CHAPTER 6
Discussion
The present study for the first time demonstrates the effects of vitamin B₁₂ deficiency/supplementation on brain fatty acids, neurotrophins, angiogenic factors and cognitive performance across three generations. This study also evaluates the effects of omega-3 fatty acid supplementation both in the presence and absence of vitamin B₁₂ on the above parameters. The following sections discuss the findings of each generation.

6.1 F₁ Generation Offspring at Birth

The present study reveals several novel and interesting key findings related to maternal vitamin B₁₂ and omega-3 fatty acid status in the pup brain at birth which are as follows: (i) Vitamin B₁₂ deficiency resulted in lower protein and mRNA levels of VEGF, higher HIF-1 alpha protein levels and lower trend for NGF protein levels (ii) Omega-3 fatty acid supplementation to a vitamin B₁₂ deficient diet improved the DHA levels, BDNF protein levels, NGF protein levels and VEGF mRNA levels iii) Vitamin B₁₂ supplementation increased the protein levels of BDNF while fatty acid profile, levels of NGF and VEGF were comparable to the control group iv) Omega-3 fatty acid supplementation together with vitamin B₁₂ increased the levels of DHA, BDNF, and NGF as compared to both the control and BS groups.

6.1.1 Effect of Vitamin B₁₂ Deficiency on Brain Fatty Acid, BDNF, NGF and VEGF Levels

Maternal vitamin B₁₂ deficiency showed reduced levels of AA and reduced trend of DHA in the brain of the offspring as compared to the control group. It has been suggested that accretion of AA and DHA occurs in the brain during the last trimester of gestation and plays a vital role in neuronal growth and function (Rogers et al. 2013). It has been suggested that low availability of LCPUFA in the brain is implicated in impaired neurodevelopment (Dijck-Brouwer et al., 2005).

Maternal vitamin B₁₂ deficiency did not affect the levels of BDNF while the trend for reduced NGF was observed in the offspring brain as compared to the control group. Earlier animal studies from our department demonstrated reduced levels of NGF and BDNF in the offspring brain as a consequence of maternal micronutrient imbalanced
(high folate and low vitamin B<sub>12</sub>) diet (Sable et al. 2011, 2012). NGF is essential for the development and survival of sensory neurons (Berry et al. 2012). Apart from neurotrophic properties, recently NGF has been described as an important angiogenic molecule (Lazarovici et al. 2006), it is known to have a cross-talk with VEGF (Calza et al. 2001; Hansen-Algenstäedt et al. 2006) and also promote endothelial cell proliferation and migration (Dolle et al. 2005).

The present study reports lower levels of VEGF in the offspring brain as a consequence of maternal vitamin B<sub>12</sub> deficiency. The crucial role of VEGF in vascularization and neuronal cell migration has been well implicated in the developing brain (Schwarz et al. 2004). It has been reported that the developing brain requires a good vascular system for the delivery of oxygen and nutrients (Mackenzie and Ruhrberg, 2012). The lower levels of VEGF in the present study may be indicative of hampered brain vasculature. Reduced VEGF levels are known to be associated with neurodegenerative diseases like amyotrophic lateral sclerosis (ALS) in mice as well as in humans (Lambrechts et al. 2003).

An animal study carried out in our department have reported high levels of homocysteine and low vitamin B<sub>12</sub> levels in the dam plasma as a consequence of vitamin B<sub>12</sub> deficiency (Khaire et al. 2015b) which is likely to influence angiogenesis. It has been demonstrated that homocysteine inhibits angiogenesis through the inhibition of VEGF/VEGFR, Akt, and ERK1/2 mechanisms (Zhang et al. 2012). Hyperhomocysteinemia through the mediation of oxidative stress has been shown to be associated with changes in the structure and function of cerebral blood vessels (Faraci and Lentz, 2004).

Further, the protein levels of HIF-1 alpha were higher in the pup brain from the maternal vitamin B<sub>12</sub> deficient group. It has been demonstrated that higher HIF-1 alpha levels may be responsible for hypoxia-induced growth arrest and apoptosis (Goda et al. 2003). It has also been shown to regulate local brain hypoxia and influence brain vascularization (Giordano et al. 2001). In the current study, VEGF levels were lower although HIF-1 alpha levels were higher suggesting that neuroprotective genes like VEGF may also be regulated by another transcription factor, independent of HIF-1alpha.
(Benderro et al. 2012). However, the underlying mechanisms are not fully understood and need to be explored.

6.1.2 Effect of Vitamin B$_{12}$ Supplementation on Brain Fatty Acid, BDNF, NGF and VEGF Levels

The data of the present study indicates that maternal vitamin B$_{12}$ supplementation showed similar fatty acid profile in the offspring brain at birth as that of the control group, although increased trend for DHA was observed. The increased levels of BDNF were also observed in this group. The role of BDNF in influencing neuronal proliferation, migration, survival and neurogenesis in developing brain is widely established (Tapia-Arancibia et al. 2004). Thus, increased levels of BDNF may contribute to the optimal neuronal development. Further, the levels of brain NGF and VEGF were not altered in this group and remained comparable to the control group.

6.1.3 Effect of Omega-3 Fatty Acid Supplementation to the vitamin B$_{12}$ deficient/supplemented diet on Brain Fatty Acid, BDNF, NGF and VEGF Levels

Maternal omega-3 fatty acid supplemented groups with and without vitamin B$_{12}$ showed higher levels of DHA and omega-3 fatty acids and lower levels of AA and omega-6 fatty acids in the brain of the offspring. Omega-3 fatty acids are an important modulator of neuronal membrane function, neurogenesis and neurotransmitters (Innis, 2008). It has been suggested that increased brain DHA levels are compensated by decreased brain AA levels (Balogun and Cheema, 2014) possibly because the metabolism of LCPUFAs shares the common set of enzymes (Innis, 2008).

The levels of nervonic acid (marker of myelination) were lower in the omega-3 fatty acid supplemented groups, indicative of delayed myelination and continued enhanced brain development. A study by Elsherbiny et al. has also reported the lower levels of brain nervonic acid in the animals fed with DHA-rich diet (Elsherbiny et al., 2015). A report suggests that low levels of nervonic acid are indicative of increased risk for psychosis which was prevented by supplementation with omega-3 fatty acids suggesting that omega-3 fatty acids may offset the risk conferred by decreased levels of nervonic acid (Amminger and McGorry, 2012).
Omega-3 fatty acid supplementation to the vitamin B\textsubscript{12} deficient diet normalized the levels of NGF and BDNF suggesting its role in the regulation of neurotrophins in the brain. Further, it was also able to restore HIF-1 alpha protein levels. Our results are in accordance with another recent study which demonstrates that omega-3 fatty acid consumption decreases the protein levels of HIF-1 alpha in subcutaneous adipose tissue of obese adolescents (Mejia-Barradas et al. 2014). However, the underlying mechanisms are not fully understood and need to be explored.

The combined vitamin B\textsubscript{12} and omega-3 fatty acid supplemented group showed higher levels of BDNF and NGF in the offspring brain as compared to both the control and vitamin B\textsubscript{12} supplemented groups. This could be attributed to higher levels of DHA observed in this group which is known to regulate the levels of neurotrophins in the brain. Several studies have highlighted that DHA may increase the BDNF levels in the brain due to release of neuroprotectin D1 and antioxidant capacity of DHA (Wu et al., 2008). Further, the levels of VEGF and HIF-1 alpha were maintained in this group to that of the control. The role of omega-3 fatty acids in promoting post-stroke angiogenesis in the brain has also been reported (Wang et al. 2014b). However, future studies need to examine the effect of combined supplementation of vitamin B\textsubscript{12} and omega-3 fatty acids on brain angiogenesis and neurogenesis.

To conclude this section, our data suggests that an insufficient intake of vitamin B\textsubscript{12} can result in dysregulation of NGF and VEGF levels which may increase susceptibility to neurological disorders. Vitamin B\textsubscript{12} and omega-3 fatty acid supplementation maintained the levels and expression of VEGF and NGF in the offspring brain. This may lead to novel dietary approaches for disease prevention and discovery of new therapeutic targets for mental illnesses.

It is likely that these adaptations made by the fetus in response to nutritional inadequacies in early-life may be reflected only in later life in the form of functional disabilities. Hence, it is of importance to examine whether these effects persist until adult life. The next section discusses the results of the F\textsubscript{1} generation offspring at 3 mo of age, wherein the analysis was carried out in two different regions of the brain i.e. cortex and hippocampus, associated with learning and memory processes.
6.2  F1 Generation Offspring at 3 mo of Age

The following important findings were observed in the F1 generation offspring at 3 mo of age: (i) The offspring from vitamin B12 deficiency group demonstrated lower levels of DHA (in both cortex and hippocampus), BDNF (in cortex and hippocampus), NGF (in cortex), VEGF (in hippocampus) and lower plasma MDA levels as compared to the control group. The trend for the poor cognitive performance was observed in this group. (ii) Omega-3 fatty acid supplementation to vitamin B12 deficient group improved the levels of DHA (in both cortex and hippocampus), BDNF and VEGF mRNA levels (only in hippocampus) and plasma MDA levels while NGF protein levels were not altered. This group of animals improved cognitive performance evidenced by less number of errors as compared to the vitamin B12 deficient group (iii) The offspring from vitamin B12 supplemented group had normal fatty acid profile, BDNF, NGF and VEGF levels in both the cortex and hippocampus regions and were comparable to the control group iv) Omega-3 fatty acid supplementation together with vitamin B12 improved levels of DHA, BDNF, NGF and cognitive performance as compared to control and vitamin B12 supplemented group.

6.2.1 Effect of Vitamin B12 Deficiency on Brain Fatty Acid, BDNF, NGF, VEGF Levels and Cognitive Performance

Maternal vitamin B12 deficiency reduced the levels of DHA in the cortex and hippocampus region of the adult offspring and as discussed above reduced trend for DHA was also demonstrated in the offspring at birth. Similar findings have been reported by our departmental studies in the plasma and liver of the offspring as a consequence of maternal vitamin B12 deficiency (Khaire et al. 2015a; Meher et al. 2014; Wadhwani et al. 2012). Reports indicate that B vitamins may modify methylation of phosphatidylethanolamine (PE) to phosphatidylcholine (PC) by PE-N-methyltransferase (PEMT) enzyme in the liver (van Wijk et al. 2012; Pynn et al. 2011). Studies have demonstrated that the PEMT pathway is required for the transport of DHA from the liver into the plasma and other tissues. It is likely that a deficiency of maternal vitamin B12 results in reduced DHA levels in the pup brain possibly due to the impaired conversion of PE-DHA to PC-DHA. The PC/PE ratio is known to modify the activity of Δ5 and Δ6
desaturases involved in the synthesis of omega-3 and omega-6 fatty acids (Smith et al. 2008). In support of our data, a study reports that maternal vitamin B deficient (folate, vitamin B₁₂ and B₆) diets led to accumulation of homocysteine and reduced levels of plasma DHA (van Wijk et al., 2012).

Lower mRNA and protein levels of BDNF were demonstrated in the cortex and hippocampus of the vitamin B₁₂ deficient group. Further, vitamin B₁₂ deficiency led to reduced NGF protein and VEGF mRNA levels in the cortex and hippocampus respectively. We have demonstrated similar findings in the offspring brain at birth suggesting consistent adverse effects of vitamin B₁₂ deficiency. Maternal vitamin B₁₂ deficiency also led to higher plasma MDA levels in the offspring at 3 mo of age. MDA is considered as a marker for oxidative stress-induced lipid peroxidation. It is known that the brain is highly susceptible to oxidative cellular damage due to high metabolic load and poor antioxidant defense system. Furthermore, it has been proposed that oxidative stress has been widely characterized in cerebrovascular dysfunction and cognitive decline (Marlatt et al. 2008).

The poor cognitive performance was observed in the vitamin B₁₂ deficient group demonstrated by more number of reference and working memory errors (RWME) and lower trend for correct choices in the radial eight arm maze test. This may possibly be attributed to the low DHA and neurotrophin levels which are known to be associated with a decline in cognitive abilities (Muldoon et al. 2010). It has been proposed that the adverse effects of a maternal vitamin B₁₂ deficiency on cognition in the offspring could be due to a reduction in total brain volume or improper myelination (Casella et al. 2005).

6.2.2 Effect of Vitamin B₁₂ supplementation on Brain Fatty Acid, BDNF, NGF, VEGF Levels and Cognitive Performance

Vitamin B₁₂ supplemented group showed similar fatty acid profile in both hippocampus and cortex regions of the brain as that of the control group. Similar results were demonstrated in the offspring at birth. The levels of NGF and VEGF were not altered and remained comparable to the control group. Further, the higher trend for BDNF protein levels were demonstrated in the hippocampus region of the brain. Similarly, the higher levels of BDNF in the offspring brain at birth were observed in this
group. The effect of vitamin B$_{12}$ supplementation along with dexamethasone has been reported to upregulate BDNF expression in a rat model of sciatic nerve injury (Sun et al. 2012). Similarly, in the same animal model, it has been demonstrated that methylcobalamin promotes neurite outgrowth and survival through the methylation cycle (Okada et al. 2010). The animals from this group showed similar performance in the radial eight arm maze task as that of the control group suggesting that vitamin B$_{12}$ supplemented group showed almost similar results as that of the control group and may be involved in optimal brain functioning.

### 6.2.3 Effect of Omega-3 Fatty Acid Supplementation on Brain Fatty Acid, BDNF, NGF, VEGF Levels and Cognitive Performance

Omega-3 fatty acid supplementation to vitamin B$_{12}$ deficient/supplemented diet increases the levels of DHA and decreases the levels of AA in the hippocampus of the offspring at 3mo of age. As discussed earlier, studies have reported that there might be a competition for the enzymes like delta-5 and delta-6-desaturase involved in the metabolism of omega-3 and omega-6 fatty acids which could be the possible explanation for decreased AA levels and increased DHA levels (Schmitz and Ecker, 2008; Simopoulos, 2008).

A combined supplementation of vitamin B$_{12}$ and omega-3 fatty acids showed higher levels of neurotrophins and these results are consistent with the findings obtained in the offspring at birth suggesting that these effects were sustained till adult age. Studies have indicated that there is a probable association of learning and memory functions with the brain fatty acid and neurotrophin status (Bhatia et al., 2011; Sharma et al., 2012). Thus, it was of interest to examine the cognitive assessment in the adult offspring as a result of the combined supplementation of vitamin B$_{12}$ and omega-3 fatty acids.

In the present study, the adult offspring were tested for spatial memory and learning by two different tasks: Morris water maze and radial eight arm maze. In the Morris water maze, there was no difference in the performance of rats in any group. In the radial eight arm maze, the difference was found for working memory (WME) and reference working memory errors (RWME) between the groups. In the working memory task, the animals had to remember from which arm they had removed a food pellet and
offspring from the BDO and BSO group made less number of WME as compared to the vitamin B\textsubscript{12} deficient and control group respectively. This reflects a good learning ability of the offspring and also good performance to sustain information within the given trial. In terms of reference and working memory error (RWME) which is also called as double memory error, the animals with omega-3 fatty acid supplemented groups (BDO and BSO) performed better than vitamin B\textsubscript{12} deficient/supplemented groups. It could be due to high levels of DHA and BDNF in the hippocampus and cortex of these groups since both are involved in learning and memory formation. A study has also reported the role of DHA in improving spatial memory of rats in terms of reduction of reference and working memory errors in an animal model of Alzheimer’s disease (Hashimoto et al. 2005). These observations suggest that DHA by being incorporated into neural membrane phospholipids increases neuronal cell functions (Perez et al. 2013).

The advantage of the radial-arm maze is that the training protocol and data interpretation of the basic version are simple (Sharma et al., 2010) and this test induces only a moderate level of stress (Hodges, 1996). In contrast, an earlier study reports that there are different systems of learning and memory, and performance on one type of learning task may have no effect on another (Wainwright et al., 1999).

To the best of our knowledge, there are no studies which have investigated the effects of a combination of both vitamin B\textsubscript{12} and omega-3 fatty acid supplementation on cognition using an animal model. Human studies report that multiple micronutrients and omega-3 fatty acids may influence learning and memory in children (Osendarp et al., 2007; Muthayya et al., 2009).

Further, omega-3 fatty acid supplementation to the vitamin B\textsubscript{12} deficient diet lowered the plasma MDA levels. SOD is an antioxidant enzyme with free radical scavenging activity and found to be higher in the combined supplemented group with vitamin B\textsubscript{12} and omega-3 fatty acids which may play an important role in neuroprotection.

To summarize this section, our findings indicate that maternal vitamin B\textsubscript{12} deficiency adversely influences the levels of brain DHA and neurotrophins. Omega-3 fatty acid supplementation to the vitamin B\textsubscript{12} deficient diet ameliorates some of the adverse effects. Vitamin B\textsubscript{12} supplementation did not alter the fatty acid profile and
levels of neurotrophins. However, the combined supplementation of vitamin B\textsubscript{12} and omega-3 fatty acids enhanced the levels of DHA, neurotrophins and cognitive performance. The present study suggests that omega-3 fatty acid together with vitamin B\textsubscript{12} may show beneficial effects on brain development and prevent early cognitive deficits and later neurobehavioral disorders.

The next section describes the results of the second (F\textsubscript{2}) generation offspring to examine whether the effects of the F\textsubscript{1} generation offspring are sustained in the F\textsubscript{2} generation.

### 6.3 F\textsubscript{2} Generation Offspring at 3 mo of age

The key findings are as follows: 1) Vitamin B\textsubscript{12} deficiency lowered the mRNA and protein levels of VEGF and NGF in the hippocampus and increased plasma MDA levels. It showed lower trend for BDNF levels while TrkB and CREB mRNA levels were not altered. Further, these animals demonstrated poor cognition 2) Omega-3 fatty acid supplementation to vitamin B\textsubscript{12} deficient diets normalized the levels of NGF, VEGF, BDNF and MDA and showed improved cognition 3) Supplementation of vitamin B\textsubscript{12} showed comparable levels of DHA, BDNF, CREB, NGF and VEGF to that of the control group 4) Supplementation of vitamin B\textsubscript{12} and omega-3 fatty acids together further enhanced the levels of DHA, BDNF, NGF and VEGF in the hippocampus and CREB mRNA levels and NGF protein levels in the cortex. Further, the cognitive performance of these animals was also improved as compared to the vitamin B\textsubscript{12} supplemented animals.

#### 6.3.1 Effect of Vitamin B\textsubscript{12} Deficient Diet on Brain mRNA Levels of BDNF, TrkB CREB and NGF

In the present study, lower (but not statistically significant) protein and mRNA levels of BDNF were demonstrated in the vitamin B\textsubscript{12} deficient group. However, vitamin B\textsubscript{12} deficiency did not alter the mRNA levels of TrkB and CREB as they remained comparable to their respective control groups. These results are in accordance with earlier findings which suggest that the expression of TrkB does not always depend on the tissue levels of BDNF (Frank et al. 1996; Knusel et al. 1997).
Vitamin B<sub>12</sub> deficiency also demonstrated lower NGF mRNA and protein levels in the hippocampus region of the brain. The critical role of NGF in the survival and functioning of the cholinergic neurons in the CNS is well known (Aloe et al. 2012). It has been proposed that reduced NGF signaling occurs in psychiatric disorders (Castren et al. 2007; Duman and Monteggia. 2006; Cirulli and Alleva. 2009). NGF has also been reported to modulate angiogenesis in the brain. It has been suggested that NGF promotes neuron-induced angiogenesis by stimulating VEGF production (Calza et al. 2001). It has been reported that NGF through the activation of its receptor tropomyosin receptor kinase A increases the production of VEGF and HIF-1α in the neuronal cells (Nakamura et al. 2011).

Vitamin B<sub>12</sub> deficiency also showed lower VEGF mRNA and protein levels in the hippocampus region of the brain. Our findings are similar to earlier studies which report lower plasma levels of VEGF in patients with neurodegenerative disorders (Lambrchts et al. 2003). VEGF expression is also reported to be lower in the superior temporal, hippocampal, and brainstem regions in the brain of patients with Alzheimer's disease (Provias and Jeynes, 2014). Reports indicate that in the adult brain, VEGF is involved in the neurogenesis which takes place in two defined regions, subventricular zone and the dentate gyrus of the hippocampus (Rosenstein et al. 2010). VEGF has been regarded as neurogenic factor involved in the cell proliferation, neuroblastoma production and neuronal differentiation in the hippocampus (Cao et al. 2004). It is also known to affect learning and memory processes through angiogenesis and neurogenesis (Licht et al. 2011).

6.3.2 Effect of Vitamin B<sub>12</sub> Deficient Diet on Oxidative Stress Markers and Cognitive Performance

Vitamin B<sub>12</sub> deficiency led to increased plasma MDA levels. It is known that the brain is highly susceptible to oxidative cellular damage due to high metabolic load and poor antioxidant defense system. Furthermore, it has been proposed that oxidative stress has been widely characterized in vascular dysfunction (Marlatt et al. 2008).

The offspring from the vitamin B<sub>12</sub> deficient group showed impaired cognition as they took a longer time to reach the platform in the Morris water maze test suggesting
that these offspring may have deficits in spatial learning. Further, these offspring when tested using the eight arm radial maze made less percentage of correct choices and more errors suggesting impairment in working and reference memory. We have earlier demonstrated that maternal vitamin B₁₂ deficiency showed increased homocysteine levels in the offspring and the dams (Sable et al. 2013; Khaire et al. 2015b). Reports indicate that increase in homocysteine levels also contributes to cognitive impairment (Feng et al. 2011). Our results are in accordance with other studies which have reported that deficiency of micronutrients like folic acid and vitamin B₁₂ cause hippocampal damage, improper differentiation of neuronal cells and cognitive deficits in rodents (Blaise et al. 2007; Troen et al. 2008). Further, report also suggests that altered VEGF levels are related to the severity of cognitive impairment (Ke and Zhang, 2013). Similarly, NGF deficit has also been associated with hampered cognitive processes (Conner et al. 2009). Thus, impaired cognitive performance observed in the vitamin B₁₂ deficient group may be attributed to lower VEGF and NGF levels.

6.3.3 Effect of Omega-3 Fatty Acid Supplementation on Oxidative Stress Markers and Cognitive Performance

Supplementation of omega-3 fatty acids to the vitamin B₁₂ deficient animals showed improved DHA levels in the cortex and hippocampus region, maintained BDNF levels in the cortex region, showed higher TrKB mRNA levels. These findings are of relevance since DHA is known to play a key role in cognitive development, membrane fluidity, neurogenesis, neuroplasticity and release of neurotransmitters, all processes involved in brain cell maturation and normal functions (Hashimoto, 2014). Several animal studies have also shown the positive effect of omega-3 fatty acids on BDNF levels (Wu et al. 2004a; Cysneiros et al. 2010; Gama et al. 2012). It has been speculated that high concentrations of omega-3 fatty acids provide fluidity to the membrane and may be involved in the regulation of transmembrane receptor function (Sharma et al. 2012). It is known that DHA regulates TrkB along with increased expression of CREB (Wu et al. 2008; Bhatia et al. 2011) and numerous studies have highlighted the role of these molecules in learning and memory processes (Cunha et al. 2010; Kandel, 2012). In line
with this, we demonstrated improved cognitive performance in this group as compared to the animals fed a vitamin B₁₂ deficient diet.

Omega-3 fatty acid supplementation to a vitamin B₁₂ deficient group normalized both levels of VEGF (mRNA) and NGF (protein and mRNA). Omega-3 fatty acid supplementation is reported to stimulate VEGF signalling and facilitate endothelial cell proliferation against cerebral ischemia (Wang et al. 2014b). Studies suggests that omega-3 fatty acids have protective effects in several stroke models against ischemic brain injury (Zhang et al. 2010; Hu et al. 2013). It has also been demonstrated that high dose of omega-3 fatty acid supplementation increases cortex NGF mRNA levels in animals (Balogun and Cheema, 2014).

6.3.4 Effect of Omega-3 Fatty Acid Supplementation on Neurotrophins, Oxidative Stress Markers and Cognitive Performance

In the present study, long-term vitamin B₁₂ supplementation maintains the levels of DHA, BDNF and CREB similar to that of control. However, in this group the mRNA levels of Trk-B were higher and may be attributed to the fact that there are other truncated isoforms like TrkB-T1, TrkB-T2, and TrkB-T-Shc which may be involved in TrkB modulation (Ninkina et al. 1997). It has been reported that truncated isoforms like TrkB-T1 opposes TrkB function via competition for BDNF binding or the formation of inactive heterodimers (Haapasalo et al. 2002).

Vitamin B₁₂ supplementation maintained the levels of brain NGF and VEGF to that of the control group. However, it showed lower plasma MDA levels and higher erythrocyte SOD levels as compared to the control group suggesting antioxidant properties of vitamin B₁₂. Further, the cognitive performance of the offspring in the vitamin B₁₂ supplemented group was comparable to the control group. A previous study has reported that maternal vitamin B₁₂ status influences cognitive function in children (Bhate et al. 2008). However, there are no studies examining the exclusive effects of vitamin B₁₂ supplementation on BDNF levels, its receptor TrkB and cognition across two generations.
6.3.5 Effect of Combined Vitamin B<sub>12</sub> and Omega-3 Fatty Acid Supplementation on Neurotrophins, Oxidative Stress Markers and Cognitive Performance

Studies indicate a need for supplementation of vitamin B<sub>12</sub> and omega-3 fatty acids during pregnancy to reduce the risk of neurodevelopmental disorders in the offspring, although the results of intervention trials using these nutrients in isolation are inconclusive (van de Rest et al. 2012). Our results indicate that vitamin B<sub>12</sub> and omega-3 fatty acid supplementation together over two generations enhances the levels of DHA and BDNF in the hippocampus and CREB mRNA levels in the cortex. The cognitive performance of these animals was also higher as compared to the vitamin B<sub>12</sub> supplemented animals. This group also showed increased levels of NGF and VEGF in the cortex and hippocampus region as compared to the control and the vitamin B<sub>12</sub> supplemented group. It is likely that increased NGF, VEGF and BDNF levels together contribute to the enhancing effects of cognition observed in this combined supplemented group.

In the present study, two regions of the brain i.e. hippocampus and cortex were used because these are widely implicated in learning and memory processes. Reports indicate that the hippocampus and olfactory bulbs are more effective in DHA accumulation, more resistant to DHA deficiency and showed better DHA retrieval after dietary DHA repletion as compared to other regions of the brain (Chung et al. 2008). In the current study, both hippocampus and cortex regions have shown higher levels of DHA in omega-3 fatty acid supplemented groups.

Vitamin B<sub>12</sub> and omega-3 fatty acids especially DHA are important nutrients required for optimal brain functioning. These nutrients are mostly found in animal-based foods and hence the populations consuming a vegetarian diet have sub-optimal levels of both these nutrients (Key et al. 2007; Pawlak et al. 2013). Studies have suggested a need for supplementation/fortification of these nutrients (Dror and Allen, 2012; Pawlak et al. 2013; Ganesan et al. 2014) which may reduce the risk for brain disorders. Studies have shown inconsistent results regarding the association of vitamin B<sub>12</sub> supplementation with cognitive performance. Some studies report a positive association (Bhate et al. 2008), some have shown no effects (Veena et al. 2010; Dangour et al. 2015) while few studies have reported inverse association (Eilander et al. 2010a). Similarly, studies have shown
conflicting results for the effects of omega-3 fatty acids on cognitive functioning wherein some have reported positive (Karr et al. 2012; Stonehouse et al. 2013) while few studies have shown non-significant impact on cognition (Antypa et al. 2009; Chew et al. 2015).

To conclude this section, our data suggests that long-term vitamin B\textsubscript{12} deficiency for two generations increased oxidative stress and adversely influenced NGF and VEGF protein and mRNA levels in the brain and showed impaired cognition. Vitamin B\textsubscript{12} supplementation maintained these levels while combined vitamin B\textsubscript{12} and omega-3 fatty acid supplementation together increased the levels of BDNF, NGF and VEGF in the brain. Overall, our data highlights the beneficial effects of long-term supplementation of omega-3 fatty acids and vitamin B\textsubscript{12} on brain development and function which may help in reducing the risk of neurodevelopmental disorders.

The next section discusses the results observed in the F\textsubscript{3} generation offspring at 3 mo of age.

6.4 F\textsubscript{3} Generation Offspring at 3 mo of age

The key findings of the F\textsubscript{3} generation offspring are as follows: 1) Lower NGF protein levels in the cortex and impaired cognition was observed in the vitamin B\textsubscript{12} deficient group 2) Omega-3 fatty acid supplementation to this diet showed higher NGF levels in the hippocampus and improved cognitive performance 3) Vitamin B\textsubscript{12} supplementation showed comparable NGF protein levels in both hippocampus and cortex, however, cortex BDNF levels were lower. These animals made more number of errors especially RWME as compared to the control group 4) Supplementation of vitamin B\textsubscript{12} and omega-3 fatty acids was beneficial since it demonstrated higher levels of DHA, NGF, BDNF and also improved cognitive performance.

6.4.1 Effect of Vitamin B\textsubscript{12} Deficient Diet on Neurotrophins and Cognition

NGF levels were lower in the cortex in the vitamin B\textsubscript{12} deficient group. NGF is involved in the development and survival of cholinergic neurons and it also regulates synaptic plasticity in the adult brain (Berry et al. 2012). Reduced NGF levels in the adult brain has been reported to be involved in neuropsychiatric disorders (Cirulli and Alleva,
The animals from the vitamin B₁₂ deficient group showed impaired cognition as evidenced by higher escape latency in the Morris water maze test and less percentage of correct choices were recorded in the radial eight arm maze. These findings are similar to our findings reported in the earlier generations.

6.4.2 Effect of Omega-3 Fatty Acid Supplementation to the Vitamin B₁₂ Deficient Diet on Neurotrophins and Cognition

Omega-3 fatty acid supplementation to the vitamin B₁₂ deficient diet showed higher DHA levels in both the cortex and hippocampus as compared to the vitamin B₁₂ deficient group and is consistent with our results in the earlier generations. Further, in this group, BDNF and NGF protein levels were maintained in the hippocampus. However, the levels of NGF in the cortex remained low as compared to the control group suggesting more profound effects of vitamin B₁₂ deficiency in the third generation offspring. The cognitive performance of animals in this group improved as compared to the vitamin B₁₂ deficient group and may be attributed to higher DHA levels which is known to improve cognition and synaptic plasticity (Wu et al. 2011).

6.4.3 Effect of Vitamin B₁₂ Supplemented Diet on Neurotrophins and Cognition

Vitamin B₁₂ supplementation showed comparable levels of DHA to that of the control group in both the cortex and hippocampus and is consistent to the results of previous generations. Further, the levels of BDNF and NGF in the hippocampus were also comparable to that of the control group. Interestingly, the levels of BDNF were lower in the cortex region as compared to the control group. The animals from the vitamin B₁₂ supplemented group demonstrated cognitive performance comparable to that of the control group in the Morris water maze test. In the radial eight arm maze test, correct choice, reference memory errors and working memory errors were also comparable to the control group. In contrast, these animals made more number of RWME. It is also termed as working memory-incorrect error/double error and is considered as another component of working memory error (Schmitt et al. 2003; Bimonte-Nelson et al. 2003). This kind of error is commonly considered as ‘perseveration error’ and may reflect motor perseveration (Avdesh et al. 2013). However,
these differences were observed only on day 5 of the protocol. Further studies are needed to assess the performance with longer duration of training session.

Medial prefrontal cortex, part of cerebral cortex is the main site of information storage and processing while hippocampus is involved in consolidating short-term memory into long-term memory in the cerebral cortex (Yoon et al. 2008; Valiant, 2012). The prefrontal cortex has been strongly associated with working memory processes (Dalley et al. 2004) and reduced levels of BDNF have been demonstrated in prefrontal cortex in schizophrenic patients (Issa et al. 2010). Lower frontal cortex BDNF levels are known to be associated with working memory impairment in an animal model of Down’s syndrome (Bimonte-Nelson et al. 2003). Thus, it is possible that lower cortex BDNF levels found in the present study may be associated with the deficits in working memory in the vitamin B_{12} supplemented group. However, this possibility warrants further investigation to examine BDNF levels in different parts of the cortex like prefrontal cortex, frontal cortex which are mainly involved in working memory processes.

A cross-sectional study reports an inverse association of vitamin B_{12} with short-term memory and mental development in Indian school children aged 6-10 years (Eilander et al. 2010b). Studies reporting the effects of vitamin B_{12} supplementation on cognitive function are inconsistent and more studies are warranted. Also, there is a need to standardize the dose and duration of vitamin B_{12} to better understand its role in influencing brain functioning across generations.

6.4.4 Effect of Combined Supplementation of Vitamin B_{12} and Omega-3 Fatty Acids on Neurotrophins and Cognition

The combined supplementation of vitamin B_{12} and omega-3 fatty acids demonstrated higher DHA and NGF levels in the hippocampus, higher BDNF levels in both hippocampus and cortex and improved cognitive performance. Omega-3 fatty acids act as precursors for active mediators and regulate various processes within the brain like neurotransmission, inflammation, immune reaction and neuronal survival (SanGiovanni and Chew, 2005). Studies have suggested that omega-3 fatty acids influence brain functioning through neurotrophic factors and are also known to have positive effects on cognition (Bhatia et al. 2011; Wu et al. 2011). Thus, it is likely that
vitamin B₁₂ and omega-3 fatty acids may have a synergistic effect on brain development and cognitive functioning.

Recent reports demonstrate that high dose of B vitamin (folate, vitamin B₁₂ and B₆) supplementation for 2 years showed reduced brain atrophy rates and improved cognitive performance in subjects with mild cognitive impairment having pre-existing high baseline levels of plasma omega-3 fatty acids (Jerneren et al. 2015; Oulhaj et al. 2016). These studies suggest a possible interlink between B vitamins and omega-3 fatty acids (Blasko, 2015). Another study reports the synergistic effect of vitamin B₁₂ and fish oil on lowering plasma concentrations of homocysteine (Huang et al. 2015). However, no study has evaluated the combined effects of vitamin B₁₂ and omega-3 fatty acids across three generations on brain biochemical parameters and cognitive performance.

An interlink between micronutrients (vitamin B₁₂, folic acid) and omega-3 fatty acids especially DHA in the one carbon cycle has been discussed by us earlier (Kulkarni et al. 2011; Khot et al. 2014, 2015). The one carbon cycle is vital for the generation of methyl groups which are accepted by neurotransmitters, DNA, RNA and phospholipids (Stover, 2009). We have proposed that the unavailability of phospholipids may lead to aberrant methylation patterns resulting in modified gene expression (Khot et al. 2015). Studies suggest that the dysregulation of epigenetic modifications may alter gene expression pattern in the brain thereby leading to cognitive deficits (Lockett et al. 2010; Labrie et al. 2012; Morris and Monteggia, 2014). Thus, it is likely that altered vitamin B₁₂ status influences epigenetic mechanisms resulting in altered brain functioning.

To summarize this section, our data suggests that prolonged vitamin B₁₂ deficiency adversely influences learning and cognition. The long-term vitamin B₁₂ supplementation may cause gradual and progressive changes in the brain function leading to lower cortical BDNF levels and impairment in working memory. This is in contrast to the findings observed in earlier generations wherein vitamin B₁₂ supplementation did not show any adverse effects on brain function. The combined supplementation of vitamin B₁₂ and omega-3 fatty acids demonstrated both short and long term benefits. This study provides clues for the long-term supplementation/fortification of both vitamin B₁₂ and omega-3 fatty acids to improve brain functioning and cognition.