INVESTIGATING THE RELATIONSHIP BETWEEN LONG CHAIN OMEGA-3 FATTY ACIDS AND PREFRONTAL CORTEX DEVELOPMENT DURING ADOLESCENCE

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ABSTRACT

Delivery of building blocks essential for brain development is critical for the normal development of the brain and behavior. Omega-3 (N3) fatty acids are essential nutrients, which can only be obtained from the diet. Docosahexaenoic acid (DHA), a long chain omega-3 fatty acid found in cold-water seafood, is highly enriched in neuronal membranes of the prefrontal cortex (PFC). During the time between infancy and young adulthood, when DHA accrues rapidly in the PFC, the PFC undergoes dramatic but protracted changes and is comparatively one of the last of the parts of the brain to fully develop. During early life, the PFC initially undergoes progressive changes (i.e., over-production of synapses, dendritic arborization, and growth of cell size) reflected by increasing gray matter volume, which is followed by a protracted period of regressive events (i.e., pruning of superfluous synapses, reduction in dendrites, glia and associated microvasculature) manifesting as cortical thinning. These progressive and regressive cortical changes are supported by DHA and are associated with improvements in intelligence and executive function including impulse control. The ability to exercise inhibitory control has been associated with multiple long-term outcomes such as career/earning potential and health. There is evidence that N3 status is related to both impulse control in clinical populations and prefrontal cortical structure and function. Given DHA's role in cortical development and its importance in PFC development, concern is raised by the trend showing net decreases in N3 fatty acid intake in the

United States. The present study examines the relationship between N3 status and the function and structure of the prefrontal cortex with respect to ability to inhibit impulses in typically developing adolescents. We used maternal infant feeding practices (i.e., breastfeeding duration) to examine whether differential exposure to N3 fatty acids in infancy has an impact on impulse control and related prefrontal function during adolescence. Additionally, we examined whether current N3 status (assessed via dietary report and whole blood levels) was related to impulse regulation, prefrontal function and gray matter volume. Our results suggest that long-chain omega-3 fatty acid status both during infancy and during adolescence has an impact on adolescent brain development and behavioral outcomes.

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This thesis is dedicated to my dear late big brother, Sebastian, who will forever inspire me and my career. You were always so proud of your little sister's accomplishments. Here is one more for you, Sebbie!

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CHAPTER I: Introduction

Overview

This dissertation focuses on the role that an essential nutrient called Omega-3 (N3) fatty acids can have with regards to brain development. A multi-prong approach is taken to assess the impact of this fatty acid on impulse control examining both behavioral and neuronal functional/structural outcome measures during adolescence. The chapter provides a detailed overview of this fatty acid and the role it plays with regards to brain development.

Omega-3 fatty acids

Polyunsaturated fatty acids are a class of essential nutrients that must be obtained through the diet to prevent deficiency. As their name implies, these fatty acids contain more than one double bond on their carbon chain. The position of the first double bond from the methyl end of the molecule further determines its biological function. Omega-3 (N3) fatty acids are a type of polyunsaturated fatty acid where the position of the first double bond is at the 3rd carbon from the methyl (omega) end. Polyunsaturated fatty acids are further distinguished by the number of double bonds they contain. N3 fatty acids include alpha-linolenic acid (ALA; 18 carbon chain, 3 double bonds), eicosapentaenoic acid (EPA; 20 carbon chain, 5 double bonds) and docosahexaenoic acid (DHA; 22 carbon chain, 6 double bonds) (Bradbury, 2011).

Of the three types of N3 fatty acids, DHA is the most prominent in neuronal membranes and as such has been determined to be the main N3 fatty acid of importance to the central nervous system (Stillwell & Wassall, 2003). Within the central nervous system, the distribution of DHA is particularly concentrated in the neuronal membranes of the prefrontal cortex (PFC) comprising

10-20% of total fatty acids (Bradbury, 2011), a level which is conserved across species, highlighting its importance in evolutionary biology of neural function (McNamara & Carlson, 2006). That DHA is enriched in the frontal lobe also suggests its importance in a region that is critical for executive function. DHA accrues rapidly in the PFC from the perinatal period though the first 18 years of life, with little increase in PFC DHA content after the second decade of life (Carver, Benford, Han, & Cantor, 2001) suggesting that the adolescent years are a crucial time to ensure adequate accrual of DHA in the PFC.

Long chain N3 fatty acids (EPA and DHA) exert beneficial effects on the brain in general. These fatty acids facilitate vasodilation and promote vascular tone (Cottin, Sanders, & Hall, 2011) and promote production of brain derived neurotrophic factor for neuroprotection (Wu, Ying, & Gomez-Pinilla, 2004). EPA is a backbone of the eicosanoids, signaling molecules involved in the resolution of inflammation (Bazinet & Layé, 2014). DHA is also recognized for its role in the resolution of inflammation and as a second messenger in signal transduction pathways (Alessandri et al., 2005; McNamara & Carlson, 2006; Mitchell, Gawrisch, Litman, & Salem, 1998). One of DHA's unique functions, however, is as a component of phospholipids within the neuronal membrane. Due in part to its long and highly unsaturated structure, DHA plays a key role in the biophysical properties of the neuronal membrane, promoting membrane fluidity, structural transitions of embedded proteins, and neurotransmission (Delion, Chalon, Guilloteau, Besnard, & Durand, 1996; Stillwell & Wassall, 2003). Further, DHA has been demonstrated to be critical in promoting dendritic arborization (Calderon & Kim, 2004), enhancing neuronal size (Ahmad, Moriguchi, & Salem, 2002), synaptogenesis (Wurtman, Cansev, & Ulus, 2009), pruning of

superfluous synapses (de Velasco et al., 2012) and ultimately development of large scale functional networks (Grayson, Kroenke, Neuringer, & Fair, 2014).

Membrane fatty acid composition is highly regulated. Brain levels of DHA are correlated with blood levels of DHA, which are in turn highly influenced by dietary intake. As such, blood levels of N3 fatty acids display a high level of variability across individuals (20-30% in adults) (Harris, 2013). Indeed, changing intake of dietary essential fatty acids is reflected in a change in both blood levels and brain/synapse essential fatty acid levels (Figure 1) (Connor, Neuringer, & Lin, 1990; Galli, White, & Paoletti, 1970; Hulbert, Turner, Storlien, & Else, 2005; Moriguchi & Salem, 2003).

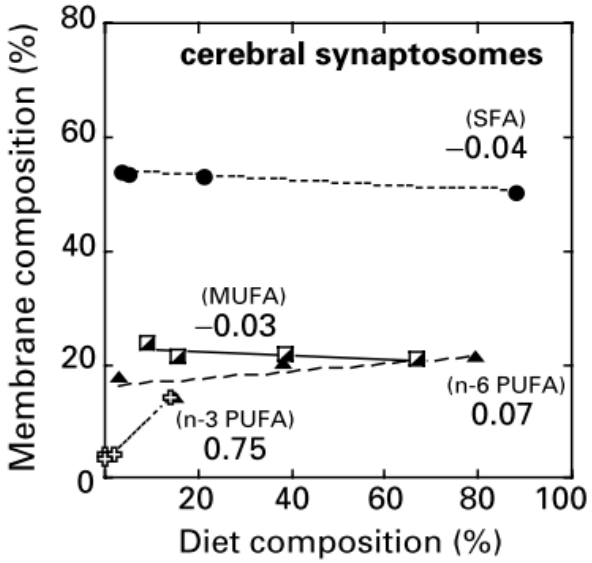

Figure 1. Responsiveness of synaptosomes to dietary fat manipulation. Fatty acid composition of the cerebral synaptosome membrane is highly responsive to changes in dietary N3 fatty acids, as indicated by a slope of 0.75 (i.e., almost a 1-for-1 increase in membrane composition) for PUFA when diet composition is increased from 0% to almost 20%. (Hulbert et al., 2005). [N3 fatty acid, n-3 PUFA; omega-6 fatty acids, n-6 PUFA; saturated fatty acids, SFA; monounsaturated fatty acid, MUFA]

Sources of omega-3 fatty acids. Given that polyunsaturated fatty acids are *essential* nutrients (i.e., our bodies cannot manufacture them *de novo*), the only source of this fatty acid is through diet. During infancy, long-chain polyunsaturated fatty acids are delivered through a diet of breastmilk

or formula. Though the essential fatty acid content of breastmilk varies with maternal diet (Liu et al., 2016), breastmilk in general results in greater cortical DHA content than either formula with or without DHA (Diau et al., 2005), owing in part due to its biophysical properties (e.g., larger size lipid droplets enveloped in triple layer membrane versus small-size protein-coated droplets found in formula) (Schipper et al., 2016). DHA accrual in the infant cortex has been shown to be proportional to breastfeeding duration (Makrides, Neumann, Byard, Simmer, & Gibson, 1994).

After weaning, table food provides sources of N3 fatty acids. Sources of ALA are plant-based foods including leafy greens, walnuts, and flaxseed while sources of preformed EPA and DHA are primarily marine sources, particularly from cold-water fish (e.g., tuna, salmon). While it is biologically possible to convert the shorter chain precursor ALA, through a series of enzymatic elongations and desaturations steps to EPA and then to DHA (Schmitz & Ecker, 2008), in reality, the shorter chain varieties meet other fates. In particular, ALA is often used for energy (via β-oxidation) in order to spare longer chain DHA for phospholipid. Moreover, there is some controversy as to whether sufficient DHA can be converted from the shorter chain ALA (Domenichiello, Kitson, & Bazinet, 2015) as this conversion is not efficient in humans (Plourde & Cunnane, 2007). Adding to the complexity, this ***biosynthetic capacity seems to be further modulated by sex and/or sexual maturity*** as estrogen facilitates conversion to DHA (Kitson, Stroud, & Stark, 2010) whereas testosterone depresses conversion (Marra & de Alaniz, 1989). Consuming preformed DHA is the only reliable way to increase DHA in membranes (Barceló-Coblijn et al., 2008). Further, due potentially to nutrient form and interactions, consuming preformed DHA in the form of *seafood* may be a particularly potent way of affecting membrane DHA (Marangoni, Colombo, Martiello, Negri, & Galli, 2007).

Effects of dietary patterns on omega-3 fatty acid availability/critical window. Of equal importance to the absolute amount of omega-3 in the diet is their ratio to another class of polyunsaturated fatty acids, omega-6 fatty acids. These polyunsaturated fats compete for the enzyme systems required for elongation and desaturation (Schmitz & Ecker, 2008). The balance between these enzymatic processes and subsequent incorporation into phospholipids is dependent on the balance of substrate supplied by diet (Lin, Shah, Salem, & Jr., 2011). It is of concern then that the dietary profile of polyunsaturated fat intake by Americans has changed dramatically over the last century, resulting in a net decrease in effective dietary omega-3 fatty acids, due almost entirely to the increased proportion of omega-6 relative to omega-3 in the diet and thereby promoting omega-6 metabolism (Blasbalg, Hibbeln, Ramsden, Majchrzak, & Rawlings, 2011). Likewise, an analysis of the diets of middle and high school adolescents revealed dietary patterns that were poor in sources of N3 fatty acids (Cutler, Flood, Hannan, & Neumark-Sztainer, 2009). Decreased intake of N3 fatty acids among adolescents may be of particular concern given that DHA rapidly accumulates in membranes of PFC gray matter primarily during the first two decades of life (Carver et al., 2001). Low intake during a critical window of DHA accrual in a brain region undergoing major dynamic development has the potential to impact cortical function and related behaviors such as impulse control, reviewed below.

Impact of insufficient omega-3 fatty acids during critical windows. A robust, reproducible example of the impact of insufficient DHA supply during a critical period comes from the development of visual acuity. DHA rapidly accumulates in the phospholipid membranes of rods in the retina and facilitates interaction of the proteins involved in photon signal transduction (Stillwell & Wassall, 2003). Infants deprived of sufficient DHA develop poorer visual acuity than infants supplied

adequate DHA (Agostoni, 2008). Importantly, attempts to correct visual acuity after the critical period has ended are largely unsuccessful. While supplementation with omega-3 to correct a deficiency induced in juvenile primates increases retinal membrane DHA levels to control levels, visual acuity was still impaired (Neuringer, 2000). Other animal studies have demonstrated difficulty in remediating low DHA levels (Bourre, Durand, Pascal, & Youyou, 1989; Li et al., 2006), particularly as the age of the animal increases. These studies suggest that insufficient DHA supply during a sensitive period has functional consequences that may impact brain function beyond the developmental window. Given the concentration of DHA in the PFC and DHA's involvement in a number of aspects of neuronal structure and function, insufficient DHA during adolescence, a stage of development during which the PFC undergoes profound changes, may have serious negative consequences.

Development of the prefrontal cortex

Brain development over the first few decades of life involves major, dynamic, non-linear changes in gray matter (e.g., Giedd et al., 1999). Early in life, gray matter, consisting primarily of neuronal cell bodies, their dendrites and synapses as well as glial cells, undergoes substantial expansion (Huttenlocher, 1990) thought to reflect increases in neuronal cell size, dendritic arborization, synaptogenesis and proliferation of glia. Gray matter expansion eventually peaks after which gray matter begins to thin and decrease in volume. While there is some debate as to the nature of the observed loss of gray matter during this period (Giedd, Keshavan, & Paus, 2008; Gogtay & Thompson, 2010; Tamnes et al., 2010), the reduction of gray matter volume may reflect, at least in part, synaptic pruning and reduction in supporting processes/glia as superfluous synapses are

eliminated and remaining synapses are strengthened. Cortical thinning decelerates and becomes relatively stable into adulthood (Tamnes et al., 2010).

Adding to the complexity of brain development is that this process occurs in a temporally and regionally dependent manner (Gogtay et al., 2004; Remer et al., 2017; Shaw et al., 2008). Primary sensory and motor cortices reach maximum synaptic density and peak gray matter thickness earlier than higher-order association cortices including the frontal cortex, which does not peak until adolescence. Cortical thinning in these higher-order regions continues through young adulthood (Huttenlocher & Dabholkar, 1997; Tamnes et al., 2010). This period of synaptic refinement is associated with improvements in cognitive function and behavioral changes (Casey, Giedd, & Thomas, 2000; Sowell et al., 2004).

The medial PFC. One of the prefrontal regions reported to reach peak cortical thickness relatively late is the medial prefrontal cortex, a region that includes cingulate cortex. An investigation of the developmental trajectories of sub-lobular regions revealed that the cingulate attains peak cortical thickness at 11.2 (left hemisphere) and 13.8 (right hemisphere) years of age (Shaw et al., 2008). The medial PFC encompasses regions including ventromedial PFC (vmPFC), rostral anterior cingulate cortex (rACC), and dorsal anterior cingulate cortex (dACC) (Figure 2). Of relevance to the present work are the vmPFC and the dACC. A brief review of their purported functions follows. While midline PFC structures are thought to develop earlier than more lateral PFC regions (Shaw et al., 2008) a detailed account of the chronology of development within the midline structures has yet to be published. However, given the early importance of social valuation in interpersonal relationships (as reviewed by Nelson & Guyer, 2011), and the differential functional development

of valuation and control systems (Casey, Jones, & Hare, 2008) one can infer that vmPFC development begins earlier than does rACC or dACC development.

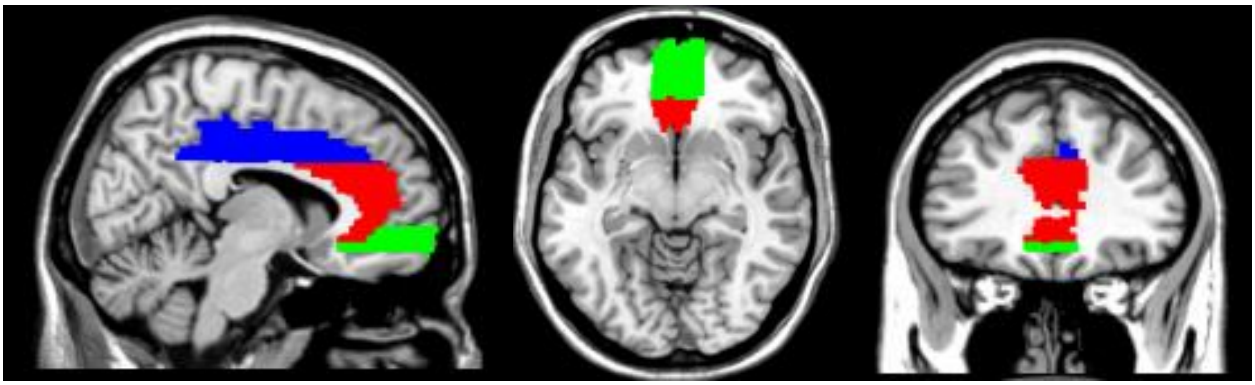

Figure 2. Regions within the mPFC highlighted in subsequent chapters. For the purposes of this dissertation the anterior cingulate is subdivided into a rostral portion (rACC, red) and dorsal portion (dACC, blue). In addition, the ventromedial prefrontal cortex (vmPFC) is shown in green.

The vmPFC and ACC are part of the medial prefrontal network (Price, 2007) and interconnected with emotion-regulated limbic regions (amygdala, ventral striatum, and periaqueductal gray) (Nelson & Guyer, 2011). The function of the vmPFC is thought to be the expression and control of emotion and instinctual behaviors in general (Fuster, 2003), with particular relevance to *valuation* especially with regards to emotional and rewarding stimuli. While more lateral regions of the ventral PFC contribute to *inhibition* (i.e., inhibiting responses not in line with goals), and *rule acquisition* (i.e., learning new context-dependent contingencies of behavior) (Nelson & Guyer, 2011). With respect to *valuation*, the vmPFC is implicated in computing information about the expected outcome magnitude and value of either a previously neutral stimulus or one with inherent biological value (Nelson & Guyer, 2011). Information about expected outcomes are relayed to the rostral rACC and then to the dACC to inform the "action-outcome" learning process implemented in the dACC (Rolls & Grabenhorst, 2008). With respect to impulse control, the vmPFC relays integrated information regarding value/motivation to lateral regions of the ventral

PFC to inform output (i.e., inhibition) (Nelson & Guyer, 2011) and its functional connectivity with dorsolateral PFC has been shown to underlie self-restraint (Steinbeis, Haushofer, Fehr, & Singer, 2014). On the other hand, the function of the dACC is generally implicated in reactive cognitive control in that it is engaged in situations where there is high likelihood of error or competing responses. Activity in the dACC occurs in response to high conflict situations (where errors are likely to occur (Carter et al., 1998) or to errors themselves which ultimately results in a subsequent increase in cognitive control and behavioral adjustments (Kerns et al., 2004).

Omega-3 fatty acids and the medial PFC. There is mounting evidence that N3 fatty acids are also associated with PFC structure and function. Blood levels of N3 fatty acids have been found to be related to cortical thickness in the rostral anterior cingulate cortex (ACC) in older adults (Zamroziewicz, Paul, Rubin, & Barbey, 2015). Using a voxel-based morphometry and a region of interest analysis, Conklin et al. (2007) reported that ACC volume was positively related to reported long-chain N3 fatty acid intake in adults (Conklin, Gianaros, et al., 2007). The relationship between N3 fatty acids and PFC function is also observed in children. Using a small sample (n=33) of 8-10 year old boys, McNamara et al. (2010) found that blood levels of DHA were related to PFC activation during blocks of sustained attention in regions including dorsolateral PFC (dlPFC). Further, eight weeks of DHA supplementation resulted in increased activation in the dlPFC, insula, and cingulate gyrus (McNamara et al., 2010). In a related analysis of MR spectroscopy data, boys with high blood DHA levels (defined by median split) had a greater concentration of metabolites such as myo-inositol and N-acetylaspartate in the ACC but not the dlPFC (McNamara et al., 2013). Finally, while there were no observable differences in ACC activation during sustained attention blocks, boys with low blood DHA displayed reduced functional connectivity between the right ACC and regions including the right ventrolateral PFC, middle occipital, inferior frontal gyrus,

and superior parietal lobule during sustained attention compared to boys with high blood DHA (Almeida, Jandacek, Weber, & McNamara, 2016). These studies suggest that N3 fatty acids may be important for prefrontal structure and function, particularly of midline cortical structures.

The aggressive rate of brain growth consisting of progressive and regressive changes in brain structure over development require continual supply of long chain N3 fatty acids to support developmental processes. Given the concentration of DHA in the PFC and DHA's involvement in a number of aspects of neuronal structure and function, insufficient DHA during adolescence, a stage of development during which the PFC undergoes profound changes, may have negative consequences on core functions of the PFC such as impulse control.

Development of impulse control

Executive functions are higher order cognitive processes, generally categorized as *cognitive flexibility, working memory and inhibition* (Diamond, 2013). Inhibition, in addition to referring to *interference control* (i.e., cognitive inhibition and selective attention) also encompasses *behavioral inhibition* (i.e., self-control of behavior, emotions, drives or impulses), which refers to the ability to delay or withhold an impulse, habit or prepotent/dominant response in the service of a goal, particularly in the presence of competing responses (Diamond, 2013). As a critical ingredient in goal-directed behavior, the ability to inhibit habitual or inappropriate responses is central to long-term outcomes over the lifespan. Children who had better inhibitory control are more likely, as teenagers, to be resilient to frustration/stress, make better academic gains as well as fewer risky decisions, and as adults, have better health, be happier and earn more money (Mischel, Shoda, & Rodriguez, 1989; Moffitt et al., 2011; Tangney, Baumeister, & Boone, 2004).

Though the underlying process is the same throughout development, the expression of self-regulation and inhibitory control changes across development (Petersen, Hoyniak, McQuillan, Bates, & Staples, 2016) . Initially inhibitory control emerges by the end of the first year of life and rapidly improves. For example, when instructed to withhold a dominant response during a Go/No-Go task, which requires inhibition of a prepotent response, 3-4 year old children cannot inhibit their response, despite understanding the instructions; children only begin to succeed later (beginning at 4.5 years of age) (as reviewed by Diamond 2002). Indeed, multiple groups have shown that inhibitory control improves dramatically over the course of childhood and adolescence through young adulthood (Diamond, 2002; Luna, Padmanabhan, & O'Hearn, 2010).

Measuring response inhibition. One way to assess inhibitory control is though survey either of self-report or third-party observations. The Behavior Rating Inventory of Executive Function is an 86-item psychometrically validated, standardized questionnaire (Gioia, Isquith, Guy, & Kenworthy, 2000) used to assess facets of executive abilities in youth ages 5-18. Caregivers are instructed to rate the frequency that their child's behaviors were problematic: "never", "sometimes", or "often". Since caregivers are instructed to consider the child's general behavior in real world situations over the 6 months prior to their visit, this method is considered naturalistic assessment of general inhibitory control temperament (Petersen et al., 2016). The questionnaire yields 8 non-overlapping scales concerning a child's ability to control impulses (*Inhibit*), tolerate change and switch attention (*Shift*), regulate emotional responses (*Emotional Control*), independently start or generate problem solving (*Initiate*), hold information when generating plans (*Working Memory*), anticipate future events/grasp main points (*Plan/Organize*), put work/belongings in order (*Organization of Materials*), and ability to track own

performance/impact on others (*Monitor*). Subscale scores may also be grouped into two sub-composite scales, a *Behavior Regulation* Scale and a Metacognition scale, as well as one overall score. Higher scores suggest higher level of dysfunctional behavior. This assessment has normative values for age and sex (t-scores and percentiles) and higher scores are indicative of more problematic behavior. The Inhibit subscale, reflecting the ability to control impulses or stop behavior, was of interest to the current studies.

Structured laboratory tasks of response inhibition generally assess one of two types of control: i*mpulsive choice* (assess the capacity to delay a prepotent response) or *impulsive action* (assessing the capacity to withhold a prepotent response). Among tasks that assess *impulsive action*, the Go/No-Go task is a commonly used task that requires the participant to be vigilant while responding to stimuli to ensure they can inhibit a response to the target when it appears. Go/No-Go tasks may be simple or complex in their design, the latter of which involves more than one contingency to maintain in working memory (i.e., "conflict" paradigms requiring not only inhibiting a prepotent response but also providing a conflicting correct response) (Petersen et al., 2016; Simmonds, Pekar, & Mostofsky, 2008). The present work used a simple response inhibition task with minimal working memory demands (i.e., only a single cue signaling to inhibit the prepotent response) in which a series of 30 letters is presented for 200 ms each, followed by a 1300 ms fixation. Subjects are instructed to press the button in their right hand as quickly as possible for every letter (“Go” trials) except the letter ‘Q’ (“No-Go” trials). A total of 150 trials are presented in this design of which 18% are No-Go trials.

While there are a number of ways to look at performance on a Go/No-Go task involving Signal Detection Theory metrics (e.g., bias toward responding a particular way and sensitivity to target stimulus) (Stanislaw & Todorov, 1999), the most commonplace metrics are the rate of correct Go's (hit rate; to measure task compliance), rate of missed Go's (omission errors; a measure of inattention), rate of Incorrect No-Go's (commission errors, or false alarms; a measure of ability to inhibit a prepotent response) (Riccio, Reynolds, Lowe, & Moore, 2002) and response time on Go trials (reaction time; a measure of psychomotor processing speed and efficiency of response preparation/selection) (Simmonds et al., 2008)).

Response inhibition in Go/No-Go tasks recruits a distributed network including right inferior frontal gyrus, caudate, and the ACC (Menon, Adleman, White, Glover, & Reiss, 2001; Ogg et al., 2008; Rubia et al., 2006; Simmonds et al., 2008; Tana, Montin, Cerutti, & Bianchi, 2010). The ACC, ostensibly engaged to enact reactive control upon detection of the target stimulus, demonstrates a protracted age-related increase in activity over adolescence during situations requiring error vigilance (Rubia, Smith, Taylor, & Brammer, 2007) – interpreted as a compensatory reliance on this reactive mechanism until the dorsolateral PFC-based proactive control comes online (Andrews-Hanna et al., 2011). As the medial PFC is one of the last regions to mature functionally (Ordaz, Foran, Velanova, & Luna, 2013) and structurally (Shaw et al., 2008), its protracted developmental window may heighten its vulnerability to variations in long-chain omega-3 fatty acids.

Omega-3 fatty acids and response inhibition. There is some evidence to support the association between omega-3 fatty acids and impulse control. Animal models of DHA depletion producing low levels of DHA in the frontal cortex produce behaviors consistent with clinical presentation of

attention deficit hyperactivity disorder (ADHD) (Levant, Zarcone, & Fowler, 2010; Vancassel et al., 2007). Evidence in humans, however, is mixed likely owing to variations in methodology. Among the positive associations are reports of children with ADHD having lower levels of DHA in the blood (Burgess, Stevens, Zhang, & Peck, 2000; Stevens et al., 1995); adults without ADHD reporting higher levels of impulsivity with lower blood levels of omega-3 fatty acids (Conklin, Harris, et al., 2007); and omega-3 supplementation improving both accuracy and reaction time on a Go/No-Go task (Fontani et al., 2005). However, large cohort studies have mixed results regarding the relationship between fish or long chain N3 intake and behavioral disorders (e.g., externalizing behavior) (Gispert-llaurado et al., 2016; Waylen, Ford, Goodman, Samara, & Wolke, 2009). Overall, the mixed findings concerning a link between omega-3 fatty acids and impulse control warrant additional investigation.

Investigational overview

In the current work, we use three methods of assessing long chain N3 fatty acids to assess the impact on adolescent inhibitory control at two critical windows of development. Briefly, to assess exposure to long chain fatty acids during infancy (Study 1), we used maternal report of infant feeding practices. To assess exposure to long chain fatty acids during adolescence we used both dietary report (Study 2) and analysis of fatty acids in whole blood (Study 3). Inhibitory control was measured using both a parent-reported behavioral assessment and a laboratory assessment of impulsive action using the Go/No-Go task. Finally, a combination of neuroimaging techniques was used to assess the impact of N3 fatty acids on brain development. First, functional MRI was conducted while the adolescents performed the Go/No-Go task. Second, voxel-based morphometry (VBM) was used to assess the degree to which N3 fatty acids are related to gray-

matter volume. Together, these studies highlight the need for this essential nutrient during these two critical windows.

CHAPTER II: Breastfeeding and development of impulse control and PFC in adolescence (Study 1)

Introduction

The postnatal period is a time of rapid brain growth and development. A human infant's brain size will grow to 83% of its adult mass by 2 years of age, with the majority of this growth happening in the first 12 months of life (Knickmeyer et al., 2008). During this period, gray matter volume expands due to the growth of cell size, proliferation of supporting glia, arborization of dendritic processes and rapid synaptogenesis (Huttenlocher & Dabholkar, 1997; Lenroot & Giedd, 2006). The building blocks for this rapid cortical growth come from the infant's diet. Among the myriad of known qualities in human breastmilk, the profile of fatty acids – particularly the long chain omega-3 fatty acid, DHA – are increasingly being identified as relevant for healthy infant brain development (Ballard & Morrow, 2013; Martin, Ling, & Blackburn, 2016).

Arguably one of the most important components for brain development is the content of long-chain omega-3 fatty acids, including arachidonic acid and docosahexaenoic acid (DHA), both of which are enriched in neural tissue membranes (Diau et al., 2005). In particular, DHA plays a key role in the biophysical properties of the neuronal phospholipid membrane, promoting membrane fluidity and facilitating structural transitions of proteins embedded in membranes (Stillwell & Wassall, 2003). DHA is recognized for its role in the resolution of inflammation and as a second messenger in signal transduction pathways (Alessandri et al., 2005; McNamara & Carlson, 2006; Mitchell et al., 1998). In addition, DHA plays a critical role in supporting developmental processes. DHA enhances neuronal size (Ahmad et al., 2002), promotes formation of synapses and dendritic spines (Wurtman et al., 2009), and facilitates cortical pruning (de Velasco et al., 2012). DHA rapidly accumulates in gray matter during development and continues until young adulthood

(Carver et al., 2001). Though the essential fatty acid content of breastmilk varies with maternal diet (Liu et al., 2016), owing in part due to its biophysical properties (e.g., larger size lipid droplets enveloped in triple layer membrane versus small-size protein-coated droplets found in formula) (Schipper et al., 2016), breastmilk in general results in greater cortical DHA content than either formula with or without DHA (Diau et al., 2005). DHA accrual in the infant cortex is proportional to breastfeeding duration (Makrides et al., 1994) and duration of breastfeeding appears to be a driver of the observed effect on cognition (Anderson, Johnstone, & Remley, 1999).

While the evidence regarding whether breastfeeding provides a benefit to cognitive development is mixed (Walfisch, Sermer, Cressman, & Koren, 2013), the studies demonstrating benefits of breastmilk on cognition and intelligence report benefits in both the short and long term. As early as 7-10 days after birth, exclusively breastfed infants are more alert and are better able to attend to stimuli compared to peers exclusively fed formula (Hart, Boylan, Carroll, Musick, & Lampe, 2003). After accounting for confounding variables including maternal education and age, breastfeeding duration has also been associated with verbal IQ scores at 6 years old (Oddy et al., 2003), higher standardized academic test scores at 10 years old (Oddy et al., 2010), multiple measures of scholastic abilities including teacher ratings of school performance (8-12 years) (Horwood & Fergusson, 1998), cognitive performance in 11-16 year olds (Greene, Lucas, Livingstone, Harland, & Baker, 1995), and psychomotor speed in males at 17 years old (Nyaradi, Oddy, Hickling, Li, & Foster, 2015). The benefit of breastmilk during infancy has even been extended to intelligence at 27 years of age (Mortensen, Michaelsen, Sanders, & Reinisch, 2002) and earning potential at 30 years of age (Victora et al., 2017). These associations are supported by a large randomized controlled trial which demonstrating causal results: that longer duration of

any breastfeeding in the first year and higher rates of exclusive breastfeeding at 3 months produces higher IQ and teacher ratings of scholastic ability at 6.5 years of age (Kramer et al., 2008). These associations and causal finding speak to the impact of breastfeeding duration on cognitive development in both the short and long term.

In addition to the benefits on cognitive development, longer duration of breastmilk exposure has also been associated with fewer behavioral disorders. In a retrospective analysis of a large sample of children with attention deficit hyperactivity disorder (ADHD) and controls, shorter duration of breastfeeding was found to be a significant predictor of child ADHD diagnosis (Stadler, Musser, Holton, Shannon, & Nigg, 2016). Compared to either their typically developing siblings or controls, children with ADHD were less likely to have been breastfed in the first 12 months of life (Mimouni-Bloch et al., 2013). The relationship between breastfeeding and behavior persists among typically developing individuals as well. Breastfeeding has been associated with reduced incidence of both internalizing behavioral problems in at-risk children (Krabbendam, Bakker, Hornstra, & van Os, 2007) and externalizing behavioral problems across childhood and adolescence (Oddy et al., 2010), with each additional month of breastfeeding showing an improvement in behavioral scores between ages 2-14 years (Oddy et al., 2010).

Mounting evidence suggests that breastmilk is not only beneficial to long-term cognition but also to brain structure and function. In infants born earlier than 37 weeks of gestation (i.e., abbreviated exposure to the 3rd-trimester rise in DHA cortical deposition (Martínez & Mougan, 1998)), greater breastmilk intake has been related to greater cognitive/motor functioning and larger subcortical gray matter volume at 7 years of age (Belfort et al., 2016), as well as greater verbal intelligence

scores, total brain volume and white matter volume at 15 years (Isaacs et al., 2010). Only a few studies to date have examined breastfeeding in relation to long-term brain and behavior outcomes in infants born at term (≥37 weeks of gestation), however. Deoni and colleagues (2013) found that, in children about 3.5 years old, exclusive breastfeeding (≥ 3 months; mean approximately 13.5 months) was associated with greater receptive language scores and myelin development of both early and late-maturing white matter structures associated with higher order cognitive functions, including frontal cortex (Deoni et al., 2013). Preliminary work by McNamara et al. (2015) indicates longer breastfeeding duration may be associated with indices of cortical functional integrity in the dorsolateral PFC and anterior cingulate cortex of 9 year old boys (Mcnamara, Vannest, & Valentine, 2015). Kafouri et al. report that duration of exclusive breastfeeding predicted cortical thickness in *a priori* intelligence-associated regions, including the superior and inferior parietal lobe, as well as full scale IQ, in 15 year olds (Kafouri et al., 2013). Finally, Ou et al. (2016) report that 8-year-old children who were predominantly breastfed (mean 12.6 months) displayed greater gray matter volume in a whole brain volumetric analysis in left inferior temporal and left superior parietal lobes compared with predominantly formula fed children. Additionally, breastfed children displayed greater activation during perceptual and visual language tasks, which was interpreted as better function (Ou et al., 2016).

Despite the observed relationships between breastmilk during infancy and improved behavioral control during childhood and adolescence, as well as mounting evidence of a link between an early diet of breastmilk and subsequent brain development, the relationship between infant diet and the neural bases of inhibitory control has yet to be studied. Response inhibition, an individual's ability to inhibit pre-potent actions, is an executive function that is integral to attention, self/emotional-

regulation and goal-directed behavior (Diamond, 2013). Response inhibition improves from childhood, through adolescence, and into young adulthood (Luna et al., 2010) in concert with protracted PFC structural/functional development (Casey, Giedd, & Thomas, 2000). Interestingly, the PFC undergoes an aggressive period of gray matter expansion in the first 12 months of life (Gilmore et al., 2012). Given that DHA is involved in developmental processes supporting gray matter expansion, and breastmilk is a rich source of DHA, the aim of the current study was to explore whether duration of breastfeeding during the first year of life – a proxy for cortical DHA accrual – is related to prefrontal function and response inhibition.

To our knowledge, only one group has previously reported on task-based inhibitory control, an executive function largely attributed to the prefrontal cortex, in relation to breastfeeding. Using task based assessment of response inhibition (Continuous Performance Task), Forns and colleagues (2012) report that, after adjusting for confounding variables including maternal intelligence, duration of any breastfeeding (mean 4.13 months) during infancy predicted better response inhibition (commission errors, also known as false alarms) in 11 year-olds (Forns et al., 2012). These findings suggest that behavioral development, including development of response inhibition, may benefit from longer durations of breastfeeding, but the relationship with prefrontal function is still unclear.

To address our aim, we used duration of breastmilk consumption during infancy as a proxy for exposure to highly bioavailable DHA (Diau et al., 2005). Additionally, since DHA accumulates in the prefrontal cortex from the perinatal period to roughly 18 years of age, we focused our investigation on activation differences within the prefrontal cortex. We hypothesized that

adolescents who were exposed to breastmilk for longer would better able to inhibit a prepotent responses and have faster psychomotor speed on an fMRI Go/No-Go task as well and exhibit less impulsive behaviors as rated by a caregiver (BRIEF). Additionally, given the trophic influence of DHA, we hypothesized that adolescents who had longer duration of breastmilk exposure would display differential prefrontal function (i.e., activation) than their same age peers who were exposed to breastmilk for shorter duration when successfully exercising inhibitory control.

Methods

Participants were recruited as a part of a longitudinal neuroimaging study, the Adolescent Development Study, aimed at identifying neurobiological precursors and consequences of early drug and alcohol initiation and escalation. Full details of the methods are described in detail elsewhere (Fishbein, Rose, Darcey, Belcher, & VanMeter, 2016). In brief, adolescents in a narrow age range (11-13 years of age) were recruited to minimize age-related differences in neurodevelopment (N=135, 54% female). Main exclusions included prior substance use, left-handedness, conditions rendering MRI unsafe, history of head trauma, and neurodevelopmental disorders. Participants taking psychostimulant (centrally acting) medications were permitted to enroll in the study if study visits could be scheduled during normally occurring medication holidays. Demographic, neurocognitive, drug and alcohol use surveys, and imaging assessments were conducted at baseline and repeated 18- and 36-months later. Georgetown Institutional Review Board approved the study and adolescents and their caregivers provided assent and consent prior to all data collection.

Family socioeconomic status index (SES). SES was calculated using a z-score of the average z-scores of total annual household income prior to taxes (ranging from less than $5,000 to greater than or equal to $200,000) and mean parental cumulative years of education (method adapted from (Manuck, Phillips, Gianaros, Flory, & Muldoon, 2010)).

Behavior Rating Inventory of Executive Function (BRIEF). The BRIEF (Gioia et al., 2000) is an 86-item psychometrically validated questionnaire to assess facets of executive abilities and was administered to the caregiver who came with the participant for the study visits. Each item asks the caregiver to rate the child's behaviors as "never", "sometimes", or "often" a problem. The questionnaire yields 8 non-overlapping scales, of which the Inhibit subscale, reflecting the ability to control impulses or stop behavior, was of interest to the current study. Higher scores suggest higher level of dysfunctional behavior. Normative values for age and sex (t-scores) are reported.

Assessment of infant diet and perinatal factors. Biological mothers of participants were invited to complete a questionnaire assessing information about their pregnancy with regards to their enrolled adolescent. Of 135 adolescents in the main study, 129 mothers were approached and consented to provide responses. Infant feeding practices were retrospectively assessed using a questionnaire modified with permission from that of the Growing Up Today Study (Gillman et al., 2001). Mothers were asked to indicate information regarding infant feeding practices, including predominance of breastmilk or formula in the six months after the infant's birth on a 5-point scale ranging from (1) breastmilk only to (5) formula only. Duration of breastmilk exposure was assessed (zero; less than 1 month, 1 to 3 months; 4-6 months; 7-9 months; greater than 9 months). Age during infancy at which the adolescent was introduced to solids, cow's milk, and formula (if

any, including type) was also assessed. Mothers also indicated their age at time of delivery, child's birth weight and length, whether they used alcohol, tobacco, had obstetric complications (yes/no) and whether their child was delivered prior to their due date and, if so, number of weeks premature.

Intelligence and physical development. Intelligence was assessed using Kauffman Brief Intelligence Questionnaire; K-BIT (Kaufman & Kaufman, 1990). In order to account for potential differences in current physical stature of adolescents, body mass index (BMI) (kg/m^2) sex and age-specific z-scores and percentiles were calculated using weight measured with a digital scale (Health-O-Meter Professional 394KLX) and height measured via a stadiometer (SECA 216 wall-mount Mechanical measuring rod; triplicate measures within 0.5 cm, averaged) applied to 2000 CDC Growth Charts (Kuczmarski et al., 2000). Additionally, pubertal development was assessed via self-report using the Pubertal Development Scale (Carskadon & Acebo, 1993; Petersen, Crockett, Richards, & Boxer, 1988) consisting of a series of questions to evaluate the degree to which a specific physical change such as skin/voice changes, growth spurt, breast development, and facial hair has occurred. Responses are averaged and resulting scores range from (1) prepubertal to (4) postpubertal.

Go/No-Go fMRI task. Adolescents completed a simple Go/No-Go functional MRI task, which elicits neuronal activity related to response inhibition (Menon et al., 2001; Simmonds et al., 2008). This task uses a hybrid design with alternating blocks of event-related Go/No-Go (45 seconds) and Fixation (12-16 seconds) each repeated 5 times. During the Go/No-Go blocks, a series of 30 letters is presented for 200 ms each, followed by a 1300 ms fixation. Subjects are instructed to press the button in their right hand as quickly as possible for every letter ("Go" trials) except the letter 'Q'

("No-Go" trials). A total of 150 trials are presented in this design of which 18% are No-Go trials. Trials were included in analyses if response time (processing speed) was at least 150 ms, indicative of an intentional, rather than anticipatory response. Ability to inhibit response for the No-Go trials was used as an indication of impulse control/ability to inhibit a prepotent response (Riccio et al., 2002). The task was implemented in E-prime and completed during MR imaging. Calculated metrics of interest include *successful inhibition rate* (Correct No-Go/ Total No-Go), *false alarms* (Incorrect No-Go/Total No-Go) (reflecting impulsivity), and reaction time to Go trials (reflecting processing speed). *Hit rate* (Correct Go/Total Go) was used to determine whether the participant was adequately engaged with the task (at least 70% of Go trials with response).

MRI data acquisition. MRI scans were performed on a 3T scanner (Siemens Tim Trio) at the Center for Functional and Molecular Imaging, Georgetown University (Washington, DC) using a 12-channel radio frequency head coil. For anatomical localization and spatial normalization a structural MRI acquisition was collected using a 3D T1-weighted MPRAGE image with the following parameters: TR/TE=1900/2.52 ms, TI=900 ms, 176 slices, slice resolution= 1.0 mm^3. FMRI acquisition used T2*-weighted gradient-echo planar imaging (EPI). The blood oxygenation level dependent (BOLD) functional MRI acquisition parameters were: TR/TE=2500/30 ms, 90^o flip angle, in-plane resolution 3.0 mm^2, 47 slices, slice thickness=3.0 mm. Data for the Go/No-Go task were collected in one run.

fMRI preprocessing. Image processing and statistical analysis was carried out using the SPM8 software (http://www.fil.ion.ucl.ac.uk/spm) including correction for sequential slice timing and realignment of all the images to mean fMRI image to correct for head motion. Realigned images

were then co-registered with the anatomical MPRAGE. The MPRAGE was then segmented and transformed into the Montreal Neurological Institute (MNI) standard stereotactic space using affine regularization. Lastly, the affine regularization parameters were applied to normalize the fMRI images into MNI space, and the data were spatially smoothed using a Gaussian kernel of 6 mm^3 full-width half maximum (FWHM). A scrubbing algorithm utilizing framewise displacement (FD) was used to assess participant movement during the fMRI scans (Power, Barnes, Snyder, Schlaggar, & Petersen, 2012). Participants were excluded from analyses if they had more than 1 mm framewise displacement in over 20% of their volumes during the fMRI scans.

First-level analysis. Regressors for the first-level analysis included vectors coding for correct Go and No-Go and incorrect Go and No-Go trials as well as six regressors of no interest from the motion correction parameters to remove any remaining signal changes related to head movement. The regressors were convolved with the canonical hemodynamic response function and a 128-s temporal high-pass filter was applied to the data to exclude low-frequency artifacts such as MRI signal drift. The contrast of interest was successful inhibitions to examine activation associated with successful control of behavior (i.e., Correct No-Go > Incorrect No-Go).

fMRI statistical analysis. First-level contrasts were entered into a 2-sample t-test statistical model comparing LD and SD groups. Given the relative importance of DHA to PFC function, second-level group analyses were constrained to a specific search region with an explicit mask of bilateral frontal lobe gray matter created using Automated Anatomical Labeling atlas via Wake Forest Pick Atlas (Maldjian, Laurienti, Kraft, & Burdette, 2003). To correct for multiple comparisons, 3dClustSim (https://afni.nimh.nih.gov/pub/dist/doc/program_help/3dClustSim.html) was used for

cluster-level correction utilizing a minimum uncorrected threshold of $p < 0.001$ to determine significant clusters ($p< 0.05$). Peak MNI coordinates were extracted from each surviving cluster and anatomical descriptions were identified using the AAL atlas.

Data reduction. Analyses were restricted to participants born at term, without exposure to perinatal complications or in utero exposure to tobacco, and with eligible imaging and behavioral data as follows.

Of 129 questionnaire responses, 4.7% (n=6) indicated smoking during their pregnancy, 7% (n=9) indicated delivery prior to 37 weeks of gestation, and 17.1% (n=21 and n=1 missing response) indicated having been diagnosed with an obstetric complication while pregnant with the enrolled child. Given the potential impact of these conditions on fetal and postnatal development (e.g., smoking (Ernst, Moolchan, & Robinson, 2001), preterm birth (Counsell & Boardman, 2005), obstetric complications (Newby, Myers, & Ducsay, 2015)), only participants whose mothers indicated a negative response to all 3 of these questions were include in this analysis (76.7%; n=99).

The focus of this analysis was duration of exposure to breastmilk, however, as this was not the primary aim of the parent study, responses were largely dichotomous with 50.5% (n=50) indicating feeding breastmilk to their child for greater than 9 months (Figure 3). Those indicating 9 months or less (n=49) had the following distribution: *7-9 months:* 10.1% (n=10), *4-6 months*: 15.2% (n=15), *1-3 months*: 11.1% (n=11), *less than 1 month*: 3% (n=3), *never fed breastmilk*: 10.1% (n=10). Thus, we created two groups for our analyses using duration of breastmilk exposure as a

simple binary variable: greater than 9 month's exposure (50.5%) (*Longer Duration, LD)* versus less than or equal to 9 months exposure including never breastfed (*Shorter Duration, SD)* (49.5%), similar to Oddy et al., (2010), though their dichotomization centered around 6 months exposure.

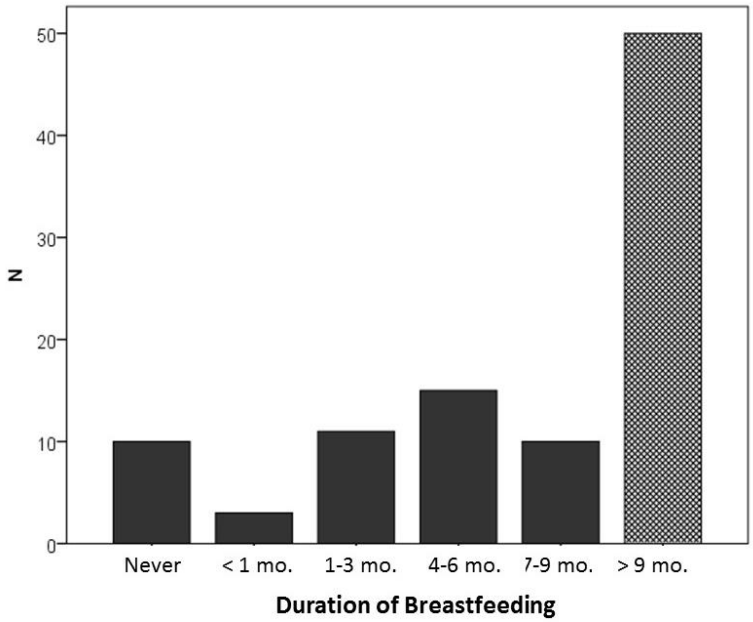

Figure 3. Distribution of duration of breastfeeding responses, after exclusions. Exclusions included obstetric complications, premature birth and tobacco exposure in utero. Bimodal distribution used to create Longer Duration (gray bar) and Shorter Duration (black bars) groups.

Of the n=99 adolescents eligible for inclusion in analysis based on perinatal factors, 19 additional adolescents were excluded for the following reasons: drug and/or alcohol use prior to study enrollment (n=2); neurological condition disclosed after enrollment (Tourette's; n=1); technical issues with task (n=2), MRI collection issues (n=4 missing part of brain); over 1mm movement in over 20% of images (n=8); and insufficiently engaged with task (n=2; hit rate less than 70%). Exclusion reason type did not differ by breastmilk exposure duration group (χ^2=6.441, df 4, p=0.169). Analyses from this point forward pertain to participants with eligible perinatal history

and eligible scan/behavioral data (n=80) with n=45 in the LD group and n=35 in SD group. Participant characteristics indicated in Table 1.

Behavioral analysis. Data analysis was conducted in IBM SPSS Statistics 24 (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). The distributions of dependent variables (DVs) were confirmed via Shapiro-Wilk test and non-parametric analyses were used for DVs where indicated as transformations did not correct distribution (BRIEF Inhibit t-score, Shapiro-Wilk 0.929, df 80, $p<0.001$). For DVs with normal distributions, Successful inhibition rate (correct No-Go), Unsuccessful inhibition rate (False alarms; incorrect No-Go), and Reaction time (correct Go), data were confirmed to be free from outliers.

The two duration groups were similar in many respects on other items related to infant diet including timeline for adding complementary foods to breastmilk/formula (χ^2 (3) =0.690, p=0.875), with most (52%) beginning between 4-6 months. By design, formula use differed between the groups with 94. 3% of the SD group adolescents and 32.7% of the LD group adolescents having been given formula at some point in first 9 months (χ^2 (1) =26.912, $p<0.001$). Formula users in both groups reported similar use of formula types (χ^2 (2) =2.471, p=0.291) with most (81%) reporting cow's milk based formula. As expected, the adolescents in the LD group were from families of greater SES, which has been demonstrated previously (Johnson, Riis, & Noble, 2016). Since SES differed significantly between groups and was significantly, albeit weakly, related to behavioral dependent variables (BRIEF Inhibit *t*-score r_S =-0.359, p=0.001, n=77; Go/No-Go False Alarm rate r= -0.257, p=0.024, n=77; Go/No-Go Successful inhibition rate

r= 0.290, p=0.011, n=77), SES Index was entered as a covariate of no-interest at the group-level functional analysis. Normally distributed behavioral dependent variables were compared using an ANCOVA where SES was included as a covariate. Given that BRIEF Inhibit *t*-score was not normally distributed, the variable was rank transformed to enable entry into ANCOVA. The ANCOVA analysis included SES Index as a covariate to control for group differences in SES.

Table 1. Participant characteristics (Study 1). Adolescents exposed to breastmilk for > 9 months (Longer Duration; LD) compared to those exposed for 9 months or less (Shorter Duration; SD). Groups are similar in many features except for SES Index.

	Longer Duration Group (LD) (>9 months)	**Shorter Duration Group (SD) (≤ 9 months)**	**p**
N	45	35	
Participant age	12.8(0.7)	12.5(0.8)	0.108
Female sex	21(46.7%)	21(60%)	0.236
Race/Ethnicity			0.074
African American	*17.8%*	*42.9%*	
Hispanic/Latino	*8.9%*	*8.6%*	
Caucasian	*66.7%*	*40.0%*	
Other	*6.7%*	*8.6%*	
Pubertal development score	2.3(0.7)	2.1(0.7)	0.306
BMI z-score	0.24(0.87)	0.45(0.72)	0.256
BMI Percentile	*56.6(26.8)*	*64.0(21.6)*	
Intelligence (Kaufman Brief Intelligence Questionnaire)	113.1(17.0)	106.2(13.3)	0.055
Maternal age at childbirth (years)	31.5(5.5)	29.9(6.5)	0.224
Family socioeconomic status index	0.37(0.88)	-0.18(0.98)	0.012
Average Cumulative Education (years)	*17.3(2.9)*	*15.4(2.3)*	
Annual Income Level (median)	*$50,000-$74,000*	*$50,000-$74,000*	
(range)	*≤ $4,999 - >$200,000*	*≤ $4,999 - >$199,999*	
Participant birth weight (grams)	3410.5(418.1)	3327.9(409.4)	0.388
Participant birth length (cm)	51.5(2.5)	51.1(3.3)	0.660

Results

BRIEF Inhibit subscale. SES-adjusted means for SD and LD groups were 59.7 and 60.8 for the Inhibit subscale. Results of the ANCOVA using the rank-transformed Inhibit t-score and controlling for SES Index revealed that there were no significant differences between groups in Inhibit t-score (p=0.729).

Go/No-Go performance. Results of the ANCOVA, controlling for SES, reveal that groups did not differ in false alarm rate (incorrect No-Go's [SES-adjusted means and standard error]; LD: 46.0(2.6), SD: 43.1(3.1), p=0.482) or rate of successful inhibitions (correct No-Go [SES-adjusted means and standard error]; LD: 51.6(2.9), SD: 55.6(3.8), p=0.384). However, adolescents in the LD group displayed significantly faster reaction time to hits (correct Go's) [SES-adjusted means *SD* 329.9(7.8SE) ms, *LD* 308.1(6.7SE) ms; F=4.290, df 1, p=0.042; partial eta squared 0.055] (Figure 4).

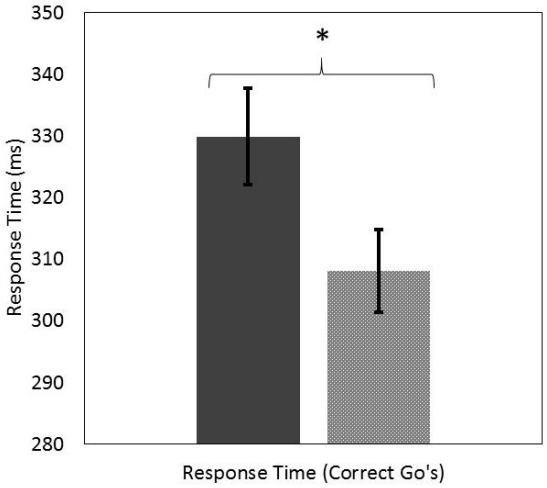

Figure 4. Performance on Go/No-Go task: Response time. Adolescents who were exposed to breastmilk for longer than 9 months (light gray bar) responded significantly faster to Go trials than adolescents exposed to breastmilk for 9 months or less (black bar) (p=0.042, SES-adjusted means).

BOLD activation during successfully inhibited trials. Table 2 and Figure 5 detail activations where the LD group exhibited greater neural activity in the prefrontal cortex in response to successful inhibitions than the shorter duration group, controlling for SES index. Only activation in the ventromedial prefrontal cortex (vmPFC) survived correction for multiple comparisons using AFNI's 3DClustSim (131 voxels minimum). There were no clusters where activation in the shorter duration group exceeded that of the longer duration group (reverse contrast). Beta weights were

extracted from this cluster using MarsBar and graphed for visualization purposes and to examine heterogeneity of activation (Figure 6). Examination of distribution of β regression weights confirmed data were free from outliers.

Table 2. Summary of BOLD activation associated with successful inhibition (Study 1). Contrast of interest: successful inhibitions (correct No-Go> Incorrect No-Go). Socioeconomic status index included as covariate of no-interest. Coordinates are reported in MNI space. Max t indicates max value of the cluster t-statistic. Comparisons at $p<0.05$ corrected using 3DClustSim (* indicates significant cluster).

Region	***x***	***y***	***z***	**Max *t***	**Volume (mm3)**
Medial frontal gyrus (L)*	-12	42	-10	3.85*	131
Inferior frontal gyrus (L)	-28	32	-12	3.69	21
Middle frontal gyrus (L)	-40	52	-6	3.83	75
Inferior frontal gyrus (R) [BA 47]	48	18	0	3.68	24

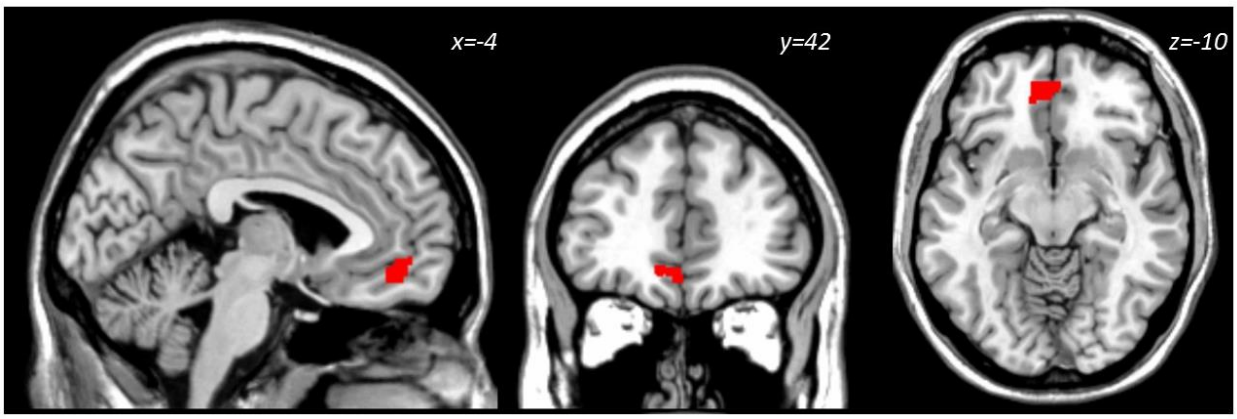

Figure 5. fMRI results (Study 1). Adolescents in the Longer Duration group exhibited greater activation during successful inhibitions in the ventromedial PFC than adolescents in the Shorter Duration group.

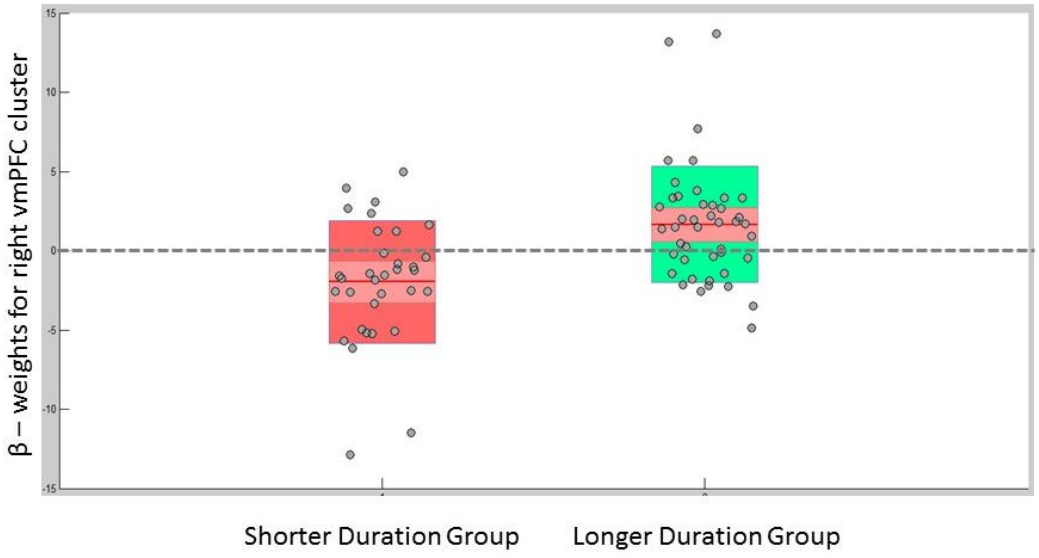

Figure 6. Extracted β-weights for vmPFC cluster by group. vmPFC activation during successfully inhibited trials was significantly greater in the LD group (green bar) than in the SD group. Dashed line at zero line for reference.

Discussion

In order to investigate the impact of duration of breastmilk exposure during infancy on adolescent impulse control and related brain activity, we compared adolescents who were exposed to breastmilk for 9 months or less with those exposed for longer than 9 months based on two measures of inhibitory control: caregiver ratings (BRIEF inhibit subscale) and performance on a Go/No-Go task. After adjustment for disparity in SES Index between groups, contrary to our hypothesis, we found that groups were similar in their inhibitory control, as rated by caregivers and successful inhibitions rate on the Go/No-Go task. However, adolescents breastfed for longer than 9 months displayed significantly faster task processing speed than their counterparts. Finally, adolescents breastfed for longer than 9 months displayed significantly greater activation in the ventromedial PFC during successfully inhibited trials than adolescents breastfed for a shorter duration. To our knowledge, this study represents the first examination of brain differences during successfully inhibited behavior based on breastmilk exposure duration.

The main finding is a difference in vmPFC activation while exercising cognitive control during successfully inhibited trials. While we expected differences in PFC activation between groups, given longer duration of breastmilk intake during infancy allows more time for DHA accrual in PFC facilitating neurodevelopment, the vmPFC is not a canonical region associated with inhibitory control, which include the pre-supplementary motor area, dorsolateral PFC, right inferior frontal gyrus and anterior cingulate (Menon et al., 2001; Simmonds et al., 2008). Aside from its role in affective regulation and social cognition/self-judgements (Delgado et al., 2016), the vmPFC is frequently referred to as locus of the valuation system (Hare, Camerer, & Rangel, 2009). Activity in this region is correlated with subjective goal value of the stimulus in adults, where 'goal value' refers to the "amount of expected reward associated with [the stimulus]"(Hare et al., 2009). Activation in the vmPFC is parametrically modulated by subjective value in valuation and choice paradigms in children and early adolescents (Steinbeis et al., 2014). The vmPFC may not be directly involved in response inhibition but evidence suggests that its functional connectivity with dorsolateral PFC is related to the ability to inhibit a prepotent response (Steinbeis et al., 2014), and vmPFC connectivity is reduced in non-human primates deprived of DHA beginning in the perinatal period (Grayson et al., 2014). Aside from the potential for reduced vmPFC-dlPFC connectivity in SD adolescents, we propose that, given vmPFC's association with valuation and reward (e.g., see (Rolls & Grabenhorst, 2008), and the effort (cost) required to achieve control of behavior (Kool, McGuire, Wang, & Botvinick, 2013), heightened vmPFC activation observed in longer-duration adolescents during successfully inhibited events may reflect a greater 'goal value' intrinsic to an achievement and/or the related satisfaction or positive affective state derived from achieving a goal. Low valuation of self-control success may translate ultimately to increased risk

taking. Lower vmPFC activity was correlated with increased risk taking in abstinent drug abusers (Fishbein et al., 2005) and may provide a mechanism to help explain the small but robust link between short duration of breastfeeding and lifetime alcohol use disorders (Alati, Van Dooren, Najman, Williams, & Clavarino, 2009; Goodwin et al., 1999; Sorensen, Mortensen, Reinisch, & Mednick, 2006).

As a potential mechanism, the essential fatty acid DHA accumulates in the neonatal brain following the general trajectory of development. DHA rapidly accumulates in cortical gray matter from the perinatal period through the second decade of life (Carver et al., 2001) but regional concentration is specific to time point in maturation. Post mortem examination of non-human primates at four weeks indicates that the density of DHA is greatest in regions undergoing development at that time (i.e., primary motor regions and basal ganglia in the non-human primate) (Diau et al., 2005). Between roughly 1 month and 12 months after birth, association cortices, typically considered to be late-developing, display some of the largest increases in gray matter (Gilmore et al., 2012). As speculated by Diau et al., (2005), aggressive cortical growth in regions under development may render them susceptible to DHA insufficiency. While others have described the trajectory of regional neuroanatomical development to involve maturation of primary sensory/motor regions in advance of higher order association cortices (Giedd et al., 1999; Gogtay et al., 2004; Sowell et al., 2004), and others have examined anatomical changes in brains of children under 4 years old (Deoni et al., 2013; Gilmore et al., 2012; Knickmeyer et al., 2008) the temporal dynamics and fine-grained details of neurodevelopmental trajectory within the medial PFC from infancy through young adulthood are as yet unavailable. However, given the emergence of associated behaviors, that is, relatively early emergence of valuation for social and other rewards

and protracted development of impulse control (as reviewed in Casey, Jones, & Hare, 2008), the ventromedial aspect of the PFC may begin development earlier (Fuster, 2003) and thus require more DHA earlier than the dorsal and lateral aspects involved in impulse control. Following this, it is possible that extended breastmilk exposure facilitated the synaptic development of this region and its connections, resulting in the greater levels of activity to successful inhibitions observed in this study. Moreover, the vmPFC receives dopaminergic projections from the ventral tegmental area of the brainstem, and animal models of DHA depletion have been shown to decrease dopaminergic neurons in the ventral tegmental area (Ahmad, Park, Radel, & Levant, 2008; Delion, Chalon, Guilloteau, Besnard, & Durand, 1996), which intriguingly suggests that abbreviated breastmilk (and thus DHA) exposure during infancy may provide insufficient support to tegmental innervation of vmPFC function.

In addition, the neurodevelopmental trajectory of the vmPFC corresponds to heightened responses to incentives during adolescence in comparison to both children and adults resulting in an inverted U-shaped functional trajectory (Van Leijenhorst et al., 2010). Thus, given that omega-3 deficiency delays the refinement in the rodent visual system (de Velasco et al., 2012) it is also possible that adolescents with shorter exposure to DHA in infancy are delayed in their vmPFC functional development in relation to adolescents exposed to breastmilk for longer. This potentially would demonstrate increased vmPFC activation to successful inhibitions, presumably reflecting heightened valuation of self-control, compared to their peers at a later age. While we speculate that the reduced vmPFC activation in the SD group is reflective of the left-side of the inverted U (i.e. closer to child-like function), the possibility remains that the lower vmPFC activity in the SD group is actually on the right-side of this curve (i.e. closer to adult-like function). Future studies

should explore the longitudinal trajectory of vmPFC function to value-associated stimuli in relation to duration of breastmilk exposure during infancy.

Adolescents with longer exposure to breastmilk were quicker in processing speed (correct go reaction time) without a detriment to performance, suggesting quicker psychomotor processing than peers exposed to a shorter duration of breastmilk. Despite faster processing speed, longer duration adolescents were not less accurate (i.e., their false alarm rate was similar to the shorter duration adolescents), so they did not trade speed for accuracy. Faster processing speed with longer duration of breastmilk consumption has been reported by at least one other study. Nyaradi et al. (2015) found that longer duration of breastmilk consumption (longer than 4 months) displayed faster psychomotor speed at 17 years of age in males, though this effect was not found in females. (Nyaradi et al., 2015). Importantly, response times to Go trials become demonstrably faster over development (Williams, Ponesse, Schachar, Logan, & Tannock, 1999). Taken together, our results fit with other findings and suggest that longer duration of breastfeeding may enhance maturation of psychomotor processing speeds through adolescence potentially via support of synaptogenesis and myelination.

In the current study, contrary to our hypothesis, breastfeeding duration groups were similar in their response inhibition capabilities as rated by caregivers or as measured through Go/No-Go task performance. The only other study to our knowledge to examine breastfeeding and performance on Go/No-Go found that duration of any breastfeeding (mean 4.13 months) during infancy predicted better response inhibition (Forns et al., 2012). However, methodological differences may explain the disparity in findings with the current study. Our task parameters were such that 18%

of trials were No-Go's (producing a false alarm rate of 45%) whereas Forns and colleagues had 10% of trials as No-Go, resulting in a stronger pre-potent response requiring inhibition. The increased infrequency of the No-Go trials combined with the slightly younger (age 11-12, versus 12-13 here) and much larger sample (n=393) may have facilitated detection of an effect of breastfeeding on inhibitory control performance. Although an indirect measure of response inhibition, reduced externalizing behaviors as measured by parent report on the Child Behavior Checklist was related to greater breastfeeding duration in a large sample (n=2,366) (Oddy et al., 2010). In light of these studies, that in the current study the groups were similar on both measures suggests that we may have been underpowered to detect an effect on behavior. Given that there are other facets to impulse regulation (e.g., de Wit, 2009), it is also possible that other facets of impulse control may be more substantially impacted, although these were not tested in the current analysis. We speculate that given group differences in vmPFC activation to successful self-control, future studies should examine degree of impulsive choices and vmPFC-dorsolateral PFC connectivity (Hare, Hakimi, & Rangel, 2014; Steinbeis et al., 2014) through variation in temporal discounting behavior in typically developing adolescents.

The prefrontal cortex is under development through the second decade of life. Thus, one might speculate whether lower levels of PFC DHA as a result of shorter duration of breastfeeding could be rectified with diet later on in the developmental window. It is possible that increasing consumption of omega-3 fatty acids during adolescence could reduce differences in reaction time and brain response to successful inhibition. However, animal models suggest that the extent of functional recovery may be dependent on timing of dietary repletion. Examples from the visual system, with a more finite critical window for development, suggest that insufficient DHA supply

during infancy can impair development of neural visual circuits, creating long lasting neurobiological changes that may be difficult to correct post-critical period with later diet (Bourre et al., 1989; Li et al., 2006; Neuringer, 2000). In systems with longer developmental windows, evidence for restoration of function is mixed. Beginning dietary repletion at the equivalent of young adulthood results in partial recovery of brain DHA and the associated spatial learning task performance, though a longer duration of repletion was needed for the effect, 6 vs 2 weeks (Moriguchi & Salem, 2003). On the other hand, beginning dietary repletion at the equivalent of childhood is unable to restore dopaminergic function in the prefrontal cortex and nucleus accumbens in rats previously fed a DHA deficient diet beginning in the perinatal period (Kodas, Vancassel, Lejeune, Guilloteau, & Chalon, 2002). We believe the effects observed in the current study are attributable to early infant diet and not current intake of omega-3 fatty acids (data not shown: groups report similar levels of energy-adjusted omega-3 index (EPA+DHA) via food frequency questionnaire at the time of neuroimaging and behavioral assessments (SD group, n=17; LD group, n=31; Mann-Whitney U=373.5, p=0.994). However, it remains to be determined whether adolescents exposed to breastmilk for shorter duration would derive a psychomotor or vmPFC-neural benefit from further increase in dietary long-chain omega-3 fatty acids.

This study has a number of notable strengths. Study exclusions attempted to account for confounders known to influence perinatal brain development. DHA accrual in the fetal brain is greatest during the 3rd trimester (Martínez & Mougan, 1998) thus we attempted to minimize influence of disparate length of time for DHA accrual in utero by excluding participants born earlier than 37 weeks of gestation. Likewise, infant birth weight and length – a proxy for head circumference and thus brain size at birth – was not significantly different between groups

minimizing differences in later cognitive development attributable to differences in birth weight (Bakker et al., 2003; Richards, Hardy, Kuh, & Wadsworth, 2001). While the persistence of the relationship between breastfeeding duration and cognitive development in low- and middle-income countries supports a causal relationship (reviewed by Prado and Dewey, 2014), maternal IQ and socioeconomic class are factors which confound the breastfeeding-cognitive development relationship in high-income countries (Walfisch et al., 2013). Therefore, we used an SES index, which included both maternal cumulative years of education and household income, as a covariate of no-interest in the imaging analysis and as a covariate in ANCOVAs of the behavioral data. Additionally, participants were all born in 1999-2001, prior to the mandatory DHA-AA fortification requirement of infant formulas in the US in 2002. DHA content of breastmilk varies with DHA content of maternal diet (Liu et al., 2016) and we were unable to account for this variability. However, even formula fortified with DHA is only somewhat effective at restoring DHA content of the neonatal brain (Diau et al., 2005) and the biophysical differences between fat droplets in formula and breastmilk (Schipper et al., 2016) may contribute to this disparity. Moreover, infant formula available prior to 2002 contained ALA/LA precursors to DHA and AA respectively, and due to low conversion rates, blood DHA levels of formula-fed infants fall short of breastfed infants (Clark, Makrides, Neumann, & Gibson, 1992). Since in this study a significantly greater proportion of the SD adolescents consumed formula at some point in the first 9 months than adolescents in the LD group (94.3% vs 32.7%), and cortical accrual of DHA from infant formula is less than that from human milk, adolescent groupings in this study likely reflect a difference in DHA accrual during the post-natal period.

Despite the strengths noted above, we were limited in our ability to examine duration groups with finer resolution given the distribution of responses. The questionnaire assessing duration of infant diet would have been better served to include breastfeeding duration categories beyond 9 months. In addition, our survey relied on recall accuracy. Despite the drawbacks of a retrospective assessment (i.e., recall accuracy), a number of studies indicate that maternal recall of breastfeeding duration is highly correlated with actual documented practices through 17 years after birth (Kark, Troya, Friedlander, Slater, & Stein, 1984; Vobecky, Vobecky, & Froda, 1988) suggesting that it is an acceptable measure for the current study. It is possible that infant temperament influenced the duration of the feeding relationship (i.e., reverse causality as discussed by Kramer, 2010) and may have persisted through development only to be potentially exacerbated by infant diet. Unfortunately, the sample size in the present study was too small to reliably compare the subset of adolescents who were exclusively formula fed from birth to see if their current temperament or behavior differed from those who were weaned earlier than 9 months. Moreover, while breastmilk fed in the absence of bonding (i.e., through nasogastric tube) also produces improvements in IQ (Lucas, Morley, Cole, Lister, & Leeson-Payne, 1992), supporting the influence of nutritive factors, non-nutritive factors associated with breastfeeding may partially account for differences in vmPFC neurodevelopment observed.

In sum, while we did not detect a difference in response inhibition between duration groups in the current study, we were able to detect a significant difference in BOLD response for successful inhibition, which may support internal motivation of adolescents exposed to breastmilk for longer. Despite the American Academy of Pediatrics recommendation to breastfeed exclusively for at least 6 months and in combination with complementary foods for at least 12 months (AAP Section on

Breastfeeding, 2012), only a fraction of American mothers (25.4%) breastfeed for at least 6 months (CDC's Pediatric and Pregnancy Nutrition Surveillance System). These findings add support for policy goals aimed at supporting a woman's ability to breastfeed particularly during the first year of life when brain growth is accelerated (Knickmeyer et al., 2008) as each additional month of breastfeeding may improve behavioral outcomes long term (Oddy et al., 2010). The present results also highlights the need for educational campaigns to increase awareness that breastmilk promotes more than just brain development during infancy, as parents report viewing early infant diet as not highly relevant to their child's behavior later in life (Gage et al., 2014). Furthermore, given that there are certain situations where women cannot or simply do not want to breastfeed, increasing the amount of DHA in formula to meet those levels bioavailable in human milk may be recommended (Salem, 2007).

CHAPTER III: Adolescent diet and development of impulse control and PFC (Study 2)

Introduction

Cortical gray matter thickness and volume follow a nonlinear pattern of development, the timing of which is regionally dependent (Shaw et al., 2008). Gray matter volume generally increases in childhood, peaks in late childhood/early adolescence and declines into young adulthood (Giedd et al., 1999; Gogtay et al., 2004; Lenroot & Giedd, 2006). The gray matter thinning during adolescence reflects synaptic pruning and refinement (Huttenlocher & Dabholkar, 1997), and is associated with improvements in cognitive function and behavior (Casey et al., 2000). Within the PFC, these dynamic developments occur rapidly during the adolescent years and are thought to underlie improvements in executive function such as impulse control (Rubia et al., 2000; Tamm, Menon, & Reiss, 2002). Response inhibition, an individual's ability to inhibit his/her actions, is one such example of an executive function that is integral to developing the ability to delay gratification (Steinbeis et al., 2014), and a cornerstone of long-term achievements (Mischel et al., 1989). Response inhibition improves from childhood, through adolescence, and into young adulthood (Luna et al., 2010). This maturational process is supported by regionally specific changes in activation within the PFC (Ordaz et al., 2013; Rubia et al., 2006) and the protracted development of the PFC may reflect a period of vulnerability to various environmental and biological factors.

Omega-3 fatty acids are a class of long-chain polyunsaturated fats that can only be obtained via diet. Docosahexaenoic acid (DHA), an omega-3 fatty acid found (hereafter, N3) in marine sources, is the only fatty acid of its class relevant to the central nervous system (Stillwell & Wassall, 2003). Of equal importance to the absolute amount of omega-3 in the diet is the ratio of omega-6 fatty

acids to omega-3. These polyunsaturated fats compete for the enzyme systems required for elongation and desaturation (Schmitz & Ecker, 2008). The balance between these enzymatic processes and subsequent incorporation into phospholipids is dependent on the balance of substrate supplied by diet (Lin et al., 2011). Indeed, variation in dietary DHA is reflected in variation of DHA content of phospholipids measured both in the periphery and in the central nervous system (Connor et al., 1990; Moriguchi & Salem, 2003). Within the central nervous system, the distribution of DHA is particularly concentrated in the neuronal membranes of the PFC (Bradbury, 2011), highlighting its importance in a region that is critical for executive function. DHA accrues rapidly in the PFC from the perinatal period though the first 18 years of life, with little increase in PFC DHA content after the second decade of life (Carver et al., 2001) suggesting that the adolescent years are a crucial time to ensure adequate accrual of DHA in the PFC.

DHA plays a key role in the biophysical properties of the neuronal phospholipid membrane. DHA promotes membrane fluidity, which facilitates structural transitions of proteins embedded in membranes (Stillwell & Wassall, 2003). DHA also functions as a second messenger modulating synaptic signal transduction pathways and promotes the resolution of inflammation (Alessandri et al., 2005; McNamara & Carlson, 2006; Mitchell et al., 1998). Further, DHA has been shown to support developmental processes through enhancement of neuronal size (Ahmad et al., 2002), promote synapse and dendritic spine formation (Wurtman et al., 2009), and facilitate cortical pruning (de Velasco et al., 2012). Given the concentration of DHA in the PFC and DHA's involvement in a number of aspects of neuronal structure and function, insufficient DHA during adolescence, a stage of development during which the PFC undergoes profound changes, may have negative consequences.

There is evidence that low levels of omega-3 fatty acids in the diet, and thus low levels in tissue membranes including synaptosomes (Hulbert et al., 2005), have functional consequences related to impulse control. Depleting omega-3 fatty acids from the diet produces behaviors consistent with the clinical picture of Attention Deficit Hyperactivity Disorder (ADHD) (Levant et al., 2010). Children with ADHD display lower levels of DHA in the blood (Burgess et al., 2000; Stevens et al., 1995). In adults without ADHD, blood levels of DHA were inversely related to self-reported impulsivity (Conklin, Harris, et al., 2007) and supplementation improves both accuracy and reaction time, measured as response latency, during a Go/No-Go task (Fontani et al., 2005). Additionally, a large cohort study reveals that children who reported greater consumption of fish exhibited less problem (externalizing) behavior than children reporting lower levels of fish intake (Gispert-llaurado et al., 2016).

Moreover, there is mounting evidence that N3 fatty acids are also associated with PFC structure and function. Blood levels of N3 fatty acids have been found to be related to cortical thickness in the rostral anterior cingulate cortex (ACC) in older adults (Zamroziewicz et al., 2015). Using a voxel-based morphometry and a region of interest analysis, Conklin et al. (2007) reported that ACC volume was positively related to reported long-chain N3 fatty acid intake in adults (Conklin, Gianaros, et al., 2007). The relationship between N3 fatty acids and PFC function is also observed in children. Using a small sample (n=33) of 8-10 year old boys, McNamara et al. (2010) found that blood levels of DHA were related to PFC activation during blocks of sustained attention in regions including dorsolateral PFC (dlPFC). Further, 8 weeks of DHA supplementation resulted in increased activation in the dlPFC, insula, and cingulate gyrus (McNamara et al., 2010). In a

related analysis of MR spectroscopy data, boys with high blood DHA levels (defined by median split) had greater concentration of metabolites such as myo-inositol and N-acetylaspartate in the ACC but not the dlPFC (McNamara et al., 2013). Finally, while there was no observable differences in ACC activation during sustained attention blocks, boys with low blood DHA displayed reduced functional connectivity between the right ACC and regions including the right ventrolateral PFC, middle occipital, inferior frontal gyrus, and superior parietal lobule during sustained attention compared to boys with high blood DHA (Almeida et al., 2016). These studies suggest that N3 fatty acids may be important for prefrontal structure and function, particularly of the anterior cingulate cortex.

Dietary profile of polyunsaturated fat intake by Americans has changed dramatically over the last century, resulting in a net decrease in effective dietary omega-3 fatty acids, defined as the increased proportion of omega-6 relative to omega-3 in the diet and thereby promoting omega-6 metabolism (Blasbalg et al., 2011). Likewise, an analysis of the diets of middle and high school adolescents revealed dietary patterns that were poor in sources of N3 fatty acids (Cutler et al., 2009). Decreased intake of N3 fatty acids among adolescents may be of particular concern given that DHA rapidly accumulates in membranes of PFC gray matter primarily during the first two decades of life (Carver et al., 2001). Low intake during a critical window of DHA accrual in a brain region undergoing major dynamic development has the potential to [negatively?] impact cortical function and related behaviors such as impulse control.

In the present study, we investigated the relationship between intake of long chain omega-3 fatty acids and prefrontal function during impulse control in a cross-sectional sample of typically

developing adolescents. Typically developing early adolescents completed a food frequency questionnaire from which an energy-adjusted Omega-3 Index was computed. This Index was then related to prefrontal activity and task performance during a Go/No-Go task while undergoing fMRI, as well as a caregiver's ratings of their adolescent's ability to inhibit impulses. Given the evidence reviewed above, we expected that greater intake of energy-adjusted Omega-3 Index would facilitate performance. Specifically, we hypothesized that higher levels of Omega-3 Index will be associated with lower PFC activity (i.e., greater efficiency), increased ability to inhibit prepotent responses (i.e., correct No-Go's), and better inhibitory behavior as rated by caregivers.

Methods

Participants were recruited as a part of a longitudinal neuroimaging study, the Adolescent Development Study (ADS), aimed at identifying neurobiological precursors and consequences of early drug and alcohol initiation and escalation. Full details of the methods are described elsewhere (Fishbein et al., 2016). In brief, children in a narrow age range (11-13 years old) were recruited. Main exclusions included prior substance use, left-handedness, conditions rendering MRI unsafe, history of head trauma, and neurodevelopmental disorders. Participants taking psychostimulant (centrally acting) medications were permitted to enroll in the study if study visits could be scheduled during normally occurring medication "holidays". Demographic, neurocognitive, drug and alcohol use surveys, and imaging assessments were conducted at baseline and repeated 18- and then 36-months later. The data reported here were collected during the baseline and first follow-up visits. The Georgetown Institutional Review Board approved all study procedures and adolescents and their caregivers provided assent and consent prior to all data collection.

Participants were assessed for intelligence using a developmentally appropriate battery (Kauffman Brief Intelligence Questionnaire; K-BIT) (Kaufman & Kaufman, 1990). To account for potential differences in physical maturation, adolescents completed the Pubertal Development Scale (Carskadon & Acebo, 1993; Petersen et al., 1988) consisting of a series of questions about progress of physical development, asking respondents to evaluate the degree to which a specific physical change (such as skin/voice changes, growth spurt, breast development, and facial hair) has occurred. During study visits, body mass index (BMI) (kg/m^2) sex and age-specific z-scores and percentiles were calculated using weight measured with a digital scale (Health-O-Meter Professional 394KLX) and height measured via stadiometer (SECA 216 Wall-mount Mechanical measuring rod; triplicate measures within 0.5 cm, averaged) applied to 2000 CDC Growth Charts (Kuczmarski et al., 2000).

Family socioeconomic status. Caregivers of participants were interviewed to collect information on parental education and income to calculate an index of household socioeconomic status (SES index) using a method adapted from Manuck and colleagues (2010). Maternal and paternal cumulative years of education were averaged and a standardized education z-score was calculated for each participant. Reported level of total annual household income prior to taxes (ranging from less than $5,000 to greater than or equal to $200,000) was converted to standardized income z-score for each participant. SES index was computed by averaging standardized values for the income and education variables for each participant, then subsequently re-standardizing to achieve a distribution with a 0-centered mean and standard deviation of 1 for the full sample (N=135).

Food frequency questionnaire. Adolescents completed a paper-based food frequency questionnaire called the Harvard Youth/Adolescent Food Frequency Questionnaire (YAQ) to assess usual diet over the past year. The YAQ is a widely-used, scantron questionnaire validated for ages 9-18, which provides a dietary analysis based on a retrospective assessment of usual frequency and portions of 152 food items consumed over the past 12 months (Rockett et al., 1997). Questionnaires were completed at the end of study visits. Participants were paid $15 via Amazon gift card for completing the 30-minute survey. Nutrient output was compiled using 2011 nutrient tables (Rockett et al., 1997).

While evidence suggests that DHA is the most relevant long chain omega-3 fatty acid, there have been reports of its long chain precursor, eicosapentaenoic acid (EPA) also being related to neural and cognitive outcomes (Bauer et al., 2014; Bauer, Crewther, Pipingas, Sellick, & Crewther, 2013). Thus, our analyses are based on the reported intake of DHA + EPA, known as the Omega-3 Index (Harris & Von Schacky, 2004), adjusted for total energy consumed [(EPA grams + DHA grams) / total calories] (Subar et al., 2001) and then scaled by 1000 calories to represent long chain N3 fatty acid consumption per 1000 calories of intake (hereafter, energy-adjusted N3 Index). Reported intake of DHA mg is adjusted for total energy intake to reduce extraneous variation (diets higher in total calories may also be higher in fats consumed) (Willett, Howe, & Kushi, 1997). A square root transformation was applied to the energy-adjusted N3 Index in order to minimize influence of a few participants reporting high intakes.

Behavior Rating Inventory of Executive Function (BRIEF). An 86-item psychometrically validated questionnaire (Gioia et al., 2000) to assess facets of executive abilities was administered to primary caregivers of participants. Caregivers rated the frequency that their child's behaviors were problematic as "never", "sometimes", or "often". The questionnaire yields 8 non-overlapping scales, of which the Inhibit subscale, reflecting the ability to control impulses or stop behavior, was of interest to the current study. Higher scores suggest higher level of dysfunctional behavior. Normative values for age and sex (t-scores) are reported here. No responses were classified as "inconsistent" and all were included in analysis.

Go/No-Go task. Adolescents completed a simple Go/No-Go functional MRI task, which elicits neuronal activity related to response inhibition (Menon et al., 2001; Simmonds et al., 2008). This task uses a hybrid design with alternating blocks of event-related Go/No-Go (45 seconds) and Fixation (12-16 seconds) each repeated 5 times (total time: 5:02 minutes). During the Go/No-Go blocks, a series of 30 letters is presented for 200 ms each, followed by a 1300 ms fixation. Subjects are instructed to press the button held by their right hand as quickly as possible for every letter ("Go" trials) except the letter 'Q' ("No-go" trials). A total of 150 trials are presented in this design of which 18% are No-go trials. While relative accuracy of performance on the Go/No-Go is an indication of attention, errors of commission (response to the target when the correct action is a withheld response) are an indication of poor response inhibition (Riccio et al., 2002). The task was implemented in E-prime and completed during MR imaging. Responses faster than 150 ms were excluded from behavioral analysis and not modeled in the fMRI analysis (below) to minimize analysis of anticipatory responses (i.e., unlikely to reflect true stimulus processing). Metrics of interest include number of Correct No-Go (reflecting successful response inhibition), number of

Incorrect No-Go (also known as false alarms, reflecting impulsivity), and reaction time to Go trials (reflecting processing speed). Hit rate, or response rate to Correct Go (limited to responses longer than 150 ms), was used to determine whether participants were adequately engaged with the task and a hit rate of at least 70% of Go trials was required for inclusion in the analyses.

MRI data acquisition. MRI scans were performed on a 3 T scanner (Siemens Tim Trio) at the Center for Functional and Molecular Imaging, Georgetown University (Washington, DC) using a 12-channel radio frequency head coil. For anatomical localization and spatial normalization a structural MRI acquisition was collected using a 3D T1-weighted MPRAGE image with the following parameters: TR/TE=1900/2.52 ms, TI=900 ms, 176 slices, slice resolution= 1.0 mm^3. FMRI acquisition used T2*-weighted gradient-echo planar imaging (EPI). The blood oxygenation level dependent (BOLD) functional MRI acquisition parameters were: TR/TE 2500/30 ms, 90^o flip angle, in-plane resolution 3.0 mm^2, 47 slices, slice thickness=3.0 mm. Data for the Go/No-Go task were collected in one run.

fMRI Data preprocessing. Image processing and statistical analysis was carried out using SPM8 (http://www.fil.ion.ucl.ac.uk/spm) including correction for sequential slice timing and realignment of all the images to mean fMRI image to correct for head motion artifacts between images. Realigned images were then co-registered with the anatomical MPRAGE. The MPRAGE was then segmented and transformed into the Montreal Neurological Institute (MNI) standard stereotactic space using affine regularization. Lastly, the affine regularization parameters were applied to normalize the fMRI images into MNI space, and the data were spatially smoothed using a Gaussian kernel of 6 mm^3 full-width half maximum (FWHM). A scrubbing algorithm utilizing framewise

displacement (FD) was used to assess participant movement during the fMRI scans (Power et al., 2012). Participants were excluded from analyses if they had more than 1 mm frame-wise displacement in over 20% of their volumes during the fMRI scans.

First-level analysis. Regressors for the first-level analysis included vectors coding for correct Go and No-Go and incorrect Go and No-Go trials as well as six regressors of no interest from the motion correction parameters to remove any remaining signal changes related to head movement. The fMRI responses were convolved with the canonical hemodynamic response function and a 128-s temporal high-pass filter was applied to the data to exclude low-frequency artifacts such as MRI signal drift. The contrast of interest was successful inhibitions to examine activation associated with cognitive control of behavior (i.e., Correct No-Go > Incorrect No-Go).

fMRI statistical analysis. First level contrasts were entered into a linear regression with the square root transformed energy-adjusted N3 Index. Given the relative importance of DHA to PFC function, second-level group analyses were constrained to a specific search region with an explicit mask of bilateral frontal lobe gray matter, created using AAL, Wake Forest Pick Atlas (Maldjian et al., 2003). To correct for multiple comparisons, 3dClustSim was used for cluster correction (https://afni.nimh.nih.gov/pub/dist/doc/program_help/3dClustSim.html), utilizing a minimum uncorrected cluster-defining threshold of $p < 0.001$ to determine clusters significant at the $p<0.05$ level. Peak MNI coordinates were extracted from each surviving cluster and anatomical descriptions were identified using the AAL atlas.

Data reduction/exclusions. Of 135 participants enrolled in the parent study, 126 completed the food frequency questionnaire. Seven of these responses were excluded from analysis for the following reasons: one participant for implausibly high caloric intake (>13,000 kcal per day (Cutler et al., 2009)); three for leaving excessive numbers of questions blank; three due to factors revealed post-enrollment that may affect neurodevelopment (n=2 reporting drug/alcohol prior to study enrollment, n=1 Tourette's syndrome diagnosis).

Of 119 participants with eligible food frequency questionnaire data, thirteen were excluded from the behavioral analysis (three due to technical data collection errors; ten for hit rates below the 70% threshold). Of 106 participants remaining with eligible diet surveys and Go/No-Go behavior, 18 were excluded on the basis of imaging data (4 due to braces, 2 were missing imaging data, 2 were missing part of the brain image, and 10 for excessive motion). Final analyses were restricted to participants with eligible imaging, behavioral and dietary data (n=88).

Behavioral analysis. Data analysis was conducted in IBM SPSS Statistics 24 (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). The distributions of dependent variables were confirmed via Shapiro-Wilk test and non-parametric analyses were used for DVs with skewed/kurtotic distributions, as indicated by *Spearman's Rho* (r_s). These were: BRIEF Inhibit t-score; Successful inhibition rate (correct No-Go), Unsuccessful inhibition rate (False alarms; incorrect No-Go), and Reaction time (correct Go). The distribution of the independent variable, energy-adjusted N3 Index, was transformed by taking the square root in order to minimize influence of a few participants reporting high intakes. Multiple linear regressions were calculated using rank-transformed data to examine the relative contribution of

energy-adjusted N3 Index and demographic predictors (i.e., age, socioeconomic status index) on behavioral outcomes for significant correlations.

Results

Participant characteristics are presented in Table 3. Energy-adjusted N3 Index was unrelated to age (r=0.189; p=0.077; n=88), SES index z-score (r_s=0.093, p=0.400, n=85), pubertal development score (r=0.053, p=0.622, n=88), BMI z-score (r=-0.080, p=0.457, n=88), KBIT IQ score (r_s=0.154, p=0.160, n=85) and intake was not different between males and females (Mann-Whitney-U test p=0.506). SES index z-score was related to BRIEF Inhibit *t*-score (r_s=-0.312, p=0.004, n=84), but not successful inhibitions (Correct No-Go r_s=0.200, p=0.066, n=85), false alarms (r_s=-0.137, p=0.212, n=85) or Correct Go Reaction Time (r_s=0.111, p=0.313, n=85). Age was unrelated to BRIEF Inhibit *t*-score (r_s=-0.004, p=0.974, n=87), successful inhibitions (Correct No-Go r_s=0.131, p = 0.224, n=88), false alarms (r_s=-0.145, p=0.178, n=88) or Correct Go Reaction Time (r_s=-0.128, p=0.235, n=88).

Table 3. Participant characteristics (Study 2).

	Central Tendency		
	Mean(SD)	**Median**	**Range**
N	88		
Sex (% Females)	45 (51.1%)		
Age	13.3(1.1)	13.3	11.1 – 16.1
Race and Ethnicity			
Caucasian	53.4%		
African American	28.4%		
Hispanic or Latino	9.1%		
Other	9.1%		
Socioeconomic Status index (z-score) [n=85]	0.116(0.962)	0.359	-2.589 – 1.511
Parental education (years, mean)	16.5(2.7)	17	7 – 22
Household income (median)	$50,000–$74,999	$100,000–$149,999	<$5,000->$200,000
Pubertal development	2.4(0.7)	2.4	1 – 3.8
BMI z-score	0.35(0.93)	0.45	-2.2 – 2.2
Percentile	60.2(28.2)	66.8	1.5 – 98.7
Intelligence [n=87]	110.8(14.6)	111	71 – 138
BRIEF Inhibit subscale, Percentile (t-scores analyzed)	59.1(23.6)	59	23 – 99
Go/No-Go Hit Rate (% Correct Go)	94.5(6.6)	97.6	71.5 – 100
Go/No-Go False Alarm Rate (% Incorrect No go)	42.5(19.7)	38.9	3.7 – 77.7
Go/No-Go Successful Inhibition Rate (% Correct No-Go)	55.1(21.5)	55.6	11.1 – 96.3
Go/No-Go Response time, ms (Correct Go)	320.4(50.3)	316.9	245.1 – 453.0
Reported daily energy intake (kcal)	1888 (885)	1743	568 - 5645
N3 Index daily intake (mg)	124(150)	45	0 – 640
N3 Index daily intake, energy adjusted (mg/1000 kcal)	68.1(89.1)	29.3	0 – 437.1

BRIEF inhibit subscale (parental report). Mean and median percentile scores for this sample were 59%, indicating that the current sample is marginally more impulsive than peers of the same gender and age. BRIEF Inhibit subscale *t*-score was inversely related to energy-adjusted N3 Index (r_s=-0.261, p=0.015; n=87) (Figure 7).

A multiple linear regression was calculated using rank-transformed data to predict the Inhibit subscale ratings based on N3 Index and SES Index. Age was not a significant correlate of the Inhibit subscale score and thus was not included in the model. A significant regression equation was found ($F(2, 81)=8.410$, $p<0.001$), with an R^2 of 0.172 (adjusted R Square 0.151). Standardized coefficients (β) for N3 Index were -0.275 (t=-2.698, p=0.008) and for SES index -0.281 (t=-2.758, p=0.007), indicating both SES index and energy adjusted N3 Index were significant predictors of BRIEF Inhibit subscale scores.

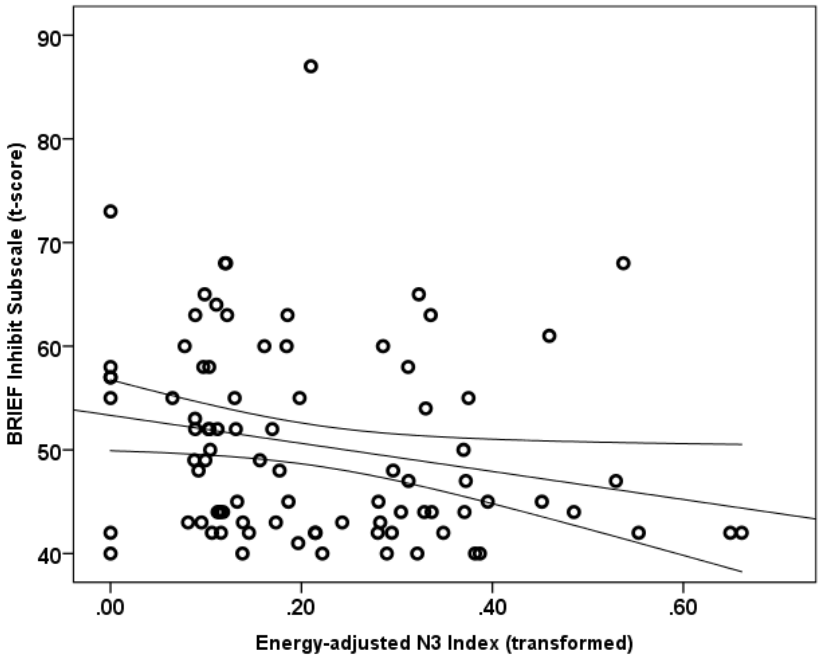

Figure 7. Energy adjusted N3 index intake is inversely related to response inhibition. Ratings by caregivers using BRIEF Inhibit subscale t-score (r_s=-0.261, p=0.015).

Go/No-Go performance. Participants were successful at inhibiting the prepotent response (i.e., Correct No-Go) most of the time (median = 55% of successfully inhibited responses). Successful inhibition rate was not significantly related to energy-adjusted N3 Index (r_s=0.206, p=0.054, n=88).

Median false alarm rate (Incorrect No-Go) was 38.9%. The relationship between false alarm rate and energy-adjusted N3 Index did not reach statistical significance (r_s=-0.196, p=0.067, n=88). Median reaction time (Correct Go's) was 316.9 milliseconds. Reaction time was not significantly related to energy-adjusted N3 Index (r_s= 0.159, p=0.140, n=88).

PFC activity during successful response inhibition. Within the PFC, voxel-wise regression analysis revealed that activation during successful inhibitions (Correct No-Go>Correct Go) was inversely associated with energy-adjusted N3 Index in four clusters (Table 4). Figure 8 shows activation in the dorsal ACC (dACC; 126 voxels; peak MNI 6, 22, 30; max *t* -4.12), which survived correction for multiple comparison at a corrected p<0.05 determined using 3DClustSim (minimum cluster size 101 voxels). There were no clusters where activation was positively correlated with N3 index. Beta weights were extracted from this cluster using MarsBar and plotted against energy-adjusted N3 Index for visualization purposes and to examine heterogeneity of activation (Figure 9). Examination of distribution of β regression weights confirmed data were free from outliers.

Table 4. Summary of BOLD activation associated with successful inhibition (Study 2). MNI Coordinates of local maxima for activation during successful inhibitions (Correct No-Go>Incorrect No-Go) inversely associated with dietary N3 Index intake (cluster defining threshold ke=10, uncorrected p=0.001, df 86). Cluster surviving p=0.05 corrected denoted.

Region	***x***	***y***	***z***	**Max *t***	**Volume (mm3)**
Inferior frontal gyrus, left	-34	18	-2	3.49	12
Insula, left	-36	0	-2	3.51	21
Anterior cingulate	0	26	22	3.51	19
Cingulate gyrus, right [BA 32/24]*	6	22	30	4.12	126*

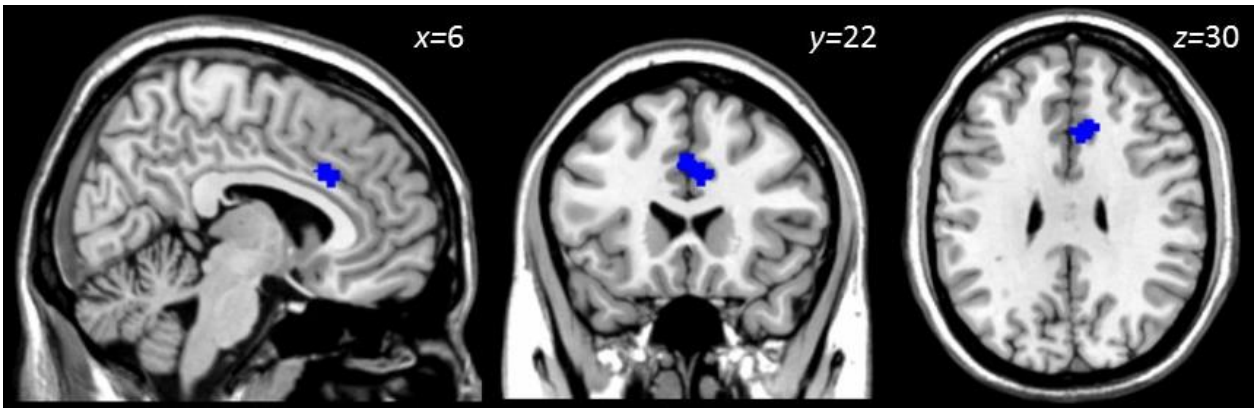

Figure 8. fMRI results (Study 2). Activation during successful inhibitions inversely related to reported N3 index.

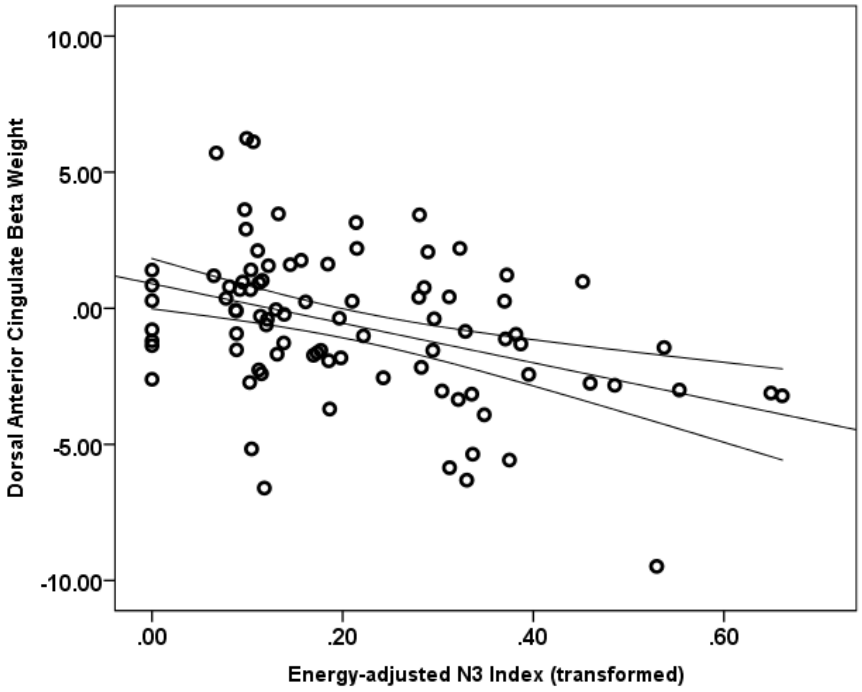

Figure 9. Relationship between dACC activation and energy adjusted N3 Index intake. Distribution of β-weights confirmed to be normal and free of outlier data.

Discussion

Adolescence is a sensitive neurodevelopmental period when major dynamic changes are occurring in the brain, particularly in the PFC which subserves executive functioning such as impulse control. The present study examines the extent to which long chain omega-3 fatty acids in the diet contribute to PFC function during successful impulse control during adolescence. We found that response inhibition as measured by successful inhibitions during a simple Go/No-Go task was not significantly associated with reported energy-adjusted N3 Index. However, caregiver ratings of their child's general ability to inhibit their impulses was significantly inversely related; adolescents reporting lower N3 intake were rated as less able to control impulses. Furthermore, we found a small but significant inverse relationship between reported intake of energy-adjusted N3 Index and activity in the dACC during successful response inhibition, such that adolescents with lower intake of N3 Index exhibit hyper-activation in the dACC to achieve similar behavioral performance as their peers reporting higher Omega-3 Index. To our knowledge, this is the first study to correlate dietary long chain omega-3 fatty acids with impulse control and prefrontal function in a sizable sample of adolescent boys and girls. Together with the extant comparable literature (reviewed below), the current findings suggest that long-chain omega-3 fatty acids may be particularly relevant to function of medial prefrontal cortex, particularly the anterior cingulate cortex.

The PFC exerts top-down control over behavior and the anterior cingulate is purported to be involved in, among many cognitive functions, performance monitoring during situations where the chance of error is high (Carter et al., 1998) or the need for heightened vigilance if there are conflicting responses possible (Brown & Braver, 2005). Indeed, adults engaging in the Go/No-Go task (compared to fixation blocks) display activity in a network of occipital, cerebellar and

frontal regions including the dACC (Ogg et al., 2008). As a specific event within Go/No-Go tasks with simple designs (i.e., tasks with constant stimulus-response associations, precluding involvement of working memory), successful inhibitions recruit a network of regions including the rostral portion of superior medial frontal cortex (Simmonds et al., 2008). Though Simmonds et al. (2008) specifically identified recruitment of a region slightly more dorsal than that reported in the present study (pre-supplementary motor area), differences in contrast and populations may contribute to the slight disparity in activation coordinates (here, Correct No-go> Incorrect No-go in adolescents rather than Correct No-go> baseline in adults). Nevertheless, dACC function during error processing has been found to be critical to improvements in inhibitory control observed over development (Ordaz et al., 2013).

In the present study, consuming lower amounts of long chain omega-3 fatty acids was related to greater activity in the dACC, but unrelated to rate of successful inhibitions. This result may suggest that greater dACC neural activity is required to accomplish the same level of vigilance in the face of conflicting responses as their high omega-3 peers. Omega-3 fatty acids are involved in functions at the cellular level that may represent a putative mechanism for this cortical inefficiency. Interestingly, omega-3 deficiency has been shown to impair cortical glucose transport and utilization (Pifferi et al., 2005; Ximenes da Silva et al., 2002) and a recent study in boys reports indices of metabolic dysfunction in the ACC of boys with low omega-3 status (McNamara et al., 2013). Supplementation with omega-3s resulted in increased frontocortical efficiency in a rodent ADHD model (Liso Navarro et al., 2014). Relatedly, dACC activity attenuates over the course of engagement with the task (Tana et al., 2010). Thus, it is possible that cingulate function in adolescents reporting a lower N3 Index have an inefficient metabolism/protracted habituation to

the effort level required to perform the task, potentially signifying decreased efficiency. Additionally, as DHA restriction leads to the impairment of pruning of superfluous axonal connections (de Velasco et al., 2012), it is possible that adolescents with lower N3 Index have greater activity in the ACC because this network has not yet undergone pruning of superfluous synapses, a concept proposed by Berl, Vaidya, & Gaillard (2006). Thus, inefficiency in metabolism and/or impaired cortical pruning may contribute to the greater ACC activity to compensate for lower intake of long-chain omega-3 fatty acids.

The present results confirm previous reports of a relationship between omega-3 fatty acid status and the anterior cingulate and because both boys and girls were included, these results extend the association to adolescent girls' diet, dACC function and caregiver ratings of impulse control. It is notable that the current study also found a relationship between dietary N3 Index and cingulum activation without an *a priori* cingulate ROI. This confirms previous reports utilizing ROIs and suggests that midline structures may be sensitive to omega-3 levels (Almeida et al., 2016; Conklin, Gianaros, et al., 2007; McNamara et al., 2013). It should be noted that the region of interest for both McNamara et al. (2013) and Almeda et al. (2016) was more rostral and anterior to the cluster observed after a voxel-wise PFC analysis in the current study. Location of ROI and other slight methodological differences may explain why we report a task-related signal difference while Almeida et al. (2016) did not observe a difference in ACC BOLD signal during task blocks requiring response vigilance/inhibition between boys with low and high blood omega-3 levels. The current study distinguished between successful and unsuccessful trials in a hybrid design, rather than a block design, specifically to examine activation associated with successful response inhibition rather than attention *per se*. Additionally, the current study examined older adolescents

(mean 13 years versus 9 years) and included both males and females, both characteristics demonstrated to have influence on developmental status/trajectory of BOLD signal (Ordaz et al., 2013) and neuroanatomical development (Giedd et al., 1999).

Consistent with our hypothesis, caregivers' ratings of adolescent inhibitory control inversely related to their adolescent's self-report of their intake of long chain omega-3 fatty acids. Given that the BRIEF is not subject to adolescents' self-report bias, our finding is notable in that it lends a degree of external validity. Self-report or third-party reports of behavioral regulation have been previously related to omega-3 fatty acid status. Together with the findings of self-reported impulsivity in adults (Conklin, Harris, et al., 2007) and in children (Gispert-llaurado et al., 2016), our results support a role for omega-3 fatty acid status in generalized impulse regulation.

Contrary to our hypothesis, however, response inhibition as measured by ability to inhibit prepotent responses on the Go/No-Go task was unrelated to intake of N3 Index in the diet. Disparity between BRIEF subscales and presumably related task performance has been reported previously (McAuley, Chen, Goos, Schachar, & Crosbie, 2010). It may have been that the measure of response inhibition assessed by a task in a laboratory setting was too specific of a behavior, while caregivers are reporting on general inhibitory control ability in real-world settings over the six months prior to the study visit. As acknowledged by Aron (2011), "the stopping of motor responses, no matter how sophisticated the model, will only be relevant for impulse control some of the time" (Aron, 2011). Consistent with our study, McNamara and colleagues also did not find differences between high and low DHA groups on false alarm rate using a similar task measuring response inhibition and sustained attention in boys (McNamara et al., 2010). Given that others

have observed improved Go/No-Go performance (accuracy and response time) with supplementation in adults (Fontani et al., 2005), and have seen associations between (posterior) cingulate activation and performance during difficult but not easy task conditions (Boespflug, McNamara, Eliassen, Schidler, & Krikorian, 2016), it is possible that the intake levels reported in this study are too low and/or the current task is too easy (median hit rate 97.6%; median successful response inhibition rate 55%) to detect associations with performance. Additionally, given that only a small, but significant, portion of the variance in attention (processing speed and omission errors) was attributable to Omega-3 Index in a large (n=266) cohort of adolescents, it is possible that the current sample size (n=88) was underpowered to detect behavioral associations with task metrics (van der Wurff et al., 2016).

Some strengths of the present study include the inclusion of both male and female adolescents and a relatively restricted age range to minimize the influence of brain development. One of the main limitations of the current study is that the food frequency questionnaire is dependent on recall of usual diet. Other methods (24-hour recall, quantitative 7-food records) may increase accuracy, however are often difficult to implement with large samples and limited resources. The food frequency questionnaire is the preferable method for measuring intake of nutrients with very high day-to-day variability, and its output represents habitual intake of the respondent. It is worth noting that reported intake has been previously shown to correlate well with serum biomarkers (Kuratko & Salem, 2009; Sun, Ma, Campos, Hankinson, & Hu, 2007; Vandevijvere et al., 2012); however, the food frequency questionnaire estimates chronic/usual nutrient intake over previous 12 months and not absolute values reliably (Subar et al., 2001). Nonetheless, the present results demonstrate the feasibility of assessing diet in a large sample neuroimaging cohort.

Summary and Implications. Dietary N3 Index intake was significantly inversely related to both activity in the dACC during successfully inhibited trials and to a measure of impulsivity, albeit only accounting for 6.8% of the variance in behavior. It is unlikely that dietary nutrients will exert substantial direct influence over behavior or brain function. However, the degree of contribution found in the current study is in line with another known environmental factor, SES. Results of a multiple linear regression indicate that energy-adjusted N3 Index accounts for a similar amount of unique variance in caregivers' ratings of adolescent inhibitory control as does socioeconomic status, an established correlate of brain structure and function (Johnson et al., 2016).

Two factors cause concern that adolescent brains may be receiving insufficient levels of omega-3: decreased effective intake and increased requirements. Effective intake of omega-3 fatty acids, compared to omega-6 fatty acids, a competitive substrate for metabolism, has been on the decline in the U.S. over the past century (Blasbalg et al., 2011). A review of reported global intake of dietary DHA found that 12-19 year-olds consume 30-50 mg/day in the U.S. (Flock, Harris, & Kris-Etherton, 2013), which is comparable to our sample (median reported daily intake DHA 30mg, data not shown). While there are no dietary reference intakes for long chain omega-3 fatty acids (EPA and DHA), the consensus among experts is that this level is far below that desired for optimal health (Flock et al., 2013). Compounding the issue of relative consumption is the fact that the adolescent period of rapid PFC maturation may represent a critical period where omega-3 needs are elevated compared to the adult brain. That the central nervous system's DHA requirements are heightened during rapid growth of specific tissues is a general theme in development. For example, early in postnatal development the retina has increased requirements for DHA. Deficiency of DHA in this period of development reliably results in poor visual acuity (Agostoni, 2008). It is

conceivable that because PFC development occurs over a longer period, the PFC may be comparably sensitive to insufficiency of DHA during adolescence. Post mortem examinations indicate that DHA content increases significantly until 18 years of age (Carver et al., 2001), suggesting protracted accumulation in cortex. A PET study of adults estimates that cortical DHA levels would fall by 5% within a few months of an omega-3 deficient diet (Umhau et al., 2008). The metabolic needs of the developing adolescent brain may render it more sensitive to DHA levels. Thus, a marginal deficiency during adolescence is likely to impact brain development significantly. Reduced DHA content of membranes may be difficult to remediate, especially in younger animals (Bourre et al., 1989) where anabolic growth increases nutritional requirements. Thus, given turnover rates and the likely impoverished DHA intake in adolescents, PFC may be undersupplied with DHA during this critical period in development, potentially delaying cortical maturation and producing cognitive deficits.

Identifying factors that modulate the development of the neural basis of response inhibition during adolescence is critical for minimizing poor outcomes. To our knowledge this is the first large-scale neuroimaging study in a sample of typically developing male and female adolescents to show a relationship between N3 Index and the ability to inhibit responses as well as associated neural activity. While not evidence of a causal relationship, taken together these results suggest that intake of long chain Omega-3 fatty acids is related to caregiver perceptions of their adolescent's ability to control impulses and function of the dACC, a prefrontal region implicated in a number of executive functions including monitoring errors and performance. Unlike other comparable contributors like socioeconomic status *per se*, diet is a factor that may be easily modified in the service of catalyzing morphological and functional neurodevelopment, specifically to increase

behavioral self-control, which ultimately may have an impact on preventing maladaptive outcomes.

CHAPTER IV: Adolescent blood levels and development of impulse control and PFC (Study 3)

Introduction

The long-chain omega-3 (N3) fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are essential nutrients in the diet that are critical for normal development and function of the central nervous system. Found in cold-water marine sources, these fatty acids particularly DHA accrue in the neuronal phospholipid membrane between the perinatal period and young adulthood (Carver et al., 2001). As an integral component of phospholipids, due in part to a highly unsaturated structure, they promote membrane fluidity and neurotransmission (Stillwell & Wassall, 2003), dendritic arborization (Calderon & Kim, 2004), neuronal size (Ahmad et al., 2002), synaptogenesis (Wurtman et al., 2009), pruning of superfluous synapses (de Velasco et al., 2012) and ultimately development of large scale functional networks (Grayson et al., 2014). Brain levels of DHA are correlated with blood levels of DHA, which are in turn highly influenced by dietary intake. As such, blood levels display a high level of variability within person (20-30% in adults) (Harris, 2013). Indeed, changing intake of dietary essential fatty acids is reflected in a change in both blood levels and brain/synapse essential fatty acid levels (Connor et al., 1990; Galli et al., 1970; Hulbert et al., 2005; Moriguchi & Salem, 2003).

Animal evidence suggests that the prefrontal cortex (PFC) may be one of the most sensitive regions to change in dietary long-chain essential fatty acids (Favrelière, Barrier, Durand, Chalon, & Tallineau, 1998). The PFC undergoes substantial structural development during the adolescent years when, after an increase in gray matter through childhood, gray matter volume peaks and

begins to decline through young adulthood (Gogtay et al., 2004; Shaw et al., 2008; Tamnes et al., 2010). In general, the process of thinning gray matter, thought to be due at least in part to pruning of superfluous synapses (Huttenlocher & Dabholkar, 1997), is associated with skill refinement (Lu et al., 2007) and intelligence (Shaw et al., 2006; Sowell et al., 2004). One PFC region that may be particularly impacted by N3 fatty acids during childhood and adolescence is the cingulate gyrus. The anterior cingulate, particularly the dorsal region (dACC), has been implicated in integrating context/task-information to monitor performance and guide behavior (Heilbronner & Hayden, 2016).

Within the PFC, protracted development contemporaneous with cortical thinning is associated with improvements in impulse control (Steinbeis, Bernhardt, & Singer, 2012), a core executive function. The ability to regulate one's own thoughts and inhibit habitual or inappropriate responses is central to academic and long term success (Tangney et al., 2004). While animal models of DHA depletion producing low levels of DHA in the frontal cortex have been shown to result in hyperactivity (Vancassel et al., 2007) evidence in humans of a relationship between long chain N3 fatty acids and impulse control is mixed likely owing to variations in methodology. Large cohort studies show mixed evidence with dietary reports regarding relationship between fish or long chain N3 intake and behavioral disorders (e.g., externalizing) (Gispert-llaurado et al., 2016; Waylen et al., 2009). In Study 2, we showed that dietary intake of EPA+DHA was inversely related to impulsivity in adolescents as rated by caregivers. While we were unable to show that dietary intake of EPA+DHA was related to inhibition as measured using a Go/No-Go task (i.e., ability to inhibit a prepotent response), we did find an inverse relationship between dietary long chain N3 intake and activation in the dorsal ACC during successfully inhibited events.

Studies based on blood levels of DHA, considered a more direct measure of available N3, have shown the impact of N3 on PFC function and development. For example, blood levels of N3 fatty acids were inversely related to self-reported cognitive impulsivity in adults (Conklin, Harris, et al., 2007) although were not related to a specific measure of response inhibition (Go/No-Go task) in boys (Almeida et al., 2016; McNamara et al., 2010, 2013). There is much evidence to suggest that the impact of blood levels of N3 on the ACC development and function might be greatest during adolescence most likely corresponding to the cubic developmental trajectory of the medial prefrontal and cingulate cortices, which attain peak thickness in early adolescence (Shaw et al., 2008). Boys with high blood DHA levels (defined by median split) had greater concentration of metabolites such as myo-inositol and N-acetylaspartate in the ACC but not the dlPFC (McNamara et al., 2013). Additionally, while there were no observable differences in ACC activation during sustained attention blocks, boys with low blood DHA displayed reduced functional connectivity between the right ACC and regions including the right ventrolateral PFC, middle occipital, inferior frontal gyrus, and superior parietal lobule during sustained attention compared to boys with high blood DHA (Almeida et al., 2016). It has been speculated that aggressive cortical growth in regions under development may render them susceptible to DHA insufficiency (Diau et al., 2005). The diets of middle and high school adolescents are typically poor in sources of N3 fatty acids (Cutler et al., 2009). These findings suggest that the anterior cingulate, under development during this period, may be particularly sensitive to inadequate intake of long chain N3 fatty acids.

To date most studies of task-based response inhibition in typically developing youth have only included boys (Almeida et al., 2016; McNamara et al., 2010, 2013). Data on the nature of the

relationship in girls is lacking. The prevalence of neurodevelopmental disorders involving high degrees of impulsivity such as ADHD is higher in males (Scahill & Schwab-Stone, 2000), and there is evidence of sexual dimorphism of the PFC cortical surface and its association with ADHD (Dirlikov et al., 2015). Additionally, as reviewed by Silva, Barazzoni, & Singer (2014), the influence of sex may be an important consideration in tissue levels of N3 fatty acids as sex hormones have been demonstrated to modulate the conversion between precursor forms of omega-3 (alpha-linolenic acid, EPA, DPA) and the neurally relevant product, DHA. These findings highlight the importance of investigating the relationship between long chain N3 fatty acids and neuroanatomical and behavioral development in adolescent females as well as males.

The present study addresses this empirical gap through a cross-sectional study examining the relationship between blood levels of N3 fatty acids, gray matter volume of cingulate gyrus and behavioral control in adolescent boys and girls. We used voxel-based morphometry (VBM), which is sensitive to morphological characteristics of gray matter such as thickness and cortical folding (Hutton, De Vita, Ashburner, Deichmann, & Turner, 2008), to quantify differences in gray matter volume (GMV). We restricted our analyses to an *a priori* area of interest, the dACC. As a primary objective, we sought to explore the relationship between whole blood long chain omega-3 fatty acids and structural characteristics of the cingulate cortex. We hypothesize that whole blood N3 index would be associated with dorsal cingulate gray matter volume in a sample of male and female adolescents, though we expect differences in this association between males and females given evidence reviewed above. Our secondary aim was to explore whether a biomarker of long chain N3 fatty acids, percent of fatty acids comprised of EPA+DHA in whole blood (hereafter, *N3 Index)* relates to response inhibition behavior, as measured by caregiver report and performance on a task

requiring behavioral control (Go/No-Go). We hypothesize that N3 Index would be related to impulse control on both measures.

Methods

Participants were recruited as a part of a longitudinal neuroimaging study, the Adolescent Development Study, aimed at identifying neurobiological precursors and consequences of early drug and alcohol initiation and escalation. Full details of the methods are described in detail elsewhere (Fishbein et al., 2016). In brief, children in a narrow age range (11-13 years old) were recruited. Main exclusions included prior substance use, left-handedness, conditions rendering MRI unsafe, history of head trauma, and neurodevelopmental disorders. Participants taking psychostimulant (centrally acting) medications were not enrolled if study visits could not be scheduled during normally occurring medication holidays. Demographic, neurocognitive, drug and alcohol use surveys, and imaging assessments were conducted at baseline and repeated 18- and 36-months later. The data reported here were collected during the first follow-up visit. The Georgetown Institutional Review Board approved all study procedures and adolescents and their caregivers provided assent and consent prior to all data collection.

Participant Characteristics. Upon enrollment into the study, intelligence was assessed using the Kauffman Brief Intelligence Questionnaire (K-BIT) (Kaufman & Kaufman, 1990). Additionally, family socioeconomic status index was estimated using parental education and household income provided by one of the caregivers (method adapted from Manuck, Phillips, Gianaros, Flory, & Muldoon, 2010). Average parental cumulative years of education was standardized and averaged with a standardized total annual household income level. SES index was computed by re-

standardizing the average of these two standard scores to achieve a distribution with a 0-centered mean and a standard deviation of 1 for the full sample (N=135).

At the time of the visit, physical maturation and pubertal status were assessed via the Pubertal Development Scale, which consists of a series of questions about the degree to which progress of physical change has occurred (e.g., breast development, skin/voice changes) (Petersen, Crockett, Richards, & Boxer, 1988). Responses are averaged and resulting scores range from (1) prepubertal to (4) postpubertal. In addition, body mass index (BMI) (kg/m^2) sex and age-specific z-scores and percentiles were calculated using weight measured with a digital scale (Health-O-Meter Professional 394KLX) and height measured via stadiometer (SECA 216 Wall-mount Mechanical measuring rod; triplicate measures within 0.5 cm, averaged) applied to 2000 CDC Growth Charts (Kuczmarski et al., 2000).

Dried blood spot collection and analysis. Blood collection and analysis procedures followed those described in Bell et al. (2011). In brief, droplets of whole blood were absorbed onto two circular collection spots on Whatman 903 blood collection cards from the middle finger tip using a blood lance (Accu-Chek®, Safe-T-Pro Plus, Roche Diagnostics GmbH, Mannheim, Germany). The procedure lasted approximately 10 minutes and was collected just prior to the end of visit on same day as neuroimaging and behavior were collected. Participants were given $25 Amazon gift cards for their participation in this sub-study.

Blood spot cards were air dried for 3 hours, sealed in polythene bags with desiccant packets, and stored at -80^{o}C until shipment (up to 6 months). Samples were shipped to Nutrition Group

Laboratories at the University of Stirling via first overnight delivery at ambient temperature. Samples were subjected to transmethlyation and fatty acid methyl esters were separated and quantified by gas liquid chromatography as detailed by Bell et al. (2011). Fatty acids, including EPA and DHA, were classified using reference standards and expressed as percentage of total fatty acids. While evidence suggests that DHA is the most relevant long chain omega-3 fatty acid, there have been reports of its long chain precursor EPA also being related to neural and cognitive outcomes (Bauer et al., 2014, 2013). Thus, all subsequent analyses are based on percentages of DHA + EPA in whole blood, akin to the Omega-3 Index (Harris & Von Schacky, 2004), hereafter *N3 Index.* A log-base10 transformation of this value successfully corrected originally skewed distribution.

Behavior Rating Inventory of Executive Function (BRIEF). The BRIEF (Gioia et al., 2000) is an 86-item psychometrically validated questionnaire to assess facets of executive abilities and was administered to the caregiver who came with the participant for the study visits. Each item asks the caregiver to rate the child's behaviors as "never", "sometimes", or "often" a problem. The questionnaire yields 8 non-overlapping scales, of which the Inhibit subscale, reflecting the ability to control impulses or stop behavior, was of interest to the current study. Higher scores suggest higher level of dysfunctional behavior. Normative values for age and sex (t-scores) are reported.

Go/No-Go task. To measure response inhibition performance, adolescents completed a simple Go/No-Go task. Alternating blocks of event-related Go/No-Go (45 seconds) and rest (12-16 seconds) were each repeated 5 times. During the Go/No-Go blocks, a series of 30 letters is presented for 200 ms each, followed by a 1300 ms rest. Subjects are instructed to press the button

in their right hand as quickly as possible for every letter ("Go" trials) except the letter 'Q' ("No-Go" trials). A total of 150 trials are presented in this design of which 18% are No-Go trials. Trials were included in analyses if response time (processing speed) was at least 150ms, indicative of an intentional, rather than anticipatory, response. Ability to inhibit response for the No-Go trials was used as an indication of impulse control/ability to inhibit a prepotent response (Riccio et al., 2002)**.** The task was implemented in E-prime and completed during MR imaging, although only behavioral results are reported in the current study. Calculated metrics of interest include *successful inhibition rate* (Correct No-Go/ Total No-Go), *false alarms* (Incorrect No-Go/Total No-Go) (reflecting impulsivity), and reaction time to Go trials (reflecting processing speed). *Hit rate* (Correct Go/Total Go) was used to determine whether the participant was adequately engaged with the task (at least 70% of Go trials with response).

MR image acquisition and analysis. All scanning was performed using a Siemens TIM Trio 3 T scanner with a 12-channel head coil. The high-resolution structural magnetic resonance images were collected using a T1-weighted MPRAGE. A total of 176 sagittal slices were collected with the following sequence parameters: TR/TE/TI=1920/2.25/900 ms, flip angle =9^{o}, slices thickness = 1.0 mm, FOV= 250x250 mm^2 and a matrix of 256x256 for an effect spatial resolution of 0.97x0.97x1.0 mm^3.

Quality control for MPRAGEs. Three independent raters blind to the participant identity visually verified quality of structural scans for inclusion in a Voxel-Based Morphometry (VBM). Raters scored structural scans for degree of artifacts, which may impact the tissue classification algorithm (i.e., ringing, gross quality problems such as phase wrap or clipped brain, and movement related

artifacts) on a 0 – 5 point scale. Summary scores were in good to excellent agreement (intraclass correlation coefficient=0.873, 95% confidence interval= 0.798-0.919 [SPSS 24 based on mean-rating (*k*=3), absolute-agreement, 2-way mixed-effects model]) (Koo & Li, 2016).

Image preprocessing. Preprocessing for voxel based morphometry was performed using SPM8 (http://www.fil.ion.ucl.ac.uk/spm/software/spm8/). Tissues were segmented using the *New Segment* option using the default parameters to generate the native tissue segmentation as well as lower resolution versions for the DARTEL registration. Individual images were spatially normalized to a study-specific whole-brain template (i.e., semi-optimized) using DARTEL toolbox to increase accuracy of inter-subject alignment. Images were spatially normalized to MNI space and modulated by the determinant of the Jacobian of the transformation to preserve tissue volume and create images corresponding to gray matter volume (GMV). The GMV images were smoothed using convolution with a gaussian kernel with a FWHM of 12 mm to reduce spatial noise and minor differences in anatomic variability following spatial normalization (Ridgway et al., 2008). Measures of GMV were normalized by total intracranial volume in order to account for inter-individual differences in brain size.

Region of interest (ROI) selection. Given an association between energy-adjusted dietary N3 Index and activation in the dorsal cingulate reported in Study 2, separate left and right mid-cingulate and anterior cingulate masks were generated using AAL via the Wake Forest Pick Atlas (Figure 10) (Maldjian et al., 2003). Masks were imported into MarsBaR and used to extract mean gray matter volume from these regions, GMV from bilateral dorsal cingulate ROIs were subsequently analyzed in the statistical analyses as described below.

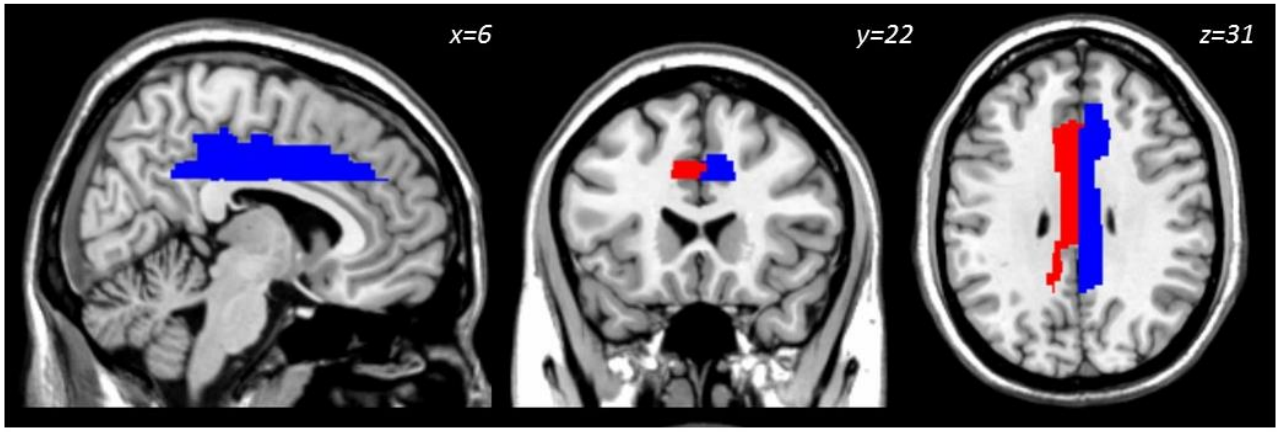

Figure 10. Location of *a priori* Mid-Cingulate ROI for analysis of gray matter volume. Right hemisphere ROI (blue), left hemisphere ROI (red).

Statistical analysis and data quality control. Of the 135 participants enrolled in the parent study, 93 were approached to provide blood samples as this sub-study was added after the parent study was underway. Ten participants declined to provide blood. The remaining 83 gave assent and caregivers provided informed consent to provide blood samples. A total of 81 participants blood samples were successfully collected (n=2 failed due to insufficient blood extracted for analysis).

Of the 81 participants providing blood, 4 had to be eliminated (n=3 not attending to task (hit rate <70%); n=1 technical data collection issue). Of these participants, 13 do not have structural images (due to braces at time of visit) and an additional 2 did not meet quality control standards for the MPRAGE, resulting in 62 participants (33 females, 29 males) eligible for analysis.

Distributions of dependent variables were confirmed prior to analysis to ensure assumptions of normality. Examination of dependent variables confirmed no outliers were present in any of the measures (Hoaglin & Iglewicz, 1987). As stated previously, our main predictor variable N3 index was log-base10 transformed before being used in any analyses. Males and females were compared

on characteristics and dependent variables (cingulate GMV and response inhibition measures) using independent samples Student's T-test and Mann-Whitney U-test where noted. Sexes were significantly different on pubertal status. Thus, partial correlations between whole blood N3 Index and dependent variables, controlling for pubertal status, by sex are reported for whole group correlations. Data were analyzed in SPSS 24 (IBM Statistics).

The distribution of two dependent variables, BRIEF Inhibit t-score and Correct Go reaction time, were non-normal (Shapiro-Wilk 0.875, df=62, p<0.001, and Shapiro-Wilk 0.867, df=62, p<0.001, respectively). Standard transformations were unsuccessful at normalizing distributions, so these DVs were used in non-parametric Spearman's rank correlations and rank-transformed for use in linear modeling.

Results

Participant characteristics as a whole group and by sex are presented in Table 1. Males and females were similar in characteristics except for pubertal development, with females reporting being farther along in development than males (*t* -3.068, *df*=55, p=0.003).

N3 index was unrelated to potentially confounding variables amongst the whole group (age, r=0.18, p=0.890; pubertal development, r=-0.134, p=0.321; SES Index z-score r_s=-0.91, p=0.483; BMI z-score r=-0.104, p=0.419; IQ r=-0.157, p=0.232, n=60). N3 Index was also not significantly different between males and females (t=-0.685, df=60, p=0.496) (Table 5).

As a whole group, since pubertal status was related to certain dependent variables (Go/No-Go False Alarms r=0.350, p=0.008, n=57; Correct inhibition rate r=-0.374, p=0.004, n=57; but not Go/No-Go reaction time r_s=10.108, n=57, p=0.425; BRIEF Inhibit t-score r_s =-0.24, p=0.093, n=57, or mid cingulate volumes left r=-0.051, p=0.704, n=57; right r=-0.090, p=0.509, n=57) and pubertal status was significantly different between sexes, we entered pubertal development score as a covariate in the subsequent whole group correlation analyses.

Table 5. Participant characteristics (Study 3). Group means and standard deviations listed, except where indicated. ([a.] Mann-Whitney U-test used to examine difference between male and female means).

	Whole sample	**Males**	**Females**	**p**
N	62	29	33	
Age	14.4(0.7)	14.3(0.6)	14.4(0.8)	0.514
Race				0.202
Caucasian %	*56.5%*	*55.2%*	*57.6%*	
African American	*32.3%*	*41.4%*	*24.2%*	
Hispanic/Latino	*3.2%*	*0%*	*6.1%*	
Other	*8.1%*	*3.4%*	*12.1%*	
Socioeconomic status index (z-score)[a]	0.024(0.980)	0.016(1.025)	0.031(0.956)	0.972
Parental education (years, mean)	*16.3(2.7)*	*16.6(2.9)*	*16.1(2.5)*	
Household income (median)	*$100,00-$149,999*	*$75,000-$99,999*	*$100,00-$149,999*	
Pubertal development	2.82(0.58)	2.59(0.46)	3.03(0.61)	0.003
BMI z-score	0.34(1.04)	0.19(1.03)	0.47(1.05)	0.294
Percentile[a]	*59.9(29.6)*	*55.1(30.5)*	*64.1(28.6)*	*0.244*
Intelligence (K-BIT)	110.2(14.2)	111.4(17.7)	109.0(10.0)	0.528
Blood DHA (% of total fatty acids)[a]	1.87(0.50)	1.81(0.42)	1.93(0.56)	0.521
N3 index (% of total fatty acids)[a]	2.17(0.60)	2.10(0.47)	2.24(0.70)	0.667

Cingulate GMV. Regarding relationships between N3 Index and cingulate GMV, among the whole group, controlling for pubertal status, N3 index was not related to gray matter volume in the mid-cingulate gyrus (left*: r*=-0.107, *df*=54, *p*=0.431; right: r=-0.161, df=54, p=0.234).

Males and females were similar in ICV-adjusted GMV for mid-cingulate volumes on both left (t=-0.895, df=60, p=0.374) and right (t=-1.295, df=60, p=0.475) (Table 6). However, while N3 Index was unrelated to cingulate GMV in females (left: *r*=0.253, n=33, p=0.156; right: r=0.124, n=33, p=0.439), N3 index was significantly inversely related to mid cingulate gray matter volume in males (left: r= -0.556, n=29, p=0.002; right: r= -0.503, n=29, p=0.005) (Figure 11).

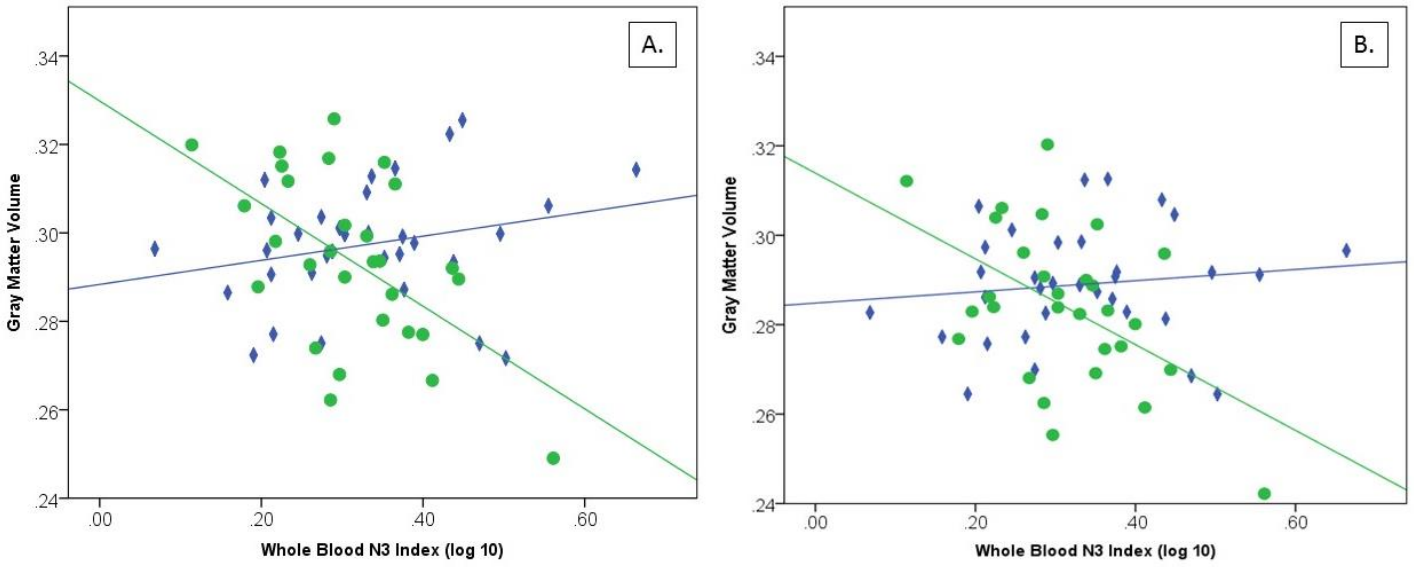

Figure 11. Structural MRI results (Study 3). Relationship between whole blood N3 Index and gray matter volume by sex. Pearson's correlations in (A) left mid cingulate and (B) right mid cingulate. While no test reached significance in females (blue diamonds), bilateral male cingulate GMV was significantly inversely related to N3 index (green circles).

Response Inhibition: Go/No-Go performance. Among the whole sample, controlling for pubertal status, blood N3 index was neither related to false alarm rate (r=-0.174, df=54, p=0.199) nor reaction time for Correct Go's (rank-transformed; r = 0.074, df=54, p=0.588). Males and females

were similar in mean reaction time for correct Go's (Mann-Whitney U=480.0, p=0.983), and the difference between sexes for successful inhibitions (t=1.744, df=60, p=0.086) and false alarms (t= -1.843, df=60, p=0.070) did not reach statistical significance (Table 6). Similarly, there were no significant relationships for either sex between N3 index and successful inhibitions (females: r=0.205, n=33, p=0.251; males: r=0.236, n=29, p=0.217), false alarms (females: r= -0.178, n=33, p=0.321; males: r=-0.291, n=29, p=0.125) or reaction time (females: r_s=0.273, n=33, p=0.124; males: r_s =-0.111, n=29, p=0.566).

Response Inhibition: BRIEF Inhibit Subscale. Among the whole sample, controlling for pubertal status, the relationship between rank transformed blood N3 index and rank-transformed BRIEF Inhibit subscale did not reach statistical significance (r=-0.231; df=54 p=0.087). Males and females were similar in mean BRIEF inhibit t-score (Mann-Whitney U=493.5; p=0.832) (Table 6). Inhibit t-scores were not related to N3 Index in either females (r_s=-0.061, n=33, p=0.738) or males (r_s=-0.220, n=29, p=0.253).

Among males only, however, BRIEF Inhibit t-scores were positively related to GMV in the right (but not left, p=0.264) cingulate (r_s=0.440, n=29, p=0.017) such that less gray matter volume was associated with lower t-scores indicative of better impulse control.

Table 6. Behavioral outcomes and brain structure by sex (Study 3). Group means and standard deviations, except where indicated. ([a.] Mann-Whitney U-test used to examine difference between male and female means.)

	Whole sample	**Males**	**Females**	**p**
N	62	29	33	
BRIEF Inhibit subscale				
t-score[a]	50.4(9.7)	49.9(9.6)	50.8(9.9)	0.832
median	48	48	50	
range	40 – 87	40 – 72	41 – 87	
Percentile [a]	59.5(23.0)	57.9(24.7)	60.9(21.7)	0.916
median	60	59	65	
range	23 – 99	23 – 96	28 – 99	
Go/No-Go False Alarm Rate (% Incorrect No go)	32.5(15.4)	28.7(13.3)	35.8(16.5)	0.070
Go/No-Go Successful Inhibition Rate (% Correct No-Go)	66.2(16.3)	70.1(14.7)	62.8(17.7)	0.086
Go/No-Go Response time, ms (Correct Go)[a]	331.1(62.7)	331.4(63.6)	330.9(63.0)	0.983
median	311.0	310.0	320.4	
range	248.5 – 558.4	255.0 – 558.4	243.5 –519.8	
Total Intercranial Volume (cubic liters)	1.53(0.14)	1.61(0.10)	1.44(0.12)	<0.001
Mid-cingulum GMV (ICV adjusted)				
Mid, left	0.296(0.016)	0.294(0.019)	0.297(0.013)	0.374
Mid, right	0.287(0.015)	0.284(0.018)	0.289(0.013)	0.475

Discussion

This study set out to explore the relationship between N3 fatty acid status, structural development of the cingulate gyrus, and control of impulsive action in both adolescent males and females. To this end, we collected dried blood spot samples for whole blood N3 Index, used voxel-based morphometry to quantify ROI-based gray matter volume in the cingulate, and assessed response inhibition via both parental report (BRIEF) and task performance data (Go/No-Go successful inhibition and false alarms). We found that, while there was no relationship between whole blood N3 Index and gray matter volume of the dorsal cingulate in females, there was an inverse relationship in males such that higher N3 Index was associated with less dorsal cingulate gray

matter volume bilaterally. Contrary to our hypothesis, N3 index was neither related to caregiver's ratings of inhibitory control nor to response inhibition performance metrics (successful inhibitions, false alarms or reaction time) either in the group or within each sex. These results suggest a sex-specific link between blood levels of long-chain N3 fatty acids and the structural development of cingulate cortex in boys but not girls.

Our main finding was that whole blood N3 Index was significantly inversely associated with dorsal cingulate gray matter volume in males. To our knowledge, while others have reported relationships between the morphology of more rostral cingulate regions and long-chain omega-3 fatty acids in adults (Conklin, Gianaros, et al., 2007; Zamroziewicz et al., 2015), this is the first report of a relationship between omega-3 fatty acids and gray matter volume in adolescent boys. Conklin and colleagues reported that greater reported average dietary intake of long-chain N3 fatty acids was associated with greater sub-genual anterior cingulate GMV in a sample of male and female middle-aged adults (Conklin, Gianaros, et al., 2007). Similarly, Zamroziewicz and colleagues report that in their sample of cognitively intact older adult APOEe4 carriers, greater plasma levels of omega-3 fatty acids were related to greater GMV of left rostral anterior cingulate (Zamroziewicz et al., 2015). The fact that these studies used adults may explain the seemingly disparate direction of findings from the current study, where greater blood levels of the N3 Index were associated with less GMV in the cingulate. Long chain N3 fatty acids, particularly DHA, are involved in trophic support of gray matter over the entire life span (e.g., supporting neurogenesis and morphological maturity of neurons (Kawakita, Hashimoto, & Shido, 2006), enhanced neuronal size (Ahmad et al., 2002) and prevention of apoptosis (Salem, Litman, Kim, & Gawrisch, 2001). However, another critical function of DHA is its role in the pruning of superfluous axonal connections (de Velasco

et al., 2012), which takes place primarily during development. Subsequent to the general increase in cortical gray matter volume in childhood, peak volume is reached in late childhood/early adolescence and then declines into young adulthood (Giedd et al., 1999; Gogtay et al., 2004; Lenroot & Giedd, 2006). The medial prefrontal and cingulate cortices follow a cubic trajectory and attain peak thickness relatively late, with the dorsal supracollosal anterior cingulate estimated to reach peak cortical thickness at 13.8 years (Shaw et al., 2008), suggesting that cingulate volume for adolescents in the current study may be on the descent. A possible implication of this is that adolescent boys with greater blood N3 Index levels may be more further along in neuroanatomical development of the dACC than those with lower blood levels.

During adolescence, the reduction of gray matter volume reflects synaptic pruning and refinement (Huttenlocher & Dabholkar, 1997; Tamnes et al., 2010), and is associated with improvements in cognitive function and behavior (Casey et al., 2000). Indeed, in the present study, among males, GMV in the right (but not left, $p=0.264$) cingulate was positively related to BRIEF Inhibit t-scores ($rs=0.440$, $n=29$, $p=0.017$) (data not shown), such that less gray matter volume was associated with better impulse control. There is some inconsistency with respect to cingulate morphology and better response inhibition, though differing participant ages, populations and methods of assessing impulse control may contribute to disparities. For example, in adults, lower ACC GMV was associated with higher self-reported impulsivity (Matsuo et al., 2009), but greater ACC GMV in young adults is associated with impulsive action (Wang et al., 2016) while children with ADHD have less ACC surface area compared to controls (Dirlikov et al., 2015) and boys with disruptive behavioral disorders have thinner cingulate coritices than controls (Fahim et al., 2011). Our findings lend support to the notion that, in typically developing adolescent males, less cingulate

GMV is associated with better impulse control, and less cingulate GMV is associated with higher blood levels of long chain N3 fatty acids. Whether these findings suggest that low N3 adolescent boys are at the same neurodevelopmental point but have impaired/less pruning/network refinement than their high N3 same-age peers or are delayed in their neurodevelopmental progress remains to be determined.

The current study is consistent with previously reported findings with respect to an association between long chain N3 fatty acids and cingulate characteristics. The anterior cingulate of boys with low blood levels of DHA was reported to have reduced indices of metabolic health (McNamara et al., 2013) and also reduced functional connectivity during sustained attention to regions including ventrolateral prefrontal cortex and insula (Almeida et al., 2016). Moreover, we have previously reported that low energy-adjusted long-chain N3 intake is associated with greater activation in this region (during successful inhibitory control) (Darcey et al., in preparation). Thus, inefficiency in metabolism and/or impaired cortical pruning may contribute to the greater ACC activity to compensate for lower intake of long-chain omega-3 fatty acids. Together with the current finding that boys with lower blood N3 index have greater gray matter volume in the dorsal cingulate (although in a region more caudal to that used by McNamara's group), these studies suggest that long-chain omega-3 fatty acids are related to the structural and metabolic development and ensuing network development of the cingulate gyrus in males.

The present study also extends the available literature in humans with the demonstration that, contrary to our hypotheses, dorsal cingulate cortical structural development in female adolescents is not related to long-chain N3 fatty acids. Examination of the relationship between blood levels

of long chain N3 fatty acids and cingulate morphology has not been previously reported in the literature. That this relationship is not observed in girls, to our knowledge, has not been previously reported, though this is consistent with animal evidence that brain volume in males may be particularly sensitive to N3 variation (e.g., Galli et al., 1970). Level of long chain omega-3 fatty acids in whole blood samples were similar between males and females, which has been reported by others (Johnston, Deuster, Harris, Macrae, & Dretsch, 2013; Marangoni et al., 2007; Ogura et al., 2010; van der Wurff et al., 2016). Levels of N3 Index components, EPA and DHA, and/or intermediary DPA, however, may differ between sexes. Despite similar levels of product (DHA), levels of immediate precursor (DPA) are higher in males (Marangoni et al., 2007). Furthermore, 15-24 year old women had lower EPA but higher DHA in serum phospholipids than same age males, despite reporting similar intakes reported on dietary assessment (Crowe, Skeaff, Green, & Gray, 2008). Given evidence that estrogen facilitates synthesis of long chain polyunsaturated fatty acids (Mason et al., 2014) while testosterone downregulates these synthetic enzymes (Marra & de Alaniz, 1989), conversion of precursor omega-3 fatty acids (alpha-linolenic acid, EPA and DPA) to DHA is more efficient in young females than males (Burdge & Calder, 2005). This effect may render males more sensitive to variation in N3 index level and contribute to the disparity between rates of certain neuropsychiatric disorders between males and females (as speculated by Schuchardt, Huss, Stauss-Grabo, & Hahn, 2010). In the current study, though similar in chronological age, females were significantly more advanced in pubertal status. In Study 2, while females were also more advanced in pubertal status than males (data not shown), we did not observe a difference between sexes regarding the relationship between dietary N3 Index and cingulate function. Both males and females reported significant advancement in pubertal status between timepoints however. It is possible that advancing sexual maturity and thus estrogen status

may have contributed to the relative insensitivity of female cingulate gray matter volume to variation in blood N3 Index level. Advanced pubertal status is a reliable predictor of the divergence in emergence of internalizing symptoms between girls and boys (Hayward & Sanborn, 2002), suggesting that perhaps pubertal status in the current sample was advanced enough to elicit differences between sexes. Overall, the disparity between sexes observed in the present study supports the conclusion by Crowe et al. (2008) that males and females "may need to be considered separately when examining the association between disease risk and biomarkers of n-3 fatty acids."

Contrary to our hypotheses, we did not find a relationship between blood levels of the N3 Index and impulse control in either adolescent boys or girls, as measured either by caregiver report (BRIEF Inhibit subscale) or by ability to inhibit a prepotent response during a Go/No-Go task. There is evidence that behavioral control is related to long chain N3 fatty acids in animal models (Levant et al., 2010; Vancassel et al., 2007) and patient populations (Raz & Gabis, 2009), and others have reported an association between greater serum N3 fatty acids and lower self-reported cognitive impulsivity in cognitively healthy adults (Conklin, Harris, et al., 2007). Indeed, in Study 2, report of lower energy-adjusted long-chain N3 fatty acid intake was associated with worse ability to inhibit impulses as rated by caregivers (BRIEF Inhibit subscale). Participants were a mean age of 13.3 however at that time, a full year younger than those adolescents included in the current analysis but BRIEF scores are analyzed as age and sex-normed t-scores to account for developmental differences. It is unclear why we did not observe a similar relationship between BRIEF Inhibit scores and a biomarker, rather than reported intake, of long chain N3 intake. Regarding Go/No-Go task performance, we were unable to detect a relationship between task-based response inhibition and blood N3 Index levels, consistent with previous studies using

younger participants (Almeida et al., 2016; McNamara et al., 2013)(and Study 2). The task may not be difficult enough to elicit enough variation to explore relationship, particularly since the adolescents in this study are now slightly older and suggests there may be ceiling effects at play (Petersen, Hoyniak, McQuillan, Bates, & Staples, 2016). Given that others have observed improved Go/No-Go performance with supplementation in adults (Fontani et al., 2005), and associations between blood levels and (working memory) performance during difficult but not easy task conditions (Narendran, Frankle, Mason, Muldoon, & Moghaddam, 2012) it is possible that the current task is too easy for most participants (median hit rate 99.2%; mean successful response inhibition rate 66.2%$\pm$16.6%) to detect associations with performance. It is possible that either the range of N3 Index and/or the range of behavioral abilities skews higher in typically developing populations thus precluding a relationship with N3 fatty acids without sufficiently large samples.

Whole blood levels of long chain N3 fatty acids (i.e. N3 Index) observed in the current study are comparable to levels reported in available studies. Due to the ease of the method used to collect finger-tip blood samples (dried blood spot), an increasing number of studies utilizing this technique are being reported (Stark, Elswyk, Higgins, Weatherford, & Jr, 2016). Of the comparable studies, levels of whole blood DHA presently reported are similar to that found in male and female Italian adults (Marangoni et al., 2007). In a sample of 13-15 year old typically developing Dutch adolescents, whole blood DHA levels were slightly higher in the current study (2.58$\pm$0.49 compared to 1.87$\pm$0.50) (van der Wurff et al., 2016) but could reflect different dietary patterns. In general, the whole blood collection technique produced results comparable to similar

studies and the ease of collection facilitated gathering data on this biomarker in a neuroimaging study of adolescents.

A strength of this study is the inclusion of moderate sized samples of boys and girls with neuroimaging data. Use of a biomarker for long-chain N3 index may generally be considered a strength, though some caution that the strength in this measure is realized when used in conjunction with dietary intake data (Vandevijvere et al., 2012). We used voxel based morphometry to study relationships between gray matter volume and whole blood N3 Index, however, though large kernel smoothing of VBM data was methodologically strong, there may be the potential to further improve sensitivity to detect relationships through the use of structural data that has not been modulated by deformation field (non-Jacobian) (Radua, Canales-rodríguez, Pomarol-clotet, & Salvador, 2014). Furthermore, grey matter volume is a composite of two other traits (surface area and thickness), so it is at present difficult to determine if the nature of the relationship between N3 index and dACC structure in males is due surface area or thickness or some combination of the two. Future studies may use alternate analysis techniques to delineate specific contributions to these associations. Lastly, we studied a cross sectional sample of adolescents so are unable to explore the trajectory of dACC gray matter change or contribution of long chain N3 fatty acids to variance in this trajectory over time. Adults with clinical levels of impulsivity display decreased ACC gray matter relative to controls (Makris et al., 2010), suggesting that the inverse relationship between dACC GMV and N3 Index in the present study may not persist into adulthood. Indeed, other studies of adult cingulate GMV and long chain N3 fatty acids report a positive relationship between variables (Conklin, Gianaros, et al., 2007; Zamroziewicz et al., 2015) despite an inverse relationship between N3 fatty acids and self-reported impulsivity (Conklin, Harris, et al., 2007).

Moreover, in the present study, N3 Index explains a sizeable portion of the variance in left (30.9%) and right (25.3%) dACC volumes (as reflected by r^2 value). Higher contributions of N3 fatty acids to cingulate structure have been reported in a sample of cognitively intact older adult APOEe4 carriers where plasma levels of omega-3 fatty acids explained 68.7% of the variance in volume of left rostral ACC (Zamroziewicz et al., 2015). Different populations and methods used between studies make drawing inferences concerning the dependence of medial prefrontal gray matter on dietary N3 fatty acids at different ages difficult. Future studies using accelerated longitudinal designs may be better able to delineate the nature of long chain N3 fatty acid contribution to variance in medial PFC structure over time.

Identifying factors that modulate the development of the neural basis of response inhibition during adolescence is critical for minimizing poor outcomes. To our knowledge this is the first large-scale neuroimaging study in a sample of typically developing male and female adolescents to show a relationship between whole blood N3 index and dorsal cingulate gray matter volume in males, but not females. While not evidence of a causal relationship, taken together these results suggest that intake of long chain Omega-3 fatty acids is related to structural development of dACC in males, a prefrontal region implicated in many executive functions including monitoring errors and performance.

CHAPTER V: Discussion

Summary of dissertation goals

The main objective of this dissertation was to examine the relationship between omega-3 fatty acids, an essential dietary nutrient, prefrontal cortical structure and function and impulse control, a core component of executive function, within normative adolescent development. As the impact of omega-3 fatty acid status has differential effects across the lifespan from infancy to adolescence, we used maternal report of infant feeding practices as a proxy for omega-3 status in early life and used both dietary report and blood levels during adolescence to determine current omega-3 status. The studies presented herein provide evidence that long chain omega-3 fatty acid status in both infancy and adolescence may relate to indices of prefrontal structure and function as well as impulse regulation in typically developing adolescents.

Table 7. Summary of results across studies. **Right dACC GMV was positively related to BRIEF Inhibit t-scores in males only. Higher t-scores indicative of greater difficulty controlling impulses.

Outcome Category	Measurement	Study 1: Infant Diet	Study 2: Adolescent Diet	Study 3: Adolescent Blood
Independent Variable		Breastfeeding duration	Energy-adjusted N3-index	Whole blood levels N3 index
Age of sample		12.7±0.7 years old	13.3±1.1 years old	14.4±0.7 years old
Behavior	BRIEF			
	Inhibit (subscale)	*No difference between groups*.	Greater N3-index associated with better behavior.	*No relationship.***
	Go/No-Go performance			
	Successful inhibitions (Correct No-Go)	*No difference between groups.*	*No relationship.*	*No relationship.*
	False alarm rate (Incorrect No-Go)	*No difference between groups.*	*No relationship.*	*No relationship.*
	Reaction time (Correct Go)	Longer duration group displayed faster psychomotor speed than shorter duration group.	*No relationship.*	*No relationship.*
Brain	Functional activation			
	Successful inhibitions (Correct No-Go> Incorrect No-Go) [PFC mask]	Longer Duration group had greater activation in vmPFC. (131 voxels)	Greater N3-index associated with less dACC activity. (126 voxels)	*No relationship.*
	Gray Matter Volume			
	Dorsal Anterior Cingulate	*[Not examined]*	*[Not examined]*	Greater N3 index associated with less dACC gray matter volume, in *males only.*

Summary/Interpretation of studies

Long chain omega-3 fatty acids and behavior. While we did not find a difference in caregivers' ratings of adolescent impulse control based on duration of breastmilk exposure, we did find that reporting low current intake of long chain N3 intake was associated with worse rated behavioral control. Interestingly, we were unable to replicate this finding subsequently with the use of a biomarker, whole blood long chain N3 fatty acids. However, it is important to note these measures were collected at different timepoints in the study such that breastfeeding was assessed mostly at the first timepoint (aka W1); diet surveys were mostly collected during W1 and part of the second timepoint (W2) and blood was collected during W2 (see Table 7 for comparison of mean ages associated with each analysis). A post-hoc paired-samples analysis of BRIEF Inhibit t-scores (Pair 1 in Table 8) indicates among adolescents providing blood (Study 3), there was significant improvement in inhibitory behavior between timepoints (Related Samples Wilcoxon Signed Rank Test 277.0, p=0.001). Concomitant with this improvement, however, we also see a drop of over 56.6% in the coefficient of variation around the mean (from 38.3 to 16.6), suggesting much less variability among scores at the second timepoint. This reduced variability may have contributed to our inability to detect a relationship with whole blood N3 fatty acids at this latter timepoint.

On the other hand, we found that adolescents exposed to breastmilk (a proxy for DHA accumulation in the brain) for longer than 9 months during infancy (LD) had faster psychomotor processing speeds on the Go/No-Go task than adolescents exposed to breastmilk for 9 months or less (SD). While we found an effect of early life diet, we were unable to find an association between Go/No-Go performance and either current dietary long chain N3 fatty acids or whole blood long chain N3 fatty acids. While outside the scope of this work, there is evidence to suggest

that myelin, which serves to increase nerve conduction velocity, is highly impacted by early diet (Deoni et al., 2013), including specifically long-chain N3 fatty acids (Salvati et al., 2008) providing a potential mechanism for this observation. While caution should be used when interpreting motor speed differences in developmental studies (Petersen et al., 2016), the LD and SD groups were similar in age at the time of assessment, minimizing the influence of age on motor development. Furthermore, a supplementation study which reported improved reaction time on Go/No-Go in adults determined, via measurement of finger flex latency, that reaction time improvement was due to reduction in time to initiate movement (a central process), not via speed of movement (a peripheral motor process) (Fontani et al., 2005). That the only influence on task performance was linked to early diet suggests that the neural foundations for quicker psychomotor speed may be established during early postnatal life.

One potentially significant impediment to detecting associations with task-based response inhibition is the low difficulty of the task, with participants successfully inhibiting responses more than half of the time at the beginning of the study. Further, adolescents significantly improved their response inhibition rate on the task between timepoints (Wilcoxon=1,403.0, $p<0.001$) and became slightly more consistent as well (Pair 2, in Table 8; 32.1% drop in the coefficient of variation of successful inhibitions between timepoints), both factors may have precluded associations with long chain N3 status.

Taken together, the results of the present work suggest that neither early accumulation of long chain N3 fatty acids nor current N3 fatty acid status are related to response inhibition as measured by a simple Go/No-Go task. General impulse control, however, may be related to dietary N3 fatty

acids earlier but not later in adolescence. A more nuanced potential explanation for null results is discussed below.

Table 8. Degree of improvement in inhibitory control between timepoints, W1 and W2. Paired sample descriptive statistics. Visits spaced approximately 18 months apart.

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	W1.BRIEF.InhibitT	58.0204	49	22.20688	3.17241
	W2.BRIEF.InhibitT	48.84	49	8.115	1.159
Pair 2	W1GNG.CorrectNoGo	52.54237288	59	19.80791559	2.578770959
	W2GNG.CorrectNoGo	65.97614564	59	16.92330544	2.203226705

Long chain omega-3 fatty acids and the PFC. In terms of PFC activity during successfully response inhibition, we found that adolescents with greater exposure to breastmilk during infancy have greater activity in the vmPFC while adolescents who report greater current dietary long chain N3 intake have an associated reduction in activity in the dACC. Moreover, we also found that adolescent boys with greater levels of long chain N3 fatty acids in whole blood have an associated reduction in dACC gray matter volume, which is, in turn, associated with better general response inhibition as rated by caregivers.

These results may at first seem slightly contradictory but in actuality are in line with each other and with the available literature, as detailed in below. Omega-3 fatty acids are critical to the PFC, but timing of regional accumulation and requirements follow specific developmental trajectories. We will use this lens to interpret the results of each study with respect to the age of the sample during the assessment.

As discussed at the end of Study 1, there is evidence that regional DHA accumulation within the PFC is dependent on which regions are undergoing development at that time. The vmPFC is evolutionarily older compared to more lateral and dorsal PFC structures, and as *ontogeny recapitulates phylogeny*, and thus would be early in the developmental timeline (Shaw et al., 2008). Establishing functionality (e.g., assigning value to social relationships) (Delgado et al., 2016; Hare et al., 2009) would serve the child's needs early in life. Thus, it stands to reason that early differences in DHA accumulation (Diau et al., 2005; Makrides et al., 1994) would manifest in this region, and reduced trophic support (low DHA) (Ahmad et al., 2002; Wurtman et al., 2009) during the progressive cortical expansion phase predominating infancy (Gilmore et al., 2012) may manifest as altered levels of activity and subsequent connectivity (including connectivity with dlPFC, of relevance to self-control (Steinbeis et al., 2014)). Importantly, the direction of this relationship is specific to *when* in development vmPFC function is tested. The vmPFC's functional trajectory shows an inverted U-shaped developmental pattern, peaking in adolescence in response to high-reward choices, compared to children and adults (Van Leijenhorst et al., 2010). Given that groups in Study 1 were similar in chronological age, we interpret this increased activity during successful response inhibition to potentially suggest a higher valuation of successful self-control in adolescents exposed to breastmilk for longer than 9 months. Alternatively, or perhaps in addition, it is intriguing to consider that the longer exposure adolescents may be displaying "peak" vmPFC function in response to the (inherent) reward of success *earlier* than their age-matched peers. Only a longitudinal analysis tracking trajectory of vmPFC function beginning in early childhood to late adolescence in these groups could answer this question.

On the other hand, *during adolescence*, the predominant developmental processes in prefrontal cortex are regressive in nature, partly presumed to consist of culling extra synaptic connections as well as associated dendrites, glial cells and microvasculature (Gogtay & Thompson, 2010; Huttenlocher, 1990 but see Bourgeois & Rakic, 1993), leading eventually to a thinner structure (PFC cortical mantle). Again, there is evidence that long chain N3 fatty acid exposure during this stage would support these processes as well (e.g., reducing superfluous synaptic connections (de Velasco et al., 2012) and supporting neurotransmission (Kodas et al., 2002). Thus, low long chain N3 fatty acid status during adolescence would provide reduced support of these regressive developmental processes predominating adolescence and may manifest (structurally) as *more* gray matter volume, which we found in Study 3 in boys. Advancing pubertal status, particularly in males where testosterone can hamper enzymatic metabolism of omega-3 fatty acids (Marra & de Alaniz, 1989), may make the PFC in males particularly sensitive to long chain N3 fatty acid status. Furthermore, less dACC gray matter volume in males was associated with improved impulse control – a developmentally appropriate progression. While not a causal result, we interpret the relationship between greater whole blood long chain N3 fatty acids and less dACC gray matter volume in boys to suggest that while the dACC of girls may be more resilient to variation in N3 status, advanced neuroanatomical development in boys may be partly dependent on long-chain fatty acid status.

Furthermore, an interpretation of the reduced activation during successful inhibitions being associated with greater dietary long chain N3 fatty acids (Study 2) is that, for the same level of performance, greater N3 intake is associated with greater efficiency of function in the dACC. While the "neural efficiency" argument has drawn criticism (Poldrack, 2015), there is cellular and

metabolic evidence to support this notion in the case of omega-3 fatty acids as discussed in Study 2 (McNamara et al., 2013; Pifferi et al., 2005; Ximenes da Silva et al., 2002). Nevertheless, *at the very least*, we may conclude that the early adolescent dACC operates differently depending on if there is high or low levels of long chain N3 fatty acids in the diet.

Less activity in the dACC during successful inhibitions (Correct No-Go > Incorrect No-Go) as observed in adolescents with greater intakes of long chain N3 fatty acids (Study 2) is also a developmental pattern observed in the larger sample. To illustrate this point (Figure 12), a one-sample t-test of activation associated with this specific contrast reveals that when the adolescents are younger (Wave 1), there are no clusters of activity in the cingulate. When the adolescents return for their follow up visit roughly 18 months later, the same contrast reflecting successful inhibitions is associated with a substantial cluster of less activity in the dACC.

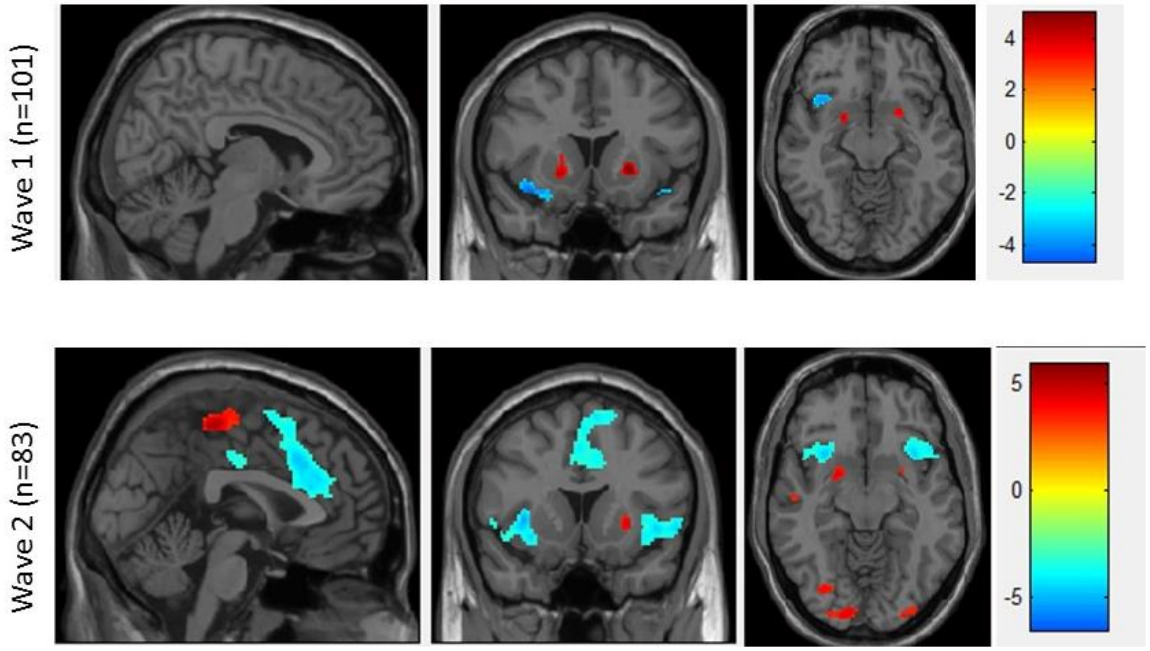

Figure 12. Activation associated with successful inhibition at Wave 1 and Wave 2. One-sample t-test of activation, spaced approximately 18 months apart. Sample sizes reflect attrition and results of quality control.

For comparison, this dACC region at the later timepoint overlaps entirely with the cluster of activity in Study 2 (see Figure 13). We interpret this evidence to be in support of the notion that higher intakes of long chain omega-3 fatty acids may be associated with one pattern consistent with advancing neurodevelopment.

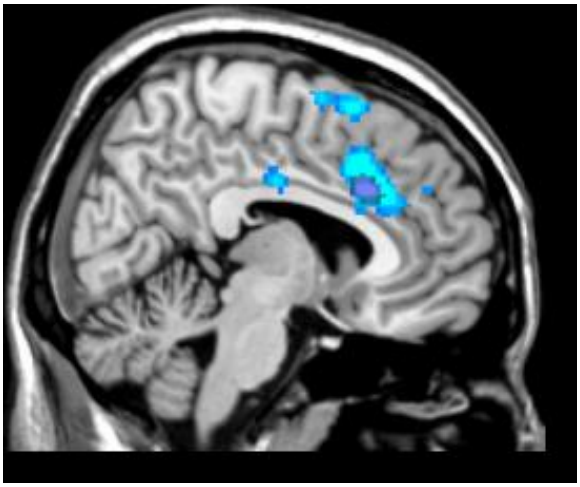

Figure 13. Overlap between Study 2 fMRI result and fMRI developmental pattern. dACC cluster where activity was inversely associated with long-chain N3 fatty acid intake (Study 2) [purple/overlap] is contained by extent of dACC deactivation to the same contrast (Correct No-Go > Incorrect No-Go) when the adolescents are older [light blue].

However, again, while it's likely that "functional maturation" is a misnomer given that even the adult brain is in flux (Berl et al., 2006; Somerville, 2016), unlike the inverted-U functional development of the vmPFC described above, the *general* functional pattern within the dACC concomitant during thinning is an *increase* in activity over development (Rubia et al., 2007). If greater long chain N3 intake is arguably going to advance neurodevelopment amongst adolescents of the same approximate age, the *reduction* in dACC activity observed in Study 2 in relation to greater N3 intake appears to stand in direct contradiction. Two points, however, are helpful in demonstrating how Study 2's results are in line with this theory: (1) dACC's presumed role in cognition and (2) the preponderance of studies examining dACC's response to *inhibitory errors* rather than response to *inhibitory successes*.

PFC exerts top-down control over behavior and the anterior cingulate is purported to be involved in, among many cognitive functions, performance monitoring during situations where the chance of error is high (Carter et al., 1998) and the need for heightened vigilance if there are conflicting

responses possible (Brown & Braver, 2005; Heilbronner & Hayden, 2016). In contrast to the proactive cognitive control sustained over the course of the task (Aron, 2011), which would involve the dorsolateral PFC when it is developmentally on-line (Andrews-Hanna et al., 2011), the ACC has been implicated in reactive control, a transient process enacted after perception of the stimulus (Braver, 2012). Over adolescence, the ACC demonstrates an age-related increase in activity during events requiring vigilance (Andrews-Hanna et al., 2011), interpreted as a compensatory reliance on this reactive mechanism until the more proactively engaged dlPFC comes online. Thus, the dACC response to errors may be of use in evaluating the current findings. Furthermore, the bulk of literature on GNG task focuses on the No-Go trials, particularly the neural activity associated with *incorrect* No-Go trials (i.e., the inability to inhibit impulses). The contrast presented throughout this work reflects the activation associated with successful inhibitions (i.e., *Correct* No-Go trials > Incorrect No-Go trials). But this contrast can conceptually be reversed to enable comparison with this literature and facilitate extrapolation of implications. In this light, **the flipped interpretation is that greater intake of dietary N3 index results in greater activation of dACC *during errors* (Study 2).** We see that between adolescence and adulthood, the strength of ACC activation increases during error monitoring (Ordaz et al., 2013; Rubia et al., 2006). This is also true for during high-response conflict (Andrews-Hanna et al., 2011). Additionally, Luna et al. report that "only adults showed robust recruitment of dorsal ACC during inhibitory errors" (Luna et al., 2010), suggesting that greater recruitment of dACC during errors is a developmentally consistent pattern. Furthermore, deviation from this pattern has been associated with behavioral consequences - those who show less activation during trials requiring inhibition are more likely to transition to heavy alcohol use (Norman et al., 2011). That the present work is cross-sectional in nature limits interpretation of 'developmental effects'. Again, we also acknowledge that due to

interindividual variation, no *single* pattern equates to functional “maturation" (Berl et al., 2006; Somerville, 2016). But taken together, based on the interpretation of results of this inverse contrast, we interpret this greater activation of dACC during inhibitory errors being linked to greater energy-adjusted intake of long chain N3 fatty acids to be suggestive of N3 fatty acid promoting neurodevelopmentally consistent dACC function compared to same age adolescents with lower intake and potentially suggestive of greater degree of vigilance during errors.

Discussion points

Only dACC, not other canonical Go/No-Go regions. We did not find Go/No-Go related-activation associated with other regions besides the dACC. Response inhibition as elicited by the Go/No-Go task has been found to generally recruit a distributed processing system involving pre-supplementary motor area, dorsolateral PFC, right inferior frontal gyrus and anterior cingulate, caudate and inferior parietal lobe (Menon et al., 2001; Simmonds et al., 2008). This is due in part to the fact that we limited out analyses to the PFC. The reasons we chose to limit our analyses are due to the evidence that DHA is highly enriched in this region, and this region is undergoing substantial remodeling during adolescence. Furthermore, radiotracer studies indicate that DHA content of striatum is not easily influenced by DHA intake in young adult rats (Kitson et al., 2016). Thus, variations in DHA are more likely to reveal differences in PFC function (specifically in regions recruited for error detection/performance monitoring, dACC) and not striatal activity.

Task differences and choice of contrast differences may also contribute to why we only found dACC and not other frontal cortex regions. As reviewed by Simmonds et al. (2008), the present work uses a simple-Go/No-Go task in which the participant only has 2 stimulus types to respond

to: Go and a less frequent No-Go trials. Complex design tasks, where additional contingencies change the meaning of potential No-Go trials thus requiring working memory, tend to recruit right dlPFC. Activation of dlPFC during simple tasks may in actuality be inefficient and counterproductive to performance. Activation of right inferior frontal gyrus may be dependent on the task and contrast used as it is typically found with No-Go > Go comparisons. Simmonds and colleagues suggest this is a potential reflection of an "oddball effect".

Null results. Contrary to our hypotheses, we did not find a direct relationship between various N3 Index measures and task behavior and the link between N3 Index measures and BRIEF Inhibit were mixed. Additionally, while we report on dorsal cingulate gray matter volume in Study 3, we did attempt to replicate functional methods in this older sample with blood N3 Index to no avail. Potential reasons why are discussed below.

There are a couple of suspected reasons why, aside from finding an association with energy-adjusted N3 fatty acids and better impulse control behavior on BRIEF (Study 2), we were unable to find a relationship with Go/No-Go performance: (1) Singular/specific facet of impulsivity and (2) Developmental sensitivity of the task.

- *Many facets of impulsivity*. Many types of response inhibition and the present work is only an examination of 2 measures. The Go/No-Go task in its current form is an assessment of behavior representing only a small slice of behavior (impulsive action). BRIEF inhibit and GNG false alarm are likely tapping into slightly different features of impulsive action as they are not related in this sample (Data not shown; Study 1: r_s=0.167, p=0.138, n=80; Study 2: r_s =0.011, p=0.916, n=87; Study 3: r_s =0.099, p=0.419, n=69). Thus, the disparity between finding an association between energy adjusted long chain N3 fatty acids in the

diet and BRIEF Inhibit but not Go/No-Go performance (Study 2) is not completely unexpected. Rather, it would be remarkable if performance on a laboratory task conducted in a calm (albeit, noisy scanner) environment without social pressures (Somerville, 2016) were strongly related to a caregiver's impressions of their adolescents general impulse control abilities in real-world settings over the six month span prior to their visit. Naturalistic assessments may be a more representative/accurate assessment of adolescent abilities than lab-based tests (Barkley, 2012).

- *Developmental sensitivity of the task (Complexity, difficulty).* Task complexity has been shown to determine if adult level performance of inhibitory control reached in childhood or adolescence (Petersen et al., 2016). Adolescents would likely approach ceiling performance at a younger age on a simple response inhibition task with minimal working memory demands ("delay or rule to inhibit prepotent response") versus complex inhibitory control paradigms ("conflict" paradigms requiring inhibition of a prepotent response but in the context of a conflicting response) (Petersen et al., 2016). Ceiling effects related to task performance may have increased our Type II error through a reduction in variability of response inhibition performance (Petersen et al., 2016). As most task-based measures of impulsivity are restricted time frame of usefulness (i.e., most tasks have a window of sensitivity in development of less than 3 years (Petersen et al., 2016)), the age ranges of participants in this work may be outside of the developmental sensitive window for this task. Indeed, many "executive" abilities are already fully developed by this stage of adolescence (Paus, 2005). In support of the notion that this task may have been too easy, others report that blood levels of N3 fatty acids are related to performance only on the most difficult levels of a task (Narendran et al., 2012). The task used in this study was identical

at each timepoint and did not vary in task difficulty. For Study 3, the adolescents are slightly older than they were in Study 2 (14.4±0.7 years old versus 13.3±1.1 years) and we did not observe a relationship between blood N3 index levels and response inhibition though, as reviewed by Luna (2010), improvement in task based response inhibition generally reaches a plateau (reflecting near-adult levels) during adolescence (Luna et al., 2010). The task parameters were identical between waves and thus it is seems reasonable that a lack of a relationship with response inhibition and dietary N3 index assessed primarily at the first timepoint would limit the degree of behavioral improvement approximately 18 months later (Petersen et al., 2016).

We were also unable to detect a direct relationship between omega-3 and BRIEF Inhibit scores in Studies 1 and 3. With respect to Study 1, it is unclear exactly why we did not find a difference in ratings of inhibitory control between longer and shorter breastfeeding duration groups. Given that the difference in PFC function between groups was localized to the vmPFC, we may speculate that perhaps the groups might differ in motivation/reward valuation rather than response inhibition per se. With respect to Study 3, while we found that, in males, greater levels of whole blood long chain N3 fatty acids are related to less dACC GMV which is in turn related to better impulse control ratings, we were unable to find a direct relationship between blood levels and behavior. Although this may appear to be a complete mediation (i.e., no direct effect, relationship completely mediated by dACC GMV), as Kenny and Judd (2014) explain, it is easier to throw a ball twice over a long distance than to cover the same long distance in one throw. It is possible, therefore, that we were underpowered to detect the direct effect between blood and behavior (Kenny & Judd, 2014).

Lack of functional results with whole blood omega-3 index. While we present structural findings for Study 3, we also attempted to replicate Study 2 using identical parameters (i.e., including both sexes) and only changing blood N3 index as the new independent variable. In an fMRI analysis restricted to bilateral frontal gray matter, we were unable to find any clusters of activation either positively or negatively associated whole blood N3 index during successful inhibitions (Correct No-Go > Incorrect No-Go). Given the result we found whereby sex influences the relationship between blood long chain N3 levels and dACC gray matter volume, it is possible that analysis using the whole group occluded the relationship with blood and dACC activity during successful inhibition. It is interesting though, that as networks continue to develop with age, it may not be activation (or magnitude of activation) within a single region that distinguishes adolescents but rather the degree of functional connectivity (Grosbras et al., 2007). This suggests a potential future direction as discussed below.

Differential influence of sex across studies. Effect of sex was largely non-existent until Study 3 when the sample was the oldest (Study 1: no effect; Study 2: no effect, Study 3: N3 inversely related to dACC GMV in males only). There are a few potential explanations for this pattern of results.

The primary determinants of DHA production and incorporation are expression and activity of the (delta6) desaturase enzyme and competition between omega-3 and omega-6 substrates for enzymatic metabolism (Kitson et al., 2010). Furthermore, sex hormones modulate the activity of delta6 desaturase (Kitson et al., 2010; Marra & de Alaniz, 1989). In Study 1, males (n=38) and females (n=42) were similarly distributed amongst duration groups (χ^2=1.404, p=0.169) and we did not observe an effect of sex when included in the model (data not shown; included in

ANCOVA as a fixed factor along with duration group). Reports of differential benefits of breastfeeding duration by sex are mixed, however. There is evidence that duration of breastfeeding has small but significant effects with similar benefits conferred to males and females on cognitive outcomes like IQ, standardized test scores, teacher ratings of school performance through high school (Horwood & Fergusson, 1998). However, others report a significantly greater benefit of longer duration of breastfeeding for males than females on white matter volume and intelligence of adolescents born prematurely (Isaacs et al., 2010), academic performance (math and spelling) at 10 years old (Oddy, Li, Whitehouse, Zubrick, & Malacova, 2011) and psychomotor speed at 17 years of age (Nyaradi et al., 2015). Unfortunately, the current sample is too small to investigate with a 2x2 (duration group * sex) design. Interestingly though, there is some evidence that the composition of mammalian milk is dependent on sex of the offspring, with the richness of milk (in fatty acids) observed to be higher for males than for females (Hinde, 2009) with potential influence on subsequent behavior (Hinde & Capitanio, 2010). Whether this is a cause of potential sex differences from breastfeeding duration or a consequence of males at a biological disadvantage for N3 conversion (i.e., a biological compensatory mechanism) is an intriguing question.

With regards to dietary intake (Study 2), males (n=43) and females (n=45) report similar intakes of long chain N3 intake (square root transform) either unadjusted (p=0.814) or adjusted for total energy intake (p=0.830) and sex was not a significant predictor of either the BRIEF Inhibit subscale (linear regression; p=0.506) or false alarm rate on the Go/No-Go task (linear regression; p=0.329). That report of nutrient intake among this age group does not differ by sex appears to be consistent with the literature. Prior research has found that diet quality scores of children and teens

does not differ by sex (Feskanich, Rockett, & Colditz, 2004; Hiza, Casavale, Guenther, & Davis, 2013) and both sexes display similar dietary patterns over time (Cutler et al., 2009).

With regards to blood levels (Study 3), adolescent boys and girls had similar levels of the N3 index (EPA+DHA) and similar levels of DHA. A subsequent analysis reveals that while EPA is also similar between groups (p=0.810), there was a trend towards males having higher median levels of the intermediate fatty acid DPA (data not shown; 0.95% vs 0.90% of fatty acids Mann-Whitney U=354.0, p=0.079). Although this difference did not reach significance, levels of the precursor fatty acids to DHA (i.e., EPA and DPA) have been found by others to be higher in adolescent males than in females (age 15+; Crowe, Skeaff, Green, & Gray, 2008), and blood levels of DHA (the end product) tend to show the reverse pattern, higher in females than in males (Crowe et al., 2008; Marangoni et al., 2007). It is unclear why this sex difference is not entirely represented in the current sample although duration of pubertal development may be a contributing factor. While the girls were significantly more advanced in pubertal status than boys in this sample (and sex hormones modulate DHA production as described earlier), the sample is still in the middle stages of pubertal development. It is possible that as more time elapses, and thus longer exposure to greater levels of sex hormones, differences in enzymatic processes evidenced by blood levels of essential fatty acids may become more prominent.

Relationship between diet and blood levels in the present sample. Others have reported mild to moderate correlations between dietary report intake of fish and/or long chain N3 fatty acids and blood levels in middle aged females (Sun et al., 2007) and in a samples of typically developing adolescent boys and girls (van der Wurff et al., 2016; Vandevijvere et al., 2012). Similarly, we have evidence of validation of the use of the diet survey within our sample. Using a subsample of

adolescents who completed both the food frequency questionnaire and those who provided a blood sample at the same timepoint (n=19), we see that energy-adjusted omega-3 index (via diet) and omega-3 index observed in blood are highly correlated (r_s=0.660, p=0.002), similar to other studies (e.g., Almeida, Jandacek, Weber, & McNamara, 2016; Dahl, Maeland, & Bjorkkjaer, 2011; Marangoni et al., 2007). We also see that reported dietary intake predicts blood levels approximately 18 months later, albeit to a lesser degree (r_s=0.318, p=0.015, n=58) suggesting that long-chain N3 fatty acid status is relatively stable in this sample (Figure 14).

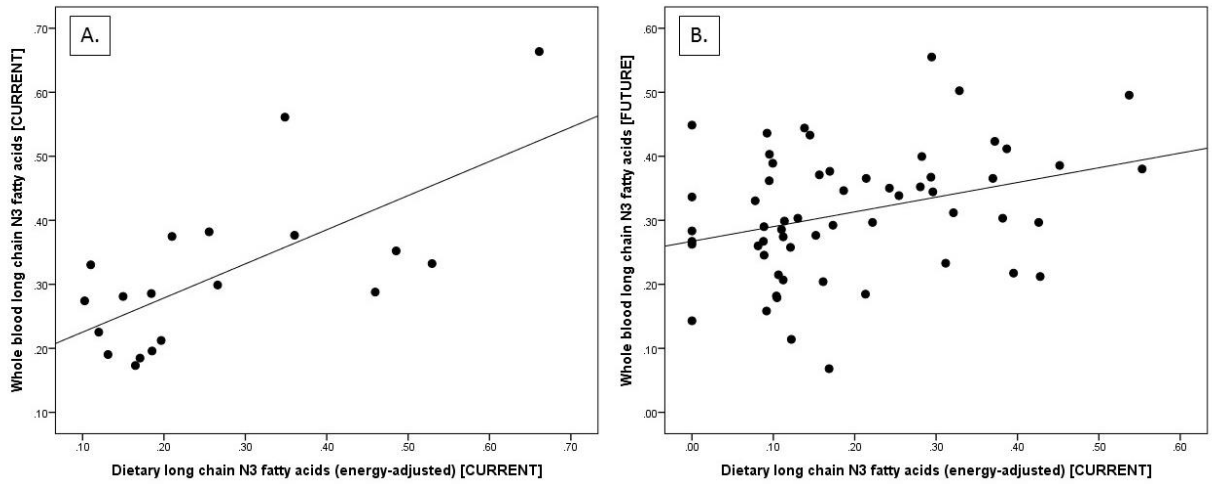

Figure 14. Relationship between dietary omega-3 and blood levels of omega-3. Current reported intake of energy-adjusted long chain N3 fatty acids (square root transform) correlates with (A) current whole blood long chain N3 fatty acids (log10 transform) in a subset of adolescents (n=19) and (B) future whole blood long chain N3 fatty acids (log 10 transform) in a larger sample providing blood approximately 18 months later (n=58).

While we have both diet and blood data on a small subset of participants collected at the same timepoint (n=19) and this data suggests high correspondence between estimated long chain N3 fatty acids calculated from reported dietary intake and whole blood long chain N3 fatty acid level, due to constraints on funding and participant burden, we were unable to collect both data points in most of the sample. As Vandevijvere and colleagues (2012) discuss, while biomarkers

and dietary intake demonstrate some degree of correlation, biomarkers, due to variation from differences in absorption and metabolism, are not always superior to diet assessment. They conclude that biomarkers should be "used in addition to and not in replacement of dietary surveys" (Vandevijvere et al., 2012).

Influence of socioeconomic status. Dietary patterns are often related to socioeconomic status, an established correlate of brain structure and function (Johnson et al., 2016). Thus, we make attempts to account for the contribution of SES to brain development and behavior in order to better isolate the unique influence of nutrition. SES and maternal IQ are factors which have been shown to confound the breastfeeding-cognitive development relationship in high-income countries (Walfisch et al., 2013). However, as reviewed by Prado and Dewey (2014), while higher SES is related to breastfeeding extent in developed countries, the opposite is observed in low- and middle-income countries. In Study 1, SES was significantly, albeit weakly, associated with both measures of inhibitory control and the Longer Duration group reported significantly greater household socioeconomic status, largely driven by greater education. Controlling for the influence of SES in behavioral and fMRI results ostensibly revealed the unique influence of infant feeding practices on brain function during inhibitory control. In Study 2, again SES was related to inhibitory control (caregiver ratings only), but energy-adjusted omega-3 index intake was unrelated to SES. Modeling SES and dietary long-chain omega-3 intake revealed that diet accounted for a similar amount of *unique* variance in caregivers' ratings of adolescent inhibitory control as did SES. In Study 3, across all participants, again SES was weakly related to inhibitory control (Go/No-Go successful inhibitions only) but SES was unrelated to either blood omega-3 index level or dACC GMV. Among males, who exhibited a strong relationship between dACC GMV and blood omega-3, there was no relationship between SES and any behavioral or structural outcome variable. Thus,

in the present work, we attempted to account for any influence of SES where applicable. Assessing the unique influence of omega-3 fatty acids on developmental outcomes is useful in that unlike SES *per* se it is a factor which may be easily modified in the service of optimizing morphological and functional neurodevelopment.

Future directions

Trajectory of functional development. While cross sectional studies are a good starting point for examination of the effects of nutritional components on brain structure/function, it remains difficult to assess how these nutrients influence changes over development using this type of study design. Thus, exploring the trajectory of medial PFC function and structure with longitudinal analyses in relation to status of long chain N3 fatty acids will help us answer questions about the long-range implications of variations in N3 status from the baseline assessment forward. Also, the *nature* of the trajectory (i.e., the *rate* of increase or decrease in activation or gray matter volume) may be an important predictor of long term, broader outcomes such as the magnitude or rate of gray matter change during development that is ultimately predictive of skill, IQ, or other outcome measures (Lu et al., 2007; Shaw et al., 2006).

Of particular interest in this respect is the duration of breastmilk exposure during infancy and inverted-U functional trajectory of the vmPFC. Given the potential for DHA to facilitate neurodevelopment, and the neurodevelopmental functional pattern of the vmPFC is heightened responses to incentives in comparison to both children and adults (inverted U-shaped functional trajectory)(Van Leijenhorst et al., 2010), future studies should explore the longitudinal trajectory of the vmPFC function to goal-associated stimuli in relation to duration of breastmilk exposure

during infancy. It is possible that adolescents with shorter exposure duration are only delayed in their vmPFC functional development in relation to adolescents exposed to breastmilk for longer, and potentially would demonstrate increased vmPFC activation to successful inhibitions compared to their peers at a later time point. Study of the vmPFC response overtime would help clarify this.

Functional connectivity. Aside from the specialization of neural regions for particular functions, one of the other core features of the brain is the degree to which it integrates information between regions. Thus, a natural extension of the work presented here is to examine the functional connectivity of regions (Study 1: vmPFC with dlPFC; Study 2: dACC with other regions implicated in response inhibition: right inferior frontal gyrus, caudate nucleus) in association with N3 fatty acid status. Since different patterns of cortical activity may enable the same task performance, and we did not observe a relationship between breastfeeding group or dietary long chain N3 fatty acids and output (Go/No-Go successful inhibitions), it would be interesting to explore differences in patterns of functional connectivity.

Conclusions and broader impact

The present work explores relationships between long chain omega-3 fatty acids and PFC This work explored the relationships between long chain omega-3 fatty acids and PFC function/structure with respect to impulse control within typically developing adolescents. Results suggest that long chain N3 fatty acids have some bearing on general impulse control in adolescence and are related to PFC function and structure and may ultimately influence the trajectory of brain development in a manner specific to age/developmental stage. At a fundamental level, the results herein suggest that dietary components might account for some variance in functional and

structural neuroimaging results in studies of typically developing adolescents. We believe the results have a broader impact, however, in that they provide additional evidence that, to paraphrase, "*moving parts need oil*" in the form of long chain omega-3 fatty acids (Giedd et al., 2008). Thus, this dissertation should stand as a public health message aimed at adolescents and their parents to consider the impact of diet as highly relevant to their behavioral development (Gage et al., 2014). Recommendations for dietary long chain omega 3 fatty acids derived from marine sources are not only important with regards to cardiovascular disease, certain mental health and neurodegenerative conditions, and infants/women of childbearing age/nursing mothers (Kris-Etherton, Grieger, & Etherton, 2009) but the results presented herein support the argument that long chain omega-3 fatty acids are important for normative development of key aspects of brain function and structure and thus behavior.

APPENDIX: AAL atlas regions included in bilateral prefrontal gray matter mask

Precentral_L,R

Frontal_Sup_L,R

Frontal_Sup_Orb_L,R

Frontal_Mid_L

Frontal_Mid_Orb_L

Frontal_Inf_Oper_L

Frontal_Inf_Tri_L

Frontal_Inf_Orb_L

Rolandic_Oper_L

Supp_Motor_Area_L

Frontal_Sup_Medial_L

Frontal_Med_Orb_L

Rectus_L

Insula_L

Cingulum_Ant_L

Cingulum_Mid_L

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