-3 fatty acid supplementation on visual and cognitive development during pregnancy and lactation, and in term and preterm infants, concluding that there is a beneficial effect of maternal ω-3 LCPUFA supplementation during pregnancy and lactation on

cognitive development of infants and children, but not for visual development. In summary, DHA supplementation during pregnancy and lactation had no beneficial effect on visual development but there was evident advantage on later mental development (Helland, Smith, Saarem, Saugstad & Drevon, 2003) suggesting that effects of DHA supplementation during pregnancy and lactation may appear later in life when cognitive functions are more mature. DHA supplementation during lactation yielded no relevant effect on visual development on three randomized controlled trials (Gibson, Neumann & Makrides, 1997; Lauritzen, Jorgensen, Mikkelsen et al., 2004; Lauritzen, Jorgensen, Olsen, Straarup & Michaelsen, 2005; Jensen, Voigt, Prager et al., 2005). For cognitive development, the results from the three trials were inconsistent; Jensen *et al* (2005) showed a positive effect of maternal DHA supplementation on psychomotor development of the offspring/infants at 30 months, while Lauritzen *et al* (2005) found a positive effect on problem solving ability only in girls at 9 months of age. In general, results from DHA supplementation studies during pregnancy and/or lactation suggest that DHA may have a beneficial role on later cognitive development but not on visual development. Two Cochrane reviews on DHA supplementation at term and preterm infants concluded that the results of most well-conducted randomized controlled trials (RCTs) have not shown any advantageous effect of LC-PUFA containing formula milk on visual or cognitive development of infants born either at term (Simmer, Patole & Rao, 2008) or prematurely (Simmer, Schulzke & Patole, 2008).

Supplementation on older children has not been studied thoroughly. A follow-up study by Bakker, Ghys, Kester, Vles, Dubas, Blanco and Hornstra (2003) investigated the relation between cognitive function at 7 years old and the DHA and AA levels in umbilical venous plasma phospholipids, representing the prenatal LCPUFA availability. Using the Kaufman Assessment Battery for Children they assessed cognitive function on 304 children. They found no significant association with either DHA or AA at birth and cognitive performance at 7 years old. They also measured LC-PUFA levels at 7 years old and again found no association of DHA or AA with cognitive performance. Zhang, Hebert and Muldoon (2005) used cross-sectional data from the Third National Health and Nutrition Survey, 1988-1994 to examine an association between dietary fat intake and cognitive and psychosocial functioning in children aged 6-16 years old, in the United States. They observed that the

consumption of PUFAs was related with better performance in the digit-span test. The South Africa Medical Research Council (2000) conducted a randomized placebo-controlled trial with ω-3 oil supplement in 6-to-11 year old primary school children over a 9 month period and found a significant increase in ω-3 fatty acids status as well as a significant advance of the ω-3 group over the control group on the total recall score of the Hopkins Verbal Learning Test (HVLT; Tichelaar, Smuts, Kvalsvig & Burgess, 2000). In a subsequent study, a bread spread containing fish flour from marine source was developed in order to assess the effects of ω-3 fatty acids on cognition of 7-to-9 year old children (Dalton, Wolmarans, Witthuhn, van Stuijvenberg, Swanevelder & Smuts, 2009). The study population consisted of 183 school-aged children from a primary school in a low socio-economic community of mixed ancestry (African-European-Malay) from the Northern Cape Province of South Africa. The specially designed bread spread supplied about 892 mg of DHA per week and was provided for 104 days over a 6-month period. Intervention took place only on school days and not on weekends or during school holidays. They assessed cognitive performance using the HVLT, a reading and a spelling test. The study showed a significant supplementation effect for HVLT *Recognition* and *Discrimination index*, as well as for the spelling test. This study suggests that supplementation with fish flour rich in ω-3 long-chain fatty acids can improve verbal learning ability and memory of children from lower socio economic status that fish consumption is rare. Results from the above two studies should be treated with caution, however, as the HVLT is a test designed to evaluate performance of Alzheimer's patients and has not been standardized on healthy children. Further, the authors did not report any differences on intelligence or maternal education/intelligence between the two groups at baseline, thus any significant post-intervention results may not be due to the intervention product but due to baseline differences on intelligence. Last but not least, the effects cannot be certainly attributed to ω-3 LCPUFA, because the fish flour also contained minerals which might also have an effect on cognition.

The above positive findings contrast with those obtained in two parallel, randomized, placebo-controlled trials in Australia and Indonesia that were designed by the NEMO (Nutrition Enhancement for Mental Optimization) study group (2007) in order to assess the effect of micronutrients, LC-PUFAs, or combination of both on cognitive performance in well-nourished and marginally nourished school-aged children. A total of 396 children (6-10 years

old) in Australia and 384 children in Indonesia were randomly assigned to receive a drink with a micronutrient mix, with DHA (88mg/day) and EPA (22mg/day), or with both or placebo for 12 months (6 d/wk). DHA and EPA treatment increase plasma DHA as well as total ω-3 fatty acids in both countries, but found no effect of LC-PUFA supplementation on cognitive performance, compare with the micronutrient treatment that increased scores on verbal learning and memory tests in Australia.

Finally, the study group of McNamara, Able, Jandacek, Rider, Tso, Eliassen, Alfieri, Weber, Jarvis, DelBello, Strakowski, and Adler (2010) examined the effects of low (400mg/d) and high (1200mg/d) doses of DHA supplementation on cortical activity during sustained attention in 33 boys 8 to 10 years of age. After eight weeks of supplementation, the DHA content in erythrocyte membranes increased significantly in the two DHA groups but not in the placebo (corn oil) group. During sustained attention, both DHA dose groups had greater changes in activation of the dorsolateral prefrontal cortex than did the placebo group; erythrocyte DHA was positively associated with activation in dorsolateral prefrontal cortex.

5.1.4. Summary, Aims and Hypothesis

In summary, very little is known about the effect of DHA supplementation on cognition in healthy school-aged children. In the Western diet, intake of ω-3 polyunsaturated fatty acids is inadequate, while that of ω-6 PUFAs is usually high, thus negatively influencing the ω-6:ω-3 ratio in the diet. Most of the research on the relationship between ω-3 PUFA and cognitive development has focused on breast-fed or formula-fed infants born either at term or pre-term. This is not surprising given of the dramatic brain growth during gestation and the first two years of life. But brain development continues throughout childhood and adolescence. Volumes of cortical gray-matter increase during childhood, reaching peak levels around the time of the puberty onset, while volumes of white matter show a linear increase throughout childhood and adolescence, with the maximum volumes reached often as late as in the third decade of life (Pfefferbaum et al., 1994).

In the current study, we assessed possible effects of ω-3 supplementation on brain and cognition in 60 healthy children between 9 and 12 years of age. Since diet supplementation may be beneficial in particular to children with poor diet, we recruited children from less well-

off neighbourhoods, as determined by the Index of Multiple Deprivation 2007, and with a low habitual intake of fatty fish.

The brain structure was assessed using magnetic resonance imaging (MRI), and cognitive abilities as well as behavioural problems were assessed using a comprehensive battery of neuropsychological tests and questionnaires. To evaluate the effectiveness of the supplementation, we collected blood samples to assess levels of fatty acids in plasma and erythrocytes. The dietary intervention included a daily use, for a period of three months, of margarine enriched (experimental group) or not (placebo group) with ω-3 fatty acids (1,000 - 1,300 mg/d). We hypothesize that this intervention will (1) improve some cognitive abilities (such as attention, processing speed) and mood/behavioural symptoms (e.g. depression, aggressiveness, impulsivity) and (2) will have noticeable effect on brain structure (e.g. white-matter properties related to myelination).

5.2. Methodology

5.2.1. Design

To evaluate possible effects of omega-3 supplementation on brain and behaviour of 60 healthy children 9-to-12 years old, the design of the ω-3 study had the following features: a) a double-blind randomized placebo controlled design; b) the placebo group (n=30) is matched according to age, sex and maternal education to the treatment group (n=30); c) recruitment is carried out in less well-off neighbourhoods, as determined by the Index of Multiple Deprivation 2007 (we assume that ω-3 supplementation may be particularly beneficial in children with poor diet, often associated with a lower socioeconomic level [Northstone, Emmett, & Rogers, 2008]); d) quantitative assessment of brain and behaviour, and e) acquisition of blood samples to assess level of fatty acids pre and post intervention. An overview of the study design is shown in Figure 3.

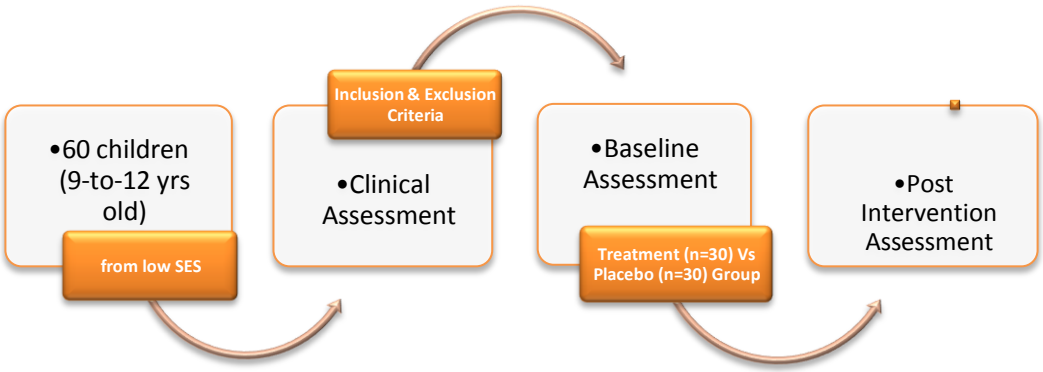


Figure 3: Overview of omega-3 study design. Note that all measurements on baseline assessment were repeated on post-intervention assessment.

5.2.1.1. Size of study population

As far as we know, there is currently no MRI data available from ω-3 supplementation studies on healthy children in order to measure the power. Longitudinal MRI studies have detected changes in white matter volume in samples of n=31 (3.5 year scanning intervals; Paus, 2005) and n= 36 (1.5 year scanning intervals, unpublished data from the Santa Fe study). Further, based on studies focusing on group differences, a sample of 25-30 participants was enough to detect changes between gray and white matter volumes in specific brain regions of ADHD children versus control children (Carmona, Vilarroya, Bielsa, Tremols, Soliva, Rovira, Tomàs, Raheb, Gispert, Batlle & Bulbena, 2005; McAlonan, Cheung, Cheung, Chua, Murphy, Suckling, Tai, Yip, Leung & Ho, 2007; Durston, Hulshoff, Schnack, Buitelaar, Steenhuis, Minderaa, Kahn, Van Engeland, 2004).

None of the previous studies have used MRI to detect changes on the brain after omega-3 supplementation, whether in non-clinical samples or children with ADHD. The present study is therefore considered to be an explorative one. It will provide us with valuable information on the variability and effect size concerning MRI data on brain structure after ω-3 supplementation and help us improve the design of follow-up studies. Due to the explorative character of the study, the sample size that is considered to be appropriate is based on estimations rather than power calculations. It is assumed that 30 children per group will be sufficient to detect effects on white matter volume or structure as measured by MRI in the intervention compared with placebo group.

5.2.2. Setting and Participants

The participants were recruited from less well-off neighbourhoods of Nottingham, as determined by the Index of Multiple Deprivation 2007 (IMD; Department for Communities and Local Government 2007). Nottingham is the largest city in the East Midlands region of United Kingdom. The city of Nottingham has a population of 286,000, while the wider Nottingham Urban Area has a population of 667,000 and is the seventh-largest urban area in United Kingdom (Pointer, 2005). According to the IMD 2007, Nottingham is one of the most deprived areas in the East Midlands region. The health of people in Nottingham is significantly worse than the England average and life expectancy is lower (by three years) for both men and women living in Nottingham compared with the England average (Association of Public Health Observatories and Department of Health, 2008). Also, early deaths from cancer, heart disease and stroke, as well as the frequency of smoking are all above the England average. Levels of binge drinking, alcohol-related hospital admissions, drug misuse and violent crime are significantly worse in Nottingham than the England average. Last but not least, levels of child poverty and children under 15 "not in good health" are worse that the England average (Association of Public Health Observatories and Department of Health, 2008).

The omega-3 study was approved by the Medical School Research Ethics Committee of the University of Nottingham. Written informed consent was obtained from the parents of the children, and assent was obtained from the children.

5.2.2.1. Selection Criteria

As aforementioned, children between 9 and 12 years of age were recruited from schools located in less well-off neighbourhoods of Nottingham, according to the IMD 2007. The IMD 2007 includes the following seven domains: Income deprivation; Employment deprivation; Health deprivation and disability; Education, skills and training deprivation; Barriers to housing and services; Crime; Living environment deprivation. The IMD is highly correlated with Townsend Index, which consists only of four domains. Those neighbourhoods in Nottingham that belong to the 20% of most deprived areas in the UK were determined. In these areas, the most disadvantaged schools (as identified on the basis of the OFSTED school inspection reports) were contacted for recruitment of children.

The main exclusion criteria were: 1) eating fatty fish/shellfish more than once a week; 2) using ω-3 and/or ω-6 supplements more than once a week; 3) consuming products fortified with ω-3 (EPA and/or DHA) in the three months prior to study participation (>100mg/day); 4) positive history of alcohol abuse during pregnancy; 5) positive history of malignancy and heart disease requiring heart surgery; 6) severe mental illness (e.g. autism, schizophrenia) or mental retardation; and 7) MRI contraindications. The full list of inclusion/exclusion criteria is presented in Table 1. The treatment group was matched to the placebo group according to age, sex, and maternal education.

Table 1: Exclusion and Inclusion criteria

		Actions
Demographics	1. Child in target age (9-12 yrs)	Inclusion
	2. Child native language English	Inclusion
	3. Child is available for providing a blood sample	Inclusion
	4. Child is available for MRI scan	Inclusion
Diet	1. Child eating fat fish/shellfish more than once a week	Exclusion
	2. Child using omega-3 and or -6 supplements	Exclusion
	3. Child consuming products fortified with ω-3 EPA and/or DHA (>100mg/day) 3 months prior to the study	Exclusion
Pregnancy and birth	1. Use of alcohol by the mother during pregnancy	Exclusion[20]
	2. Diabetes of the mother during pregnancy (onset before pregnancy, treated by insulin)	Exclusion
	3. Premature birth (< 35 weeks) and/or detached placenta	Exclusion
	4. Hyperbilirubinemia requiring transfusion	Exclusion
Child's medical history	1. Type 1 diabetes	Exclusion
	2. Systemic rheumatologic disorders (e.g. Complications of strep throat, such as glomerulonephritis or endocarditis)	Exclusion
	3. Malignant tumours requiring chemotherapy (e.g. leukaemia)	Exclusion
	4. Congenital heart defects or heart surgery	Exclusion
	5. Aneurism	Exclusion
Neurological conditions	1. Epilepsy	Exclusion
	2. Bacterial Infection of CNS	Exclusion
	3. Brain tumour	Exclusion
	4. Head trauma with loss of consciousness >30 minutes	Exclusion
	5. Muscular dystrophy, myotonic dystrophy	Exclusion
Developmental conditions	1. Nutritional and metabolic diseases (e.g. failure to thrive, phenylketonuria)	Exclusion
	2. Major neuro-developmental disorders (e.g. autism)	Exclusion
	3. Hearing deficit (requiring hearing aid)	Exclusion
	4. Vision problems (strabismus, visual deficit not correctible)	Exclusion
Mental health & abilities	1. Treatment for schizophrenia, bipolar disorder	Exclusion
	2. IQ < 70	Exclusion
MR contraindications	1. Metal implants/Braces	Exclusion
	2. Electronic implants (e.g. pacemakers)	Exclusion
	3. Severe claustrophobia	Exclusion

[20] >210 ml alcohol/week [eg. 14 bottles of beer, 9 glasses of wine, 7 glasses of hard liquor].

5.2.2.2. Recruitment

Participants were recruited through regional primary and secondary schools in less well-off neighbourhoods. Following a briefing with the teacher, we visited individual classrooms and presented the project. All students received a letter containing an information brochure and a self-addressed and stamped response card for their parents. We asked the parents to mail the response card back to the study group and indicate whether or not they are interested in participating in the project and to provide the home phone number for a follow-up call. During the follow-up call to the interested family, we verified basic eligibility for the project (diet, blood sample, MRI). The telephone interview covered all the exclusion criteria in order to verify that the child is eligible for the study. If the child was eligible for participation the next set of letter was sent to the parents including: a parent information sheet; a student information sheet; a parent consent form; a parent questionnaire form (including mother education and all exclusion and inclusion criteria); and a parent screening questionnaire (detailed screening about child's ω-3 consumption over the last 3-months). After receiving all information back to the research lab and verifying eligibility, participants were matched according to age, sex and maternal education and randomly assigned (see later) into the treatment or placebo group. The families were contacted after matching to set up an appointment for the first visit in the laboratory where the baseline assessment took place. The laboratory visit began with the signing of the consent (parents) and assent (children) forms.

Due to a limited collaboration of primary and secondary schools (from 40 schools approached only 6 showed an interest and responded back), we also employed a 2nd recruitment strategy. We used a consumer mailing list from a database and digital marketing agency, called Data HQ. The mailing list consisted of 3,000 names of low-income families with children between the ages of 9 and 12 years. We sent a new advertising leaflet (see appendix II) to these 3,000 families with a self-addressed and stamped response card. Those who responded back received a 2nd letter including: a parent information sheet; a student information sheet; a parent consent form; a parent questionnaire form (including mother education and all exclusion and inclusion criteria); and a parent screening-questionnaire (detailed screening about child's ω-3 consumption over the last 3-months). After verifying eligibility, the procedure was the same as described above.

We recruited 26 children with the 1st strategy, whereas the mailing lists provided us with another 14 children. The 2nd recruitment strategy was initiated after we had recruited and matched the first 24 children recruited using the 1st strategy. Only 2 out of the 26 "1st-strategy" children were matched with 2 of the 14 children recruited using the 2nd strategy. As such, the placebo and the intervention group did not differ in this respect.

5.2.3. Randomisation

The treatment and placebo products were similar with respect to taste and appearance and were only different in coding. Products were sent to Nottingham colour-coded from Unilever in Vlaardingen, the Netherlands. A pair-wise randomisation of participants, matched on sex, age and maternal education took place. Within each matched pair , one participant was randomized (by flipping the coin) to one of the treatments, and the other participant to the other treatment. As already reported above, the two groups did not differ with respect of recruitment strategies.

5.2.4. De-blinding

Two de-blinding envelopes were made in Vlaardingen; these contained the colour-treatment combinations (e.g. red colour = active product; blue colour = placebo). One envelope was sent to Nottingham, the second remained in Vlaardingen. In Nottingham, we created an additional overview containing a list with the ID-codes of the participants and the colour of the treatment they were assigned to; the envelope with the colour-treatment combination was deposited with a person not involved in the study. In case of (serious) adverse events the principal investigator (in consultation with the general practitioner of the affected child) would decide whether to reveal the treatment-blinding for the participant.

5.2.5. Intervention Product

5.2.5.1. Composition of the intervention product

The intervention product was full fat (80%) margarine. The active intervention product contained 500-650 mg DHA and 500-650 mg EPA per (10 gram) serving. The dose of the active ingredients DHA and EPA in the intervention product was based on the US Generally Recognized as Safe (GRAS) level (FDA 2004).The placebo product was a similar margarine with the same sensory properties, but with mono-unsaturated fatty acids (MUFA; refined plant

oils) replacing EPA and DHA; total saturated fatty acids (SAFA) and ω-6 fatty acid content were matched. For an overview of the nutrient and fatty acid content of the active intervention and placebo product see Table 2 below. The intervention products were provided by Unilever R&D Vlaardingen and produced in an approved facility in Vlaardingen, The Netherlands. Preparation of the products was performed according to relevant Good Manufacturing Practice (GMP) guidelines. The actual composition and fatty acids content of active and placebo products were tested for each batch before the products were made available to the participants.

Table 2: Nutrient and fatty acid composition of the active and placebo product

	Active product	Placebo product
MUFA (g)	1.87	4.32
oleic acid (g)	1.87	4.32
SAFA (g)	2.72	2.59
PUFA (g)	2.77	1.05
Total n-3 FA (g)	1.59	0.06
ALA (g)	0.08	0.06
EPA (g)	0.65	-
DHA (g)	0.59	-
Total n-6 FA (g)	0.99	0.99
LA (g)	0.99	0.99
GLA (g)	-	-
DGLA (g)	-	-
AA (g)	-	-
Trans FA (g)	0.11	0.04
Cholesterol (mg)	278.45	0.11
Vitamin E (mg)	8.0	1.89
Vitamin D (mg)	7.5	7.5
Vitamin A (mg)	800	800

5.2.5.2. Distribution of the intervention product

Products were produced in batches. The dietary intervention products were stored at -20°C at Unilever. Next, they were defrosted to 5°C, transported to Nottingham and distributed to participants from there. In a chilled state (4-7°C), the products had a shelf-life of three months. Participants were provided with a one-month supply of the intervention product (including

reserve products) at the onset of the intervention and received a new supply at each monthly visit. The intervention products were provided in neutral serving-sized cups, containing 10 grams of margarine. All products were marked with a code and production date to enable back-tracking of all production details within 24 hours.

5.2.5.3. Use and handling of intervention product

The child's parents were instructed to have their child consume one complete portion pack (10 g) of margarine per day and to record consumption in a compliance calendar (see Appendix III) that was given to them. The margarine had to be consumed as spread on sandwiches/ crackers / bread rolls. Parents were instructed not to use the margarine for cooking, baking or for frying and not to add the margarine to the child's hot meal. Also, parents were informed that the intervention product had to be given exclusively to the participating child. The intervention product could be consumed on various periods over the day or all at one occasion (e.g., with breakfast, lunch, dinner or in between). Every day a new portion pack had to be used, even if there was margarine left in the package of the previous day. Parents were instructed to store the intervention products in the fridge (<7^{o}C). Used packs had to be put into a re-closable plastic bag at the end of the day and be kept in the fridge or a cool place. Used containers were handed in to the investigators at the monthly visits when the new intervention products were handed out.

Participants were asked to maintain their usual diet throughout the intervention period. They were not allowed, however, to use other omega-3 or omega-6 containing food supplements or food fortified with EPA and DHA during the intervention. The participating family received a list with products fortified with EPA and/or DHA that could not be used during the study (Appendix IV). Every monthly visit the parents had to fill in a questionnaire to ensure that no use of supplements or fortified food was taking place and to check whether their diet or lifestyle had changed.

5.2.5.4. Compliance

Fatty acid levels were measured from blood samples which were obtained from children during the laboratory visits before and after the intervention period.

The average amount of intervention product consumed per day was calculated by measuring the weight difference between full/empty portion packs. Participants were instructed

to return the used (and non-used) portion packs of margarine at each monthly visit. Portion packs were weighted when distributed and when returned. The amount of margarine consumed was calculated by subtracting the weight of the returned (empty) portion packs from the weight of the distributed (full) portion packs.

In addition, participants received a compliance calendar. They were instructed to report the consumption of the product daily by noting whether the margarine was consumed in full each day or not. In a drawing scheme with pre-determined fields, participants were requested to indicate how much of the margarine was consumed. In case the margarine was not consumed in full, they were asked to note why they did not consume everything.

5.2.6. Blood collection and analysis

Blood samples were collected using a venipuncture. Blood samples of 5 ml were taken in the morning from fasting children. Samples were immediately stored on ice (placed in a cold room) and within two hours after collection blood was separated in three different 2-ml tubes. These three tubes were then centrifuged (3000 rpm, 10 min, 4 ºC) and separated cells from plasma. For the plasma analysis, we used the first tube where we took 0.5ml of plasma and stored at -80ºC for further analysis. For the erythrocyte analysis, we used the 2nd tube, took 0.5ml of red cells and transferred to another tube. We then added 0.5 ml of phosphate-buffered saline, vortex and centrifuged (3000 rpm, 10 min, 4 ºC), removed the supernatant and discarded it. Last we froze the red cell pallet at -80ºC for further analysis.

We measured the fatty acid composition of plasma phosphatidylcholine (PC), the predominant phospholipid in the circulation, and of erythrocyte total lipids. The measurements were carried out in the laboratory of Professor P.C. Calder at the University of Southampton as follows.

Plasma PC: Prior to extraction of lipid from plasma, a PC internal standard was added. Total lipid was extracted from plasma using choloroform/methanol (2:1). PC was separated from other plasma lipids and isolated by solid phase extraction chromatography on Bond-Elut cartridges. PC fatty acids were converted to their methyl esters by heating at 50^{o}C for 2 hours in the presence of sulphuric acid containing 2% methanol. Fatty acid methyl esters (FAMEs) were extracted into hexane and concentration by evaporating the hexane under a stream of nitrogen. FAMEs were separated by gas chromatography using standard conditions

and were identified by comparison of retention times with those of authentic standards run previously. Data were expressed as both relative concentration (i.e. g/100 of total fatty acids [i.e. weight %]) and absolute concentration (micrograms/ml plasma).

Erythrocyte total lipid: Total lipid was extracted from washed erythrocytes using chloroform/methanol (2:1). Erythrocyte fatty acids were converted to their methyl esters by heating at 50^{o}C for 2 hours in the presence of sulphuric acid containing 2% methanol. Fatty acid methyl esters (FAMEs) were extracted into hexane and concentration by evaporating the hexane under a stream of nitrogen. FAMEs were separated by gas chromatography using standard conditions and were identified by comparison of retention times with those of authentic standards run previously. Data were expressed as relative concentration (i.e. g/100 of total fatty acids [i.e. weight %]).

5.2.7. Measurements

5.2.7.1. Questionnaires

Demographics and measures of socioeconomic status (SES): Table 3 provides information on datasets acquired in these and other domains and the instruments used for their collection. *Medical and psychiatric history*: we collected information on the medical history of the child from conception to present (pregnancy, breastfeeding) as well as information on the parental mental health (depression, anxiety, aggression). For children, we used several questionnaires to evaluate a number of psychological issues (emotionality, hyperactivity, conduct behaviour, aggressiveness), as well as *parental monitoring*. A *Food Frequency Questionnaire* was completed by the parents on behalf of their children who participated in the study. Last, both parents and children completed an *Essential Fatty Acids Symptoms* list that was based on the EFA checklist used by Stevens, Zentall, Deck, Abate, Watkins, Lipp and Burgess (1995) by bringing together vital symptoms of essential fatty acid deficiency.

Table 3: Questionnaires

Demographics			
Domains	**Respondent**	**Instrument**	**Source**
Parent's Age/Marital Status	Mother	Parents Questionnaire 1	Developed by the research team
Parent's Ethnicity/Religion	Mother	Parents Questionnaire 1	Developed by the research team
DOB of the child/Birth order	Mother	Parents Questionnaire 1	Developed by the research team
Family Income	Mother	SES Questionnaire	Developed by the research team
Parental Education	Mother	SES Questionnaire	Developed by the research team
Parental Employment	Mother	SES Questionnaire	Developed by the research team
Handedness of the child	Child	Child's handedness Q.	Version of Crovitz & Zener 1962
Medical & Psychiatric Health			
Domains	**Respondent**	**Instrument**	**Source**
Child's Medical History	Mother	Inclusion/Exclusion Form	Adopted from SYS[21]
MR Contraindications	Mother	MRI Safety Screening Q.	Sir Peter Mansfield Magnetic Resonance Centre/ Brain & Body Centre
Pregnancies/Abortions/stillbirth	Mother	Parents	Adopted from SYS
Pregnancy and Birth	Mother	Parents	Adopted from SYS
Breastfeeding	Mother	Parents	Adopted from SYS
Child's medical history	Mother	Parents	Adopted from SYS
Child's EFA Symptoms	Mother	Essential Fatty Acids Symptoms Checklist	Stevens et al., (1995)
Child's EFA Symptoms	Child	Essential Fatty Acids Symptoms Checklist	Stevens et al., (1995)
Parent's mental health	Mother/Father	Depression, Anxiety and Stress Scale (DASS)	Lovibond & Lovibond, 1995
Parent's aggressive behaviour	Mother/Father	Aggression	Buss & Perry, 1992
Child's psychological attributes	Child	Strengths and Difficulties Questionnaire (SDQ)-Child	R.Goodman, 1997
Child's psychological attributes	Mother	Strengths and Difficulties Questionnaire (SDQ)-Parent	R.Goodman, 1997
Child's anxiety, depression	Child	DASS-short version (21 items)	Lovibond & Lovibond, 1995
Child's aggressive behaviour	Child	Aggression Questionnaire	Buss & Perry, 1992

[21] SYS: Saguenay Youth Study (Pausova, Paus, Abrahamowich, Almerigi, Arbour et al., 2007)

Child's aggressive behaviour	Child	Situational Triggers of Aggressive Responses (STAR)	C. Lawrence, 2006
Parental Monitoring	Child	Parental Monitoring Scale	Small & Kerns, 1993
Diet			
Food Frequency	Mother	Food Frequency Questionnaire	Adopted from ALSPAC[22] study

Measurements used for psychological attributes, mental health and aggressive behaviour will be described in more details below. *Depression, Anxiety and Stress Scale* (DASS) is a 42-item self report measure that was developed by Lovibond & Lovibond (1995). The psychometric properties of DASS were assessed through a non-clinical sample (*N*=2,914) and it was found that reliability, assessed using Cronbach's alpha, was acceptable for Depression (.91), Anxiety (.84) and Stress (.90). "A large student sample (*N*=717) was administered the Beck Depression Inventory (BDI; Beck, Ward, Mendelsohn, Mock, & Erbaugh, 1961), the Beck Anxiety Inventory (BAI; Beck , Epstein, Brown & Steer, 1988) and the DASS. The BAI and DASS scale were highly correlated (*r* = .81), as were the BDI and DASS depression scale (*r* = .74)" (Crawford & Henry, 2003). Interpretation of DASS is primarily based on the use of cut-off scores. In 2003, Crawford and Henry (2003) obtained normative data of DASS from a non-clinical sample (*N*=1,771) representative of the general adult UK population.

Strengths and Difficulties Questionnaire (SDQ) is a brief behavioural screening questionnaire consisting of 25 items (Goodman, 1997). The 25 items are divided in 5 scales: emotional symptoms (5 items), conduct problems (5 items), hyperactivity/inattention (5 items), peer relationship problems (5 items), and prosocial behaviour (5 items). A sum of the 25 items (except the prosocial scale) gives us the total difficulties; the interpretation of results is again based on cut-off scores. In addition to the 25 items, there is an impact supplement that measures chronicity, distress, social impairment and burden to others due to a problem that the respondent believes he/she has. There is also a follow-up version that has two additional follow-up questions for use after an intervention. SDQ has a parent and a teacher version that

[22] ALSPAC: Avon Longitudinal Study of Parents and Children (Golding, Pembrey, Jones & The Alspac Study Team, 2002

helps the clinician or the researcher gain a global idea for the child's behaviour at different domains. The SDQ was administered along with Rutter questionnaires to parents and teachers of 403 children drawn from dental and psychiatric clinics and found that scores derived from the SDQ and Rutter questionnaires were highly correlated (Goodman, 1997). In another study, mothers completed the SDQ and the Child Behaviour Checklist (CBCL) on 132 children aged 4 through 7. Scores from the SDQ and CBCL were highly correlated and equally able to discriminate psychiatric from dental cases (Goodman & Scott, 1999).

Situational Triggers of Aggressive Responses (STAR) is a self-report instrument assessing individual differences in the type of events and antecedents that make people feel aggressive (Lawrence, 2006). The scale comprises of 22 items that measure Frustration and Provocation. Both sub-scales showed convergent validity with measures of aggression (Buss & Perry, 1992).

Aggression Questionnaire is a 29-item self-report measure of aggressiveness (Buss & Perry, 1992). The 29 items yield 4 scales: Physical Aggression, Verbal Aggression, Anger, and Hostility. The various scales correlate differently with a range of personality traits (Buss & Perry, 1992).

Parental Monitoring Questionnaire is an adolescent self-report instrument (8 items) that assesses the extent to which parents know the whereabouts of their child after school and at night, and have knowledge of teen's friends and their parents (Small, & Kerns, 1993). Monitoring scale has been reported to have adequate reliability ($\alpha = .87$) and predictive validity (Small, & Kerns, 1993).

5.2.7.2. Neuropsychological Assessment

Table 4 presents a summary of the tests used for neuropsychological assessment.

Table 4: Neuropsychological Assessment

Test	Domains	Source
Wechsler Intelligence Scale for Children (WISC-IV)	Intelligence	Wechsler, 2003
Wisconsin Card Sorting Test-64 Card Version (WCST)	'Set-shifting'; Executive functioning	Kongs, Thompson, Iverson & Heaton, 2000
Self-Ordered Pointing Task (SOPT)	Working memory	Petrides & Milner, 1982
Ruff 2&7 Selective Attention Test	Selective & sustained attention	Ruff & Allen, 1996
Trail Making Test (TMT)	Visual search; Scanning; Speed of processing	Reitan, 1958

The *WISC-IV* is an individually administered clinical instrument for assessing intelligence of children aged 6 years through 16 years 11 months. WISC-IV has 4 composite scores: Verbal Comprehension, Perceptual Reasoning, Working Memory, and Processing Speed. Full Scale IQ (FSIQ) comprises of the four composite scores and has a total of 15 subtests. The WISC-IV was used at baseline assessment to obtain a full scale IQ. At post-intervention, only some of the subtests were used, including Cancellation, Symbol Search, Coding (which measure processing speed, visual-motor coordination, visual perception, visual scanning ability) and letter-number sequencing (which involves mental manipulation, attention, short-term auditory memory, visuo-spatial imaging, and processing speed).

The *WCST-64* is a neuropsychological test of "set-shifting", i.e. the ability to display flexibility in the face of changing schedules of reinforcement. Successful completion of the test relies upon a number of intact cognitive functions including attention, working memory, and visual processing. It is loosely termed a "frontal lobe" test on the basis that patients with any sort of frontal lobe lesion generally perform poorly at the test.

SOPT was used to measure participants' ability to manipulate items in working memory. The task consists of a set of 12 stimulus items (abstract pictures), which are presented on 12 different pages, their order changing quasi randomly each time the subject turns a page.

Participants have to point to a different item on each page without pointing to the same picture twice. Successful performance involves working memory and challenges the participants to organize, maintain and monitor their responses.

Ruff 2-&-7 Selective Attention Test was used to measure two types of attention: sustained and selective. The task provides information on the power of automatic *Vs* controlled information processing. In the automatic condition, capital letters are intermixed with the digits 2 and 7. In the controlled condition, the digits 2 and 7 are intermixed with other digits. In both conditions (10 blocks per condition) the adolescent has to read through each line and cross out 2 s and 7 s within a time limit of 15 s per block (each block consist of three lines with 50 items per line).

TMT is one of the most widely used neuropsychological tests. It provides information on visual search, scanning, and processing speed. It consists of two parts: TMT-A and TMT-B. For TMT-A the participant has to draw a line sequentially connecting 25 encircled numbers on a sheet of paper. TMT-B requires the participant to draw a line alternately between numbers and letters (e.g. 1-A, 2-B, 3-C). The score of each part is based on time required to complete the task.

5.2.7.3. Magnetic Resonance Imaging – Acquisition

Scanning was performed at 1.5T (Philips Achieva) equipped with whole body gradients, an 8-channel SENSE receive head coil and a whole-body transmit coil. Scanning protocol included *T1 weighted 3D spoiled-TFE* (256x256x160 mm FOV; 1 mm isotropic resolution; TE=4.6 ms; TR=9.9 ms; flip angle=8; TFE factor=161; total scanning time=6:44 min); *3D spoiled MT-FFE* (192x192x140 mm FOV; 2 mm isotropic resolution; TE=4.6 ms; TR=25 ms; flip angle=10; total scanning time=5:33 min); *Multi-slice DTI* (240x240x125 mm FOV; 2.5 mm isotropic resolution; TE=85 ms; TR=7400 ms; flip angle=90; directions=32; b-values =1000 s/mm2; total scanning time=8:45 min). We also measured brain metabolites by Magnetic Resonance Spectroscopy (MRS). For MRS, the imaging volume (box) was positioned carefully, using the three orthogonal localiser scans, to include only white matter in the left frontal lobe (Figure 4). {1}H MRS measurement was carried out using a PRESS[23] localization sequence with the following parameters: Echo Time (TE)= 31ms, repetition time

[23] PRESS: Point Resolved Spectroscopy

(TR)=2000ms, Bandwidth = 1000Hz, 512 samples, Volume of Interest (VOI) = 15x15x15mm3. 96 spectra with water-suppression were collected and averaged. 16 spectra were collected without water suppression to enable calculation of absolute metabolite concentrations, assuming constant water concentrations. The metabolites measured were: N-acetylaspartate (NAA), Creatine (Cr), Choline (Cho) and Myoinositol (mI).

T1W images provide excellent contrast between grey and white matter and, as such, are suitable for various volumetric analyses.

3D spoiled MT-FFE refers to Magnetization Transfer (MT) imaging (Wolff and Balaban, 1989); it is a quantitative MRI technique that gives information on the macromolecular content and structure of tissue. The interaction between free water and water bound to macromolecules is reflected on the contrast in MT images (McGowan, 1999). The macromolecules of myelin are the key source of the MT signal in white matter (Kucharczyk, Macdonald, Stanisz, & Henkelman, 1994).

Multi-slice DTI stands for Diffusion Tensor Imaging (DTI). DTI of water mobility in tissue could offer a more sensitive measure of the changes in the brain's microstructure (Le Bihan, 2003). DTI measures several parameters of water diffusion in live tissue. Diffusion is isotropic when it occurs equally in all directions, whereas when there is a barrier to delay the water motion (e.g. membranes in a white matter tract) then the diffusion is called anisotropic as is no longer equal to all directions (Chenevert, Brunberg & Pipe, 1999).

MR Spectroscopy: MR images are restructured from the entire proton signal from the tissue directed by water and fat proton signals. Protons from other metabolites do not add to imaging due to their minor concentration (Chavhan, 2007). The result of MR spectroscopy is a set of signals that form a MR spectrum (Hajek & Dezortova, 2008). Metabolites: NAA is a neuronal marker and any insult to the brain causing degeneration or neuronal loss decreases NAA (Chavhan, 2007); Cr is an energy metabolism marker and serves as a reference peak as it is constant; Cho is an element of phospholipids of cell membrane and it is precursor of acetyl choline and phosphatidyl choline; it is an indicator of cell membrane integrity (Chavhan, 2007); mI is a glia marker; it is dominant peak in newborns and decreases with age (Chavhan, 2007).

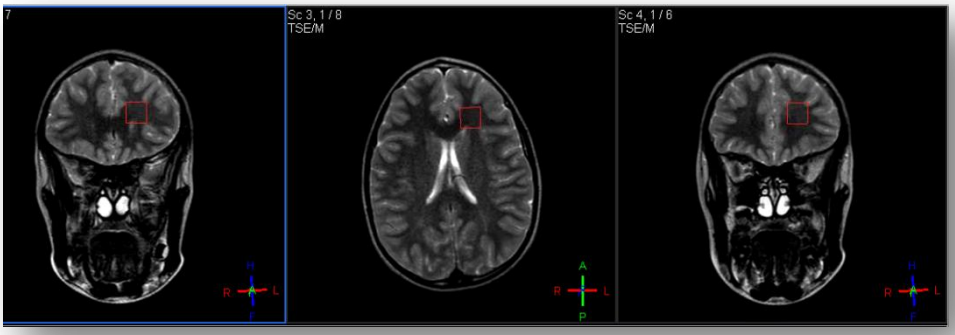


Figure 4: Positioning of imaging volume for spectroscopy analysis

5.2.7.4. MRI Analysis

White/Gray Matter (WM/GM) Volumes and MTR: T1W images were first corrected for intensity in homogeneity using the N3 algorithm (Sled et al., 1998) followed by skull stripping (Smith et al., 2002). These images were then nonlinearly warped to fit the ICBM152 nonlinear template using the ANIMAL algorithm (Collins et al., 1995). Using the ANIMAL+INSECT methodology, which utilizes tissue classification of T1W MRI data in GM, WM, and CSF classes, the nonlinearly registered data, and a probabilistic atlas, each scan was then segmented on a lobe-by-lobe basis (for both GM and WM). Lobe-wise WM masks were extracted for each subject and then re-sampled to match the dimensions of the MTR images using nearest neighbour re-sampling. Mean MTR values were then extracted from each of these WM masks.

DTI: Tract-Based Spatial Statistics (TBSS) were used to extract Fractional Anisotropy (FA) and Mean Diffusivity (MD) values of the whole brain, as well as of the corpus callosum. TBSS is part of the FSL software (Smith, Jenkinson, Johansen-Berg, Rueckert, Nichols, Mackay, Watkins, Ciccarelli, Cader, Matthews & Behrens, 2006). With the TBSS approach we identified a common registration target and aligned all participants' FA images to this target using nonlinear registration. We then created the mean of all FA images and apply "thinning" (non-maximum-suppression perpendicular to the local tract structure), to create a skeletonised mean FA image. Then, thresholded this image to restrain areas of low mean FA and/or high inter-subject variability. Last, we projected each participant's (aligned) FA image onto the

skeleton, by filling the skeleton with FA values from the nearest relevant tract centre (Smith et al., 2006).

MRS: metabolites were measured as ratios of Creatine (NAA/Cr; mI/Cr; Cho/Cr)

5.2.8. Statistical Methods

Statistical analyses were carried out using JMP (version 8) and SPSS 14 (for Windows). All variables were checked for normality by dividing the skewness with the standard error (SE) of skewness (z-score= Skewness/SE skewness; Tabachnick & Fidell, 2005). Variables that were not normally distributed were transformed using LG10, which produces a variable that contains the logarithmic (to base 10) values of the variable, in order to obtain normally distributed data. When transformation did not correct for normality then outliers defined as values three standard deviations from the mean, were excluded. The significance level applied for all statistical tests was set to the standard level of alpha=0.05.

In all analyses, raw scores of cognitive and behavioural measures were used rather than standard scores. Standard scores express the performance of an individual relative to peers of the same age.

To examine the effect of Group (treatment *Vs* placebo) on performance of all the measures, we used Analysis of Covariance (ANCOVA) with Group as the independent variable and the cognitive, behavioural, and MRI measures as well as the fatty acids levels as the dependent variables. Sex, Age and Baseline measures were used as covariates to remove any influence of them on the dependent measures. ANCOVA partials out the effect of covariate by using the regression equation to measure its influence (Brace, Kemp & Snelgar, 2006).

Thereafter, we wished to examine the relationship of the fatty acids (non group-based analysis) with the cognitive, behavioural and MRI measures to observe if DHA and/or EPA are somewhat related with specific measures that were found to be significant in the first type of analysis (group-based analysis; ANCOVA). In order to detect this relationship we used linear regressions with Age, baseline measurements, Full Scale IQ (only for cognitive measures) and parental aggression (if needed for behavioural measures) as the predictor variables and plasma and RBC DHA and EPA as the dependent variables. Sex was included in the analysis only if it was a significant predictor for any dependent variable. Due to the small number of cases and

since we do not have any strong theoretical predictions it is recommended to use the *enter* method for the linear regression (Brace et al., 2006).

5.3. Results

5.3.1. Sample Characteristics

While 60 children were planned to be recruited only 40 were available for inclusion. From 40 children tested, 5 did not come back for the post-intervention assessment. Table 5, separately for the active and the placebo group, describes the children's age, sex, maternal education, family income status, handedness, ethnicity and other prenatal and post-natal characteristics. Maternal education ranged from the lowest level, which is primary school (4-to-11 yrs old), to the highest level, which is doctoral degree. Family income status is based on a 7-level scale: 1: less than £8000; 2: £8000 to 10,000; 3: £10,000 to £12,000; 4: £12,000 to £15,000; 5: £15,000 to £20,000; 6: £20,000 to £30,000; 7: More than £30,000. As already mentioned, children in the two groups were matched according to age, sex and maternal education.

There are no considerable differences between the two groups in terms of any socio-economic status measures, or any prenatal and post-natal measures. Breastfeeding duration seems to be slightly longer for the active group but this difference does not reach significance.

Table 5: Sample Characteristics

Characteristics		Active group (n=19)	Placebo group (n=21)	*P*-value
Age (yr)	M ± SEM	11.3 ± 0.18	11.0 ± 0.18	.321
Sex	Total N of males/females	10/9	11/10	.987
Mother Education	M ± SEM	2.89 ± 0.22	2.85 ± 0.19	.901
Income	M ± SEM	4.94 ± 0.41	5.45 ± 0.43	.403
Grade	Total N of Year 7	14	15	.669
Handedness	Total N of R	16	18	.894
Ethnicity	Total N of White British	18	19	.401
BMI	M ± SEM	18.3 ± 0.64	19.5 ± 0.81	.269
Birth Weight (grams)	M ± SEM	3284.8 ± 138.1	3322.1 ± 163.7	.862
Smoking Exposure	Total N of exposed	8	4	.134
Alcohol Exposure	Total N of exposed	4	5	.769
Breastfeeding status	Total N of breastfed	11	11	.855
Breastf. duration (wks)	M ± SEM	10.8 ± 2.77	5.2 ± 1.58	.079
Full Scale IQ	M ± SEM	99.3 ± 2.65	99.2 ± 2.54	.993
Fat Intake[24]	M ± SEM	1.69 ± 0.03	1.76 ± 0.03	.170
Fish Intake[25]	M ± SEM	1.58 ± 0.07	1.66 ± 0.07	.529

5.3.2. Parental Characteristics

Table 6 describes data on parental aggression, anxiety, depression and stress. There are no considerable differences between the two groups concerning parental psychological attributes.

Table 6: Parental Characteristics

Parental Characteristics	Active Group	Placebo Group	P-value
	M ± SEM	M ± SEM	
DASS			
Depression	5.17 ± 2.20	6.57 ± 2.32	*.666*
Anxiety	1.47 ± 0.61	1.52 ± 0.36	*.935*
Stress	6.88 ± 1.76	7.31 ± 1.73	*.862*
Buss & Perry Aggression Q.			
Total Aggression	60.3 ± 4.41	58.0 ± 3.70	*.690*

[24] Fat intake was based on an 8 item questionnaire using a scale of no and yes
[25] Fish intake was based on a 5-item scale between never and more than once a day (1-5)

5.3.3. Group-based analysis (ANCOVA) on Cognitive measures

Table 7 presents results on the effects of group on the cognitive measures, as well as the effect of the covariates age and sex on the dependent measures. Variables in red are the ones that were found to be significantly ($p<.05$, uncorrected for multiple comparisons) different in each group after taking into account age, sex and baseline measures. For the WCST, instead of using seven different outcomes, we grouped the outcomes into the two factors that were extracted by the factor analysis of Kongs *et al* (2000; WCST manual). Negative numbers in "Estimates" indicate that the Active group has lower values than Placebo group. In Trail Making Test, because time (in seconds) is the outcome, positive numbers mean that the Active group has higher values than the Placebo group, thus indicating that the Active group takes more time to perform the task.

Table 7: Group-based analysis (ANCOVA) for cognitive measures with age and sex as the covariates

Cognitive measures		Group			Sex		Age	
WISC-IV	R^2 Adj.	Estimate [26]	T	*p*	t	*p*	t	*p*
Processing Speed Index	.414	-2.14	-1.47	*.152*	-0.16	*.873*	1.17	*.252*
Perceptual Reasoning Index	.534	-1.39	-1.42	*.164*	1.49	*.147*	1.41	*.170*
Block Design	**.805**	**-1.78**	**-2.15**	***.039***	0.07	*.945*	**2.61**	***.013***
Digit Span	.721	-0.53	-1.81	*.080*	0.31	*.759*	0.79	*.433*
Coding	**.529**	**-1.95**	**-1.97**	***.058***	-1.34	*.190*	1.53	*.135*
Matrix Reasoning	.417	-0.52	-1.13	*.268*	1.68	*.104*	0.55	*.583*
Symbol Search	.431	-0.45	-0.62	*.538*	0.74	*.464*	1.78	*.086*
Picture Completion	.551	0.65	1.37	*.179*	0.71	*.480*	-0.18	*.860*
Cancellation	.449	-2.35	-0.99	*.330*	0.18	*.861*	1.65	*.109*
WCST								
Factor1: Perseveration	.127	-0.01	-0.53	*.603*	-1.80	*.083*	-0.30	*.763*
Factor2: Non-Perseverative	-0.02	-0.53	-0.82	*.418*	-0.88	*.388*	-0.91	*.369*
Trail Making Test								
Trail A	.171	1.37	1.02	*.315*	-0.76	*.450*	-1.83	*.077*
Trail B	**.445**	**.047**	**2.09**	***.045***	-0.36	*.718*	0.77	*.445*
Ruff 2&7 Selective Attention								
Automatic Detection Accuracy	.528	0.68	1.41	*.168*	-0.73	*.470*	-0.55	*.583*
Controlled Search Accuracy	.505	1.17	1.72	*.095*	1.21	*.237*	-0.30	*.764*
Self Order Pointing Test								
Total Errors	.131	-0.60	-1.22	*.232*	0.92	*.365*	0.93	*.360*

[26] Estimates are only reported for the Group variable

As shown by the table, after adjusting for pre-intervention scores (baseline), age and sex, there was a significant effect of the intervention group on Block Design ([27]r=.35) and Coding (r=.32) from WISC-IV and Trail B (r=.34) from TMT. For these three tests, the placebo group improved, while the active group improved to a lesser extent or did not change over time (Figure 5). All other tests did not show a difference between active and placebo groups.

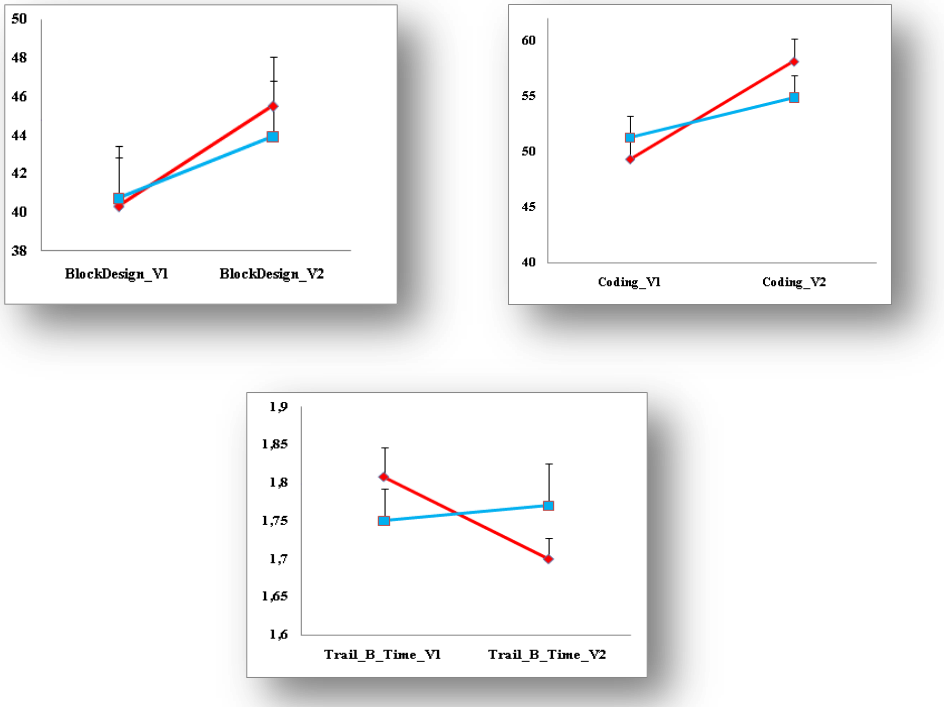


Figure 5: Blue line stands for the active group whereas red line stands for the placebo group. In Trail B test decreased scores means better performance as the Y axis reports time in seconds (after LOG10; Error Bars are SEM).

5.3.4. Group-based analysis (ANCOVA) on MRI measures

From all MRI measures only DTI and MRS showed weak but close to significant effects of the intervention. After adjusting for baseline measures, age and sex, there was a weak effect of intervention group on FA of the whole brain [F(1,29)=3.88, *p*=.059, r=.34], on MD of the whole brain [F(1,29)=4.21, *p*=.050, r=.36] and NAA/Cr [F(1,32)=3.92, *p*= .057, r=.33]. For FA the active group was decreasing FA of the whole brain, whereas MD of the active

[27] r is indicative of effect size, $\mathbf{r=\sqrt{[(t)^2/(t)^2+df]}}$

group was increasing (Figure 6). For the NAA metabolite, the active group had decreased NAA whereas the placebo group increased NAA (Figure 6). Table 8 shows results from effects of intervention group on the MRI measures.

Table 8: Effect of intervention group on MRI measures after adjusting for pre-intervention scores, age and sex (ANCOVA)

MRI		Group			Sex		Age	
	R^2 adj.	Estimate	t	*p*	t	*p*	t	*p*
White Matter Volume (Lobes)								
Frontal	.635	1355.5	0.46	*.650*	1.06	*.301*	0.15	*.878*
Parietal	.831	-1390.7	-0.90	*.376*	**2.01**	***.055***	**2.16**	***.041***
Temporal	.632	1801.7	1.08	*.289*	0.67	*.510*	-0.24	*.815*
Occipital	.606	-1868.7	-1.63	*.116*	1.45	*.158*	1.14	*.263*
Gray Matter Volume (Lobes)								
Frontal	.425	-2614.7	-0.76	*.457*	1.18	*.249*	0.93	*.363*
Parietal	.423	-2433.1	-1.55	*.133*	0.44	*.660*	0.23	*.820*
Temporal	.469	-3811.8	-1.66	*.110*	1.49	*.149*	**2.15**	***.042***
Occipital	.497	-1841.4	-1.34	*.192*	0.47	*.644*	1.48	*.151*
MTR (Lobes)								
Frontal	.153	-0.001	-0.49	*.628*	0.58	*.568*	**2.95**	***.007***
Parietal	.053	-0.000	-0.19	*.854*	0.61	*.549*	**2.26**	***.033***
Temporal	.039	0.002	0.76	*.452*	-0.26	*.794*	1.75	*.092*
Occipital	.075	-0.000	-0.17	*.865*	-1.33	*.194*	1.91	*.068*
DTI								
FA (Whole Brain)	**.618**	**-0.002**	**-1.97**	***.059***	1.08	*.289*	0.93	*.359*
MD (Whole Brain)	**.872**	**$2.3e^{-6}$**	**2.05**	***.050***	**2.30**	***.029***	**-2.94**	***.006***
FA (Corpus Callosum)	.153	-0.25	-1.43	*.163*	1.17	*.254*	**2.64**	***.013***
MD (Corpus Callosum)	.632	-0.90	-0.99	*.331*	**-2.49**	***.019***	0.34	*.733*
MRS (Height/Cr)								
N-Acetylaspartate	**.084**	**-0.06**	**-1.98**	***.057***	0.45	*.657*	1.60	*.120*
Myo-Inositol	.063	-0.04	-1.71	*.097*	0.90	*.377*	1.09	*.285*
Choline	.412	-0.003	-0.16	*.876*	1.35	*.189*	**3.65**	***.001***

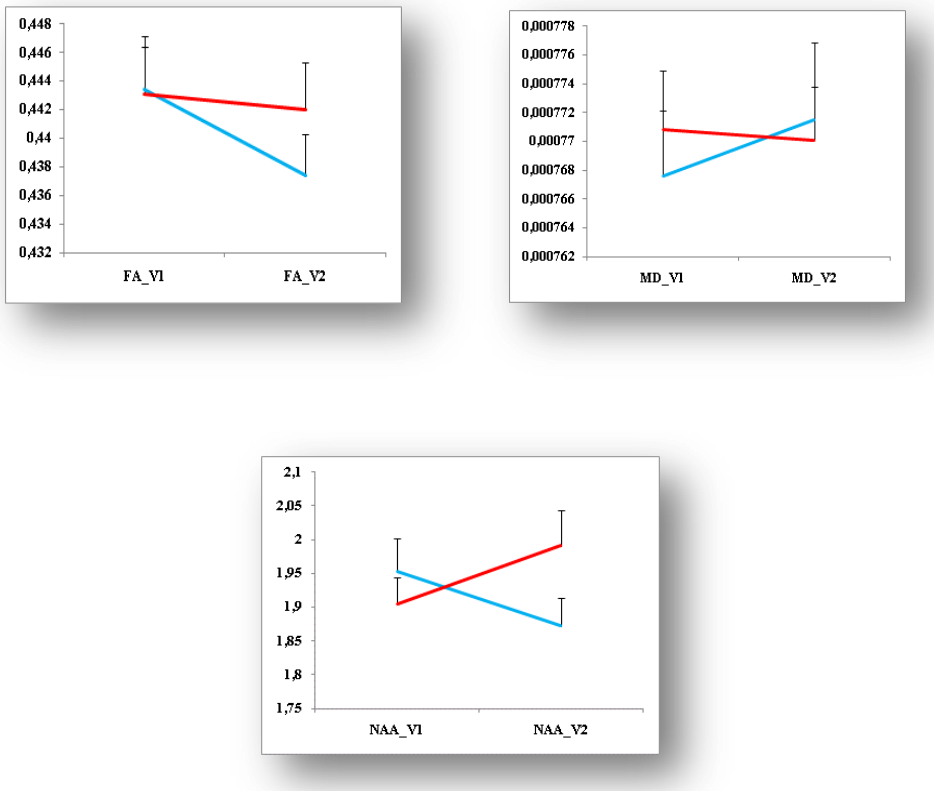


Figure 6: Intervention group differences on MRI measures: FA, MD and NAA/Cr. Blue line represents the active group, whereas red line represents the placebo group (Error Bars are SEM)

5.3.5. Group-based analysis (ANCOVA) on Behavioural measures

After adjusting for pre-intervention scores, age and sex, there was a significant effect of intervention group only on Conduct behaviour from SDQ as scored by the child [$F(1,34)=5.90$, $p=.021$, $r=.389$] with the placebo group decreasing conduct behaviour (Figure 7). There was no effect of intervention group on any of the behavioural measures that were scored by the parent. Table 9a illustrates data from the behavioural measures as scored by the child, whereas Table 9b illustrates behavioural data as scored by the parent pre and post intervention.

Table 9a: Effect of group on behavioural measures (scored by the child) after adjusting for pre-intervention scores, age and sex (ANCOVA)

Behavioural Assessment	R^2 adj.	Group			Sex		Age	
		estimate	t	*p*	t	*p*	t	*p*
SDQ								
Emotional	.210	0.22	0.95	*.351*	1.37	*.180*	-0.55	*.589*
Hyperactivity	.275	0.08	0.32	*.752*	1.16	*.256*	0.33	*.745*
Conduct	**.485**	**0.39**	**2.43**	***.021***	0.00	*.999*	0.69	*.495*
Peer Problems	.336	-0.05	-0.32	*.754*	**2.06**	***.048***	-1.31	*.199*
Prosocial	.202	-0.18	-0.78	*.440*	-1.00	*.324*	-1.05	*.304*
Total Difficulties	.454	0.63	1.31	*.199*	**2.12**	***.042***	-0.38	*.710*
STAR								
Aggression: Provocation	.225	-0.37	-0.18	*.855*	1.10	*.278*	0.23	*.818*
Aggression: Frustration	.240	-0.01	-0.54	*.595*	1.13	*.267*	-0.96	*.344*
Buss & Perry Aggression Q.								
Total Aggression	.431	2.09	0.73	*.470*	1.62	*.117*	0.15	*.880*
Essential Fatty Acids Q.								
Total EFA	.022	0.51	0.90	*.375*	0.66	*.511*	0.11	*.909*

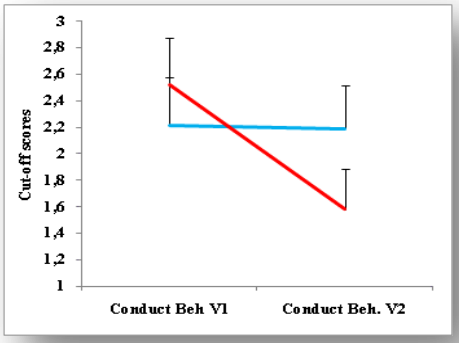


Figure 7: SDQ Conduct behaviour. Blue line represents active group whereas red line represents placebo group. Decreased scores stand for improved behaviour (Error Bars are SEM).

Table 9b: Effect of group on behavioural measures (scored by the parent) after adjusting for pre-intervention scores, age and sex (ANCOVA)

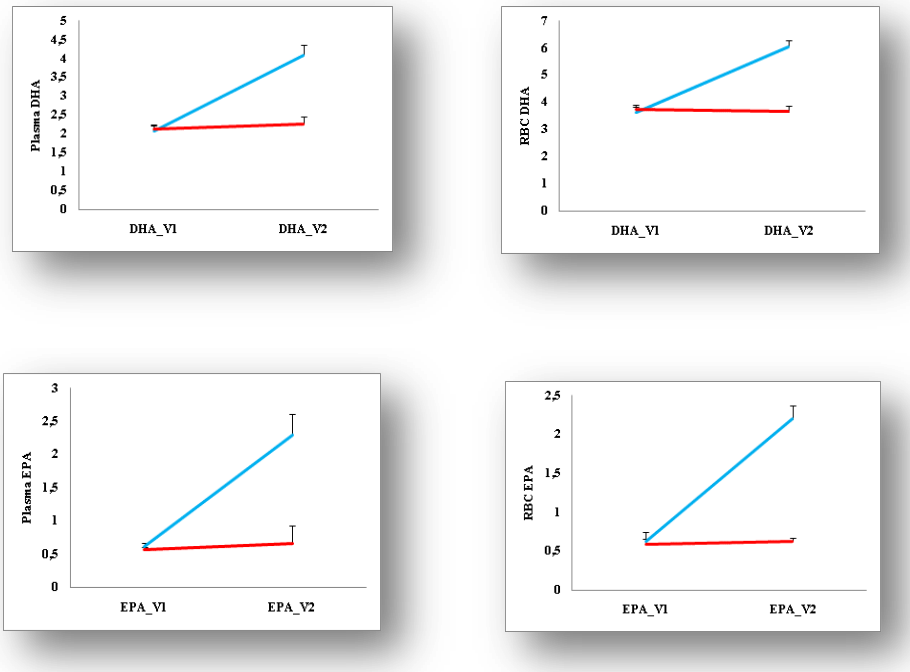
Behavioural Assessment (as scored by Parent)		Group			Sex		Age	
	R^2 adj.	estimate	t	*p*	t	*p*	t	*p*
SDQ								
Emotional	.538	-0.29	-1.64	*.112*	-1.28	*.513*	-0.62	*.400*
Hyperactivity	.432	0.45	1.48	*.149*	0.24	*.812*	0.16	*.875*
Conduct	.465	0.09	0.44	*.661*	0.50	*.618*	-0.91	*.370*
Peer Problems	.593	-0.02	-0.20	*.840*	-0.13	*.901*	-0.82	*.420*
Prosocial	.104	0.00	0.02	*.983*	0.15	*.884*	1.16	*.255*
Total Difficulties	.588	0.39	0.68	*.501*	0.10	*.923*	-0.69	*.492*
Essential Fatty Acids Q.								
Total EFA	.434	-0.06	-0.11	*.912*	-0.15	*.881*	**-2.24**	***.039***

5.3.6. Group-based Analysis on Fatty Acids Levels

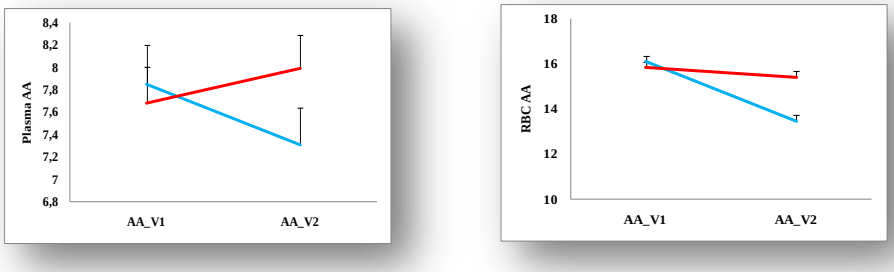
Fatty acids analysis verified that compliance of intervention product was good. ANCOVA showed that plasma and red blood cells (RBC) DHA and EPA were significantly higher in the active than the placebo group (Figure 8a), whereas ω-6 fatty acids such as plasma and RBC AA and DGLA were lower in active group (Figure 8b). Instead, plasma and RBC oleic acid (OA) were higher in the placebo group (Figure 8c). Table 10 shows data on all the fatty acids.

Table 10: Effect of Intervention group on PUFA levels (% of fatty acids) after adjusting for baseline measures, age and sex (ANCOVA)

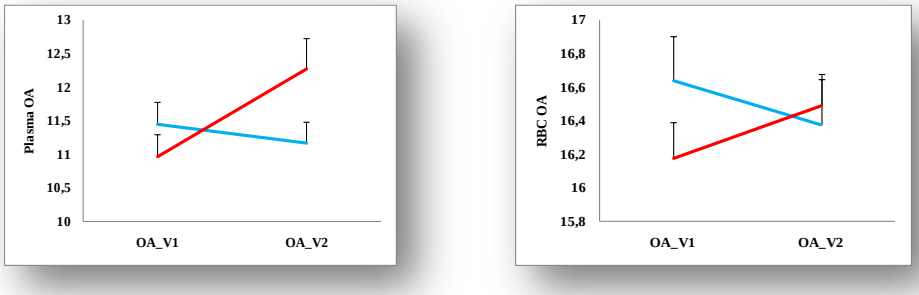
				Group		Sex		Age	
	Name	R^2adj.	Estimate	t	*p*	T	*p*	t	*p*
Plasma									
18:3 ω-3	a-Linolenic acid (ALA)	-0.04	-.020	-1.20	*.239*	0.71	*.485*	0.05	*.957*
20:5 ω-3	Eicosapentaenoic acid (EPA)	**.498**	**.819**	**5.46**	***<.0001***	1.91	*.065*	1.18	*.249*
22:5 ω-3	Docosapentaenoic acid (DPA)	**.277**	**.091**	**2.58**	***.015***	**2.46**	***.020***	1.55	*.132*
22:6 ω-3	Docosahexaenoic acid (DHA)	**.543**	**.908**	**6.02**	***<.0001***	0.49	*.628*	1.10	*.282*
18:2 ω-6	Linoleic acid (LA)	.066	-.441	-1.09	*.286*	0.20	*.843*	0.73	*.473*
20:3 ω-6	Dihomo-gamma-linolenic (DGLA)	**.333**	**-.332**	**-3.05**	***.005***	0.94	*.356*	-1.31	*.200*
20:4 ω-6	Arachidonic acid (AA)	**.616**	**-.487**	**-3.38**	***.002***	0.67	*.511*	**-2.36**	***.025***
22:5 ω-6	Docosapentaenoic acid	**.487**	**-.042**	**-5.63**	***<.0001***	0.39	*.697*	0.18	*.859*
18:1 ω-9	Oleic acid (OA)	**.324**	**-.425**	**-2.15**	***.041***	1.28	*.210*	**-2.45**	***.021***
RBC									
18:3 ω-3	a-Linolenic acid (ALA)	-0.07	-.002	-0.19	*.848*	-1.02	*.317*	-0.72	*.476*
20:5 ω-3	Eicosapentaenoic acid (EPA)	**.749**	**.774**	**9.76**	***<.0001***	1.92	*.065*	0.46	*.645*
22:5 ω-3	Docosapentaenoic acid (DPA)	**.646**	**.310**	**6.23**	***<.0001***	**2.99**	***.005***	0.32	*.754*
22:6 ω-3	Docosahexaenoic acid (DHA)	**.833**	**1.19**	**11.45**	***<.0001***	1.65	*.109*	-0.23	*.820*
18:2 ω-6	Linoleic acid (LA)	**.398**	**-.350**	**-2.61**	***.014***	-0.08	*.936*	-0.25	*.808*
20:3 ω-6	Dihomo-gamma-linolenic (DGLA)	**.755**	**-.182**	**-4.99**	***<.0001***	-0.05	*.957*	-0.85	*.401*
20:4 ω-6	Arachidonic acid (AA)	**.750**	**-1.11**	**-8.55**	***<.0001***	-1.82	*.079*	-0.23	*.819*
22:5 ω-6	Docosapentaenoic acid	**.489**	**-.065**	**-4.95**	***<.0001***	-1.03	*.311*	-0.43	*.670*
18:1 ω-9	Oleic acid (OA)	**.684**	**-.191**	**-2.10**	***.044***	-1.10	*.279*	**-2.47**	***.019***
Lipids									
NEFA	Non-esterified fatty acids	.221	-55.8	-1.74	*.092*	-0.60	*.552*	**-2.17**	***.038***
CHOL	Cholesterol	.622	.063	0.85	*.402*	0.58	*.563*	-1.47	*.152*
HDL	High-density lipoprotein	.797	.058	1.69	*.102*	-0.21	*.838*	-1.85	*.073*
LDL	Low-density lipoprotein	.757	.001	0.02	*.984*	0.66	*.512*	-1.39	*.174*
TRIGLY	Triglycerides	.206	-.080	-1.23	*.230*	0.99	*.330*	0.87	*.390*



<u>Figure 8a</u>: Effect of intervention group on Plasma and RBC DHA and EPA concentration (w%). Blue line represents the active group and red line represents the placebo group (Error Bars are SEM).



<u>Figure 8b</u>: Effect of intervention group on Plasma and RBC AA concentration (w%). Blue line represents the active group and red line represents the placebo group (Error Bars are SEM).



<u>Figure 8c</u>: Effect of intervention group on Plasma and RBC OA concentration (w%). Blue line represents the active group and red line represents the placebo group (Error Bars are SEM).

5.3.7. Associations of plasma and RBC DHA/EPA with significant outcomes

In the linear regressions we used age, baseline, Full Scale IQ (only for cognitive measures) and parental aggression (for behavioural measures) as the predictors. Using the enter method, a significant model emerged for WISC-IV Block Design [F(4,32)=36.56, *p*=<.0001, R^2 adj.=.816], and WISC-IV Coding [F(4,32)=11.99, *p*=<.0001, R^2 adj.=.578]. Table 11 gives information for the predictors that are included in the models.

Table 11: Predictors included in the models of linear regressions (plasma DHA)

Variables	Standardised Coefficients (Beta)[28]	p-value
WISC-IV Block Design V2		
Age (Months)	.320	.0007
Block Design VI (Baseline)	.619	<.0001
Full Scale IQ	.244	.027
Plasma DHA V2	-.172	.036
WISC-IV Coding V2		
Age (Months)	.273	.036
Coding VI (Baseline)	.464	.003
Full Scale IQ	.325	.031
Plasma DHA V2	-.301	.018

There was a significant model when examining at the relationship of SDQ Conduct behaviour (as scored by the child) and Age, Conduct at baseline, Parental Aggression and Plasma DHA [F(4,32)=9.03, *p*<.0001; R^2adj.=.501]; however plasma DHA (St. Beta=.194, *p*=.146) and Age (St. Beta=.031, *p*=.808) were not significant predictors of Conduct behaviour. The same was observed for Trail B; Plasma DHA (St. Beta=-.007, *p*=.957) and Age (St. Beta=-.024, *p*=.873) were not significant predictors.

Similar results were obtained for DTI FA and MD, as well as for NAA/Cr. Plasma DHA was not a significant predictor for either FA (St. Beta=-.209, *p*=.103), MD (St. Beta=.071, *p*=.400), or NAA/Cr (St. Beta=-.316, *p*=.089).

When examining at the effect of RBC DHA on cognitive, behavioural and MRI measures, there was a significant model for WISC-IV Block Design [F(4,33)=37.55, *p*<.0001; R^2adj.=.815] and WISC-IV Coding [F(4,33)=12.33, *p*<.0001; R^2adj.=.578], as well as

[28] The standardized Beta (β) Coefficients give a measure of the contribution of each variable to the model in terms of standard deviations (SD). β is the predicted change in SD of the criterion variable for a change of 1SD in the predictor, while controlling for the other predictors (Brace et al ., 2006). Hence, if age in months increases by 1SD then we can predict that performance on Block Design will increase by .320 SD.

Conduct[29] behaviour [as scored by the child; F(3,33)=13.28, *p*<.0001; R^2adj.=.527]. Table 12 reports the predictors that are included in the models.

Table 12: Predictors included in the models of linear regressions (RBC DHA)

Variables	Standardised Coefficients (Beta)	p-value
WISC-IV Block Design V2		
Age (Months)	.239	.005
Block Design VI (Baseline)	.713	<.0001
Full Scale IQ	*.176*	*.092*
RBC DHA V2	-.178	.027
WISC-IV Coding V2		
Age (Months)	*.223*	*.071*
Coding VI (Baseline)	.494	.001
Full Scale IQ	.310	.032
RBC DHA V2	-.298	.015
SDQ Conduct Behaviour V2		
RBC DHA V2	*.212*	*.099*
Conduct V1 (Baseline)	.588	.0001
Parental Aggression	.276	.045

RBC DHA was not significantly predicting Trail B (St. Beta=.09, *p*=.515), DTI FA (St. Beta=-.182, *p*=.135), or DTI MD (St. Beta=.121, *p*=.124) and conduct behaviour. There was a non significant model for NAA/Cr [F(3,31)=2.45, *p*=.084; R^2adj.=.123] with predictors RBC DHA (St. Beta=-.347, *p*=.049), Age (St. Beta=.276, *p*=.114) and NAA at baseline (St. Beta=.413, *p*=.141).

Linear regression for Plasma EPA revealed significant models for almost all dependent variables: WISC-IV Block Design [F(4,32)=34.09, *p*<.0001; R^2adj.=.805], WISC-IV Coding [F(4,32)=8.78, *p*=.0001; R^2adj.=.493], Trail B [F(4,32)=6.48, *p*=.0008; R^2adj.=.406], DTI FA [F(4,27)=15.67, *p*<.0001; R^2adj.=.684], DTI MD [F(3,27)=49.92, *p*<.0001; R^2adj.=.844], but not NAA/Cr [F(3,30)=2.07, *p*=.126; R^2adj.=.097]. Plasma EPA was a significant predictor only for DTI FA (St. Beta=-.366, *p*=.007) and DTI MD (St. Beta=.161, *p*=.051). Negative values on St.Beta indicate that the higher the EPA the lower the FA, whereas a positive St.Beta indicates a linear relationship, higher EPA higher MD.

Linear regression for RBC EPA revealed significant models for almost all dependent variables: WISC-IV Block Design [F(4,33)=37.36, *p*<.0001; R^2adj.=.815], WISC-IV Coding

[29] Linear regression for Conduct behaviour was performed after removal of predictor Age since it was not significantly contributing to the total variance.

[F(4,33)=10.44, p<.0001; R^2adj.=.533], Trail B [F(4,33)=7.08, p=.0004; R^2adj.=.424], DTI FA [F(4,28)=12.86, p<.0001; R^2adj.=.628], DTI MD [F(3,28)=58.42, p<.0001; R^2adj.=.860], but not NAA/Cr [F(3,31)=1.98, p=.138; R^2adj.=.087]. RBC EPA was a significant predictor only for WISC-IV Block Design (St. Beta=-.178, p=.029) and DTI MD (St. Beta=.185, p=.015).

Since fatty acids in the blood revealed decreased levels of AA after supplementation (section 5.3.6.), we wished to examine the relationship between plasma and RBC AA with the cognitive and MRI measures that were found to be significant on the group-based analysis. Linear regressions with Age, baseline measurements, and sex (when appropriate) as the predictors and AA as the independent variable showed no associations of either plasma or RBC AA with any of the above cognitive or MRI measurements.

5.4. Discussion

This was an exploratory study investigating the effects of a dietary supplementation of the polyunsaturated fatty acids DHA and EPA on brain and cognition in school-aged children from less well-off neighbourhoods. To obtain a more inclusive view of the effects of DHA on the brain and cognition, we used a broad range of neuropsychological measures in order to cover all domains of executive functioning, and a number of different MRI measures in order to survey possible changes in brain structure. To verify compliance vis-à-vis supplementation, we obtained blood samples to measure fatty acids levels in the blood.

Two types of analysis were performed: a group-based and a plasma/RBC-based analysis. In the group-based analysis, we wished to examine if there were any noteworthy effects of DHA and EPA supplementation on cognition and brain structure. Plasma/RBC-based analysis was performed in order to identify any associations between fatty acids (DHA/EPA) and cognition/brain structure.

5.4.1. Effects of DHA/EPA on brain and cognition: Group-based Analysis

Group-based analysis revealed that performance on Block Design and Coding from WISC-IV, as well as Trail Making B, did not change between pre and post assessment in the active group (supplemented with DHA and EPA), whereas the placebo group (OA supplemented group) showed an increase in performance over the three-month period, as one would expect in this age group. Further, there was a considerable improvement in conduct

behaviour of the placebo group but no change in the active group (as assessed by the child but not by the parent). MRI results are consistent with the above pattern in that the active group is showing changes in FA/MD (DTI) and NAA/Cr (MRS) that are contrary to what we would expect in relation to the age-related changes in these measures. Several previous DTI studies revealed age-related increases in fractional anisotropy (FA) and decreases in mean diffusivity (MD) in a number of white-matter regions during childhood and adolescence (Mukherjee, Miller, Shimony, Conturo, Lee, Almli & McKinstry, 2001; Schmithorst, Wilke, Dardzinski & Holland, 2002; Klingberg, Vaidya, Gabrieli, Moseley & Hedehus, 1999; Snook, Paulson, Roy, Philips & Beaulieu, 2005; Barnea-Goraly, Menon, Eckert, Tamm, Bammer, Karchemskiy, Dant & Reiss, 2005; Eluvathingal, Hasan, Kramer, Fletcher & Ewing-Cobbs, 2007). Thus, a decrease in FA and an increase in MD between the ages of 9-to-12 in the Active group are unexpected.

As already described in the Introduction, not many studies have examined the effect of DHA supplementation on cognition in older children. The South Africa Medical Research Council (2000) showed that ω-3 oil supplement in 6-to-11 year old primary school children increased the total recall score of the HVLT (Tichelaar et al., 2000). The study by Dalton *et al* (2009) showed a significant effect of supplementation (with fish flour) for HVLT *Recognition* and *Discrimination index* on 7-to-9 year old children from a primary school in a low socio-economic community of mixed ancestry (African-European-Malay) from the Northern Cape Province of South Africa. On the contrary, the two randomized, placebo-controlled trials in Australia and Indonesia by the NEMO study group (2007) found no effect of LC-PUFA supplementation on cognitive performance of 6-to-10 yrs old children. Positive results from the first two studies should be treated with caution as the HVLT is a test designed to evaluate performance of Alzheimer's patients and has not been standardized on healthy children. Plus, it is not known if the groups were matched on intelligence or even maternal education at baseline.

Our study found no effect of DHA and EPA on cognition and behaviour of 9-to-12 yrs old children but showed some unexpected supplementation effects on brain structure through DTI and MRS. Our intervention product was based only on DHA and EPA and not AA. Examining the fatty acids levels in the blood after supplementation, we verified that plasma

DHA and EPA increased in the active group, whereas plasma and red blood cell AA notably decreased. When humans consume fish or fish oil, the DHA and EPA from diet partly replace the omega-6 fatty acids, particularly AA, in the membranes of all cells but particularly in the membranes of platelets, erythrocytes, neurophils, monocytes and liver cells (Simopoulos, 2002). Note that PUFA composition of cell membranes is greatly dependent on the dietary intake (Simopoulos, 2002).

LA and ALA are equally competitive, since they compete for the same desaturases, and thus inappropriately high intakes of one of the ω-6 and ω-3 fatty acids can have detrimental effects on other ω-6 and ω-3 fatty acids (Georgieff & Innis, 2005). For example, high dietary intakes of DHA or EPA can result in decreased tissue AA and decreased synthesis of AA can develop eicosanoids in favour of ω-3 fatty acid derived eicosanoids (Broughton & Wade, 2002). Clinical studies reported that preterm infants fed formulas rich in DHA had lower blood lipid AA than preterm infants fed un-supplemented formulas, as well as lower growth (Carlson, Werkman, & Tolley, 1996). In addition, a clinical study with full-term infants showed that supplementation with DHA only resulted in lower scores on the MacArthur Scales (MacArthur Communicative Development Inventories for language assessment; Scott, Janowsky, Carroll, Taylor, Auestad & Montalto, 1998).

The issue of dietary imbalance between the ω-6 and ω-3 fatty acids was addressed in a series of studies where pregnant mice were fed an omega-3 diet throughout gestation and lactation (Wainwright, Jalali, Mutsaers, Bell & Cvitkovic, 1999). The diet was based on very high levels of ω-3 fatty acids, provided as DHA, with minor levels of LA (1.6%). This resulted in large increases in brain DHA, as well as decreases in AA accompanied by a retardation of growth and lower performance on behavioural measures (cliff aversion, visual placing reflex, pole grasp, forelimb and hindlimp grasp reflex) in the pups. Authors hypothesized that high levels of DHA may have inhibited the activity of Δ6-desaturase in converting LA to GLA (γ-linolenic acid; 18:3 ω-6), which is then converted to AA. In a study by Huang, Wainwright, DeMichele, Xing, Biederman, Liu, Chuang, Bobik and Hastilow (2002), animals were fed a diet with high levels of ω-3 fatty acids as ALA (40%) but with ω-6 provided as 1.6% GLA in addition to LA and there were no effects on pup growth or behavioural development. Results

from the above studies emphasize the importance of ω-6 fatty acid content of the diet when supplementing with DHA, as well as the form in which ω-6 fatty acids are provided.

Other data from intervention studies have revealed that EPA at a dose of 1 or 2g·day^{-1} has shown effectiveness in reducing symptoms of treated depressive patients (Nemets, Stahl & Belmaker, 2002) but surprisingly not at a higher dose (Peet & Horrobin, 2002a). In schizophrenic patients, EPA doses at 1 to 3g·day^{-1} shows an improvement (Peet & Horrobin, 2002b) but high doses of pure EPA (>4g·day^{-1}) are not effective for either schizophrenic patients or depressive ones (Horrobin, Jenkins, Bennett & Christie, 2002); this might be related to a decrease of the membrane levels of AA. The inhibitory effect of EPA on Δ5-desaturase activity can be responsible for the lower plasma AA found when fish oil, high in DHA and EPA, is consumed (Uauy-Dagach & Mena, 1995). Plus, as mentioned above, fish oil can inhibit Δ6-desaturase activity and thus decrease AA levels (Raz, Kamin-Belsky, Przedecki and Obukowicz, 1998).

Different to fish oil, dietary olive oil (ω-9 fatty acids) protects the ω-3 fatty acids series (Navarro, Periago, Pita & Hortelano, 1994) and does not change AA concentrations (Periago, Suarez & Pita, 1990). In a study investigating the effect of a diet supplemented with 10% fish oil versus 10% olive oil during pregnancy of the rat, it was found that AA and a-tocopherol[30] concentrations were decreased, and there was a delayed postnatal development in the offspring of rats fed the fish-oil diet (Amusquivar, Rupérez, Barbas & Herrera, 2000).

Consequently, could it be that the high intakes of DHA/EPA inhibited the AA concentrations in our sample population (as shown by the fatty acid analysis in the blood) and that resulted in the unexpected effects on brain microstructure? As already mentioned in the methodology, DTI is a technique that allows us to characterize the structural properties of white matter by estimating several parameters of water diffusion in live tissue. Fractional Anisotropy (FA) reflects the degree of directionality of water diffusion; and Mean Diffusivity (MD) provides the overall magnitude of water diffusion and is a sensitive indicator of maturational changes in the brain tissue (Eluvathingal et al., 2007). In white matter, FA is thought to rely upon the microstructural features of fibre tracts, including the arrangement of individual axons, as well as their packing "density" and myelin content (Paus, 2010). In vitro

[30] A-tocopherol is the form of vitamin E that is preferentially absorbed and accumulated in humans.

experiments showed that the difference in anisotropy between myelinated and un-myelinated fibres was unexpectedly small leading to the conclusion that anisotropy of water diffusion is not a specific marker of myelination in white matter (Beaulieu & Allen, 1994). Consequently, anisotropy is thought to reflect influences from cellular components as well as from the local microstructural texture (influences such as tissue hydration, myelination, cell-packing density and fibre diameter; Schmithorst et al., 2002). While some fibre tracts show an age-related increase/decrease in FA/MD, other tracts (such as CST) show only a decrease in MD without any difference in FA (Eluvathingal et al., 2007). Hence, FA and MD usually show an inverse relationship but this is not consistent across the brain. In our case, FA and MD do show an inverse relationship but not the expected for this age-group. Perhaps, there was a shift in the fluidity of membranes (higher MD) that goes in the opposite direction than expected during normal development and this might have lowered anisotropy.

In addition, NAA decreased over time in the active group, again a finding not expected for this age group. NAA is a metabolite found in normally functioning neurons present in neuronal cell bodies, axons and dendrites and thus it is used as a measure of neuronal density and integrity (Rao, 2008). NAA has the most prominent signal in ^{1}H MR spectra of the human brain; it is more concentrated in gray than white matter (Hajek & Dezortova, 2008) and, in white matter, its concentration correlates well with myelination during brain maturation (Dezortova & Hajek, 2008). Here the NAA results do not suggest alterations in myelination since there were no changes in MTR, an indirect index of myelination, in the active group. If myelination was not altered, then what could explain the NAA decreases? A reduction in *N*-acetylaspartate is thought to represent dysfunction and/or decreased density of neurons and axons (Deicken, Zhou, Corwin, Vinogradov & Weiner, 1997). Also, changes in NAA have been attributed to changes in neuron number, density, or neuronal metabolism (Birken and Oldendorf, 1989). As already mentioned, ω-3 fatty acids are essential for the creation of the lipid bilayer in cell membranes and thus, dietary intake is reflected in membrane levels (Marteinsdottir et al., 1998). Perhaps, neurons composed of membranes with higher amount of PUFAs could impact neuronal processes, resulting in NAA loss.

5.4.2. Fatty acids levels after supplementation

The active product contained 0.65g/day of EPA and 0.65g/day DHA. After three months of supplementation, DHA and EPA increased in plasma and red blood cell of the active group, as expected. On the contrary, plasma and red blood cell AA decreased in the active group, and increased (only plasma AA) in the placebo group. Plasma DHA increased from 2.10% to 4.10% whereas EPA increased from 0.60% to 2.30% in the active group; red blood cell DHA increased from 3.70% to 6% and red blood cell EPA increased from 0.60% to 2.20%. In the active group, red blood cell AA decreased from 16% to 13.4%, and red blood cell LA decreased from 13.1% to 12.1%. It is worthwhile mentioning that red blood cell DGLA also decreased from 3.2% to 2.5% in the active group. This may be explained by the previous statement that EPA and DHA consumption inhibits the activity of Δ5-desaturase and Δ6-desaturase, which promote the production of DGLA and AA. Results on fatty acid levels are comparable with the study of McNamara *et al* (2010) who supplemented boys at the age of 8 to 10 years old with 1200mg/d DHA and found an increase of erythrocyte DHA from 3.30% to 10%; and with the study by Dalton *et al* (2009) who showed an increase of plasma DHA from 4.6% to 6%, EPA from 0.65% to 0.77%, and a decrease of AA from 10.4% to 9.2% after supplementation of 6 months with 140mg of DHA plus EPA.

According to the WHO (2008) an adequate intake of EPA plus DHA for children at the ages between 6 and 10 years of age is 200-250mg, whereas the acceptable macronutrient distribution range (AMDR) for both EPA and DHA is 0.250g-2.0g/d. WHO (2008) suggests that the Upper level AMDR for EPA + DHA consumption is set at 2 g/d due to experimental evidence indicating that high supplement intakes of n-3 LCPUFA may increase lipid peroxidation and reduce cytokine production. On the Workshop on the Essentiality of and Recommended Dietary Intakes for Omega-6 and Omega-3 Fatty Acids (Simopoulos, Leaf and Salem, 1999) it was decided that the Adequate Intake (AI)[31], for adults, concerning DHA and EPA is 0.65g/day (0.3% energy). In general, recommendations on EPA and DHA are based on prevention of cardio-vascular disease. There is no evidence so far to indicate the recommendation intakes necessary for normal cognitive development.

[31] If sufficient scientific evidence is not available to calculate an Estimated Average Requirement, a reference intake called an Adequate Intake is used instead of a Recommended Dietary Allowance. The AI is a value based on experimentally derived intake levels by a group (or groups) of healthy people (Simopoulos et al., 1999).

The estimated daily intake of ω-3 PUFA in Western countries varies largely and the DHA intakes which depend on fish vary between countries. Overall, *white* fish represent 49% and 45% of the intake of total fish in women and men, respectively, with the greatest consumption in Spain and Greece and the least in Germany and the Netherlands. The greatest intake of *fatty* fish was in the coastal areas of northern Europe (Denmark, Sweden and Norway) and in Germany (Welch, Lund, Amiano, Dorronsoro, Brustad et al., 2002).

5.4.3. Associations of DHA and EPA with cognition and brain: Plasma and RBC-based analysis

Plasma and red blood cell DHA was negatively associated with WISC-IV Block Design and WISC-IV Coding (Figure 9a), whereas plasma EPA was negatively associated with DTI FA and positively associated with DTI MD. Red blood cell EPA was also positively associated with DTI MD (Figure 9b).

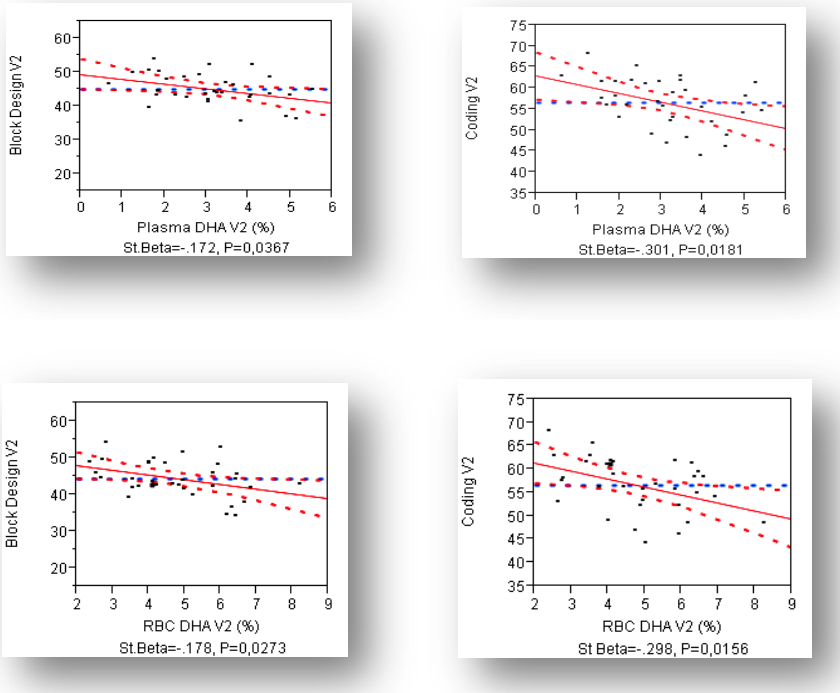
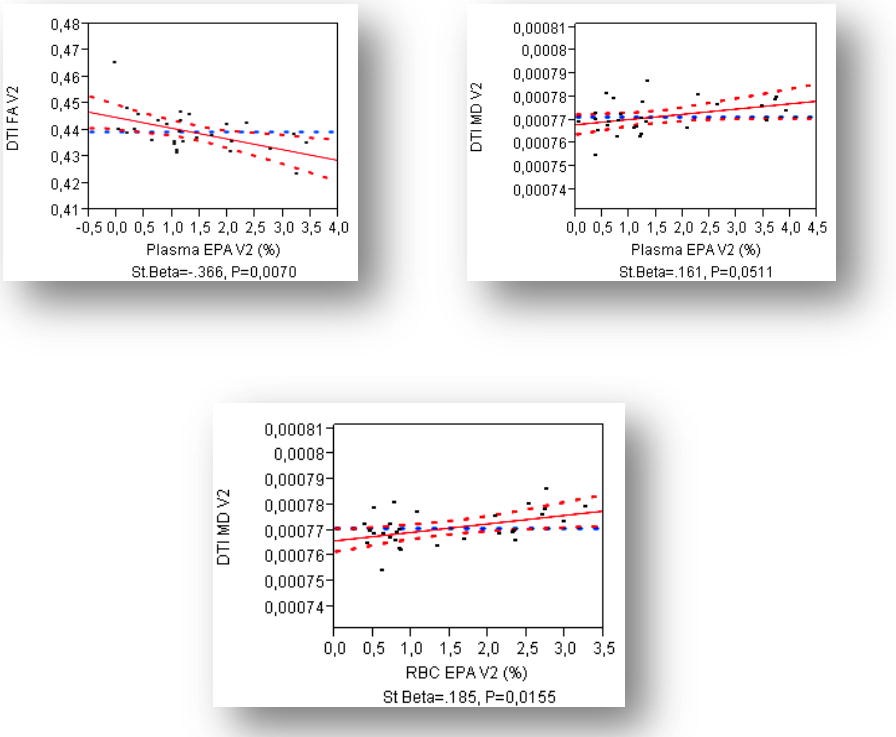


Figure 9a: Associations of plasma and red blood cell DHA with cognitive measures Block Design and Coding from WISC-IV



<u>Figure 9b</u>: Association of Plasma and red blood cell EPA with brain structure, FA and MD from DTI

Results on the associations between plasma and red blood cell DHA/EPA and cognition/brain structure may partly explain the results of the group-based analysis. In the group-based analysis, we showed that performance of the active group, on three cognitive tests was not significantly increased compare with the placebo group, and that fractional anisotropy of the active group was decreased with mean diffusivity being increased. From the plasma/red blood cell-based analysis, it is shown that plasma and red blood cell DHA is negatively associated with two of the cognitive tests that were found not to be improved by the supplementation diet. Further, the unexpected decrease of FA is also verified by the negative association of it with plasma EPA; and the MD increase could be partly explained by the positive association with plasma and red blood cell EPA.

As mentioned above, DTI studies revealed age-related increases in FA and decreases in MD in a number of white-matter regions during childhood and adolescence. The latter analysis could suggest that increased intakes of EPA, which the developing brain may not used to at this rate of intake, could somehow influence brain microstructure.

A study by Ward, Huang, Bobik, Xing, Mutsaers, Auestad, Montalto and Wainwright (1998) used an artificial rearing model in rat pups to control the DHA and AA content of diets containing adequate LA and ALA during the first two postnatal weeks. This model has the advantage of directly measuring the relationship of dietary supply and the pup brain. The pups were gastrostomized within the first few days of life and from the 5th postnatal day until the 18th they were fed rat milk substitute containing oils with 10% LA and 1% ALA and supplemented with AA and DHA from microbial cells. On the 18th day, dietary supplementation resulted in a broad range of brain DHA, with a smaller range in AA. Thus, these data showed a strong association between dietary levels of DHA and AA and those in the brain, with DHA in the diet raising DHA in the brain and decreasing AA. Plus, an exponential model provided the best fit of the association between DHA levels in the red blood cells with those in the brain (Wainwright & Martin, 2005). This implies that supplementation with DHA can affect DHA in the brain and that when supplementing with DHA to raise brain DHA, the diet should be accompanied by supplementation with AA in order to maintain a proper ratio of ω-3: ω-6 fatty acids as well as appropriate levels of AA.

Because ω-3 and ω-6 fatty acids compete for absorption into the phospholipids of neuronal membranes, increased levels of DHA/EPA in neurons can result in a decrease in the AA concentrations in phospholipids, which then reduces the synthesis of AA-derived eicosanoids (Marszalek & Lodish, 2005). In addition, DHA is converted to docosanoids, which protect neurons from oxidative stress (Bazan, 2003); DHA and docosanoids can inhibit AA conversion to eicosanoids by COX[32] and LOX enzymes (Calder, 1998).

The AA-derived eicosanoids can possibly have different actions in the brain. In particular, when sodium valproate, a mood stabilizer, (Bosetti, Weerasinghe, Rosenberger and Rapoport, 2003) and lithium chloride (Bosetti, Rintala, Seemann, Rosenberger, Contreras, Rapoport and Chang, 2002) were chronically administered in rats, the brain concentration of prostaglandin E_2 (PGE_2), a bioactive product of AA, was reduced by reducing the COX reaction. These studies imply that by decreasing PGE_2 concentrations in the brain by valproate, and lithium, can have therapeutic implications on mood disorders. AA and its metabolites can operate as second messengers and can alter brain ion channels, neurotransmitter uptake, blood

[32] Cyclooxygenase (COX) is an enzyme that is responsible for formation of important biological mediators called prostanoids, including prostaglandins, prostacyclin and thromboxane.

flow, synaptic transmission, autonomic and behavioural responses, gene transcription, pain, fever and the sleep–wake cycle (O'Banion, 1999). Also, reports showed that dietary ω-3 supplementation can be beneficial for mood disorders (Su, Huang, Chiu & Shen, 2003) because DHA and EPA can inhibit AA conversion to prostaglandins (Rubin & Laposata, 1992). Hence, if DHA and EPA can have a beneficial effect on excessive release of AA in the "affected" brain, then what consequences does excessive intake of DHA and EPA can have in normal concentrations of AA in the brain?

Even though DHA is most highly concentrated in brain and retina, and is a major constituent of neuronal membrane phospholipids, the analysis of this study indicated that plasma EPA is the one associated with decreased FA and increased MD, and not plasma DHA. Reports of EPA and DHA supplementation on mood disorders and schizophrenia treatment have demonstrated that EPA has shown efficacy in reducing symptoms of depressive patients (Nemets et al., 2002; Peet & Horrobin, 2002a), as well as improving symptoms in patients with schizophrenia (Peet, Brind, Ramchand, Shah, & Vankar, 2001; Peet & Horrobin, 2002b); whereas DHA showed no such effects (Schizophrenia: Peet et al., 2001; Depression: Marangell, Martinez, Zboyan, Kertz, Kim & Puryear, 2003; Llorente, Jensen, Voigt, Fraley, Berretta & Heird, 2003). While DHA and EPA are both from the same ω-3 family, they have different metabolic functions; DHA is a membrane structural component whereas EPA takes part in eicosanoids synthesis (Peet et al., 2001). EPA is the precursor of several eicosanoids, which act as second messengers and neuromodulators (Sumida, Graber & Nunez, 1993); it can also inhibit phospholipase A_2 (PLA_2; Finnen & Lovell, 1991) and cyclo-oxygenase (COX; Obata, Nagakura, Masaki, Masaki, Maekawa & Yamashita, 1999), as well as increase intracellular calcium levels (Okuda, Ezure, Tsukahara, Sawada, Mizutani, Katori, Bannai & Yamashita, 1994). Hence, through these functions and effects of EPA, there must be a connection that explains the negative association of EPA and anisotropy. Also, the fact that AA was not associated with either FA or MD strengthens the assumption that it is only higher intakes of EPA that somehow affects brain microstructure.

5.4.4. Effects of oleic acid on placebo-group

Placebo group was supplemented with oleic (18:1ω-9) acid which is a mono-unsaturated omega-9 fatty acid and can be found in various animals and vegetables. It is

odourless, colourless oil and the term *oleic* is derived from olive. Oleic acid can be converted in vivo to oleamide. Oleamide is a fatty-acid amide with measurable neurobiological effects in animals, including sedation and modulation of several serotonin receptor subtypes (Thomas, Cravatt, & Sutcliffe, 1999). Thus, we could suggest that an increase in oleic acid concentrations could alter the biosynthesis of oleamide with the latter acting on modulation of serotonin receptors. Further, in the myelin sheath, the amounts of DHA and AA are lower, but those of adrenic (22:4ω-6) and oleic acids are higher (O'Brien & Sampson, 1965). Martínez (1992) reported that oleic acid is another fatty acid characteristic of myelin phospholipids to the point that the accretion of oleic acid is analogous to myelination in the developing total human forebrain. The importance of oleic acid during myelination is highlighted by the fact that this mono-unsaturated fatty acid and its elongation products are typical components of myelin sphingolipids such as cerebrosides and sulfatides (Martínez & Mougan, 1998). In early brain development, while the increases in DHA parallel neuronal development, the accumulations of AA and oleic acid are good markers of myelinogenesis (Martínez, 1992). In addition, mono-unsaturated fatty acids are more resistant to lipid peroxidation, and therefore a great amount of them in the diet could be protective against the loss of vitamin E. For this reason, in our study the active and placebo product differed in Vitamin E amounts (active group had greater amount of Vitamin E than the placebo group). Taken as a whole, we could suggest that the improved performance (cognition) and increased NAA of the placebo group could be attributed to the oleic acid, and its elongation products, action on myelin and modulation of serotonin receptors. Last but not least, it has been reported that in an elderly population of Southern Italy, with a typical Mediterranean diet (high MUFA intakes), oleic acid and MUFA intakes in general, appeared to be protective against age-related cognitive decline (Solfrizzi, Panza, Torres, Mastroianni, Del Parigi, Venezia & Capurso, 1999). Thus, we could suggest that oleic acid, and in general mono-unsaturated fatty acids, are positively related with cognitive function.

5.5. Study Limitations and Strengths

This explorative double-blind randomized placebo-controlled trial was carefully designed, used a wide range of cognitive and behavioural measures, and it is the first study that used structural MRI to evaluate effects of omega-3 supplementation on brain structure and chemistry. A significant advantage of this study is the extraction of fatty acids levels in the blood which helped in verifying supplementation compliance. The matching procedure used before randomization is another benefit of this trial as we minimized the effect of age, sex and maternal education (a significant predictor of offspring's cognitive development) on supplementation effects. Participants were selected from less well-off neighbourhoods thus establishing the non frequent consumption of fish or omega-3 supplements. The screening procedure was also well designed as participants were screened twice using an omega-3 consumption questionnaire and a food frequency questionnaire.

The main limitation of this study is the number of participants in each group. Each group had 20 children. Most randomized trials on infancy have enrolled and followed 20-30 infants per treatment group (Gibson & Makrides, 2000). Although these numbers are adequate for identifying changes in the fatty acid profiles of plasma and erythrocyte membranes, they may be insufficient to detect true differences in developmental scores (Gibson & Makrides, 2000). Nonetheless, the study by McNamara *et al* (2010) found significant differences between DHA groups and placebo group on MRI activation with groups having 12 participants each. As this is an explorative study and no other study has used MRI to detect effects of DHA/EPA supplementation on brain structure, the sample size in this study should be taken as indicative and further studies should try and increase sample population.

The study was undertaken for only three months. Even though PUFAs may take up to three months to display an effect (Bryan, Osendarp, Hughes, Calvaresi, Baghurst, van Klinken, 2004), perhaps DHA and EPA needs more time to be accumulated in the brain and thus changes in children development could be captured later on. The lack of follow-up after the three months may also be another limitation. Perhaps any beneficial effects of the intervention product could be captured later on when areas that benefited the most are stimulated later in life.

5.6. Conclusion

This explorative study examined the effects of DHA and EPA supplementation on cognition and brain structure of school-aged children from less well-off neighbourhoods. We found few weak "negative" effects of DHA and EPA on cognition and behaviour of our participants; we also detected differences on brain microstructure that did not follow the expected age-related trends for the specific age-group. It is possible that our observations are related to the fact that supplementation was only based on DHA and EPA and the diet was not accompanied by supplementation with AA; hence the balance between omega-3 and omega-6 fatty acids in brain cell membranes was altered. When examining associations of fatty acids with brain microstructure, a superiority of EPA over DHA was an unexpected finding. EPA was somehow influencing anisotropy (negatively) and diffusivity (positively). More data are required to reach conclusive answers in this matter. DHA and AA play a crucial role in nerve function. The current Western diet does not provide us with the appropriate omega-3 fatty acids. Omega-6 FAs intake is much higher than intake of n-3 FAs. Intake of n-6 FAs meets recommendations whereas intake of n-3 FAs is below recommendations. The general recommendations are to increase omega-3 intake. Omega-3 supplementation is aimed at reinstating a healthy balance. Some authorities advise a specific ratio (e.g. 1:5) while the WHO advises a range (1:5 to 1:10). It is important that a healthy balance between omega-3 and omega-6 fatty acids is provided in order to avoid excess concentrations of only one of them and to promote a healthy development.

Chapter 6: General Discussion

This thesis investigated possible influences of tobacco and nutrition on the normal development of brain and cognition in children and adolescents. More specifically, we used data collected in a large sample of adolescents to investigate an association between prenatal exposure to maternal cigarette smoking and adolescents' cognition, and an association between breastfeeding and adolescent's brain and cognition. The last part of this thesis focused on short-term effects of polyunsaturated fatty acids on brain and cognition of healthy school-aged children. Three main research questions were addressed:

A. Is there an association between prenatal exposure to maternal cigarette smoking and cognitive development of adolescents when maternal education, the most known predictor of cigarette smoking during pregnancy, is held constant?

The thesis investigated the relationship between maternal cigarette smoking during pregnancy and cognitive abilities in adolescent offspring (12 to 18 years old) using an extensive 6-h battery of cognitive tests. The exposed and non-exposed adolescents were matched according to level of maternal education and school attended, thus minimizing differences in parental education between the two groups. To obtain a comprehensive view of our data, we used principal component analysis (PCA) and grouped the different cognitive tests into seven components: verbal and visuo-spatial skills, verbal and visual memory, processing speed, resistance to interference and motor dexterity. We found no association between maternal smoking during pregnancy with any of the seven cognitive components or, when tested separately, with the IQ scores. Whether or not we adjusted for a number of potential confounders (socioeconomic factors, pregnancy and post-natal environment, and parental monitoring) maternal smoking during pregnancy was not associated with cognitive abilities. Findings from this study suggest that controlling carefully for maternal education, a known predictor of cigarette smoking during pregnancy in the general population, is sufficient to eliminate possible differences between exposed and non-exposed adolescents in their cognitive abilities.

B. Is there an association between breastfeeding and adolescents' brain and cognition?

More specifically, the thesis evaluated an association between breastfeeding duration and brain structure and cognition of adolescents half of whom were exposed to maternal cigarette smoking during pregnancy. We found that mothers who smoked during pregnancy were less likely to breastfeed their infant. Given the large set of neuropsychological tests, a second principal component analysis was performed in order to verify results from the first one (Chapter 2) and to obtain a more global view of our data. The PCA revealed six components almost identical to those identified in the smaller sample of adolescents included in Chapter 2: verbal conceptualization and sequential processing, processing speed, visual and verbal memory, perceptual organization, and motor dexterity. Our results suggest that adolescents who were exclusively breastfed for over 16 weeks had noteworthy advantages in total and fluid intelligence, as well as in fine motor skills, as compare with adolescents who were never breastfed. More importantly, total breastfeeding duration was associated with a larger volume of the caudate nucleus; adolescents breastfed for more than 24 weeks had larger caudate volume than those who were breastfed for less than 6 weeks. Further, total breastfeeding duration was positively associated with cortical thickness of the left medial prefrontal cortex and the superior and inferior parietal cortex in both hemispheres. Brain size, age, and exposure status were used as covariates for the above analysis. Results from these two chapters confirm that duration of breastfeeding during the first year of life where the brain grows the fastest can be vital for brain structure and function.

C. Does supplementation by omega-3 fatty acids facilitate optimal development of brain and cognition in school-aged children from less well-off neighbourhoods?

The final part of this thesis described my work on the effects of ω-3 fatty acids supplementation on brain and cognitive development of 9-to-12 year old children. While the studies above investigated possible long-term influences of tobacco and breast milk on adolescents' cognition and brain, here we evaluated short-term effects of ω-3 fatty acids on children's cognition and brain development. We chose 9 to 12 years old children because previous studies examining effects of PUFAs supplementation focused on infants. Although

brain grows the fastest during the two first years of life, brain maturation continues throughout childhood and adolescence and, as such, nutrition is likely to continue play an important role in this process.

We collected data pre and post intervention in 35 children (9 to 12 years of age) from less well-off neighbourhoods. We detected differences in brain microstructure (Fractional Anisotropy and Mean Diffusivity) that did not follow the expected age-related trends for this age group. Specifically, we found that - after the three-month supplementation - the active group showed a decrease in FA (DTI) and NAA/Cr (MRS), and an increase in MD (DTI); no such changes were observed in the placebo group. During typical development, DTI studies have revealed age-related increases in fractional anisotropy (FA) and decreases in mean diffusivity (MD) in a number of white-matter regions during childhood and adolescence (Mukherjee et al., 2001; Schmithorst et al., 2002; Klingberg et al., 1999; Snook et al., 2005). Effects of supplementation on DHA and EPA concentrations in the blood showed that both DHA and EPA levels increased. At the same time, levels of AA decreased. When examining associations of fatty acids with brain microstructure, only EPA (and not DHA or AA) was related to FA/MD. We have suggested that limiting the supplementation to DHA and EPA, without providing also AA, shifted the balance between omega-3 and omega-6 fatty acids in the neuronal membranes and, in turn, affected brain and cognition. But given the preliminary nature of this study, these results are only suggestive and not conclusive.

The main findings of this thesis addressing the three research questions are summarised below:

6.1. The main findings of this thesis

6.1.1. Maternal education as a significant predictor when assessing cognitive abilities of adolescents exposed to tobacco during pregnancy

The first study of this thesis examined an association between maternal cigarette smoking during pregnancy and offspring's cognition; we found no significant differences between exposed and non-exposed adolescents on cognitive abilities and intelligence even after controlling for potential confounders. Importantly, our exposed sample of adolescents was

matched to non-exposed ones according to the level of maternal education and school attended. Maternal education is a known predictor of cigarette smoking during pregnancy in the general population (Cornelius et al., 2000). Thus, findings from this study suggest that controlling carefully for maternal education at the outset eliminates possible differences between exposed and non-exposed adolescents in their cognitive abilities. Results from this study are consistent with those obtained by others who found that any significant association between maternal cigarette smoking during pregnancy and offspring's intelligence was eliminated after controlling statistically for maternal education and maternal intelligence (Batty et al., 2006; Breslau et al., 2005; Fergusson & Lloyd, 1991). Fergusson and Lloyd (1991) suggested that children whose mothers smoked during pregnancy perform worse not due to possible causal effects of smoking but rather because these children tend to come from a relatively disadvantaged home environment. To our knowledge, this is the first study that matched exposed and non-exposed adolescents according to maternal education at the time of ascertainment, thus not having to rely on statistically modeling of the data to remove the effect of this important confounder on cognitive performance.

Last but not least, the fact that the sample comes for a relatively geographically isolated population increases its genetic and environmental/cultural homogeneity and, therefore, decreases the likelihood of major differences between the exposed and non-exposed individuals in these two domains.

6.1.2. Exclusive breastfeeding promotes later cognitive development

In contrast with the first study, which examined long-term associations of a harmful factor, tobacco, with adolescents' cognition, the second study of this thesis assessed an association between the duration of exclusive breastfeeding and cognitive abilities and intelligence of adolescents, half of whom were exposed to maternal cigarette smoking during pregnancy. There is a clear link between smoking during pregnancy and breastfeeding; mothers who smoked during pregnancy were less likely to breastfeed.

More specifically, this study found that duration of exclusive breastfeeding was positively associated with total and fluid intelligence, as well as perceptual organization and fine motor skills. This association remained after controlling for potential confounders such as

parental education and household income, birth weight, sex, and a number of post-natal factors. These results are consistent with other studies showing that exclusive breastfeeding was positively associated with intelligence (Clark et al., 2006; Horwood & Fergusson, 1998; Mortensen et al., 2002; Gustafsson et al., 2004). Oddy *et al* (2003) found a positive association between Verbal IQ and exclusive breastfeeding duration at 6 years old children, after adjusting for perinatal, social and family factors. Interestingly, we showed that any positive associations between exclusive breastfeeding duration and verbal intelligence were eliminated after controlling for parental education. This may suggest that verbal intelligence is highly influenced by parental education compare with fluid intelligence. This is verified by Kaufman & Doppelt (1976) who suggested that the correlation between children's IQ and parents' SES is rather higher for verbal IQ than for non-verbal IQ.

Results from this study suggest that duration of exclusive breastfeeding during the first year of life where brain grows the fastest can have important positive outcomes on later cognitive development. Surely, children's intelligence is influenced by a large number of known – and unknown - factors in the child's genes and environment. Here, we tried to control for many possible factors that could have had an influence on children's intelligence. Maternal intelligence is usually the most common confounder because intelligence has high heritability. Using maternal education as a confounder we can – in an indirect manner – also control for some genetic factors; this is because intelligence and educational attainment have nearly similar heritability (Mackintosh, 1998) and intelligence correlates with the total number of years of education (McCall, 1977).

Associations between exclusive breastfeeding duration and intelligence have small effect sizes (.118 SD); this is expected because child's intelligence is based on many heritable and environmental factors as stated above. But even small effect sizes can be important. During the first year of life, the only source of food is breast or formula milk. Exclusive breastfeeding states that the only food given to the child is breast milk; not even water is given. If exclusively breastfed adolescents have larger mean values on intelligence than those who were never breastfed, that means that elements of breast milk and breastfeeding process have a small but positive influence on later cognitive development.

6.1.3. Total breastfeeding duration is associated with larger volume of the caudate nucleus and thicker fronto- parietal cortex

Linking the long-term associations of exclusive breastfeeding duration with cognition, the third study examined associations between total breastfeeding duration and brain structure in adolescence. By using total breastfeeding duration, we have possibly increased sensitivity (and specificity) for detecting possible influences of breastfeeding on brain structure. Our results indicated that caudate volume was significantly increasing with total breastfeeding duration; the more an adolescent was breastfed as an infant, the larger the caudate nucleus during his/her adolescence. This study suggests that caudate nucleus is influenced by nutrition during early post-natal life. This is consistent with the results reported by Isaacs *et al* (2008) who showed that caudate nucleus was significantly larger in adolescent boys born preterm and fed with a high-nutrient diet (formulated to meet the increased macronutrient and micronutrient needs of this population) as infants. During the first year of life, caudate nucleus volume increases by 19% (after controlling for total brain volume, caudate nucleus still increased significantly between 1 and 2 years of age; Knickmeyer et al., 2008). Also, the caudate nucleus is known to be one of the brain regions having high DHA content in the neonatal rats (Xiao et al., 2005).

In a second set of analysis, we carried out a meta-analysis of fMRI studies of intelligence and showed that 36 brain regions are consistently activated during reasoning tasks. By grouping these regions through principal component analysis, we extracted seven (cortical) components. By examining associations between total breastfeeding duration and cortical thickness of these seven components, we uncovered a positive association between total breastfeeding duration and the thickness of the fronto-parietal cortex. Those adolescents who were breastfed for over 24 weeks had significantly thicker bilateral superior and inferior parietal lobules, as well as thicker medial prefrontal cortex, than adolescents who were never breastfed. It may worth mentioning that a study by Isaacs, Edmonds, Lucas and Gadian (2001) showed that very low birth weight children without a deficit in calculation ability had more gray matter in an area in the left parietal cortex than those children who had the deficit. This, even not directly relevant with our results, relates with the fact that our breastfed adolescents have not only thicker parietal cortex but also have better performance on fluid intelligence than

those who were never breastfed. Animal studies have shown that DHA supplementation can increase the population of neurons with longer neurite length and higher number of branches in hippocampus in culture (Calderon & Kim, 2004), as well as delaying the onset of apoptosis (Politi et al., 2001). As such, thicker parietal cortex in breastfed adolescents could theoretically reflect such early effects of DHA contained in breast milk on brain development.

6.1.4. High intakes of ω-3 fatty acids supplementation can affect brain and cognition of school-aged children

The above positive associations between breastfeeding and adolescents' brain and cognition suggest that components of breast milk, mainly polyunsaturated fatty acids, can have positive outcomes on brain and cognitive development of adolescents. Several studies have tried to verify this suggestion by supplementing children under the age of two years and/or pregnant and lactating women with ω-3 and ω-6 fatty acids. We examined short-term effects of ω-3 (DHA+EPA) supplementation on children between the ages of 9 to 12 years. We found that - after supplementation - the active group showed a decrease in FA (DTI) and NAA/Cr (MRS), and an increase in MD (DTI); results that are contrary to the expected age-related changes during normal brain development in childhood. We also found that DHA and EPA levels were increased in the blood, whereas AA (omega 6) levels were decreased. Further, when examining associations of fatty acids with brain microstructure, we found a superiority of EPA over DHA. EPA was somehow influencing anisotropy (negatively) and diffusivity (positively); DHA and AA were not associated with either FA or MD. These results suggest that it is only EPA that somehow affects brain microstructure.

Our intervention product was based only on DHA and EPA and not AA. Examining the fatty acids levels in the blood after supplementation, as stated above, we verified that plasma DHA and EPA increased in the active group, whereas plasma and red blood cell AA notably decreased. Results from studies investigating effects of DHA supplementation on rats (Wainwright et al., 1999; Huang et al., 2002) emphasize the importance of ω-6 fatty acid content of the diet when supplementing with DHA, as well as the form in which ω-6 fatty acids are provided. It has also been demonstrated that fish oil can inhibit Δ6-desaturase activity and thus decrease AA levels (Raz et al., 1998). Studies supplementing patients with schizophrenia and depression with EPA suggest that the inhibitory effect of EPA on Δ5-desaturase activity

can be responsible for the lower plasma AA found when fish oil, high in DHA and EPA, is consumed (Uauy-Dagach & Mena, 1995). Consequently, we hypothesize that the high intakes of DHA/EPA inhibited the AA concentrations in our sample population (as shown by the fatty acid analysis in the blood) and that this might have resulted in the negative changes in brain microstructure. Perhaps, there was a shift in the fluidity of membranes (higher MD) that goes in the opposite direction than expected during normal development and this might have also lowered anisotropy. Concerning the decrease in NAA metabolite in the active group after supplementation, possibly, neurons composed of membranes with excessive amount of PUFAs could have altered neuronal processes, resulting in NAA loss.

Further, the unexpected decrease of FA is also verified by the negative association of it with plasma EPA; and the MD increase could be partly explained by the positive association with plasma and red blood cell EPA. As mentioned above, DTI studies revealed age-related increases in FA and decreases in MD in a number of white-matter regions during childhood and adolescence. The latter analysis (associations of fatty acids with MRI measurements) could suggest that increased intakes of EPA, which the developing brain is not used to these intake rates, could somehow influence brain microstructure. Reports of EPA and DHA supplementation on mood disorders and schizophrenia treatment have demonstrated that EPA has shown efficacy in reducing symptoms of depressive patients (Nemets et al., 2002; Peet & Horrobin, 2002a), as well as improving symptoms in patients with schizophrenia (Peet, Brind, Ramchand, Shah, & Vankar, 2001; Peet & Horrobin, 2002b); whereas DHA showed no such effects (Schizophrenia: Peet et al., 2001; Depression: Marangell, et al., 2003; Llorente et al., 2003). DHA and EPA have different metabolic functions; DHA is a membrane structural component whereas EPA takes part in eicosanoids synthesis (Peet et al., 2001). Also, the fact that AA was not associated with either FA or MD strengthens the assumption that it is only higher intakes of EPA that somehow affects brain microstructure. However, as this is an explorative study results should be taken with caution.

6.2. Theoretical Contributions

6.2.1. Associations of tobacco exposure during pregnancy with offspring's cognition during adolescence

The incidence of cigarette smoking during pregnancy remains high and smoking effects have been underestimated and are thought to be of less significance than illicit drugs of abuse. The possible associations of maternal smoking during pregnancy with children's cognitive and neuropsychological development have been examined in a number of longitudinal studies. Part of this thesis examined long-term associations of maternal cigarette smoking during pregnancy with adolescents' cognition and found no significant differences between exposed and non-exposed adolescents. We suggest that the lack of significant differences on cognition and intelligence between exposed (cases) and non-exposed (controls) adolescents lies on the fact that cases and controls were matched according to the level of maternal education and school attended. It is widely known that maternal education is one of the most significant predictors of maternal smoking during pregnancy. Maternal education, as well as maternal intelligence, is also an important predictor for offspring's intelligence and this is because of high heritability. With this study we contribute to the "*in utero* exposure to tobacco" literature by validating that any associations of tobacco exposure during pregnancy with offspring's later IQ and cognition are hampered by confounding: there are fundamental differences between mothers who smoke during pregnancy and those who do not. While exposure to tobacco during pregnancy is associated with variations in brain structure (Toro et al., 2008; Paus et al., 2008), disadvantage on offspring's intelligence and cognition may be unlikely after controlling for important confounders such as maternal education.

6.2.2. Long-term associations of breastfeeding duration with brain structure and function

Several studies report that breastfeeding is associated with better performance on neurodevelopmental and cognitive tests in later life (Mortensen et al., 2002; Gustafsson et al., 2004; Horwood and Ferguson, 1998), suggesting that breast milk may influence early brain development. Using exclusive-breastfeeding duration, we found that Full Scale IQ and fluid intelligence, as well as fine motor skills, of adolescents were positively associated with duration of exclusive breastfeeding during infancy. As such, this part of the thesis extends the

literature by suggesting that exclusive breastfeeding for longer periods during the first year of life can have long-term influence on cognitive abilities.

While many have studied associations of breastfeeding and cognition in later life, associations of breastfeeding duration with brain structure are largely unknown. A recent study by Isaacs *et al* (2010) showed that maternal breast milk correlated significantly with verbal intelligence, as well as with white matter volume in adolescent boys who were born prematurely. Another work by Isaacs *et al* (2008) found that a high-nutrient diet group had significantly larger left and right caudate nuclei, only in boys, even when correcting for total brain volume. In our study on long-term associations of total breastfeeding duration with adolescents' brain structure, we found that the volume of caudate nucleus was positively associated with breastfeeding duration; the more the breastfed the larger the volume. Caudate nucleus is a structure that develops early and thus it may be more susceptible to environmental influences, such as nutrition, during the initial post-natal stages of brain development.

Further, we showed that the superior and inferior parietal cortex, as well as the medial prefrontal cortex, was thicker in adolescents who were breastfed for longer periods than those not breastfed at all. To our knowledge, this is the first study examining associations between breastfeeding and cortical thickness. Our results suggest that cortical thickness is also influenced by nutrition in early life. In the first year of life, in which breast or formula feeding must occur, gray-matter volume increases by 149% (Knickmeyer et al., 2008). DHA, delivered to the infant through human milk during the first year of life, may promote cellular processes with a long-lasting impact. As such, at the time of adolescence when gray matter volume and thickness are normally decreasing, adolescents who were breastfed for longer periods (over 24 weeks) have thicker parietal regions than those who were not breastfed perhaps because continuous DHA during the first year of life supported cellular processes that contribute to the overall cortical thickness. Thus, results from animal studies suggest that DHA supplementation promotes neurogenesis, delays apoptosis and increases the population of neurons with longer neurite length. Given the thicker fronto-parietal cortex in breastfed adolescents, some of these cellular events might have been promoted by DHA delivered to the nursed infant during his/her first year of life.

6.2.3. Effects of ω-3 supplementation in school-aged children: importance of ω-3 and ω-6 fatty acids balance

Very little is known about the effect of DHA/EPA supplementation on the cognition of healthy school-aged children. The ω-3 polyunsaturated fatty acids intakes are inadequate in the Western diet, while intakes of ω-6 PUFAs are usually high, thus negatively influencing the ω-6:ω-3 ratio in the diet. Most of the research on the relationship between ω-3 PUFA and cognitive development has focused on breast-fed or formula-fed infants born either at term or prematurely. Based on these studies, and studies on ω-3 deficient animal models, most research has focused its interest on effects of DHA on infants' development. Overall, it appears that DHA supplementation during pregnancy and lactation has no beneficial effect on visual development but is associated with an evident advantage vis-à-vis later mental development (Helland et al., 2003). The latter observation suggests that effects of DHA supplementation during pregnancy and lactation may appear later in life when cognitive functions are more mature and their evaluation might be more sensitive as to reveal subtle inter-individual variations. The focus of these studies was mostly on infants due to the fact that the brain grows the fastest during the two first years of life. Nonetheless, brain development is not completed by the age of two years and as such, nutrition is likely to continue play an important role in this process. For this reason, we examined effects of DHA plus EPA supplementation on cognitive and brain development in school-aged children from less well-off neighbourhoods where consumption of fish is not common. To avoid confounding effects as described in the other three studies, and since maternal education is an important predictor of children's intelligence, we matched the active and placebo groups according to age, sex and maternal education.

We detected small but noteworthy differences on brain microstructure that did not follow the expected age-related trends for this age-group thus suggesting a possible detrimental effect of the DHA/EPA supplementation. It is possible that this observation is related to the fact that the DHA/EPA supplementation was not accompanied by a supplementation with AA, thus affecting the balance between omega-3 and omega-6 fatty acids in unfavourable manner. Thus, we suggest that the high intake of DHA/EPA inhibited the AA concentrations in our sample, as indicated by the fatty-acid levels in the blood, and that this imbalance resulted in the negative effects on brain microstructure (Fractional Anisotropy, Mean Diffusivity) and brain

metabolites (N-acetyl-aspartate). Perhaps, neurons composed of membranes with excessive amounts of DHA could possibly alter fluidity of membranes that goes in the opposite direction than expected during normal development. Also, excessive amounts of DHA in membranes could alter other neuronal processes, such as neurite growth, thus affecting N-acetyl-aspartate loss. We also showed that EPA, and not DHA, is the fatty acid associated with decreased FA and increased MD. This suggests that EPA is somehow influencing anisotropy (negatively) and diffusivity (positively). This appears to be consistent with the reports of EPA and DHA supplementation on mood disorders and schizophrenia treatment in which EPA but not DHA reduced symptoms in patients with depression or schizophrenia. Thus, our study demonstrates the importance of having a proper balance between omega-3 and omega-6 fatty acids in order to avoid excess concentrations of only one of them and to promote a healthy development. But given the small sample size, our observations are preliminary and should be treated with caution.

In summary, this thesis reinforces the importance of controlling for maternal education, a significant predictor of offspring's cognition, when examining environmental variables such as tobacco exposure *in utero* and length of breastfeeding, and when designing studies which purpose is to evaluate children's or adolescents' cognition. As well, this thesis offers important knowledge on associations between breastfeeding duration and adolescent's cognition and brain structure suggesting that breastfeeding is good for optimal brain development and thus cognitive development and that mothers should be deciding with care if they will breastfeed and for how long. Last but not least, the last study of this thesis suggests in a preliminary manner that DHA plus EPA supplementation of children's diet should be carried out with caution since high intakes of DHA plus EPA without the appropriate amount of AA could alter the physiological processes of the cell membranes in unfavourable way.

6.3. Methodological Contributions

6.3.1. Controlling for maternal education: a significant predictor for child's cognitive development

As shown by the first study, associations of maternal cigarette smoking during pregnancy with offspring's cognition during adolescence is not present when maternal education is used as a matching criterion for exposed and non-exposed adolescents. Maternal education and maternal intelligence are important predictors of offspring's intelligence; this is in part due to their high heritability. By removing the effect of maternal intelligence, one can examine the effect of tobacco exposure *in utero* on child's intelligence in a manner less confounded by related factors. As mentioned earlier, it has been hypothesized that educational attainment has lower heritability than intelligence; yet, a large number of twin and adoption studies have challenged this view giving the notion that intelligence and educational attainment have practically similar heritability (Mackintosh, 1998). Furthermore, even when controlling for parental socioeconomic status, intelligence scores are still strongly related with educational attainment (Herrnstein & Murray, 1994). Thus, when examining the effects of any variable, such as maternal cigarette smoking, on child's cognitive ability, short of testing directly maternal intelligence, by controlling for maternal education, one also controls – to some extent - for maternal intelligence.

Since maternal education is a significant predictor for offspring's cognitive development and given the results from the first study, maternal education was used a covariate for the second and third study, and as a matching criterion when performing the fourth study. As such, we examined the associations of tobacco exposure *in utero*, breastfeeding duration and omega-3 supplementation with child's intelligence in a manner less confounded by factors often associated with these outcomes. In our knowledge no other study examining the association of maternal cigarette smoking with offspring's cognition has matched the exposed and non-exposed children/adolescents according to level of maternal education at ascertainment. Similarly, no other omega-3 supplementation study has matched the treatment and control group according to maternal education.

6.3.2. Principal Component Analysis of cognitive tests

The factor analytic approach has been used as early as Spearman's (1904) work to identify the general factor of intelligence. Factor analysis is a technique that tries to simplify, or identify patterns in large correlation matrices. It is based on the assumption that performance on two tests correlates because these two tests are, in part, measuring the same thing. While factor analysis describes the relationships between different IQ tests, it does not reveal the structure of human abilities (Mackintosh, 1998).

In our first study, neuropsychological assessment consisted of 33 cognitive tests. While we can uncover ample information on adolescent's cognitive abilities with 33 tests, the use of univariate approach would result in multiple comparisons leading to Type I statistical errors. For this reason, and to obtain a more global view of our data, we performed a principal component analysis with all 33 tests and extracted seven cognitive components. Due to the exploratory nature of the neuropsychological dataset a confirmatory factor analysis could not be performed.

When principal component analysis is performed in one dataset, conclusions are restricted to the sample and generalization of the results can be achieved only if analysis using different samples reveals the same factor structure (Field, 2005). Thus, the seven cognitive components derived from the first exploratory factor analysis (1st study) were not used for the 2nd study because the dataset increased (599 adolescents *Vs* 503 adolescents in the 1st study). A second exploratory factor analysis was performed on the 599 adolescents in order to evaluate if the same components from the 1st study could have been derived. Principal component analysis from the second study revealed nearly the same cognitive components; only one component was missing and that was due to a cognitive test that was excluded from further analysis on the 2nd study due to statistical reasons. Overall, principal component analyses revealed the following cognitive components: verbal conceptualization, perceptual organization, processing speed, visual and verbal memory, and motor dexterity. By having only six cognitive components instead of 33 cognitive tests, statistical analysis was more powerful and relationships between maternal cigarette smoking, as well as breastfeeding duration, with cognitive abilities were more readily understandable.

6.3.3. ALE meta-analysis: breastfeeding duration associations with "IQ-related" brain regions

One of the purposes of this thesis was to examine associations between breastfeeding duration and brain structure. Our 2nd study showed that exclusive breastfeeding duration was positively associated with Full Scale IQ and Performance IQ; other studies have shown similar results thus indicating that breastfeeding facilitates later cognitive development. Since cognitive processes mature concurrently with brain development, and taken into account results from the 2nd study, we wished to focus on brain regions that are "activated" during intelligence tasks and by extracting their cortical thickness we could evaluate breastfeeding duration association with cortical thickness of "IQ-related" brain regions. In order to perform the above, firstly, we identified 36 "IQ-related" regions through the ALE meta-analysis. Brain-imaging experiments that report 3-D coordinates in stereotactic space are used to perform an ALE meta-analysis. Once all coordinates were set in a single stereotactic space (i.e. the "Talairach" space), the ALE analysis was performed. In our knowledge, this is the first ALE meta-analysis of fMRI tasks relevant for intelligence. We identified 36 regions that are activated during intelligence tasks. We then extracted cortical thickness of these 36 regions and performed a principal component analysis to group the 36 regions into a small number of components. These 36 regions included the medial prefrontal cortex, frontal eye-field and pre-motor cortex, ventro-lateral frontal cortex, mid dorsolateral frontal cortex, lateral occipital cortex, angular gyrus, superior and inferior parietal lobule, temporal cortex and face fusiform area.

With this combination of ALE and PCA, we managed to focus on specific brain regions and examine their associations with breastfeeding duration in a rigorous and objective manner.

6.4. Questions raised and future research suggestions

Data from Studies 1 to 3 were all cross-sectional. Many studies examining maternal cigarette smoking during pregnancy are longitudinal. While the design of the Saguenay Youth Study is cross-sectional it has the advantage of having equal proportion of exposed and non-exposed adolescents matched at ascertainment by the level of maternal education. Associations of maternal cigarette smoking as well as of breastfeeding status with offspring's cognition and

brain structure should be examined in longitudinal studies while matching exposed and non exposed, breastfed and non-breastfed according to the level of maternal education, or even better, maternal intelligence. Longitudinal studies will help us understand if the associations of maternal cigarette smoking or of breastfeeding status with offspring's cognition are increasing or decreasing with time.

The last study examining effects of omega-3 supplementation on brain and cognition of school-aged children resulted in few unexpected results on brain microstructure and brain metabolites. As discussed, these results may be due to the excessive intake of DHA plus EPA without any AA intakes. As such, we suggest that any further research on omega-3 supplementation should be designed with caution on the amounts of DHA plus EPA given, as well as on the importance of ω-6 fatty acid content of the diet when supplementing with DHA.

Animal studies showed that pregnant rats supplemented with DHA, and minor levels of LA, resulted in large increases in brain DHA, as well as decreases in AA accompanied by delays in growth and behavioural development in the pups (Wainwright et al., 1999). This may suggest that high levels of DHA may have inhibited the activity of Δ6-desaturase in converting LA to GLA which is then converted to AA. Other data from human intervention studies have revealed that high doses of pure EPA ($>4 g \cdot day^{-1}$) are not effective for either schizophrenic patients or depressive ones (Horrobin et al., 2002) and this might be related to a decrease of the membrane levels of AA, as EPA inhibits Δ5-desaturase activity. Plus, fish oil can inhibit Δ6-desaturase activity and thus decrease AA levels (Raz et al., 1998). Taken together, these results indicate that excessive intake of DHA/EPA without the appropriate amount of AA in the diet, or other form of omega-6 fatty acid, could alter physiological processes and thus normal brain development. Further research should take into consideration the above results and highlight the importance of proper balance between ω-3 and ω-6 fatty acids in the diet.

6.5. Conclusion

This thesis tried to answer the question as to whether we can identify specific nutrients and toxins that alter physiological states of brain development and thus, by either employing (nutrients) or discarding (toxins) them we can reveal the secret for optimal brain development and hence, cognitive development. In order to answer this question, four studies were employed; one examining possible long-term effects of maternal cigarette smoking during

pregnancy on adolescents' cognition; the other two investigating possible long-term effects of breastfeeding on brain and cognition in adolescents; and the last study examining the short-term effects of omega-3 supplementation on brain and cognition in school-aged children. In summary, this thesis suggests that breastfeeding during the first year of life can be vital for optimal brain and cognitive development. It also proposes that adverse effects of tobacco exposure *in utero* on offspring's cognition are not present when maternal education, a significant predictor of maternal cigarette smoking during pregnancy and of offspring's cognition, is held constant. Lastly, it highlights the importance of appropriate balance between ω-3 and ω-6 fatty acids in the diet in order to avoid excessive amounts of one of them against the other.

With the first study, the thesis investigated associations of tobacco (toxin) during pregnancy with adolescences' cognition; the second and third study investigated associations of breast milk (nutrient) during the first year of life with adolescences' cognition and brain structure, and the last study investigated the effects of DHA plus EPA (nutrients) on children's cognition and brain structure during school age. We conclude that in order to achieve optimal brain and cognitive development, we do not only need to discard toxins or employ nutrients during critical periods of rapid brain development but also take into account other environmental and genetic factors that play vital role in children's development. There are many environmental factors that we should expect to influence a growing child's cognitive or intellectual development, such as health, nutrition, schooling, and parental interactions. As we cannot change genetic factors, however, we can influence environmental ones. We can protect a growing child by not exposing him/her to cigarette smoking during and after pregnancy, or while breastfeeding; we can promote the employment of breastfeeding among mothers; as well as provide a proper diet for our developing children.

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Appendices

<u>Appendix I:</u> Information brochure given to children in schools for participantion in omega-3 study

What will happen during the study?

The study involves both the parent and the child and consists of 2 visits. Each visit will consist of several sessions including: (1) Magnetic resonance imaging(MRI); (2) electroencephalography (EEG); and (3) psychological testing. Parents will also be interviewed about their child's development and psychological experiences.

During 3 months between the two visits, children will follow a diet supplementation with omega-3 fatty acids (contained, for example, in fish oil) or placebo diet. This supplement will be added to margarine that we will give to you. Other than the extra margarine, children can eat their usual foods.

What is EEG?

EEG is a way to record waves of electrical activity generated by the brain. This is done by placing many electrodes (small disks) on the scalp and connecting these with an electro-encephalograph and a computer.

What is MRI?

MRI, or magnetic resonance imaging, allows pictures to be taken of your child's brain using a large magnet, radio waves and a computer. The tunnel-like magnet around the participant establishes a strong magnetic field. It is a totally painless procedure that involves lying inside the scanner for up to 60 minutes. The child will be positioned comfortably on a scanning bed that slides into the tunnel-shaped magnet. When the scanner is turned on, it makes loud humming and knocking sounds; earplugs will be provided.

Brain & Body Centre

Our centre provides a setting for studies of environmental and genetic factors that shape the structure and function of the human brain and body.

We hope that this work will lead to new discoveries concerning nutrition and brain and cognitive development.

Who can participate?

Healthy 9 to 12 years old children may participate in this study. Before the MRI, participants are asked to fill out a form asking if there are any metal or battery-operated devices in their body. Some metal objects (such as metal plates, implants etc.) are not allowed for safety reasons. While it is safe to be scanned with other metal objects such as braces, these may distort the images.

Our study

We wish to learn more about the effect of nutrition on brain and cognitive development. We hope that the results of this study will provide useful information in developing new guidelines for healthy lifestyle.

Further information

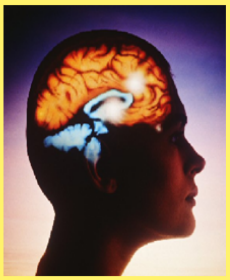
All the information obtained in this study will be kept private and strictly confidential. The name of the child will not appear on any documents, instead, a numeric code will be used.

All participants will be compensated for their time and effort.

The University of Nottingham
BRAIN & BODY CENTRE

Nutrition and Cognition:

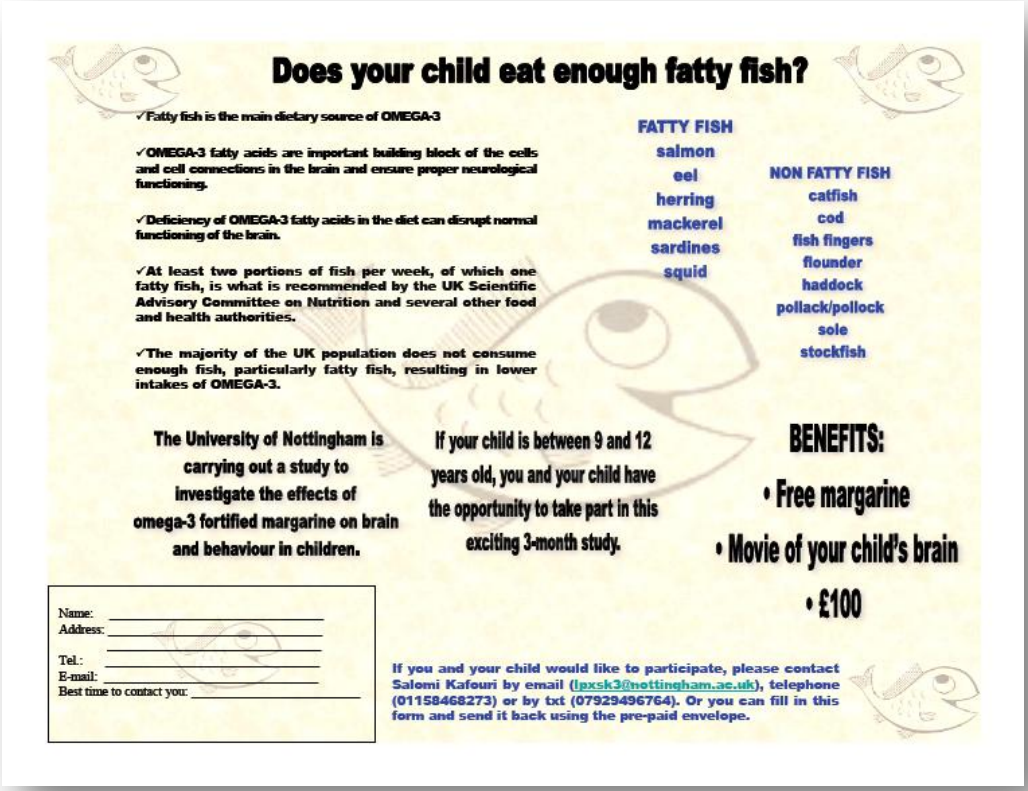
Effects of omega-3 fatty acids supplementation on brain and behavior in healthy children



Require more info?
Wish to volunteer?

Email: lpxsk3@nottingham.ac.uk
Contact tel. no.: 07929496764
Website: http://brainbody.nottingham.ac.uk/

<u>Appendix II:</u> Advertising leaflet sent to 3,000 families in Nottingham for participantion in omega-3 study



Does your child eat enough fatty fish?

✓Fatty fish is the main dietary source of OMEGA-3

✓OMEGA-3 fatty acids are important building block of the cells and cell connections in the brain and ensure proper neurological functioning.

✓Deficiency of OMEGA-3 fatty acids in the diet can disrupt normal functioning of the brain.

✓At least two portions of fish per week, of which one fatty fish, is what is recommended by the UK Scientific Advisory Committee on Nutrition and several other food and health authorities.

✓The majority of the UK population does not consume enough fish, particularly fatty fish, resulting in lower intakes of OMEGA-3.

FATTY FISH
salmon
eel
herring
mackerel
sardines
squid

NON FATTY FISH
catfish
cod
fish fingers
flounder
haddock
pollack/pollock
sole
stockfish

The University of Nottingham is carrying out a study to investigate the effects of omega-3 fortified margarine on brain and behaviour in children.

If your child is between 9 and 12 years old, you and your child have the opportunity to take part in this exciting 3-month study.

BENEFITS:
- Free margarine
- Movie of your child's brain
- £100

Name: _____
Address: _____
_____
Tel.: _____
E-mail: _____
Best time to contact you: _____

If you and your child would like to participate, please contact Salomi Kafouri by email (lpxsk3@nottingham.ac.uk), telephone (01158468273) or by txt (07929496764). Or you can fill in this form and send it back using the pre-paid envelope.

<u>Appendix III:</u> Compliance calendar given to parents and children to fill in during the supplementation months

Calendar 1st Month Pcode

DAY

1

Fill in how much margarine you ate today by colouring the parts of the circle.

If you did not eat all of the margarine you need to fill in why not:

Did you use a reserve tub of margarine today? O no O yes

If you did use a reserve tub today, please fill in why and how much you used at the bottom of this page.

2

Fill in how much margarine you ate today by colouring the parts of the circle.

If you did not eat all of the margarine you need to fill in why not:

Did you use a reserve tub of margarine today? O no O yes

If you did use a reserve tub today, please fill in why and how much you used at the bottom of this page.

3

Fill in how much margarine you ate today by colouring the parts of the circle.

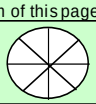
If you did not eat all of the margarine you need to fill in why not:

Did you use a reserve tub of margarine today? O no O yes

If you did use a reserve tub today, please fill in why and how much you used at the bottom of this page.

4

Fill in how much margarine you ate today by colouring the parts of the circle.



If you did not eat all of the margarine you need to fill in why not:

Did you use a reserve tub of margarine today? O no O yes

If you did use a reserve tub today, please fill in why and how much you used at the bottom of this page.

5

Fill in how much margarine you ate today by colouring the parts of the circle.

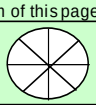
If you did not eat all of the margarine you need to fill in why not:

Did you use a reserve tub of margarine today? O no O yes

If you did use a reserve tub today, please fill in why and how much you used at the bottom of this page.

6

Fill in how much margarine you ate today by colouring the parts of the circle.

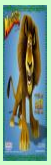


If you did not eat all of the margarine you need to fill in why not:

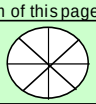
Did you use a reserve tub of margarine today? O no O yes

If you did use a reserve tub today, please fill in why and how much you used at the bottom of this page.

7



Fill in how much margarine you ate today by colouring the parts of the circle.



If you did not eat all of the margarine you need to fill in why not:

Did you use a reserve tub of margarine today? O no O yes

If you did use a reserve tub today, please fill in why and how much you used at the bottom of this page.

Beneath, fill in the day(s) you used a reserve tub, how much of it you did use and why you did use a reserve tub.

On which day?	How much?	Why?
		

Appendix IV: List of forbidden products and products fortified with EPA and/or DHA given to the parents at the beginning of the study

Forbidden supplements

Supplements containing n-3 and/or n-6 fatty acids are prohibited if consumed once a week or more often.

N-3 fatty acids:
ALA = Alpha-linolenic acid
EPA = Eicosapentaenoic acid
DHA = Docosahexaenoic acid
EPA and DHA are derived from sea fish. Therefore they are often called "**fish or marine fatty acids**".

N-6 fatty acids:
LA = Linoleic acid
GLA = Gamma-linolenic acid
DGLA = Dihomo-gamma-linolenic acid
AA = ARA = Arachidonic acid
LA and GLA are highly present in primrose oil (**teunisbloemolie**).

The following supplements which contain n-3 or n-6 fatty acids are available on the UK market. (This list is not complete! There may be more supplements available containing n-3/n-6 fatty acids).

Seven Seas Products:
Seven Seas ProBrain Pure Fish Oil - capsules
Seven Seas One-A-Day Odour Controlled Omega-3 Pure Cod Liver Oil - capsules
Seven Seas Pulse Advanced Omega-3 Pure Fish Oils with Vitamin E - capsules
Seven Seas - JointCare Advanced Glucosamine, Omega-3 & Chondrotin

EFAMOL Products:
Efamarine – omega 3 and 6
Efalex

Boots Products:
Boots Childrens Smart Omega 3 - Chews
Boots High Strength Fish Oils Capsules - Capsules
Boots Cod Liver Oil and Evening Primrose Oil – omega 3
Boots Omega Oils 3, 6 and 9 - Capsules
Boots Omega 3 Fish Oil

Other products:
Bassett's Soft and Chewy Omega-3 DHA & Vitamins A, C, D & E Pastilles
Vertese Omega Oils 3.6.9 - Capsules
Pulse Omega-3 Pure Fish Oils with Vitamin E - Capsules
Eye Q – omega 3
Equazen Eye Q - Capsules
Haliborange Teen Sense Extra Omega-3 Fish Oil
Haliborange Omega-3 Fish Oil
Haliborange Omega3 for Infants 30 Twist & Squeeze Caps

You can also have a look at the website http://www.boots.com/webapp/wcs/stores/servlet/CategoryDisplay?categoryParentId=3885&storeId=10052&categoryId=4206&catalogId=11051&langId=-1 and search for other products which contain n-3 or 6 fatty acids.

Products fortified with EPA and/or DHA

- Flora omega 3 plus drink (100ml)
- Flora Omega 3 plus (margarine)
- Branston Baked Beans omega-3 with EPA & DHA
- Dale Farm has brought out Mega Milk with EPA & DHA
- Sparky Super Fruit Bars are enriched with EPA
- St Ivel Advance Milk with omega 3 EPA & DHA
- Marks & spencer Fresh Whole Omega-3 Milk
- iQ3 Brainstorm bars are cereal and real fruit bars
- Freshlay vita egg with 100mg DHA per egg

Appendix V: Regressions with cognitive outcomes as the dependent variables and DHA (omega-3) and AA (omega-6) as the predictor variables. DHA was added first in the regression model. Analysis was repeated with AA being the first predictor – results were the same as below.

Cognitive measures	Variable	R^2 adj.	Unstandardized Coefficients (B)	Standardized coefficients (β)	*p*
WISC-IV					
Processing Speed Index	DHA	.174*	-1.73	-.191	.267
	AA		-4.401	-.498	**.006**
Perceptual Reasoning Index	DHA	.085	-.656	-.099	.584
	AA		-2.57	-.396	**.034**
Block Design	DHA	.102	-.849	-.104	.560
	AA		-3.320	-.417	**.024**
Digit Span	DHA	-.019	-.452	-.201	.293
	AA		-.330	-.151	.428
Coding	DHA	.190*	-1.91	-.279	.106
	AA		-3.33	-.499	**.006**
Matrix Reasoning	DHA	.045	-.136	-.049	.790
	AA		-.912	-.336	.074
Symbol Search	DHA	.212*	.081	.018	.916
	AA		-2.27	-.505	**.005**
Picture Completion	DHA	.309*	.936	.299	.071
	AA		-1.25	-.420	**.013**
Cancellation	DHA	.000	-2.79	-.186	.325
	AA		-3.41	-.234	.218
WCST					
Factor1: Perseveration	DHA	.007	.381	.079	.688
	AA		1.25	.262	.191
Factor2: Non-Perseverative	DHA	.038	-.416	-.163	.417
	AA		-.373	-.147	.465
Trail Making Test					
Trail A	DHA	.046	-.072	-.011	.951
	AA		1.97	.322	.087
Trail B	DHA	.030	-.023	-.198	.289
	AA		.020	.173	.353
Ruff 2&7 Selective					
Automatic Detection	**DHA**	.280*	1.92	.594	**.001**
	AA		.337	.107	.504
Controlled Search Accuracy	**DHA**	.218*	2.51	.542	**.003**

	AA		.930	.201	.219
Self Order Pointing Test					
Total Errors	DHA	.042	-.342	-.140	.468
	AA		.072	.030	.874

*Model (DHA, AA) significant at p<.05