EFFECT OF MATERNAL NUTRITION DURING THE PRE/PERI CONCEPTIONAL PERIOD ON THE TRANSCRIPTION FACTORS INVOLVED IN THE FATTY ACID METABOLISM

A thesis submitted to the

Bharati Vidyapeeth Deemed University, Pune

for the degree of

DOCTOR OF PHILOSOPHY

in Biotechnology

Under the

Faculty of Science

By

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October 2015

CERTIFICATE

This is to certify that the work incorporated in the thesis entitled "**Effect of Maternal Nutrition during the Pre/Peri Conceptional Period on the Transcription Factors Involved in the Fatty Acid Metabolism**" for the degree of '**Doctor of Philosophy**' in the subject of **Biotechnology** under the faculty of **Science** has been carried out by **Ms. Akshaya Pramod Meher** in the **Department of Nutritional Medicine, Interactive Research School for Health Affairs** at Bharati Vidyapeeth Deemed University, Katraj, Pune during the period from June 2011 to October 2015 under the guidance of **Dr. Sadhana Joshi**.

Place: Pune
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Dr. A. C. Mishra
Director

CERTIFICATION OF GUIDE

This is to certify that the work incorporated in the thesis entitled "**Effect of Maternal Nutrition during the Pre/Peri Conceptional Period on the Transcription Factors Involved in the Fatty Acid Metabolism**" Submitted by **Ms. Akshaya Pramod Meher** for the degree of '**Doctor of Philosophy**' in the subject of **Biotechnology** under the faculty of **Science** has been carried out in the **Department of Nutritional Medicine, Interactive Research School for Health Affairs**, Bharati Vidyapeeth Deemed University, Katraj, Pune during the period from June 2011 to October 2015, under my direct supervision/ guidance.

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DECLARATION BY THE CANDIDATE

I hereby declare that the thesis entitled **"Effect of Maternal Nutrition during the Pre/Peri Conceptional Period on the Transcription Factors Involved in the Fatty Acid Metabolism"** submitted by me to the Bharati Vidyapeeth University, Pune for the degree of **Doctor of Philosophy (Ph.D.)** in **Biotechnology** under the faculty of **Science** is original piece of work carried out by me under the supervision of **Dr. Sadhana R. Joshi**. I further declare that it has not been submitted to this or any other university or Institution for the award of any degree or Diploma.

I also confirm that all the material which I have borrowed from other sources and incorporated in this thesis is duly acknowledged. If any material is not duly acknowledged and found incorporated in this thesis, it is entirely my responsibility. I am fully aware of the implications of any such act which might have been committed by me advertently or inadvertently.

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Date:

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Research Student

Acknowledgement

I would like to express my highest gratitude to my beloved guide, Dr. Sadhana Joshi. She has been a wonderful mentor for me all through my Doctorate degree. I thank her for the kind and patient guidance, encouragement and precious direction which inspired a great enthusiasm in me for the completion of this project. I could not have imagined having a better advisor and mentor for my Ph.D study.

I would also like to express my heartfelt thanks & greatest gratitude to Prof. Sahebarao Mahadik, Professor Emeritus, Medical College of Georgia, USA, who has known the answer to every question I've ever asked. His brilliant comments and suggestions on both research as well as on my career have been invaluable.

I express my sincere thanks to Dr. Akhilesh Chandra Mishra, Director, IRSHA, Bharati Vidyapeeth Deemed University, Pune and former Director, Prof. Prabhakar Ranjekar, IRSHA, Bharati Vidyapeeth Deemed University, for giving me an opportunity to work at Department of Nutritional Medicine and for encouraging my research and allowing me to grow as a researcher.

I am thankful to our collaborating clinicians Prof. Dr. Savita Mehendale and Dr. Girija Wagh, Department of Obstetrics and Gynecology, Bharati Hospital, Pune for playing a key role in recruitment of pregnant women in my study. Without their precious support, it would not have been possible to conduct this research.

I reserve a word of gratitude for my senior Dr. Asmita Joshi for her guidance and moral support through the crucial years of my PhD. In addition, I would like to appreciate the suggestions of my senior colleagues Dr. Preeti, Dr. Anvita, and Dr. Anitha, throughout this study. I thank my fellow labmates, Dr. Deepali, Dr. Nisha, Ms. Harsha, Dr. Pratiksha, Dr. Vandita, Dr. Suchitra, Ms. Kamini, Ms. Hemlata, Ms. Vidya, Ms. Richa, Ms. Amrita, Ms. Nisha K, Ms. Vinita, Ms. Vrushali, Ms. Karuna, Ms. Shruti K, Ms. Akriti, Ms. Alka, Ms. Gopika, Ms. Shruti J, Ms. Surbhi S, Ms. Ankita, Ms. Anjali, Ms. Yasmin, Ms. Surbhi D, Ms. Vaishali and Mr. Mayur for creating a healthy and fun loving atmosphere in the laboratory.

This acknowledgement would be incomplete without mentioning the name of administrative & non- scientific staff, Mrs. Vaishali Kadam, Mr. Gavade, Mr. Patil, Mrs. Anjali Gajare, Mr. Nitin, Tushar, Deshmukh, Ajay and all the Maushis. I acknowledge the help of Mr. Ravindra Mulik and Mr. Vinayak Dhawale who took care of the animals. I would like to thank the staff of the Department of Obstetrics and

Gynaecology, Bharati Hospital, Pune for their cooperation in recruiting patients and collecting the samples.

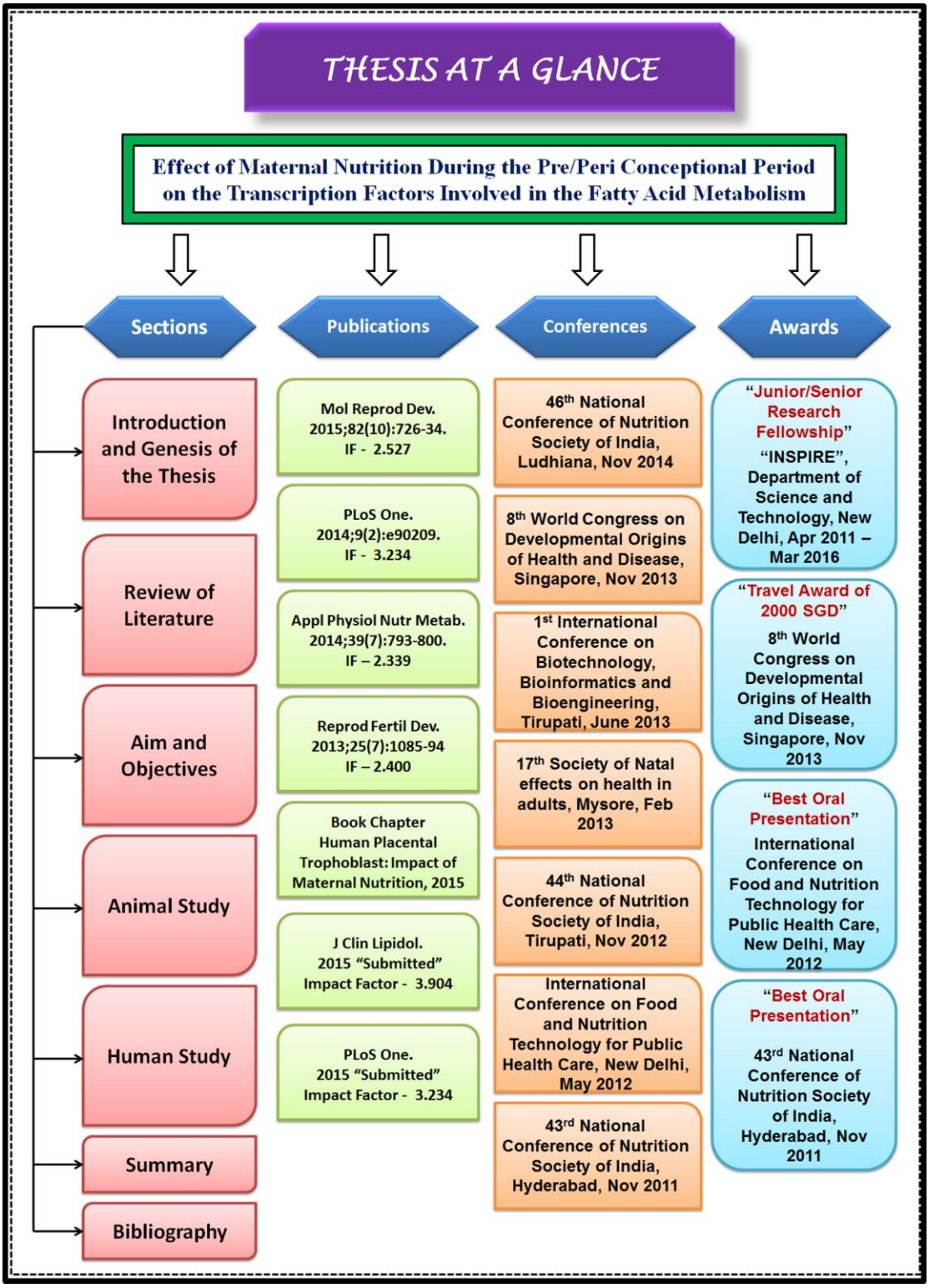
This thesis is dedicated to the lotus feet of my parents whose love and blessing is a source of inspiration to me while I raced against time for the completion of this work. I love them so much, and I would not have made it this far without them. To my siblings Shraddha and Siddhesh, I don't know what I would do without them; they were always there to support me and to give me a laugh when I needed it the most. I am grateful to my maternal grandparents for making my stay in Pune comfortable and pressure free. Thanks are also due to my paternal grandparents whose blessings and encouragement giving me the strength to complete this research work. Words cannot express how grateful I am to my mother-in law, father-in-law and grandfather in law, for all the support and care they provided me. Their prayers for me were what sustained me thus far.

The best outcome from these past five years is finding my best friend, soul-mate, and husband Dr. Ketan Gandhi. I married the best person out there for me. Ketan has been a true supporter and has unconditionally loved me during my good and bad times. He has been non-judgmental and instrumental in instilling confidence. These past several years have not been an easy ride, both academically and personally. I truly thank Ketan for sticking by my side, even when I was irritable and depressed.

Finally I thank God, for giving me strength to go through all the difficulties. I have experienced His guidance every day. He is the one who let me achieve my degree. I will keep on trusting Him for my future. Thank you, Lord.

The work presented in this thesis was performed at the Department of Nutritional Medicine, Interactive Research School for health Affairs, Bharati Vidyapeeth Deemed University, Pune. I acknowledge the Ph.D. fellowship received from Department of Science and Technology, New Delhi, India under 'INSPIRE' program. I also acknowledge the partial funding by Department of Biotechnology, New Delhi, India.

Meher Akshaya P.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PLoS One. 2014;9(2):e90209. IF - 3.234
Appl Physiol Nutr Metab. 2014;39(7):793-800. IF – 2.339
Reprod Fertil Dev. 2013;25(7):1085-94 IF – 2.400
Book Chapter Human Placental Trophoblast: Impact of Maternal Nutrition, 2015
J Clin Lipidol. 2015 "Submitted" Impact Factor - 3.904
PLoS One. 2015 "Submitted" Impact Factor - 3.234
46th National Conference of Nutrition Society of India, Ludhiana, Nov 2014
8th World Congress on Developmental Origins of Health and Disease, Singapore, Nov 2013
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43rd National Conference of Nutrition Society of India, Hyderabad, Nov 2011
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"Travel Award of 2000 SGD"
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List of Abbreviations

5-MTHF	5-Methylenetetrahydrofolate
ALA	α-Linolenic Acid
ARA	Arachidonic Acid
BD	Vitamin B_{12} Deficient
BDO	Vitamin B_{12} Deficient + Omega-3 Fatty Acid Supplemented
BHMT	Betaine Homocysteine Methyltransferase
CBS	Cystathionine Beta-Synthase
DBD	DNA Binding Domain
DGLA	Dihomo-Gamma-Linolenic Acid
DHA	Docosahexaenoic Acid
DHF	Dihydrofolate
DOHaD	Developmental Origins of Health and Disease
DPA	Docosapentaenoic Acid
EFA	Essential Fatty Acids
EPA	Eicosapentanoic Acid
FABP-4	Fatty Acid Binding Protein-4
FATP4	Fatty Acid Transport Protein-4
FD	Folic Acid Deficient
FDO	Folic Acid Deficient + Omega-3 Fatty Acid Supplemented
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GLA	Gamma Linolenic Acid
HNF-4α	Hepatic Nuclear Factor-4 Alpha
IL-6	Interleukin-6
IUGR	Intrauterine Growth Retardation
LA	Linoleic Acid

LBD	Ligand Binding Domain
LBW	Low Birth Weight
LCPUFA	Long Chain Polyunsaturated Fatty Acids
LXR	Liver X Receptors
MDA	Malondialdehyde
MS	Methionine Synthase
MTHFR	Methylenetetrahydrofolate Reductase
MUFA	Monounsaturated Fatty Acids
Myr	Myristic Acid
Myro	Myristoleic Acid
NA	Nervonic Acid
NBW	Normal Birth Weight
Ole	Oleic acid
Pal	Palmitic Acid
Palo	Palmitoleic Acid
PEMT	Phosphatidylethanolamine Methyltransferase
PPAR	Peroxisome Proliferator Activated Receptors
PPRE	Peroxisome Proliferator Response Element
PUFA	Polyunsaturated Fatty Acids
RXR	Retinoid X Receptors
SAH	S-Adenosyl-Homocysteine
SAM	S-Adenosyl Methionine
SFA	Saturated Fatty Acids
SREBP	Sterol Regulatory Binding Proteins
Ste	Stearic Acid
THF	Tetrahydrofolate
TNF-α	Tumor necrosis factor-α

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INTRODUCTION

1.1. Introduction and Genesis of the Thesis

Reports have well established that periconceptional maternal nutrition is an important determinant of embryonic, placental as well as fetal growth and development. Inadequate nutrition during this critical period can result in adverse pregnancy outcome, poor infant survival and risk of chronic diseases in later life (reviewed by Abu-Saad and Fraser, 2010). To date, several studies have investigated the role of macro- and micro-nutrients during pregnancy and its effects on fetal development (reviewed by Mistry and Williams, 2011; Gu et al. 2012; reviewed by Deshmukh et al. 2013). Folic acid deficiency during pregnancy remains a public health issue in many parts of the world (reviewed by Molloy et al. 2008). Folate deficiency is well established to be a contributor to abnormal prenatal development and/or pregnancy outcome (reviewed by Allen, 2005). Vitamin B_{12} deficiency is prevalent in women of reproductive age due to restricted consumption of animal based foods (reviewed by Simpson et al. 2010). Further, due to widespread vegetarianism, Indian population is known to be vitamin B_{12} deficient (Preedy et al. 2012).

Micronutrients like folate and vitamin B_{12} are known to regulate several processes involved in the placental development such as extravillous trophoblast invasion, angiogenesis and secretion of proteases (reviewed by Fekete et al. 2010; Williams et al. 2011). In addition to these micronutrients, fatty acids especially long-chain polyunsaturated fatty acids (LCPUFA) are also considered as important regulators of placental development. LCPUFA are used by the trophoblast cells right from early gestation for several processes like stimulation of placental angiogenesis, regulation of placental inflammation and oxidative stress (reviewed by Jones et al. 2014). Reports have demonstrated that impaired placental development and function is associated with low birth weight (LBW) babies (Kowsalya et al. 2013; Balihallimath et al. 2013). LCPUFA like docosahexaenoic acid (DHA) are interlinked with micronutrients like folic acid and vitamin B_{12} in the one carbon cycle as demonstrated by a series of animal and human studies carried out in our department (Dangat et al. 2011; Kulkarni et al. 2011c; Wadhwani et al. 2012; Sable et al. 2012; Roy et al. 2012; Wadhwani et al. 2015). Reports from our department have also demonstrated that alterations in maternal micronutrients like folate and vitamin B_{12} affects the levels of DHA in the plasma, liver, brain, milk and placenta (Dangat et al.

2011, Kulkarni et al. 2011c; Wadhwani et al. 2012; Sable et al. 2012; Roy et al. 2012).

In addition, a number of cross sectional human studies carried out in our department have extensively demonstrated lower maternal LCPUFA levels in preeclampsia and preterm pregnancy (Kilari et al. 2010; Kulkarni et al. 2011a). DHA is known to regulate the expression of several transcription factors like peroxisome proliferator activated receptors (PPAR), sterol regulatory binding proteins (SREBP), retinoid X receptors (RXR) and liver X receptors (LXR) (reviewed by Jump, 2002; reviewed by Jump, 2008). These transcription factors are expressed in the human placenta and are critical for placental development. They are involved in the fatty acid metabolism in the placenta and thus are important regulators of the lipogenic pathway (Bildirici et al. 2003; reviewed by Duttaroy, 2004; Schaiff et al. 2005).

The association of maternal micronutrients and various transcription factors has been examined on limited studies; and reports are inconsistent. Maternal folate and vitamin B_{12} deficiency during gestation and lactation are known to lower the expression of PPARα in the myocardium of weaning rats (Garcia et al. 2011). Similarly, decreased expression of SREBP-1c in the liver of the pups born to dams fed methyl deficient diets during gestation and lactation has been reported (Pooya et al. 2012). In contrast, other studies indicate that dams fed a diet deficient in folic acid and associated methyl donors during the periconception and early preimplantation periods do not alter the levels of hepatic PPARα and SREBP in the adults (Maloney et al. 2013). Further, rats fed a high multivitamin diet during pregnancy are reported to have higher levels of PPARγ in adipose tissue and PPARα in liver (Pannia et al. 2015).

It has been well established that deficiencies of folate or vitamin B_{12} are associated with elevated homocysteine concentrations (reviewed by Allen, 2005). In addition, studies carried out in our department in pregnant women report a negative association between maternal homocysteine and DHA during pregnancy (Kulkarni et al. 2011a; Wadhwani et al. 2015). Higher levels of homocysteine are known to reduce PPARα levels in the methylene tetrahydrofolate reductase deficient mice (Mikael et al. 2006). Similarly, *in-vitro* studies in pre-adipocyte cells also indicate that homocysteine downregulates PPARγ gene expression (Wang et al. 2011). A recent report indicates that higher levels of homocysteine compete for PPAR receptors, thereby altering the activation of PPAR by their ligands (Hayden, 2008; Hunt and

Tyagi, 2002). In contrast, other studies demonstrate that hyper homocysteinemia induced by dietary homocysteine supplementation does not alter the relative mRNA levels of SREBP-2 (Stangl et al. 2007).

Reports indicate that disturbances in the maternal one carbon cycle reduce liver PPARα expression, possibly through promoter hypermethylation (Mikael et al 2012). Maternal protein restriction during pregnancy in rats has also been shown to hypomethylate PPARα in the liver of their offspring (Burdge et al. 2004; Lillycrop et al. 2005). Similarly, maternal protein restriction during pregnancy has been shown to hypermethylate LXR in the liver of the offspring (van Straten et al. 2010). Further, maternal folic acid supplementation during pregnancy and lactation, is reported to decrease global and site specific DNA methylation of PPARγ in the liver of the offspring (Sie et al. 2013).

It is clear from the above mentioned studies that, maternal micronutrients and omega-3 fatty acids may regulate the activity of placental transcription factors. Any defect in the placental development may hamper the growth and function of the placenta, thereby compromising fetal growth and development. Our earlier studies in humans have demonstrated altered levels of maternal nutrients (folic acid, vitamin B_{12} and omega-3 fatty acids) in adverse pregnancy outcomes (Kulkarni et al. 2011a; Dhobale et al. 2012). A cross sectional study carried out in our department also reports lower maternal erythrocyte DHA levels at the time of delivery in mothers delivering LBW babies (Kilari et al. 2011).

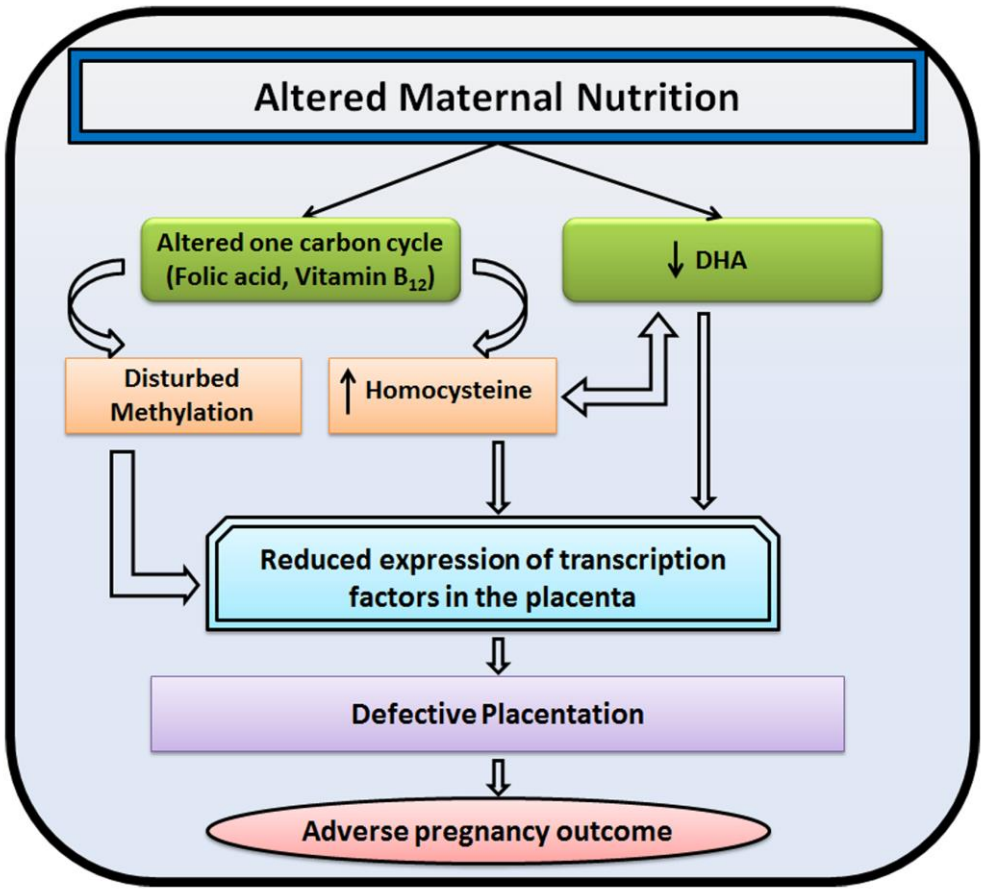
Despite the importance of transcription factors in placental development, their role in adverse pregnancy outcome resulting in LBW is not completely understood. Further, the association of fatty acids and transcription factors in placental development remains to be established.

With this background, we have recently published a hypothesis proposing an association of altered maternal micronutrient metabolism and the expression of transcription factors involved in the placental development (reviewed by Meher et al. 2015).

1.2. Hypothesis:

Maternal folic acid, vitamin B_{12} and DHA modulate the activity of key transcription factors involved in the placental development thereby affecting fetal growth (Fig 1).

Fig 1: Maternal One Carbon Metabolism and Possible Mechanisms Involved in the Regulation of Transcription Factors in the Placental Development



DHA, *Docosahexaenoic acid.*

In order to test the above hypothesis, the current thesis is divided into two parts i.e. an animal study as well as a human study. The animal study examines the effect of maternal nutrition (folic acid, vitamin B_{12} and DHA) from the pre-conception period on the reproductive performance and pregnancy outcome. This study for the first time also examines the role of above nutrients in influencing the transcription factors involved in the placental development as well as in the fatty acid metabolism in the offspring liver. The human study is a prospective study which examines the association of maternal fatty acids across the gestation with cord blood with birth outcome measures. This study for the first time also compares the placental mRNA levels of transcription factors in mothers delivering normal birth weight (NBW) and LBW babies.

AIM AND OBJECTIVES

2.1. Aim:

To examine the effect of maternal nutrition during the pre/periconceptional period on the transcription factors involved in the fatty acid metabolism.

2.2. Objectives of the Animal Study:

1. To examine the effect of folic acid / vitamin B_{12} deficiency and omega-3 fatty acid supplementation starting from the pre-conception period and continuing throughout pregnancy and lactation on

   A. Number and phases of the oestrous cycle; morphology of the mammary gland and ovaries; and birth outcome.
   B. Oxidative stress marker (MDA); placental levels of proinflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor- α (TNF-α); plasma, erythrocyte, placental and liver fatty acids in dams at end of pregnancy

2. To examine changes in placental PPARγ expression as a consequence of altered maternal micronutrients.

3. To investigate whether maternal micronutrients and omega-3 fatty acids affect global methylation patterns in the placenta and liver of the offspring at postnatal d22.

4. To examine the effect of omega-3 fatty acid supplementation on the expression of PPARγ, PPARα, LXR, RXR and SREBP-1c in the liver of the offspring at postnatal d22.

2.3. Objectives of the Human Study:

1. To compare the levels of long chain polyunsaturated fatty acids at various time points across the gestation in women delivering low birth weight (LBW) babies and normal birth weight babies (NBW).

2. To compare the placental fatty acid profile of women delivering LBW and NBW babies.

3. To examine the placental mRNA levels of PPARγ, PPARα, SREBP-1c, LXR and RXR in women delivering LBW and NBW babies.

REVIEW OF LITERATURE

Overview:

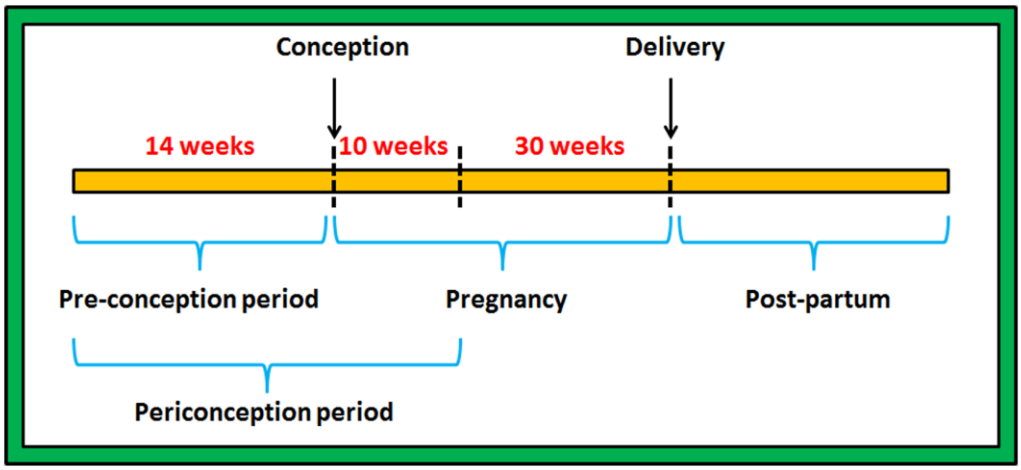
The Indian population is known to be deficient in micronutrients, especially those that are part of the one carbon cycle, i.e. folic acid and vitamin B_{12}. These micronutrients are critical for the establishment of pregnancy as well as for the placental and fetal growth. As discussed in the introduction, these micronutrients influence various transcription factors thereby leading to altered fetal growth and development. This chapter reviews various studies on maternal nutrition and transcription factors reported in the literature.

3.1. Importance of Maternal Nutrition during the Periconception Period:

3.1.1. Periconception Period:

It is well known that each individual exists as a result of reproduction, and therefore, the process of reproduction is considered as the basis of life (Johnson, 2012). Human reproduction involves several events which are highly interrelated and time dependent processes (reviewed by Louis et al. 2008). Most of the reproductive failures are known to originate during the periconception period. The periconceptional period is defined as the time period around pregnancy i.e. the pre-conception period which is around 14 weeks prior to conception followed by the conception and post-conceptional phase lasting around 10 weeks after conception (reviewed by Steegers Theunisen et al. 2013). The periconceptional period in humans is shown in Fig 2. The periconceptional period is known to include events like gametogenesis, fertilization, embryogenesis, implantation and placentation (reviewed by Yajnik, 2014).

Fig 2: Periconception Period in Humans



3.1.1.1. Gametogenesis:

Gametogenesis is the process of formation of gametes and consists of meiotic cell divisions namely, first and second meiotic cell divisions. One paternal chromosome and one maternal chromosome (homologous chromosomes) form a pair inside the cell during the first meiotic division. Before the first meiotic division, crossing over takes place between the small parts of the homologous chromosome, where they overlap with each other (reviewed by Fragouli et al. 2014). The first meiotic division involves different phases like prophase, metaphase, anaphase and telophase (Wassarman, 2012). The end of the first meiotic division is marked by an independent assortment of chromosomes; whereby chromosomes separate and move towards the two poles of the cells. At this stage the chromosome number is reduced to half. During the second meiotic division, each chromosome splits into two chromatids which move apart and pass to the daughter cells (Handel, 1998).

Oogenesis:

Oogenesis takes place in the cortex of the ovary. It begins with the mitotic division, where one oogonium divides into large number of oogonia, which then enlarge and form the primary oocyte. At puberty, the primary oocyte begins its first unequal meiotic division, where most of the cytoplasm goes to one daughter cell forming the secondary oocyte. The cell which receives the minimal cytoplasm forms the first polar body (reviewed by Fragouli et al. 2011). The secondary oocyte enters the second unequal meiotic division, which is completed only after the penetration by the sperm. At the end of the second meiotic division, there is a formation of ovum and second polar body (Starr and McMillan, 2014). Oogenesis is accompanied by the development of follicles, where the cells covering the primary oocyte multiply and form secondary follicle, antral follicle and ultimately the Graafian follicle (Leung and Adashi, 2003).

Ovulation:

The Graafian follicle is converted into the corpus luteum by bursting on the surface of the ovary and the ovum is further released into the peritoneal cavity. If the ovum is fertilized at this stage, the corpus luteum lasts for 2-3 months and pregnancy continues, alternatively the corpus luteum regresses after 10-12 days (Singh, 2014). All the other follicles degenerate after ovulation. After the formation of ovum, it is

carried by the fallopian tube from the peritoneal cavity to the uterus, which is the site of fertilization.

3.1.1.2. Fertilization:

At the time of fertilization, the secondary oocyte is arrested in the metaphase of the second meiotic division. This secondary oocyte contains chromosomes arranged on a spindle and is covered by a layer called as zona pellucida, which is further covered by cells of corona radiata (Coticchio et al. 2013). Before the sperm fertilizes the ovum, it undergoes capacitation, which involves removal of the outer glycoprotein layer, overlying the acrosomal cap (reviewed by Aitken and Nixon, 2013). Further, this capacitated sperm comes in contact with the corona radiata cells of the secondary oocyte and undergoes acrosome reaction. This acrosome reaction involves the contact of the acrosomal cap and the cell membrane of the secondary oocyte at multiple points, thereby making multiple perforations (Pollard, 1994).

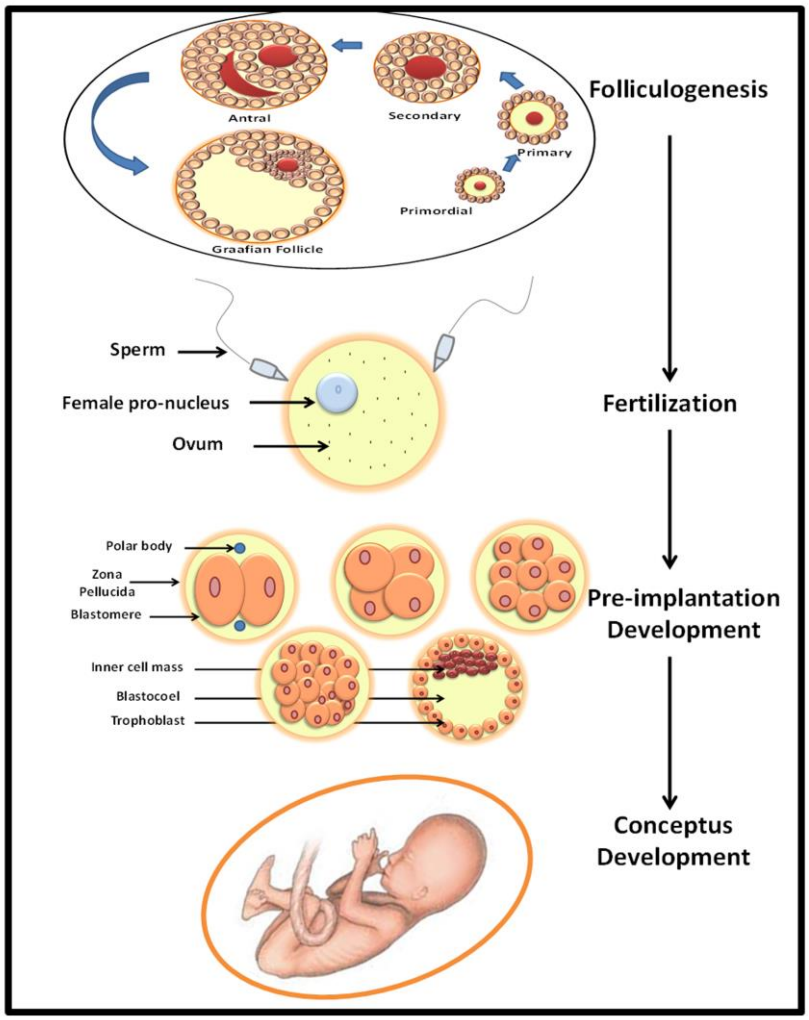
The sperm later reaches the zona pellucida layer of the secondary oocyte. The secondary oocyte releases enzymes which restrict the entry of other sperms. This reaction is called as the "zona reaction" (Brauer, 2003). The fusion of cell membranes of oocyte and sperm takes place, allowing the oocyte to complete the second meiotic division and thereby forms the ovum. The nucleus of both the sperm and ovum enlarge to form the male and female pronucleus, which further come together and lose their nuclear membrane, thereby forming the zygote. Thus, there is a formation of new life. The zygote further undergoes rapid cell (mitotic) divisions and the process is called as cleavage (O'Day, 2012).

3.1.1.3. Embryogenesis:

The zygote undergoes repeated mitotic divisions and forms two cells, called as the two cell stage embryo. These cells further divide to form four, eight and sixteen cells which are contained within the zona pellucida. These cells are called as blastomeres and the 16 celled embryo is called as morula, which is then converted into the blastocyst. The uterine fluid enters into the morula and separates it into the outer cell mass and inner cell mass (Brookes and Zietman, 1998). The cavity between the outer and inner cell mass enlarges with more fluid entering, thereby making the cells of the outer cell mass flattened and are called as trophoblast. This structure is called as blastocyst (Gilbert, 2000).

The cells of the inner cell mass which are present towards the cavity of the blastocyst become flat and are arranged in one layer. These cells are called as endoderm, while the remaining cells become columnar and are called as ectoderm. The yolk sac is formed by the cells of endoderm proliferating and lining the cavity of blastocyst. Between the endoderm and ectoderm, the embryo is now in the form of bilaminar embryonic disc. Further, the cells lining the yolk sac proliferate and form the extra embryonic mesoderm (Jirasek, 2000). Thus, with the formation of the three germ layers, the development of the embryo takes place which involves the formation of notochord, neural tube, folding of the embryo and the formation of tissues and organs of the body. The summary of the above described events is shown in Fig 3.

Fig 3: Summary of the Events Taking Place from the Pre-Conception Period to the Conceptus Development



***Source**: Modified from Fleming et al. 2011, Reproduction Fertility and Development 24(1): 35-44.*

3.1.1.4. Implantation:

After the formation of the blastocyst, it enlarges in size and the zona pellucida disappears. The blastocyst is implanted on the wall of the uterus and embedded in the endometrium (Durham and Chapman, 2014). The cells of the trophoblast which are in contact with the endometrium of the uterus proliferate to form a layer of the cells with loose cell membrane and multiple nuclei and are called as syncytiotrophoblast. The syncytiotrophoblast along with the columns of proliferating cytotrophoblast erode the endometrial epithelium, moving the blastocyst gradually deeper in the endometrium. Once the blastocyst is completely buried in the endometrium, the implantation is complete. This type of implantation is termed as "interstitial implantation" (Singh, 2014).

3.1.1.5. Placentation:

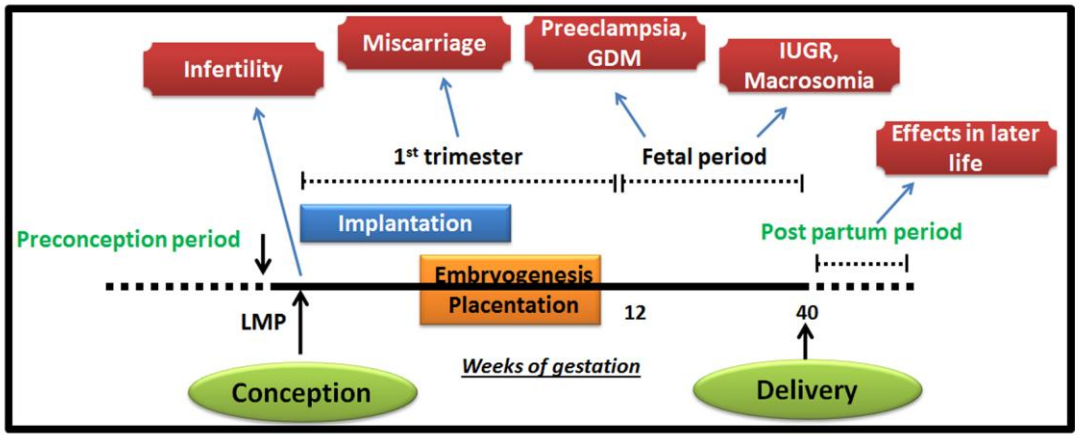
The cytotrophoblast cells which proliferate and penetrate beyond the syncytiotrophoblast shell become the extravillous cytotrophoblast lineage. At this stage, there is a formation of columnar structure made up of outer synctiotrophoblast and central core of cytotrophoblast, called as primary villi. The extra embryonic mesoderm invades the central part of primary villi, forming the secondary villi which develop the blood vessels connected to the fetal vascular system. The villi are termed as tertiary villi (Benirschke et al. 2012). The tertiary villi at one end are attached to the chorion while on the maternal side are attached to the decidua of the endometrium and are therefore called as anchoring villi. The villi which are present in the intervillous space allow the exchange of gases and nutrients and are called as floating villi (Bacon and Niles, 2012).

The placental membrane or barrier is a thick membrane which separates the maternal blood in the intervillous space from the fetal blood. This membrane becomes thinner and more efficient, as the fetus grows and the demand for nutrition increases. In the villi, the extravillous cytotrophoblast forms the "trophoblast cell columns". These cell columns proliferate and invade the decidua and superficial layer of the myometrium, where they transform the spiral arteries. These are the terminal branch of the uterine arteries that reach the endometrial surface. Therefore, in order to allow the normal blood flow to the fetoplacental unit, complete transformation of spiral arteries is required (reviewed by Carvajal, 2014).

Thus, a fully developed placenta consists of two components, the fetal component made up of chorionic plate and chorionic villi; and the maternal component made up of decidua basalis. When viewed from maternal side, the placenta appears to be round in shape, disc like and divided into 15-20 lobes (cotyledons). It shows a smooth surface, when viewed from the fetal side and has an umbilical cord attached approximately in the center (reviewed by Mihu et al. 2009).

Therefore, based on the events discussed above, it is clear that the time during which the women should start caring for her pregnancy is not after, but before it happens (reviewed by Berghella et al. 2010). Reports indicate that every year around 273,500 women of reproductive age die due to pregnancy complications, while around 15 million suffer from poor long-term health (Lozano et al. 2011). This has a great impact in developing countries (reviewed by Dean et al. 2013). A recent review suggests that fetal growth restriction (FGR) and placental-related disorders may have their origin during the periconceptional period (reviewed by Steegers Theunisen et al. 2013). Specific malformations and pregnancy related disorders originating during the periconception period are shown in Fig 4. Therefore, strategies to reduce risks of early fetal maldevelopment after the women has become pregnant, might be too late (Weisman et al. 2011). Preconception care has therefore been identified as an important aspect to improve pregnancy outcome by the Centers for Disease Control and Prevention, USA (CDC, 2006).

Fig 4: Specific Malformations and Pregnancy Related Disorders Originating during Periconception Period and Later



***LMP**, Last Menstrual Period; **GDM**, Gestational Diabetes Mellitus; **IUGR**, Intrauterine Growth Restriction.*
***Source**: Adapted from Cetin et al. 2010, Human Reproduction Update, 16(1): 80–95.*

It has been well established that the maternal nutritional status is one of the most crucial environmental factor affecting maternal, embryonic, placental as well as fetal growth and development (reviewed by Cetin et al. 2010). An optimal nutritional status before and during pregnancy is known to influence fertility and prevent adverse pregnancy outcomes (reviewed by Shepherd, 2008); and this has been discussed below.

3.1.2. Preconception Nutrition and Effects on Fertility:

A healthy uterine environment is a pre-requisite for the development of healthy embryos and progeny (reviewed by Hales et al. 2011). Reports have demonstrated the role of different nutrients like vitamin D, calcium, zinc, folic acid and LCPUFA during various reproductive phases (reviewed by Lewis et al. 2010; Bernhardt et al. 2011; reviewed by Mmbaga and Luk, 2012; Tian and Diaz, 2012; Kong et al. 2012; Tian and Diaz, 2013).

Reports from animal and human studies have identified folate to be indispensible during mammalian folliculogenesis and fetal development. Severe maternal folate deficiency during the preconceptional and gestational period has been reported to hamper female fertility as well as embryo and fetal viability in animals (reviewed by Forges et al. 2007; reviewed by Laanpere et al. 2010). Folic acid deficiency in Rhesus monkeys is reported to lead to irregular menstrual cycles with degenerated Graafian follicles, an increased number of atretic and cystic follicles, depleted granulosa cells and reduced or absent corpora lutea (Mohanty and Das, 1982).

It is well known that folic acid deficiency in humans is associated with higher levels of homocysteine (reviewed by Allen, 2005). Studies have reported a negative association between high levels of follicular fluid homocysteine and the maturation of oocytes as well as the embryo quality in patients undergoing *in-vitro* fertilization (Szymanski and Kazdepka-Zieminska, 2003; Ebisch et al. 2006). It has been suggested that molecular mechanisms of these homocysteine induced effects include inflammatory cytokine production, altered nitric oxide metabolism, oxidative stress, apoptosis and defective methylation reactions (reviewed by Forges et al. 2007).

In addition to folic acid, few studies have also examined the role of vitamin B_{12} and its impact on fertility. It has been reported that patients undergoing *in-vitro* fertilization, demonstrate a positive association between plasma cobalamin

concentrations and better embryo quality (Boxmeer et al. 2009). Rats fed a diet deficient with vitamin B_{12}, vitamin B_2, folate, methionine and choline for a period of one month prior to mating are reported to have normal fertility although the pups had growth retardation and 25% perinatal mortality (Blaise et al. 2005).

Long chain poly unsaturated fatty acids (LCPUFA) like omega-3 fatty acids are also well studied for their role in different reproductive events prior to pregnancy. High intake of α- Linolenic acid (ALA) and docosahexaenoic acid (DHA) in women during the pre-conception period has been shown to improve embryo morphology (Hammiche et al. 2011). In animals, cows fed unsaturated fatty acids high in ALA are reported to have higher blastomere numbers than cows fed saturated fatty acids (Thangavelu et al. 2007). Further, increased ovulations have also been reported in animals on a high omega-3 diet in comparison with rats on a control diet (reviewed by Abayasekara and Wathes, 1999). In murines, a lifelong consumption of a diet rich in omega-6 fatty acids has shown to be associated with a poor reproductive success at advanced maternal age, while short-term treatment with omega-3 fatty acids demonstrated improved oocyte quality (Nehra et al. 2012). The effect of pre-conceptional nutrient deficiency on fertility is shown in Fig 5.

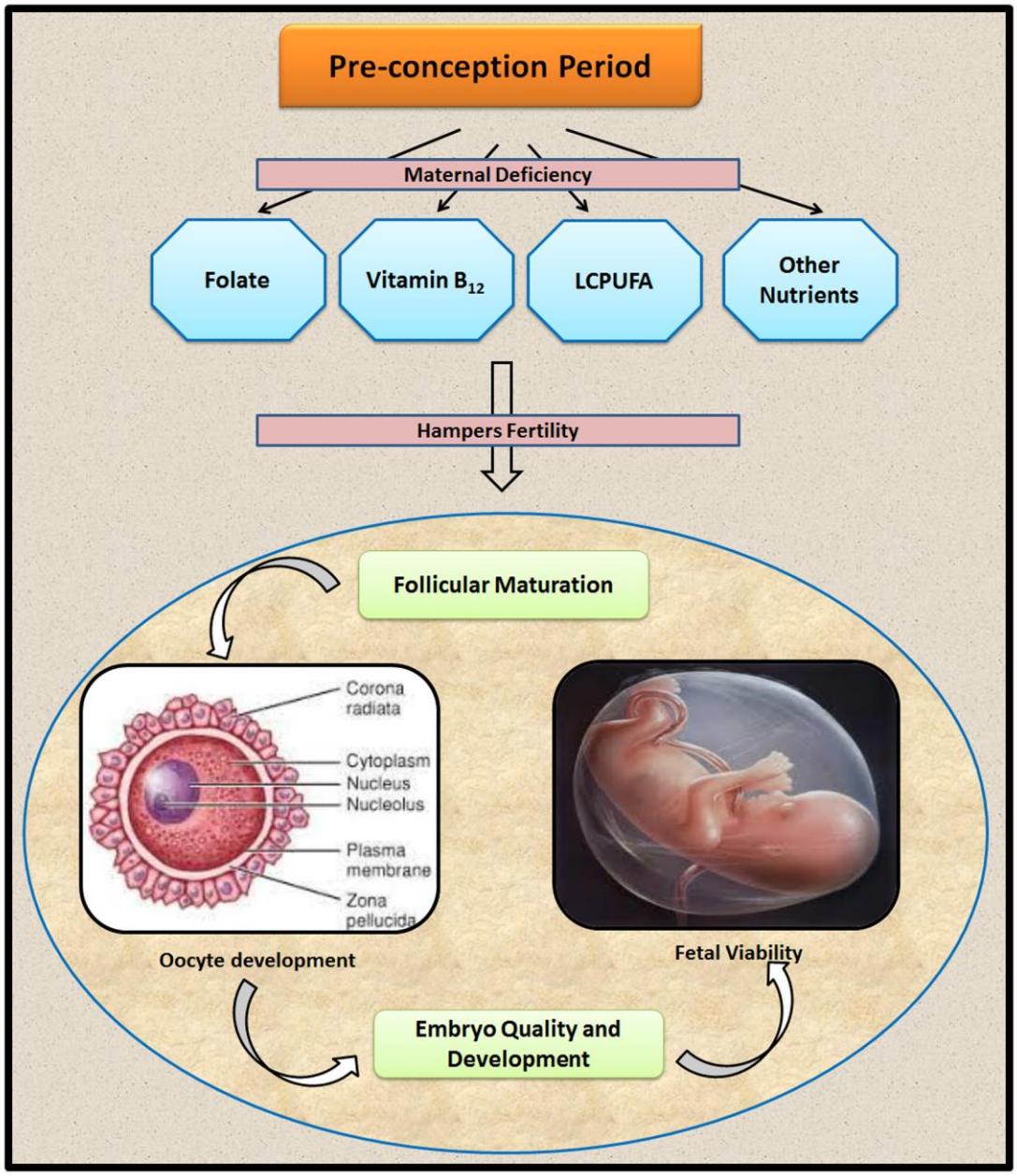
3.2. Maternal Nutrition:

Maternal nutrition before and during pregnancy is important in determining the fetal outcome. Micronutrients like folic acid and vitamin B_{12}, are critical since their deficiency is associated with numerous adverse outcomes including pregnancy loss, LBW and neural tube defects in the offspring (reviewed by Berti et al. 2011; Nasri et al. 2015). The next section therefore describes micronutrients like folate and vitamin B_{12} and their importance during the periconceptional period.

3.2.1. Folate:

Folate is a water-soluble B vitamin which was initially isolated from spinach leaves in 1941 and later synthesized in 1946 (Mitchell et al. 1941; Hall and Solehdin, 1998). Folate is the natural form occurring in foods, while the oxidized form is the synthetic folic acid used in supplements and fortified foods. The naturally occurring folate is in the form of polyglutamates (pteroylpolyglutamate) whereas the synthetic folic acid is in the monoglutamate (pteroylmonoglutamate) form (reviewed by Almeida and Cardoso, 2010).

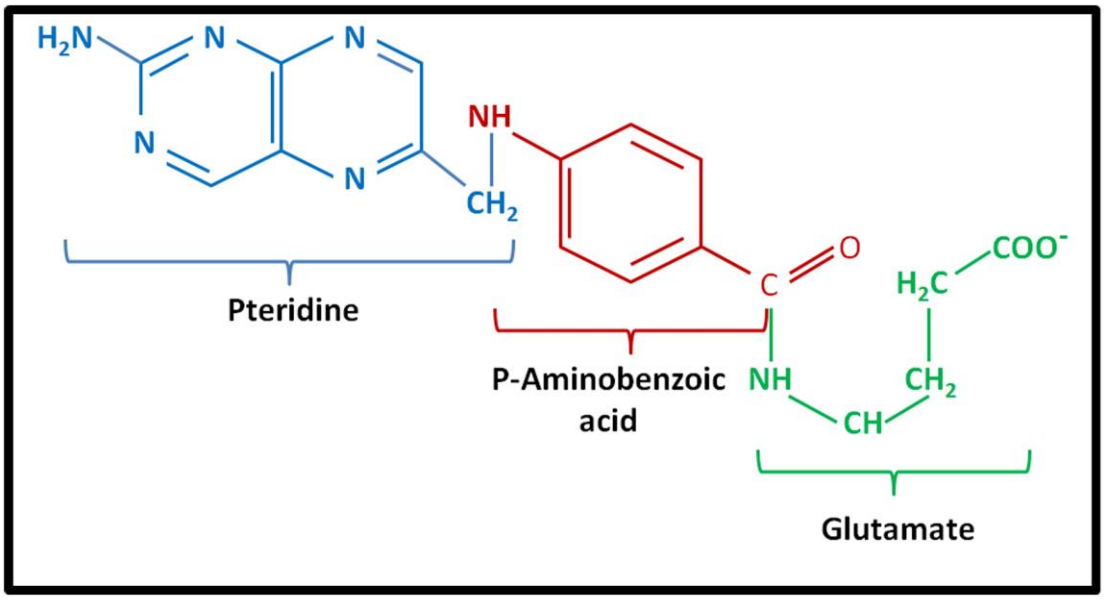
Fig 5: Effects of Pre-Conceptional Nutrient Deficiency on Fertility



***LCPUFA**, Long chain poly unsaturated fatty acids*

This synthetic folic acid consists of three parts: p-aminobenzoic acid, a pteridine ring and one molecule of L-glutamic acid (Fig 6). Humans possess the ability to synthesize the pteridine ring (reviewed by Birn, 2006) and depend on different exogenous sources of preformed folate, including dietary folate as well as that synthesized by the normal microflora present in the large intestine (Dudeja et al. 2001).

Fig 6: Structure of Folic Acid



***Source**: reviewed by Shane, 2008, Food and Nutrition Bulletin. 29:S5-16.*

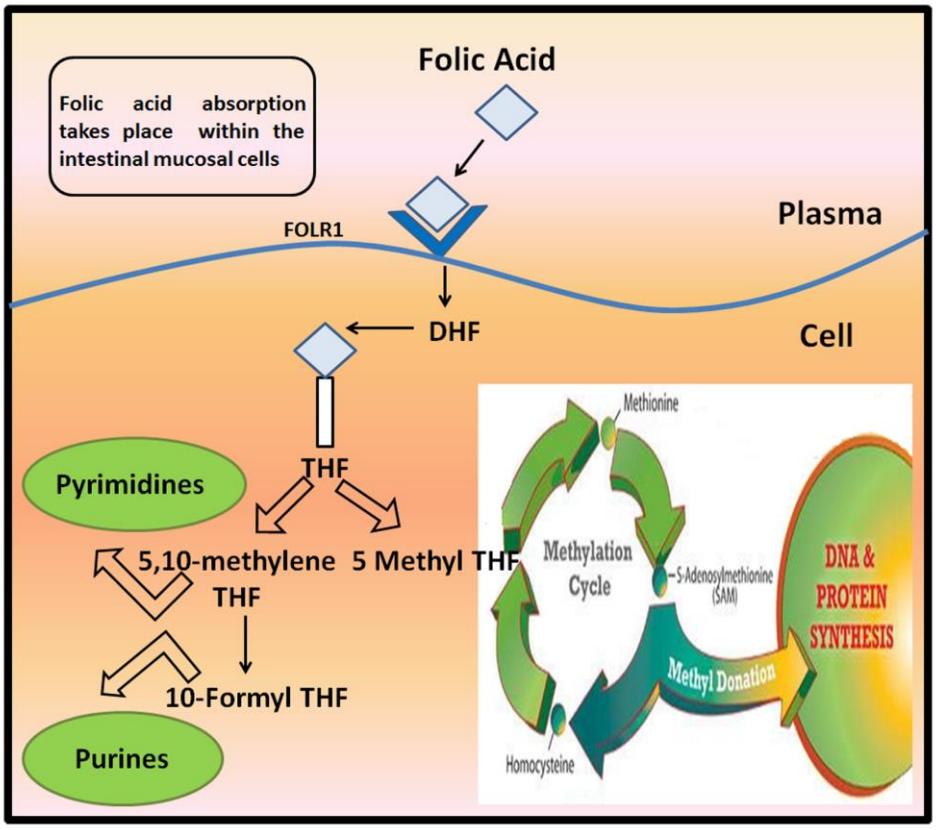
The different food sources of folate include cooked dried beans, green leafy vegetables and fortified cereals, fruits and fruit juices, nuts, beans, peas, dairy products, poultry and meat, eggs, seafood, grains, avocado, spinach, liver, yeast and asparagus (reviewed by Scholl and Johnson, 2000; NIH 2011). In addition to these, multivitamin tablets are also an additional source of folate used widely (reviewed by Scholl and Johnson, 2000). As compared to folic acid, natural folates are inherently less stable and show incomplete bioavailability. One of the possible reasons for this could be the rapid passage of the monoglutamates through the cell walls as compared to polyglutamates (reviewed by Almeida and Cardoso, 2010). Further, the metabolism of polyglutamates requires their deconjugation to monoglutamates in the enterocytes, making them less bioavailable (~50%) compared to monoglutamates (~85%) (reviewed by Sanderson et al. 2003). In addition, factors like exposure to light and heat at the time of cooking and storage, as well as the anti folate components in the vegetables contribute to the low folate availability (reviewed by McNulty and Scott, 2008).

Functions of Folic Acid:

Pregnancy is a period of increase in cell division accompanied by the growing fetus and placenta; and the increase in the number of maternal red cells as well as the size of the reproductive organs. This involves the increase in the one carbon

metabolism reactions, required for nucleotide synthesis and cell division (reviewed by Bailey, 2000). Folate, along with vitamin B_{12} is an important vitamin in cellular one carbon metabolism. This one carbon metabolism is involved in the de novo synthesis of purines and pyrimidines and for the remethylation of homocysteine to methionine (reviewed by Simpson et al. 2010) (Fig 7).

Fig 7: Functions of Folic Acid



FOLR1: *Folate receptor 1;* **DHF**: *Dihydrofolate*; **THF**: *Tetrahydrofolate*
Source: *Modified from www.pinterest.com/monikafaulhaber/methylation-cycle/*

Subsequently, the methyl groups produced through the one carbon metabolism are used for various methylation reactions including the methylation of proteins (including histones), cytosine bases on DNA, neurotransmitters, phospholipids and other small molecules (reviewed by Deshmukh et al. 2013). Folate is also a substrate for transmethylation and transsulfuration pathways and affects the metabolism of several amino acids (Duggan et al. 2008). Due to the higher requirement of the folate for the DNA synthesis and other one carbon metabolism reactions, pregnant women are at an increased risk of developing folate deficiency as compared to the non-pregnant women (European Food Safety Authority, 2015).

Requirement of Folate:

The required daily allowance (RDA) of a particular nutrient during pregnancy is nothing but the average daily dietary intake that is sufficient to meet the nutrient requirement of nearly all healthy pregnant women (97–98%) (NIH 2012). The RDA for women of reproductive age worldwide is 400μg/day folic acid from preconception until the end of the first trimester of pregnancy (Hanson et al. 2015).

Assessment of Folic Acid:

The assessment of folate status is routinely done by measuring the folate concentrations in serum/plasma or in red blood cells. Erythrocyte folate is an indicator of long-term folate status since folate is only taken up during the process of erythropoiesis and released at erythrocyte breakdown after a lifetime of 120 days (reviewed by Lamers, 2011). The serum/plasma folate reflects the recent dietary intake and is the transport form of folate to the tissue and during pregnancy to the fetus (European Food Safety Authority, 2015). The normal levels are between 10 and 15 nmol/L (reviewed by Laanpere et al. 2010).

Maternal Folic Acid Deficiency:

Dietary folate deficiency is predominant in developing countries, and reports indicate that about 25% of pregnant women in India are folate deficient (reviewed by Hovdenak and Haram, 2012). Different dietary, metabolic as well as behavioral factors affect the status of folic acid. In humans, folate deficiency can occur as a result of poor dietary intake (reviewed by Allen, 2008). It has been reported that insufficient levels of micronutrients necessary for folate metabolism, such as vitamins B_2 (McNulty et al. 2006), B_6 (Perry et al. 2007) and vitamin B_{12} (reviewed by Brosnan and Brosnan, 2006) may also cause functional folate deficiency. Inefficient folate utilization due to defects in folate-metabolizing genes is also one of the reasons for functional folate deficiency (reviewed by Scaglione and Panzavolta, 2014). Different behavioral factors such as smoking, alcohol or using oral contraceptives are also reported to be associated with poor folate status (reviewed by Scholl and Johnson 2000). The poor stability and incomplete bioavailability of natural food folates is also considered as one of the factors responsible for poor folate status (reviewed by McNulty and Pentieva, 2004).

Several reports have indicated that folate deficiency during pregnancy may lead to congenital malformations (neural tube damage, orofacial clefts, cardial

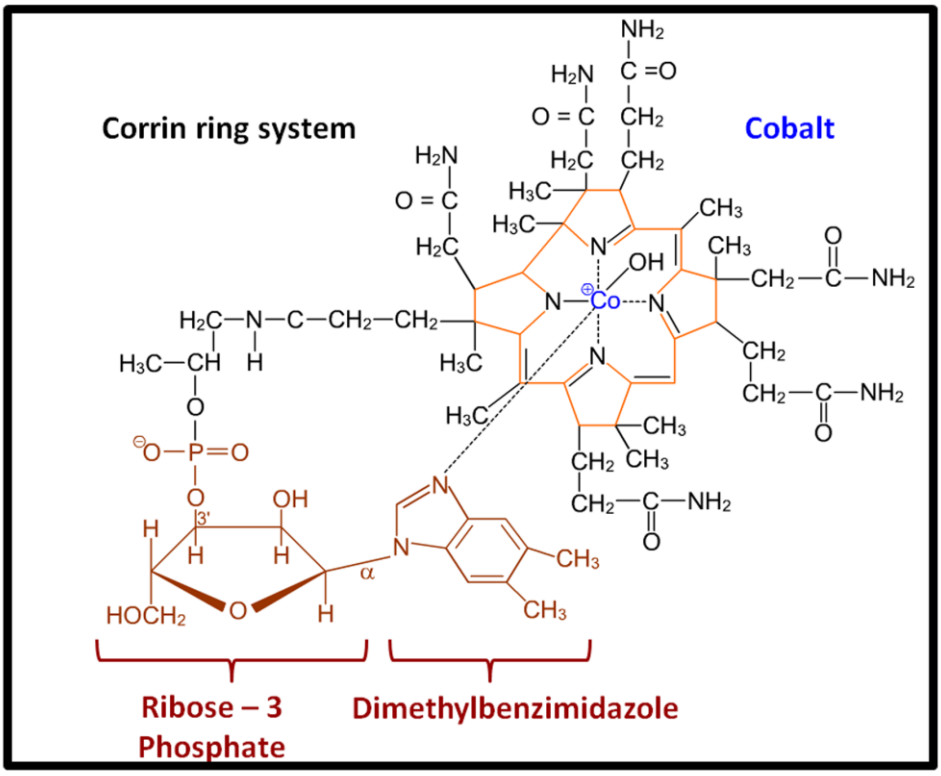
anomalies), anemia and certain complications during pregnancy like spontaneous abortions, bleeding, preeclampsia, stillbirth, preterm delivery and IUGR (reviewed by Black, 2001; Burgoon et al. 2002; Li et al. 2005). Maternal folate deficiency is one of the established risk factor contributing to the increased the incidence of neural tube defects (reviewed by Czeizel et al. 2013). Different maternal factors such as obesity, hyperthermia, race, ethnicity, smoking, alcohol abuse, malabsorption, intestinal disease and liver or renal failure, also contribute to genesis of neural tube defects (NTDs) either directly or indirectly by folate deficiency (reviewed by Safi et al. 2012). The neural tube usually closes within four weeks of conception and therefore it is necessary to have adequate maternal folate status before and around the establishment of pregnancy (reviewed by Imbruglia et al. 2009; reviewed by Czeizel et al. 2013).

It has been established that folate deficiency is responsible for elevated homocysteine levels which is known to be linked with a wide range of abnormalities in vascular function (reviewed by Molloy et al. 2008). Reports have demonstrated the association between higher maternal homocysteine levels and preeclampsia, intrauterine growth restriction, recurrent pregnancy loss and preterm birth (reviewed by Obeid and Herrmann, 2005). Increased embryonic losses and severe placental defects, including placental abruption and disturbed patterning of placental layers (Pickell et al. 2009) and altered expression of the inflammatory mediators ApoAI and IFN-γ in placenta (Mikael et al. 2013) have been reported in folate-deficient mice. This pro-inflammatory environment during pregnancy, contributes to the adverse reproductive outcomes.

3.2.2. Vitamin B_{12}:

Vitamin B_{12} also known as cobalamin (molecular weight = 1355.4), was discovered in early 1920s from studies of pernicious anemia, a previously incurable disease (reviewed by Herrmann and Geisel, 2002). Vitamin B_{12} is a complex and large water soluble vitamin. It is a group of complex molecules with cobalt containing corrin ring synthesized only by microorganisms (reviewed by Stabler and Allen, 2004) and coupled to a phosphoribo-5,6-dimethylbenzimidazolyl side group (reviewed by Simpson et al. 2010) (Fig 8).

Fig 8: Structure of Vitamin B_{12}



***Source**: reviewed by Shane, 2008, Food and Nutrition Bulletin. 29:S5-16.*

The corrin ring present in vitamin B_{12} is similar to the porphyrin ring present in the cytochrome, heme and chlorophyll. Vitamin B_{12} contains a total of six co-ordination sites, out of which four are provided by the corrin ring and a fifth by a dimethylbenzimidazole group. The sixth coordination site, which is the center of reactivity, is variable, being a methyl group (-CH3), a 5'-deoxyadenosyl group, a cyano group (-CN) or a hydroxyl group (-OH) respectively, to yield the four B_{12} forms as follows: methylcobalamin, adenosylcobalamin, cyanocobalamin and hydroxocobalamin (reviewed by Gruber et al. 2011).

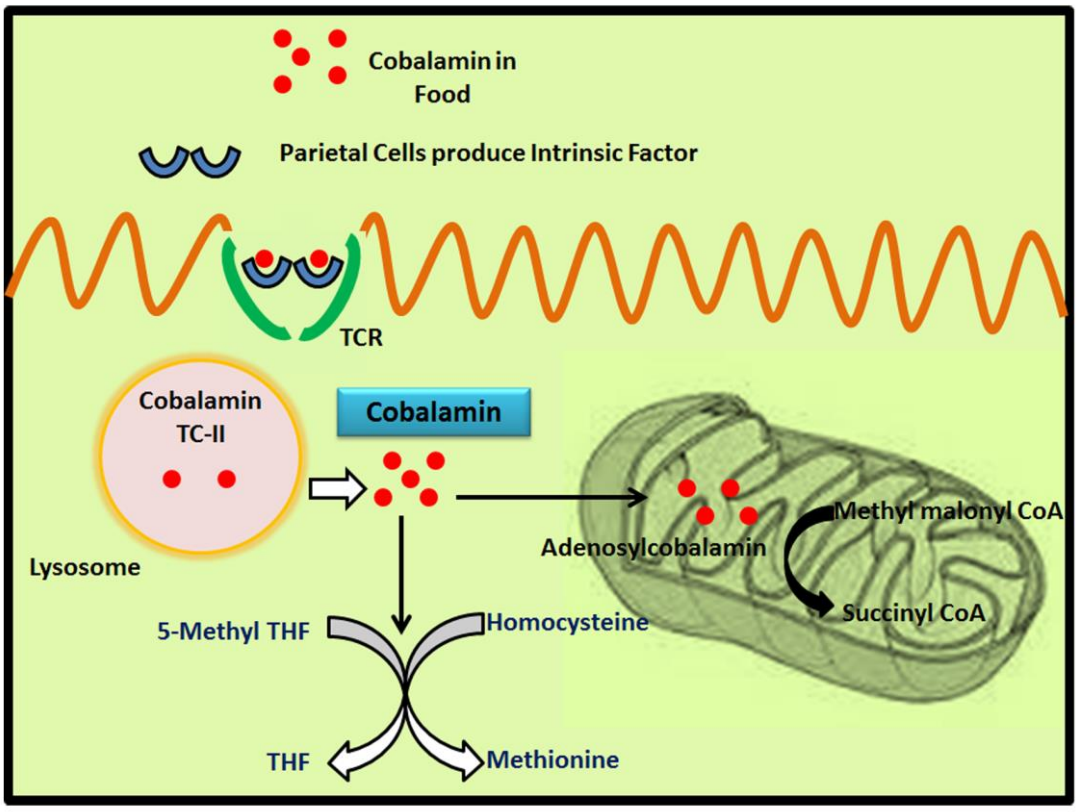
Vitamin B_{12} is synthesized entirely by microorganisms. Dietary sources include those of theanimal origin (such as meat, fish, poultry, shellfish, milk, cheese and eggs), fortified foods (most commonly ready to eat breakfast cereals) and B_{12} supplements (reviewed by Da Silva and McCray, 2009). Uncooked plant-based foods exposed to specific bacterial action and fermented foods are also other sources of vitamin B_{12}. Vitamin B_{12} is also produced by vitamin B_{12} synthesizing bacteria in the human large bowel; however, it is not yet clear whether these are sufficient to meet the needs of the individual (reviewed by Elmadfa and Singer, 2009). Seafood rich in

vitamin B_{12} include clams, oysters, octopus, fish and fish roe. Reports have demonstrated that cow's milk provide an average of 4.5µg/L of vitamin B_{12} (Park and Haenlein, 2013).

Functions of Vitamin B_{12}:

Vitamin B_{12} carries out different physiological functions in the body including erythropoiesis, synthesis and maintenance of myelin sheath and the synthesis of nucleic acid (DNA) (National Institute of Health, 2011). It plays a vital role in maintaining normal cellular intermediary metabolism (reviewed by Li et al. 2009). It acts as a cofactor in two metabolic pathways in mammalian cells: 1) the methylation of homocysteine to form methionine using cytoplasmic enzyme methionine synthase and 2) conversion of methylmalonyl CoA to succinylCoA using the mitochondrial enzyme methylmalonyl CoA mutase (Watkins et al. 2008) (Fig 9).

Fig 9: Functions of Vitamin B_{12}



THF: *Tetrahydrofolate;* **TC-II:** *Transcobalamine II-Cobalamine;* **TCR:** *Transcobalamin receptor*
Source: *Modified from Paulev and Zubieta, 2004 "Metabolism & Nutritional Disorders" In "New Human Physiology", 2nd Edition and http://products.mercola.com/vitamin-b12-spray/*

Requirement of Vitamin B_{12}:

The Institute of Medicine, USA has set the current Recommended Dietary Allowance for vitamin B_{12} as 2.4 µg per day for male and female subjects, aged 14 years and older. In order to meet fetal demands, the recommended intake of B_{12} for pregnant women is increased to 2.6 µg, versus 2.4 µg/day for adults. For infants, the RDA has been set as 0.4 µg/day (Institute of Medicine, 1998). The Indian Council of Medical Research recommends 1 µg/day which allows enough margins of safety for cooking losses, uncertainties in absorption and a small amount for storage (ICMR, 2009). A recent report by Center for Food Safety and Applied Nutrition recommends 6 µg per day for adults and children, considering 2,000 calories of the caloric intake (FDA, 2013).

Reports have demonstrated no adverse consequences of consumption of vitamin B_{12} above the RDA, (reviewed by Simpson et al. 2010) however; more studies are needed in order to draw an appropriate conclusion regarding the consumption of higher vitamin B_{12} during pregnancy.

Assessment of Vitamin B_{12}:

Different methods are used for the screening of the vitamin B_{12} deficiency, each having its own advantages and disadvantages. Serum total vitamin B_{12} levels is often used as a biomarker of vitamin B_{12} status. Serum vitamin B_{12} levels does not necessarily reflect the dietary levels since they are also known to be affected by liver disease, certain cancers, chronic bone disease, or acute illness (reviewed by Ermens and Vlasveld, 2003). Another promising marker for vitamin B_{12} status is serum holotranscobalamin (Holo TC), which in combination with total vitamin B_{12} provides a better assessment of vitamin B_{12} deficiency (Al Aisari et al. 2010). The less efficient assay for screening and diagnosis of vitamin B_{12} deficiency includes the measurement of mean corpuscular volume; however it is known to miss more than 80% of cases (reviewed by Oosterhuis et al. 2000).

Another important biomarker for evaluating the vitamin B_{12} status is the methylmalonic acid (MMA). Vitamin B_{12} acts as a cofactor for the conversion of MMA to succinyl-CoA and therefore, deficiency of vitamin B_{12} will increase the levels of MMA. However, due to the higher cost required for carrying out the MMA assay, its utility has been questioned (reviewed by Da Silva and McCray, 2009). In addition, plasma homocysteine and serum cystathionine are also the indicators of

vitamin B_{12} status, however vitamin B_6 or folate deficiencies also affect the levels of homocysteine or cystathionine (Ubbink et al. 1996; reviewed by Strain et al. 2004).

The recommended cut-offs for diagnosing vitamin B_{12} deficiency and depletion are 5148pmol/L (200 pg/ml) and 5221 pmol/L (5300 pg/ml), respectively, in plasma or serum (reviewed by Simpson et al. 2010).

Maternal Vitamin B_{12} Deficiency:

Globally, vitamin B_{12} deficiency is more prevalent in poorer populations around the world. Over the years this deficiency was neglected due to several inaccurate beliefs i.e. deficiency is unlikely except in strict vegetarians or patients with pernicious anemia, and that it usually takes 20 years for stores of the vitamin to become depleted (reviewed by Allen, 2009). Vitamin B_{12} deficiency has now become a potential health problem during pregnancy affecting the fetal development (reviewed by Rush et al. 2014). A recent review reports the global prevalence of vitamin B_{12} deficiency in the vegetarian population. Depending on the trimester, deficiency among pregnant women ranges from 17 to 39% (reviewed by Pawlak et al. 2014). Further, it has been reported that low vitamin B_{12} concentrations are common in Indians and contribute to hyperhomocysteinemia. A study carried out in south India reports that 51.1% pregnant women had low plasma vitamin B_{12} concentration (Samuel et al. 2013); while the study carried out in north India reports that 74.1% of the pregnant women had poor vitamin B_{12} stores (Pathak et al. 2007).

There are two main causes of the maternal vitamin B_{12} deficiency. Vitamin B_{12} is exclusively found in animal food products and therefore nutritional deficiency can occur if the animal food source consumption is low. Another important cause for vitamin B_{12} deficiency is malabsorption. Tropical sprue, gastrointestinal infestations, Helicobacter pylori infections are the major contributors to the low vitamin B_{12} concentrations (reviewed by Allen, 2008; reviewed by Varbanova et al. 2014). In addition to these, intestinal diseases like Crohn's or celiac disease also result in decreased vitamin B_{12} absorption (Yakut et al. 2010). It has been reported that pernicious anemia causes malabsorption of vitamin B_{12} by reducing the levels of intrinsic factor. Reports have also suggested that maternal short gut syndrome and gastric bypass surgery can result in fetal vitamin B_{12} deficiency (reviewed by Pepper and Black, 2011). Gene polymorphism in transcobalamins is also one of the factors affecting plasma vitamin B_{12} concentrations (reviewed by Allen, 2008).

Several cross-sectional, case-control and longitudinal studies report that blood concentration of vitamin B_{12} drops during normal pregnancy due to factors like hemodilution, altered renal function, hormonal changes, changes in the concentration of vitamin B_{12}-binding proteins and materno-fetal vitamin B_{12} transfer (Murphy et al. 2007). The lower levels of vitamin B_{12} during pregnancy are often associated with severe symptoms such as failure to thrive, inability to accept solid foods, stunting and developmental delays in children (reviewed by Pawlak et al. 2014).

Reports indicate that maternal vitamin B_{12} deficiency can have profound effects such as defective DNA synthesis, megaloblastic anemia and neurological abnormalities in the fetus (reviewed by Hovdenak and Haram, 2012). Studies carried out in Asia have demonstrated that inadequate vitamin B_{12} status is associated with increased risk of preterm birth and higher prevalence of developing preeclampsia (reviewed by Molloy et al. 2008). Similar to folic acid, maternal vitamin B_{12} status was found to be an independent risk factor for NTD (Suarez et al. 2003). A study carried out in South India reports that women in the lowest tertile for serum vitamin B_{12} concentration during each of the three trimesters of pregnancy had a higher risk of delivering an IUGR baby (Muthayya et al. 2006). A case control study also reports an association between P259R polymorphism in transcobalamin and spontaneous abortions (Zetterberg et al. 2002).

Several studies have also linked vitamin B_{12} deficiency with increased risk of abnormal embryo-fetal brain development, cleft palate and metabolic syndromes (reviewed by Moreno-Garcia et al. 2013). Studies carried out in India and Nepal report the association between poor maternal vitamin B_{12} status and increased insulin resistance in children (Yajnik et al. 2008; Stewart et al. 2011). It is well known that lower vitamin B_{12} levels are associated with raised plasma homocysteine levels, which is a risk factor for cardiovascular disease (Mahalle et al. 2013).

3.2.3. Fatty Acids:

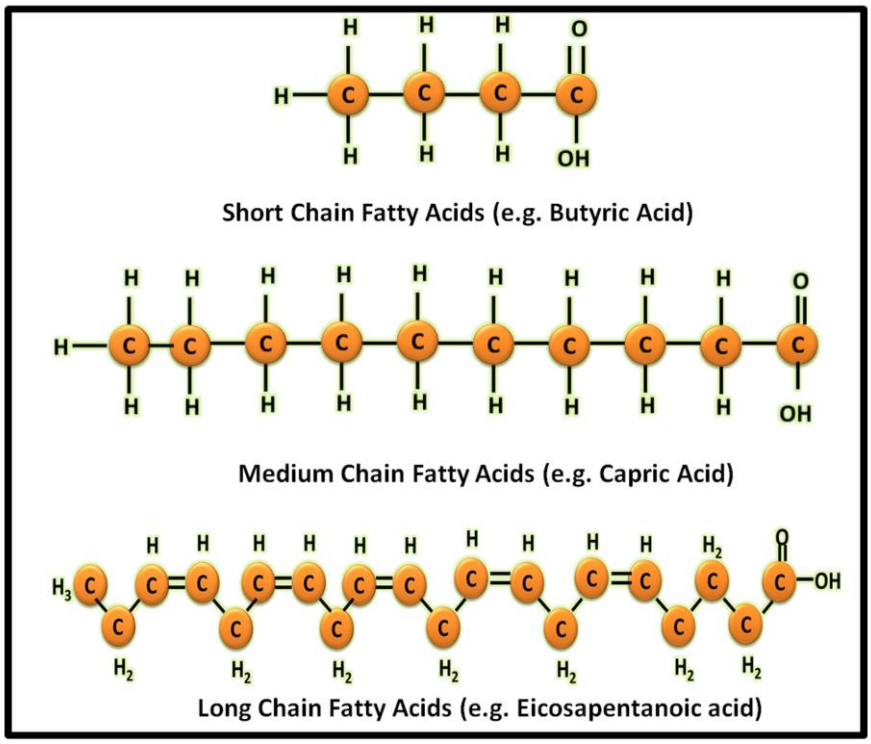
Fats or lipids consist of numerous chemical compounds like monoglycerides, diglycerides, triglycerides, phosphatides, cerebrosides, sterols, terpenes, fatty alcohols and fatty acids. Fatty acids have carboxylic acids at the polar end and hydrocarbon chains of 4 to 36 carbons long (C4 to C36) at non polar end. Due to the hydrophilic nature of the carboxylic group and hydrophobic nature of the hydrocarbons, fatty acids are considered as the amphipathic compounds (Campbell and Farrell, 2014).

They occur either as esters in natural fats and oils or as free fatty acids in the unesterified form, those found in the plasma. Fatty acids occuring in natural fats are mostly straight-chain fatty acids comprising an even number of carbon atoms. The chain may be unsaturated (containing one or more double bonds) or saturated (containing no double bonds) (Nelson and Cox, 2005).

Classification of Fatty Acids:

Fatty acids can be classified based on the chain length. Short chain fatty acids have fewer than 8 carbon atoms; medium chain fatty acids have 8 to 12 carbon atoms; long chain fatty acids have 12 to 22 carbon atoms (Fig 10) while very long chain fatty acids have more than 22 carbon atoms (O'Brien, 2008).

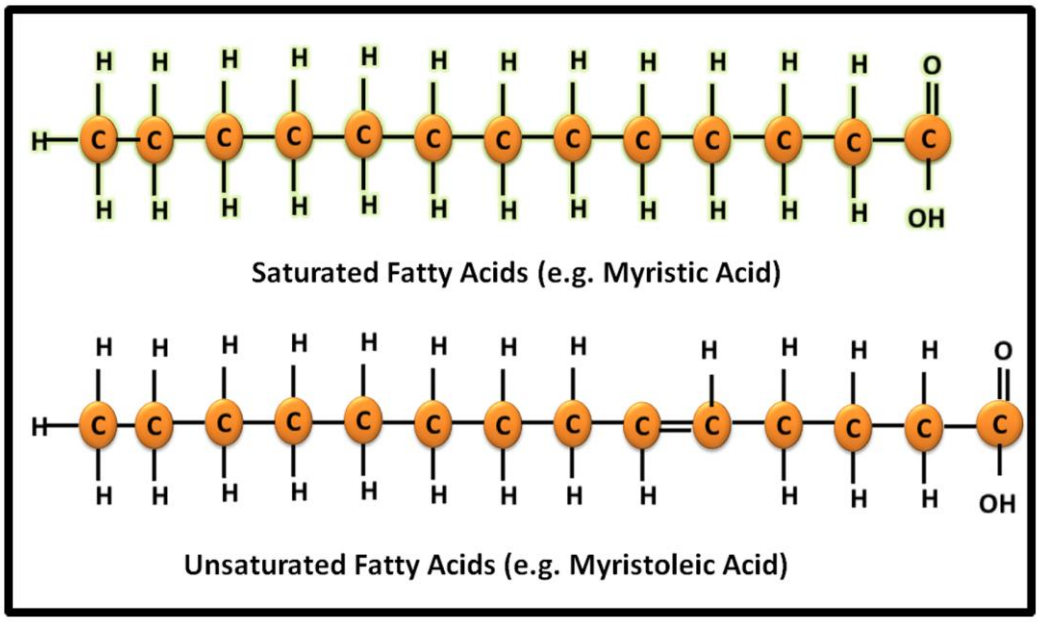
Fig 10: Short, Medium and Long Chain Fatty Acids



Some of the common short chain fatty acids include acetic acid (C2), propionic acid (C3), butyric acid (C4) and valeric acid (C5). Medium chain fatty acids include caprylic acid (C8), capric acid (C10) and lauric acid (C12); while long chain fatty acids include DHA, ARA and EPA. Based on the presence or absence of double bonds, these are classified as either saturated (absence of double bond) or unsaturated (presence of double bond) fatty acids (Fig 11). The saturated fatty acids (SFA) have only single bonds, each carbon atom within the chain has 2 hydrogen atoms (McGuire

and Beerman, 2012). The common saturated fatty acids are myristic, palmitic and stearic acid.

Fig 11: Saturated and Unsaturated Fatty Acids



The unsaturated fatty acids are monounsaturated fatty acids (MUFA) (contains single double bond) and polyunsaturated fatty acids (PUFA) (contains multiple double bond) (Ruston and Drevon, 2005) (Fig 17). Oleic acid is the common example of monounsaturated fatty acid. The PUFA are further classified into two families depending on the position of the double bond on the methyl terminal (ω; n-) end (reviewed by Bazinet and Laye, 2014). The omega-3 PUFA have their first double bond 3 carbons away from the methyl end while the omega-6 PUFA have their first double bond 6 carbons away from the methyl end (Fig 12).

Some of the omega-3 PUFA include ALA, EPA and DHA while omega-6 PUFAs are LA and ARA (Fig 12, 13). DHA is a carboxylic acid containing 22-carbon chain and six cis double bonds with the first double bond situated at the third carbon from the omega end. EPA has 20-carbon chain, five cis double bonds; the first double bond is situated at the third carbon from the omega end. ARA is a carboxylic acid with a 20-carbon chain and four cis-double bonds; the first double bond is situated at the sixth carbon from the omega end (Guo, 2014).

Fig 12: Monounsaturated and Polyunsaturated Fatty Acids

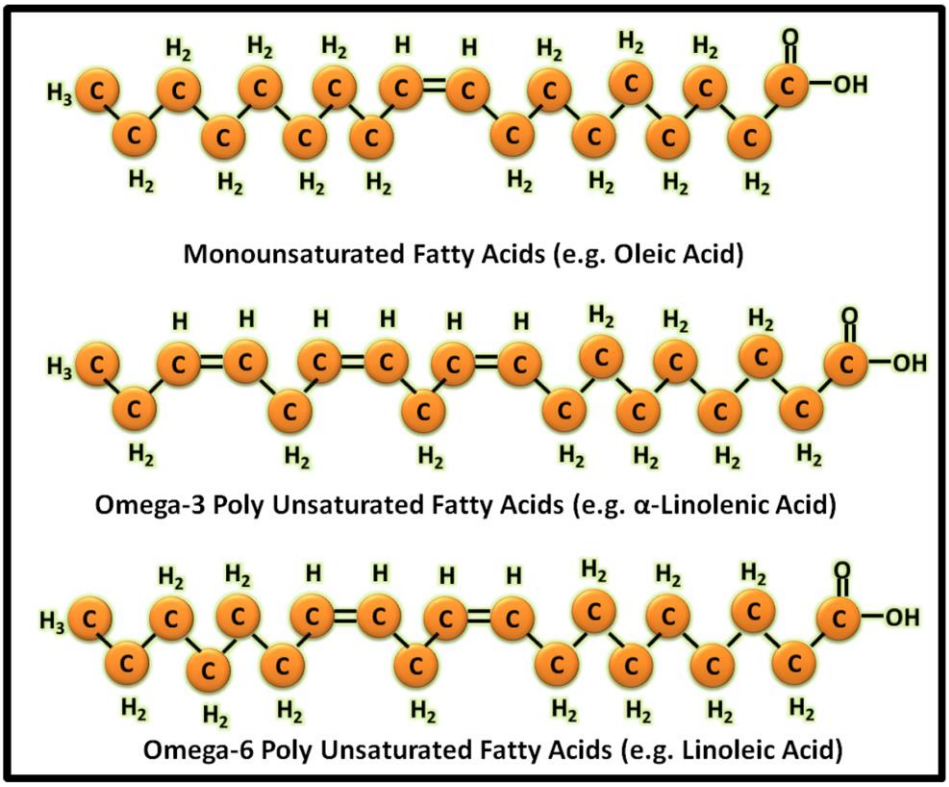
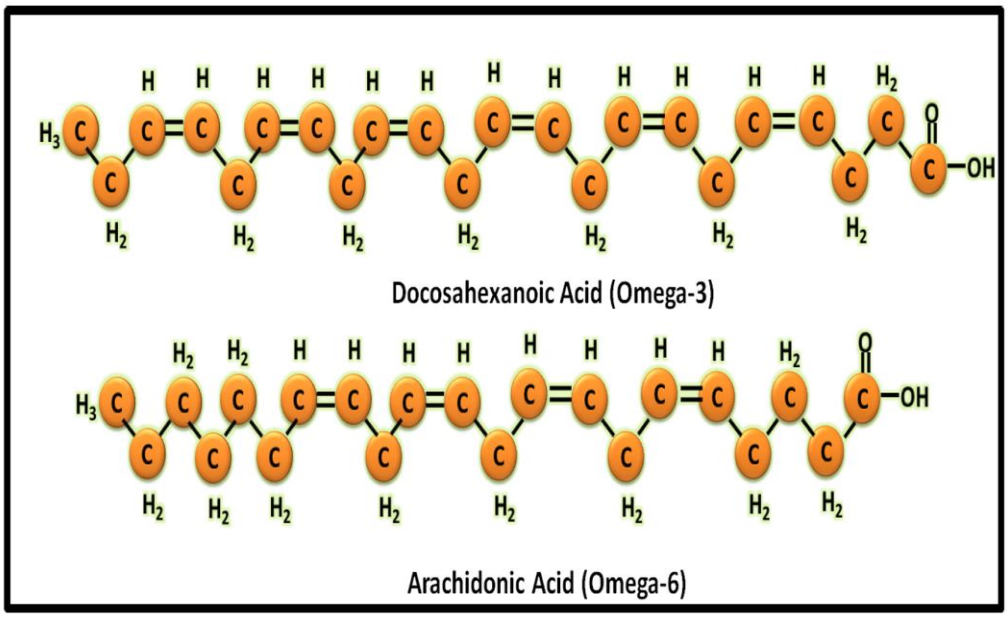


Fig 13: Long Chain Poly Unsaturated Fatty Acids



The PUFAs are also classified based on the position of the hydrogen atoms adjacent to the double bond. If the two hydrogen atoms adjacent to the double bond are on the same side of the chain, then they are said to be in the '*cis*' configuration; while if they lie on opposite sides of the chain, they are said to be in the '*trans*' configuration (Ruston and Drevon, 2005).

Nomenclature:

Various nomenclature systems are used for fatty acids. The common nomenclature includes adding the suffix –ic to a root indicative of natural source or some property of acids, eg: acetic acid (Chow, 2007). A simplified systematic nomenclature specifies the chain length and number of double bonds, separated by a colon. For example, the 16-carbon saturated palmitic acid is abbreviated as 16:0 (Nelson and Cox, 2005).

The greek letters delta (Δ) and omega (ω) are also used for naming the fatty acids. Δ is used to indicate the positions of any double bonds specified by superscript numbers; for example a 20-carbon fatty acid with one double bond between C-9 and C-10 (C-1 being the carboxyl carbon) and another between C-12 and C-13 is designated 20:2($\Delta^{9,12}$) (Nelson and Cox, 2005). The term ω or n is used to refer the position of the first double bond in the carbon backbone from the methyl end of the molecule (Lucas and Southgate, 2012). For example, oleic acid; which has its double bond on the 9^{th} carbon from the methyl end is denoted as ω-9 or n-9. One of the nomenclatures of the fatty acids is as follows: arachidonic acid, C20:4 ω-6. The first number indicates the number of carbon units, the second number indicates the number of double bonds and ω-6 refers to the position of the last double bond.

Sources of Fatty Acids:

Foods rich in saturated fats include animal fat products such as cream, cheese, butter, ghee, tallow, lard and fatty meats. Certain vegetable products like coconut, cottonseed and palm kernel oil are also high in saturated fats. In addition to this, foods which are high in saturated fat content include pizza, desserts, bacon and sausage. Unsaturated fatty acids are found in almost all plants and animals but they are highly concentrated in seeds, nuts, certain fruits and fish. The sources of MUFA include olives, avocados, rapeseed, peanuts, ricebran, soyabean and certain hydrogenated oils like olive, canola and peanut.

The animal sources of omega-3 fatty acids include oily fish such as salmon, sardines, eggs and meats such as lean beef and chicken. The plant sources include linseed/flaxseed, walnuts, soybeans and canola oil. Specifically, foods sources of ALA include flax seed, hemp, walnut and soybean (reviewed by Hunter, 1990). ALA is mostly found in the chloroplast of green leafy vegetables. EPA is present in amounts ranging from 39 to 50 % in both fresh and salt water fish. Food source rich in

DHA include fish oil and red brown algae. Omega-6 fat sources include margarine spreads, sunflower, soybean, sesame oils, sunflower seeds, nuts such as walnuts, pecans, brazil and pine nuts (reviewed by Kaur et al. 2012). Specifically, LA is abundant in safflower, sunflower and corn; it is present in medium quantities in soybean, sesame and almonds and in small quantities in canola, peanut and olive oils. It is very low in coconut and palm kernel. ARA is found in meat, eggs and dairy products.

Metabolism of Fatty Acids:

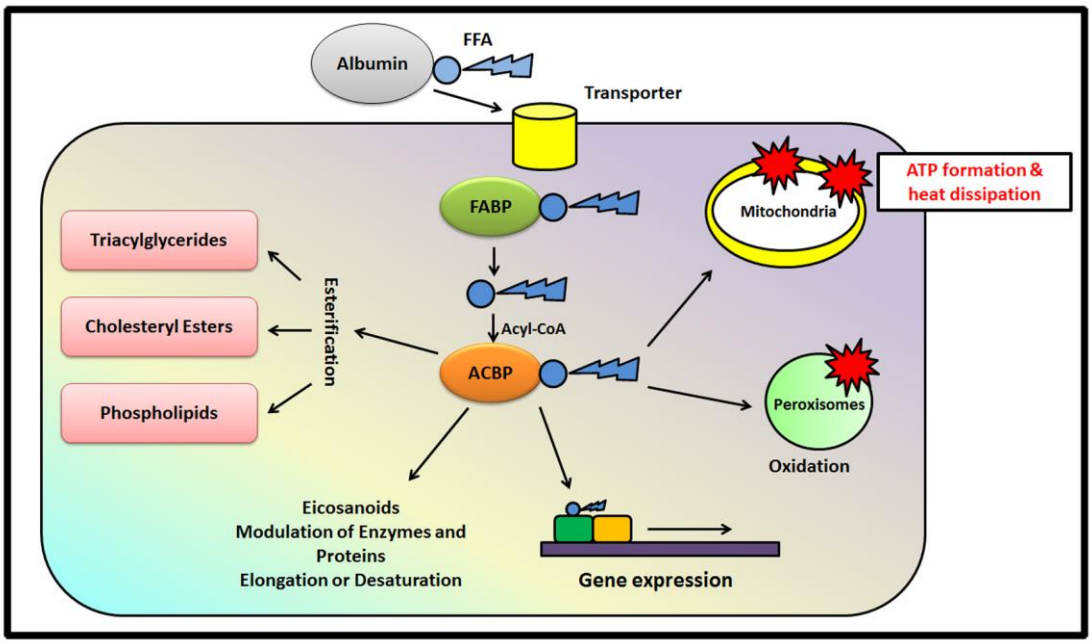
Omega-3 and omega-6 fatty acids when consumed in the diet from various food sources is mostly in the form of triacylglycerols. Free fatty acids (FFA) and monoacylglycerols are then released from the triacylglycerols during the process of digestion (Gropper et al. 2012). These FFA and monoacylglycerols are absorbed in the small intestine where they are re-esterified to triacylglycerols in the intestinal mucosa cells. The triacylglycerols are then transported either as bound to albumin or as part of lipoproteins/chylomicrons, to the circulation via lymphatic vessels (reviewed by Iqbal and Hussain, 2009). With the help of different protein transporters present on the plasma membranes, these FFA are taken up by the cells and are intracellularly transported through fatty acid-binding proteins (FABP) (Ruston and Drevon, 2005). The FFA are converted in the activated form acyl CoA and diverted to one of the following paths: 1) mitochondria for β-oxidation 2) peroxisomes for β-oxidation to provide energy in the form of adenosine triphosphate (ATP) 3) endoplasmic reticulum for esterification 4) bind to transcription factors that regulate gene expression 5) converted to signal molecules (eicosanoids) (reviewed by Kaur et al. 2012) (Fig 14).

Biomarkers of Fatty Acid Status:

The precise assessment of fatty acid intake is required for nutritional epidemiologic studies. There are several methods like food-frequency questionnaires (FFQs) and diet records through which fatty acid intake can be assessed (Broadfield et al. 2003). However, several limitations of these methods are reported. Poor recalling of food habits, inconsistency in the composition of foods from season-to-season and the inability of the nutrient databases to adequately reflect temporal changes have raised issues in adapting these assessment methods (reviewed by Cantwell, 2000).

Moreover fatty acid status depends not only on dietary intake, but also on endogenous metabolism (Assies et al. 2010).

Fig 14: Metabolism of Fatty Acids



***FFA**, Free fatty acids; **FABP**, fatty acid-binding proteins; **ACBP**, Acyl-CoA binding protein.*
Source: Modified from Ruston and Drebon 2005, John Wiley & Sons, Ltd.

Biomarkers of fatty acids serve as an independent measure of intake which is unbiased by self-recalling or changes in the fatty acid composition of the food supply from season-to-season. Hence, biomarkers of fatty acid intake have been widely used which makes it possible to compare food reports collected by dietary assessment methods (reviewed by Shim et al. 2014). Further, the blood lipid levels of LA and DHA are shown to reflect the dietary intake and therefore can be used as biomarkers of intake. Thus fatty acids are measured in various blood fractions like plasma, serum, erythrocyte and tissues, especially adipose tissue (reviewed by Lauritzen and Carlson, 2011).

Plasma fatty acid levels are known to be the indicators of the recent dietary intake (reviewed by Hodson et al. 2008). Erythrocytes have a life span of approximately 120 days (Ebaugh et al. 1953) and therefore, erythrocyte fatty acid levels are known to be the indicators of long term fatty acid status. These biomarkers of fatty acids are confounded by the fact that fat deposition and mobilization differ between and within individuals according to endogenous factors such as metabolic state (especially pregnant women) and synthesis. In addition, the results may also be

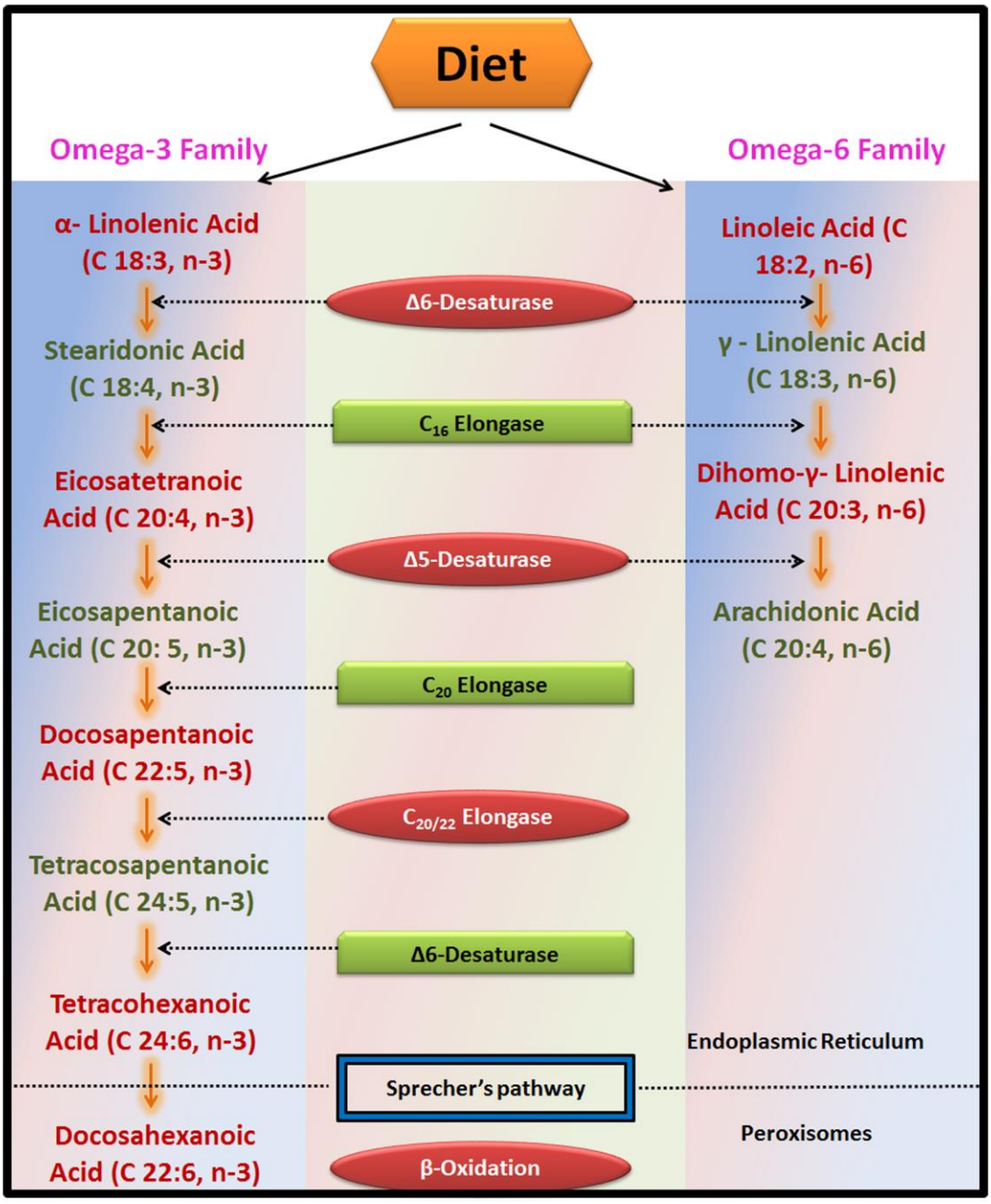
influenced by the method of collection, sampling site, tissue choice and analytical technique (reviewed by Arab and Akbar, 2002). Despite these limitations, plasma and erythrocyte levels are considered to be useful for assessing the fatty acid status of the individual.

Biosynthesis of LCPUFA:

In humans, omega-6 and omega-3 LCPUFAs are either derived from the diet or from the metabolism of dietary linoleic acid and α-linolenic acid. The pathways involved in this biosynthesis were established by Ralph Holman (1986) in the 1950s and have since been refined by Howard Sprecher et al. (1995) (reviewed by Gibson et al. 2011). Liver is the main site of LCPUFA biosynthesis (reviewed by Williams and Burdge, 2006). Both omega-6 and omega-3 fatty acids use the single set of destaurase and elongase enzymes and fatty acids from one series cannot be converted to another (reviewed by Calder, 2012).

The first step in this process involves the desaturation of LA and ALA to γ-linolenic acid (GLA) and stearidonic acid (SA) using the enzyme Δ6-desaturase (reviewed by Nakamura and Nara, 2003). This is the rate limiting step of the pathway and therefore, high dietary intakes of omega-6 PUFA have been proposed to be a limiting factor in the conversion of ALA to its long chain products EPA and DHA (reviewed by Williams and Burdge, 2006). GLA and SA are further converted into dihomo-g-linolenic acid (DGLA) or eicosatetranoic acid (C20:4n-3) respectively by elongases (reviewed by Warude et al. 2006). The next step in the LCPUFA formation involves the enzyme Δ5-desaturase which converts DGLA and eicosatetranoic acid into ARA and eicosapentaenoic acid (EPA) respectively (reviewed by Nakamura and Nara, 2003). The final conversion to ARA and EPA to docosapentaenoic acid (DPA, 22:5n-6) and DHA respectively occurs either through a direct Δ4-desaturation or an elongation, followed by a Δ6-desaturation and then partial β-oxidation to form n-6 DPA and DHA. This process is known as retro-conversion or the "Sprecher" pathway (reviewed by Bradbury, 2011) (Fig 15). The enzymes Δ5- and Δ6-desaturases are reported to be dependent on numerous nutritional and hormonal factors (reviewed by Jump et al. 2005). Reports have indicated that the conversion efficiency of ALA to EPA varies between 0.2 to 21% and that of ALA to DHA varies between 0 to 9% (reviewed by Abedi and Sahari, 2014).

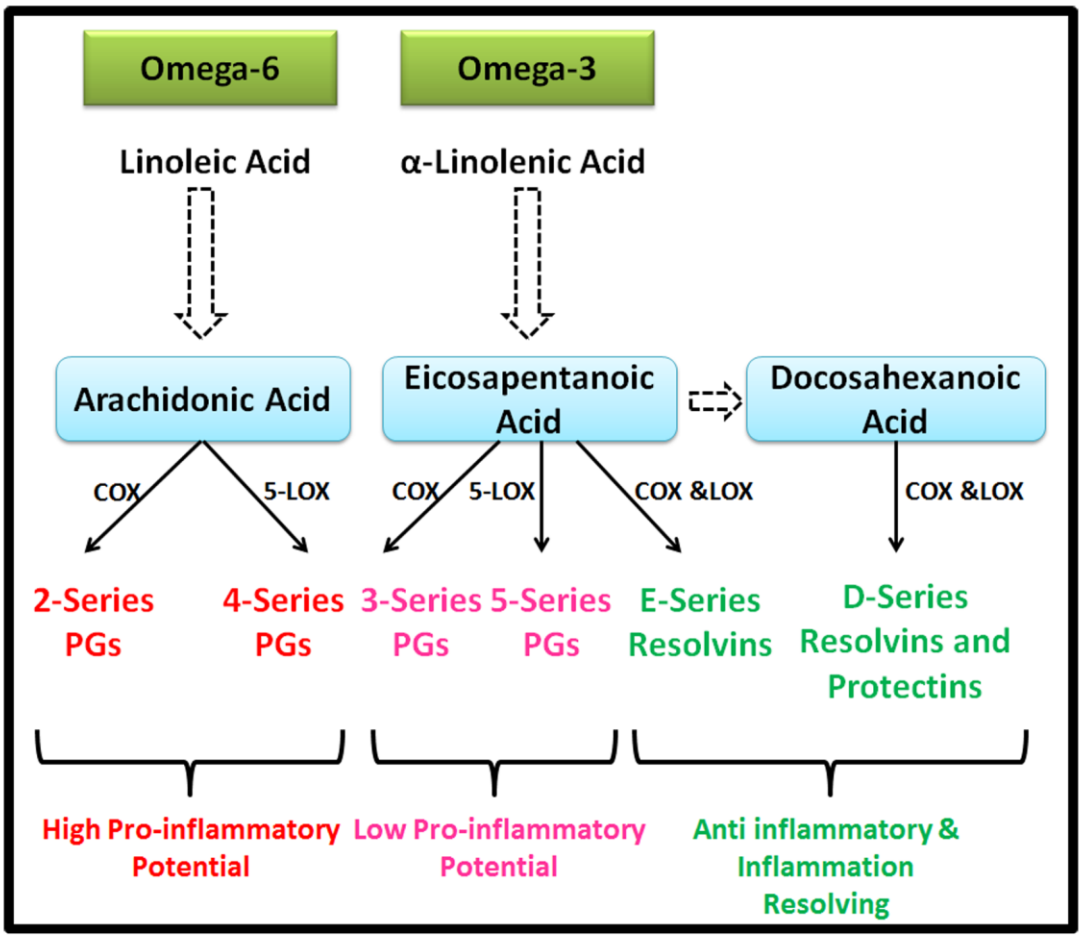
Fig 15: Biosynthesis of Long Chain Polyunsaturated Fatty Acids



Source: *Modified from Warude et al. 2006, Critical Reviews in Biotechnology. 26(2):83-93.*

The LCPUFA thus formed serve as precursors to eicosanoids including prostaglandins and prostacyclins; and play key roles in various biological functions, like inflammatory response, fetal growth and development, retinal function and brain development (Fig 16).

Fig 16: Synthesis of Prostaglandins and Eicosanoids through LCPUFA



***COX**- cyclooxygenase, **LOX**- Lipooxygenase, **PG**- Prostaglandin*
Source: Modified from Abeywardena and Head 2001, Cardiovascular Research. 52(3):361-71.

LCPUFA during Pregnancy:

Omega-3 LCPUFAs are of particular interest during pregnancy due to their beneficial effects in terms of pregnancy outcomes, such as improving gestation duration and enhancing short- and long-term development of the offspring (reviewed by Larque´ et al. 2012). Studies have shown that maternal omega-3 fatty acid supplementation during pregnancy results in a modest increase in birth size and length of gestation, reducing the risk of early preterm and low birth weight (reviewed by Coletta et al. 2010; reviewed by De Giuseppe et al. 2014; reviewed by Haggarty, 2014). This finding has been confirmed in a study carried out in Kansas city, USA which examined the effect of DHA supplementation (600 mg/day) in the last half of gestation and found an increase in the length of gestation and infant size (Carlson et al. 2013).

It is important to take adequate EPA by the pregnant women since EPA is known to be the pre-cursor of 3 series prostaglandins PGE3 and PGI3, which promote relaxation of myometrium (reviewed by Greenberg et al. 2008). It has been reported that maternal EPA concentrations may increase FATP-4 expression which increases the levels of DHA in the cord blood (Larque et al. 2006). Further, studies have reported that EPA and DHA competitively displace ARA in the membrane phospholipids and hence decrease the production of 2-series eicosanoids (reviewed by Greenberg et al 2008). Therefore, a diet with optimal omega-3 and omega-6 ratio is as important to pregnant women as the absolute individual levels of these fatty acids.

LCPUFA are one of the major nutrients transported from mother's blood to the fetal blood. Optimized delivery of LCPUFA to the fetus includes several adaptive mechanisms occurring during pregnancy like mobilization of maternal stores, LCPUFA synthesis and selective delivery of maternal circulating LCPUFA to the fetus (reviewed by Haggarty, 2014). These are required by the fetus to maintain the fluidity, permeability and conformation of membranes, as a source of energy and as precursors of important bioactive compounds (reviewed by Haggarty, 2004). Studies have indicated that the fetus accumulates about 67 mg a day of DHA during the last trimester of pregnancy (reviewed by Morse, 2012). Therefore, the availability of maternal LCPUFA needs to be adequate. Reports have also indicated that percentage of LCPUFA (DHA and ARA) in the fetal circulation is higher than the maternal circulation (reviewed by Cetin et al. 2009). LCPUFAs like ARA and DHA are critical to fetal central nervous system (CNS) growth and development (reviewed by Rombaldi Bernardi et al. 2012). ARA is known to be involved in cell division and cell signaling pathways, and functions as a precursor for inflammatory eicosanoids (reviewed by Komsa-Penkova et al. 2012). Concentration of DHA is known to be high in retinal and brain membrane phospholipids and is therefore involved in visual and neural function (reviewed by Valenzuela, 2009).

Studies carried out in our department have extensively shown that there is an interlinkage between the fatty acids and micronutrients like folate and vitamin B_{12} in the one carbon cycle (Kulkarni et al. 2011c; reviewed by Sundrani et al. 2012; reviewed by Dhobale and Joshi, 2012; Wadhwani et al. 2015).

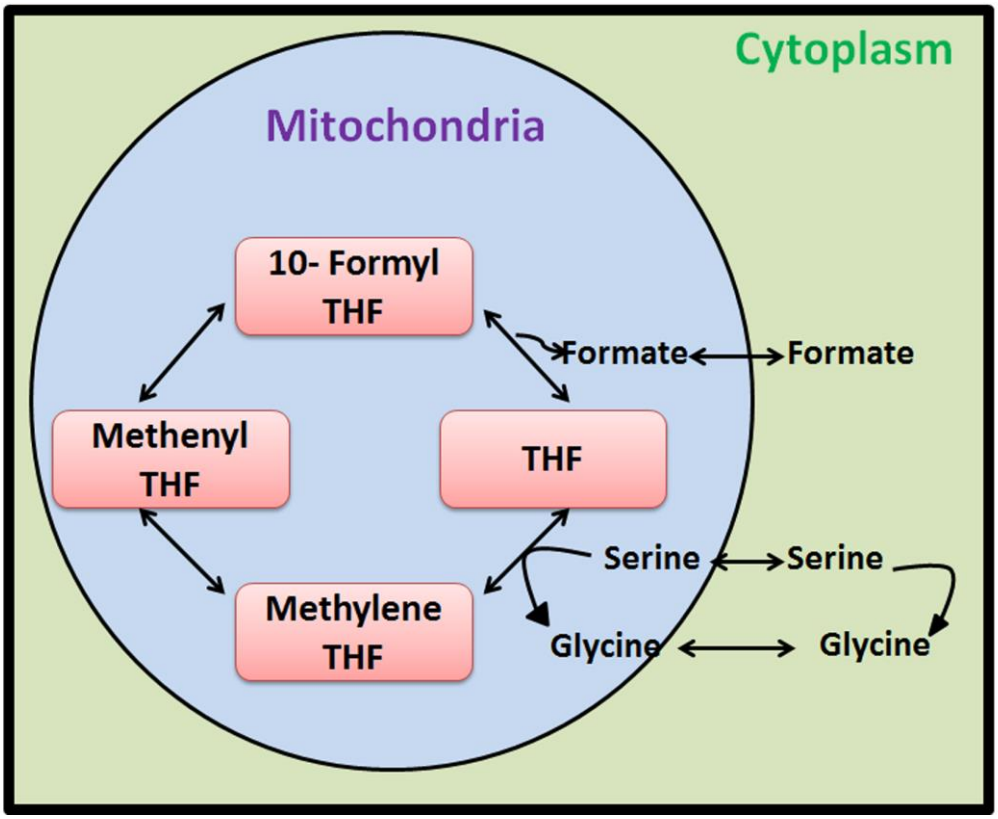
3.3. One Carbon Cycle:

One carbon metabolism is a complex metabolic network of pathways that transfer methyl groups from one compound to another (reviewed by Kruman and Fowler, 2014). Physiologically, one carbon metabolism serves two critically important functions: (i) de novo nucleotide biosynthesis, essential for DNA replication and repair and (ii) provision of methyl groups for methylation, e.g DNA methylation (reviewed by Stover, 2009). Dietary components of interest includes most of the B vitamins viz folate, vitamin B_{12}, riboflavin, vitamin B_6, choline and methionine (reviewed by Brosnan and Brosnan, 2006). In addition, enzymes such as methionine synthase (MS), methylene THF reductase (MTHFR) and cystathionine beta-synthase (CBS) represent the essential one carbon metabolism components (reviewed by Singh and Jaiswal, 2013). Folate plays an important role in one carbon cycle wherein it serves as a catalytic substrate for the transfer of one-carbon units (reviewed by da Silva et al. 2014). Out of the total cellular folate, approximately half of it is located in the mitochondria and the rest of the large part is located in the cytoplasm. Folate exists in other organelles, including nucleus; however these do not make an important contribution to the total cellular folate concentrations (reviewed by Coşar et al. 2014). Thus, the folate mediated one carbon cycle occurs primarily within the mitochondria and the cytoplasm.

Mitochondrial Folate Mediated One Carbon Cycle:

The primary role of mitochondrial one carbon metabolism is to generate one carbon units in the form of formate for cytoplasmic one carbon metabolism (reviewed by Beaudin and Stover, 2007). Formate is produced through a series of reactions involving the catabolism of serine to formaldehyde. This formaldehyde is activated by condensing with tetrahydrofolate (THF) to form methylene THF. The methylene THF is then oxidized to form 10-formyl-THF which is further hydrolyzed to THF and formate (reviewed by Stover, 2009). The formate thus produced in the mitochondria, now enters the cytoplasm (Fig 17).

Fig 17: Mitochondrial One Carbon Cycle



THF*: Tetrahydrofolate*
Source: *Modified from Anderson and Stover 2009, PLoS One. 4(6):e5839.*

Cytoplasmic Folate Mediated One Carbon Cycle:

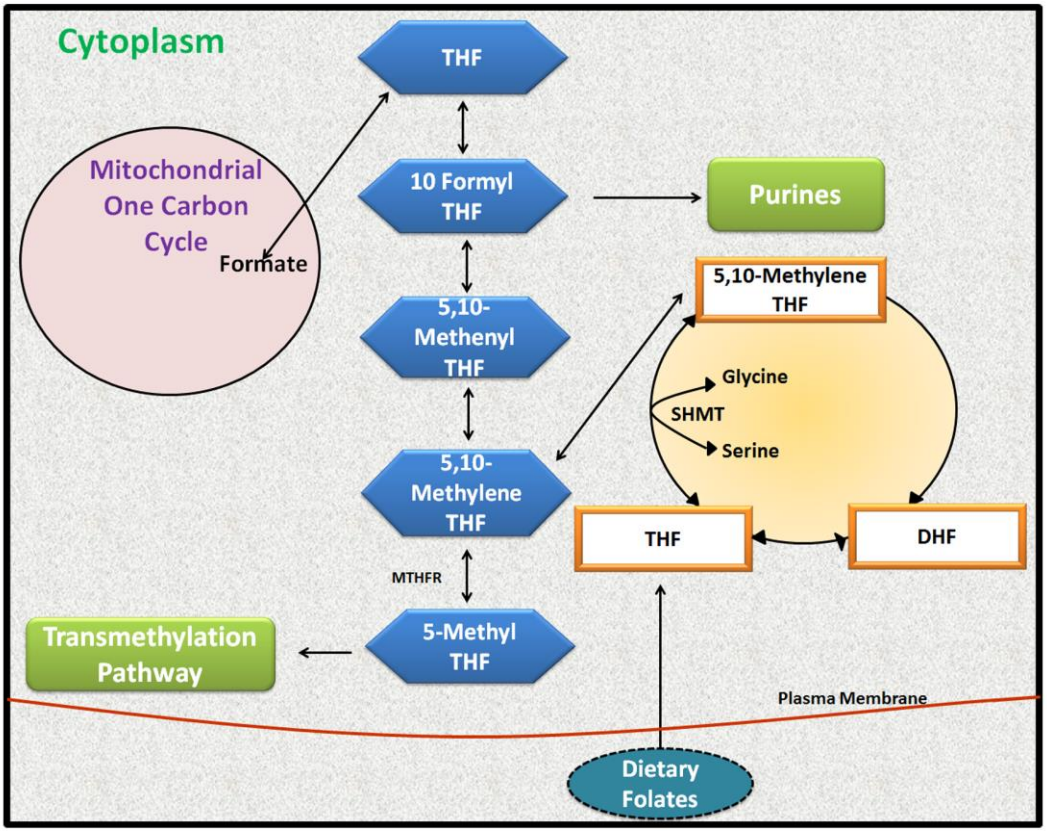
In the cytoplasm, formate enters the one carbon cycle and gets converted into 10-formyl-THF catalyzed by enzyme 10-formyl-THF synthetase. This 10-Formyl-THF-is used for the biosynthesis of the purines only if purine nucleotides are not generated from synthesis through the purine nucleotide salvage pathway. 10-Formyl-THF is converted to methylene-THF by the enzyme methenyltetrahydrofolate dehydrogense. Alternatively, methylene-THF (and glycine) can also be generated in the cytoplasm from serine and THF using the enzyme serine hydroxymethyltransferase (SHMT) (reviewed by Stover, 2009).

The methylene-THF thus formed, acts as both a one carbon donor (in the form of formaldehyde) and the donor of 2 electrons. It donates 2 electrons to generate dihydrofolate (DHF), which further gets converted into THF using the enzyme dihydrofolate reductase. The THF generated is the functional THF cofactor. Alternatively, the methylene THF is converted to 5-methyl-THF (5-MTHF) in a NADPH dependent reaction catalyzed by the enzyme MTHFR (reviewed by Stover and Field, 2011). The 5-MTHF is also formed by the 5,10-methylenetetrahydrofolate

using the enzyme MTHFR. Dietary folates are known to participate in the one carbon cycle, mainly in the form of tetrahydrofolates.

The enzyme S-adenosyl methionine (SAM) is the negative regulator of the MTHFR activity and thus controls the levels of 5,10-MTHF. Therefore, in conditions of low SAM activity, as may occur with folate deficiency; the activity of 5,10-MTHF is reduced, increasing the risk of nucleotide imbalance, apoptosis and carcinogenesis (reviewed by Halsted, 2013). The formation of 5-MTHF irreversibly transfers the one-carbon units to the methionine/homocysteine remethylation cycle (Fig 18).

Fig 18: Cytoplasmic One Carbon Cycle



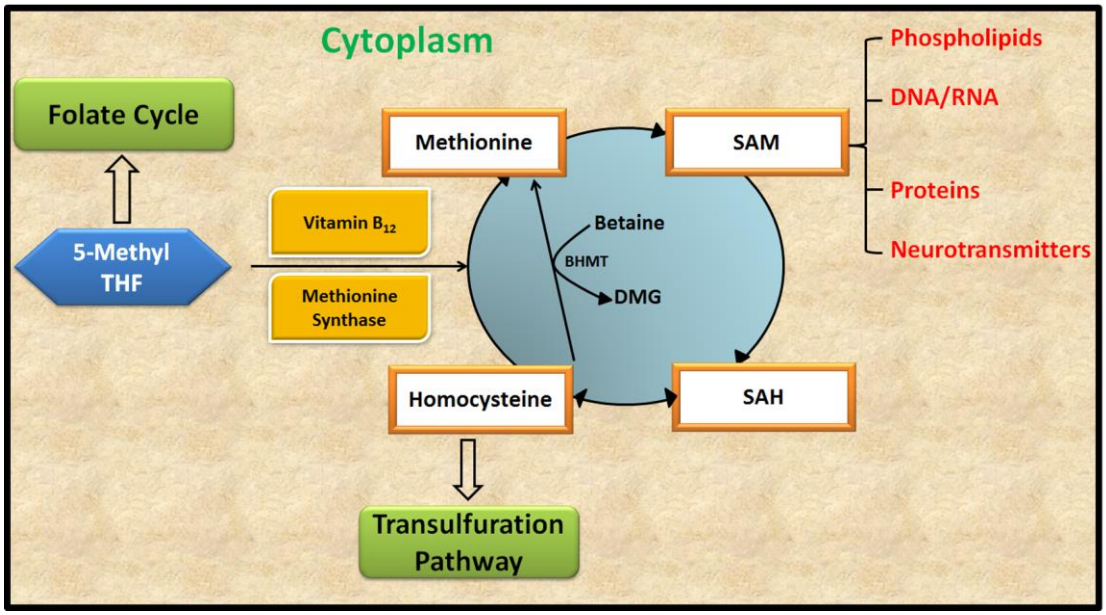
MTHFR: *Methylenetetrahydrofolate Reductase;* ***THF:*** *Tetrahydrofolate;* ***DHF:*** *Dihydrofolate;* ***SHMT:*** *serine hydroxymethyltransferase.*
Source: Modified from Stover 2009, Journal of Nutrition 139(12): 2402-2405.

Re-Methylation/Transmethylation Pathway:

5-MTHF serves as a one carbon donor to the remethylation of homocysteine to form methionine, catalyzed by the enzyme MS and requires vitamin B_{12} as a co-factor (reviewed by Finer et al. 2014). During this reaction, the reduced form of vitamin B_{12} i.e. cob(I)alamin can be oxidized to cob(II)alamin, resulting in the inactivation of the MS-cobalamin complex. The activity of MS is restored by methionine synthase reductase, encoded by the gene 5-methyltetrahydrofolate-homocysteine

methyltransferase reductase (MTRR), which catalyzes the reductive methylation of cob(II)alamin (reviewed by Beaudin and Stover, 2007). Homocysteine is also converted to methionine from betaine in a vitamin B_{12} independent reaction using the enzyme betaine-homocysteine methyltransferase (BHMT) (reviewed by Coşar et al. 2014). This reaction with betaine is confined mainly to the liver. Methionine (an essential amino acid) thus formed is either used for protein synthesis, or along with ATP is adenylated to form the direct methyl donor SAM using the enzyme methionine adenosyl transferase (reviewed by Selhub et al. 1999). SAM serves as the universal methyl donor for numerous cellular methylation reactions, including the methylation of lipids, histones, DNA, RNA and neurotransmitters (reviewed by Stover, 2004). SAM donates methyl groups and converts to S-adenosyl-homocysteine (SAH) which is subsequently hydrolyzed, thus regenerating homocysteine and completing the cycle (reviewed by Kruman and Fowler, 2014). The hydrolysis of SAH to homocysteine is a reversible reaction and therefore elevated cellular concentrations of SAH are likely to precede and accompany all forms of hyperhomocysteinemia (reviewed by Halsted, 2013). Homocysteine is either recycled via the remethylation pathway to methionine or enters the transsulfuration pathway (Fig 19).

Fig 19: Transmethylation Pathway

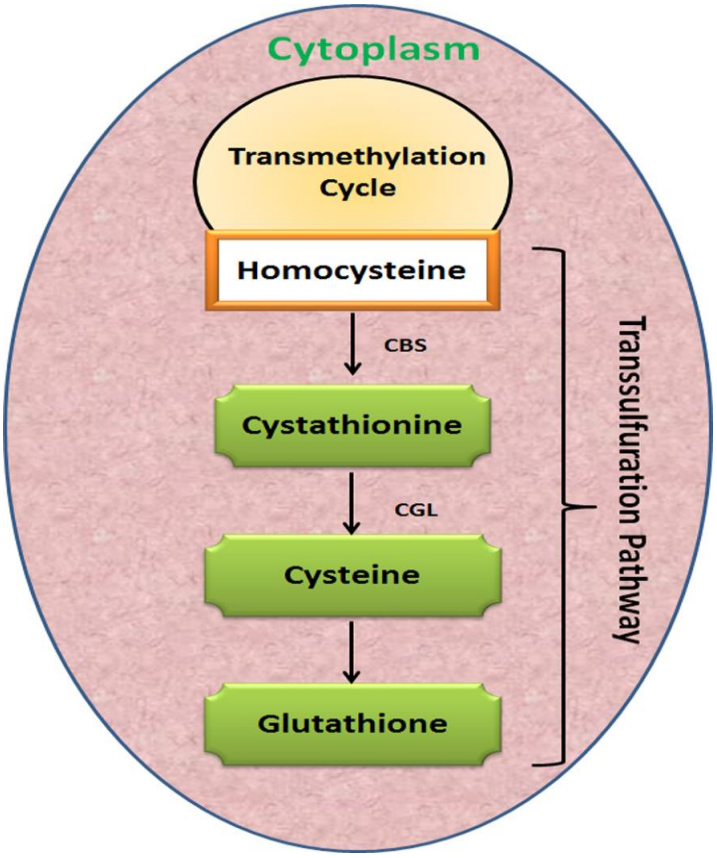


SAH: *S-Adenosyl Homocysteine;* ***SAM:*** *S-Adenosyl Methionine;* ***THF:*** *Tetrahydrofolate;* ***BHMT:*** *betaine-homocysteine methyltransferase.*
Source: *Modified from Coşar et al. 2014, Turkish Journal of Medical Sciences 44(1):1-9.*

Trans-sulfuration Pathway:

Homocysteine along with serine is catabolized via the trans-sulfuration pathway to produce cystathionine. This reaction involves vitamin B_6 (pyridoxine) as a co-factor and is catalyzed by the enzyme CBS (reviewed by Bhatia et al. 2014). After the conversion to cystathionine, homocysteine can no longer enter the transmethylation pathway. Using the enzyme cystathionine-γ-lyase, cystathionine is hydrolyzed into cysteine, ammonia and α-ketobutyrate thereby forming a metabolic bridge between the methionine cycle and cysteine (reviewed by Banerjee et al. 2003). The cysteine is further utilized in the formation of glutathione which acts as an important antioxidant (reviewed by Lushchak, 2012) (Fig 20).

Fig 20: Transsulfuration Pathway



CBS: *Cystathionine β Synthase;* ***CGL:*** *Cystathionine γ Lyase.*
Source: Modified from Coşar et al. 2014, Turkish Journal of Medical Sciences. 44(1):1-9.

Interlinkage between Fatty acids and One Carbon Cycle:

As mentioned earlier, the methyl groups produced by the methionine-homocysteine cycle are donated to carry out methylation of various biomolecules. One of the major methyl group acceptors includes phospholipids (reviewed by Khot et

al. 2015). These phospholipids accept the methyl groups for the synthesis of phosphatidylcholine (PC) from phosphatidylethanolamine (PE) catalyzed by the enzyme phosphatidylethanolamine methyltransferase (PEMT) (reviewed by Assies and Pouwer, 2008). The PC molecule thus synthesized is enriched in LCPUFA such as DHA and ARA, and is critical for the delivery of important PUFAs from the liver to the plasma and distribution to peripheral tissues (DeLong et al. 1999; Watkins et al. 2003; Pynn et al. 2011). Reports have demonstrated that any alteration in the PC/PE ratio modulates the activity of Δ-5 and Δ-6 desaturases involved in omega-3 and omega-6 PUFA synthesis, thereby affecting the fatty acid composition of newly synthesized PC and the amount of PC-DHA produced (reviewed by Assies and Pouwer, 2008). Further, the erythrocyte PC-DHA is known to be correlated with the liver, brain, retina and adipose tissue DHA content (Elizondo et al. 2007; Huang et al. 2007a).

Implications of an Altered One Carbon Cycle:

The importance of one carbon metabolites during pregnancy is well established. Different dietary micronutrients are the major determinants of the one carbon cycle. In every cell of the body, the methionine and folate cycles are ubiquitously present and participate in key metabolic reactions (reviewed by Kalhan and Marczewski, 2012). Therefore, altered one carbon metabolism due to either nutrient deficiency or by nutrient, hormonal and environmental interactions can have profound impact on the cell function, metabolism, growth and proliferation (reviewed by Kalhan, 2013). It is now clear that deficiency of key one carbon metabolites like folate or vitamin B_{12} will affect the methyl transfer by influencing methylation of homocysteine (reviewed by Mason, 2003). Studies have indicated that altered methyl transfers in the mother and the fetus, due to both folate and vitamin B_{12} deficiencies can influence maternal-fetal metabolism and cause long term consequences (reviewed by Cetin et al. 2010).

3.4. Maternal Nutrition and Pregnancy Outcome:

During pregnancy there are changes in the woman's physiology to meet the increased metabolic demands and the requirements of the growing fetus (Pipkin, 2011). Reports indicate that every year, out of 160 million pregnant women all over the world, around 15% of them develop serious preventable complications (reviewed

by Almeida and Cardoso, 2010). Several studies have provided evidence to support the link between the maternal nutritional status and adverse pregnancy outcomes (reviewed by Scholl, 2008; reviewed by Black et al. 2008). Adequate nutrition during this period is critical because the foundations for a healthy life are laid in the womb (reviewed by Elmadfa and Meyer, 2012).

Deficiency of both macro- and micro-nutrients is known to affect the process of fetal development. It has been reported that maternal global restriction of feed intake results in offspring having low birth weight (LBW) and mental disabilities such as anxiety and poor cognitive function (Akitake et al. 2015). In contrast, animal studies indicate minor or no effects on birth weight, while others demonstrate significant decrease in birth weight in dams fed low protein diets (reviewed by Kind et al. 2006). Micronutrients like folic acid, vitamin B_{12} and LCPUFA are also known to affect pregnancy outcome and are discussed below.

Folic acid is required for fetal growth and development. A study carried out in North India reports that 26.3% of pregnant women had lower stores of folic acid (Pathak et al. 2007). Inadequate maternal folate status is known to be associated with several conditions like abruptio placentae, preeclampsia, spontaneous abortion, stillbirth, preterm delivery and LBW (reviewed by Molloy et al. 2008). The role of folic acid in preventing neural tube defects has been widely accepted and therefore folic acid fortification or supplementation is implemented all over the world (reviewed by Safi et al. 2012).

Apart from folate, another vitamin which is critical during pregnancy is vitamin B_{12}. Due to predominant vegetarianism in India, vitamin B_{12} deficiency during pregnancy is a major cause of concern (Refsum et al. 2001). Reports indicate that 74.1% of women in northern India have lower stores of vitamin B_{12} during pregnancy (Pathak et al. 2007). A study carried out in western India reports that vegetarians have a 4-fold higher risk of low vitamin B_{12} concentrations compared with those who frequently eat egg and meat products (Yajnik et al. 2006). Reports indicate a global prevalence of low plasma vitamin B_{12} concentrations during pregnancy (reviewed by Hovdenak and Haram, 2012). It has been suggested that a decline in plasma vitamin B_{12} can also be attributed to alterations in haptocorrin-bound cobalamin, in pregnant women consuming an adequate diet (Greibe et al. 2011).

Maternal vitamin B_{12} is independently reported to be associated with neural tube defects (Groenen et al. 2004; Suarez et al. 2003). It is also associated with adverse pregnancy outcomes like preterm birth (Ronnenberg et al. 2002), intrauterine growth retardation (IUGR) (Muthayya et al. 2006) and recurrent miscarriage (Reznikoff-Etievant et al. 2002). A case control study also reports the P259R polymorphism in transcobalamin influencing the risk of spontaneous abortions (Zetterberg et al. 2002).

There is an increased requirement for fatty acids especially LCPUFA during pregnancy. The fetus depends upon the transfer of LCPUFA from maternal to fetal circulation and therefore adequate maternal LCPUFA levels are critical for the development of fetus (reviewed by Gil-Sanchez et al. 2011). A number of human studies from our department have demonstrated the association of LCPUFA levels at the end of pregnancy with adverse pregnancy outcome (Kilari et al. 2010; Kulkarni et al. 2011a). Further, several studies have shown the association between maternal omega-3 LCPUFA supplementation during pregnancy with length of gestation and birth weight (reviewed by Larque et al. 2012). A study carried out on south Indian subjects also reports the association between the lower intake of fish during the third trimester of pregnancy and higher risk of delivering LBW babies (Muthayya et al. 2009). Reports have demonstrated that other micronutrients like vitamin D, zinc, manganese and iron are also involved in the fetal growth and development. Maternal vitamin D status has been reported to be associated with risk of LBW and small-for-gestational age (SGA) infants (Baker et al. 2009; reviewed by Wood, 2009; Leffelaar et al. 2010; reviewed by Uriu-Adams and Keen, 2010).

3.5. Low Birth Weight:

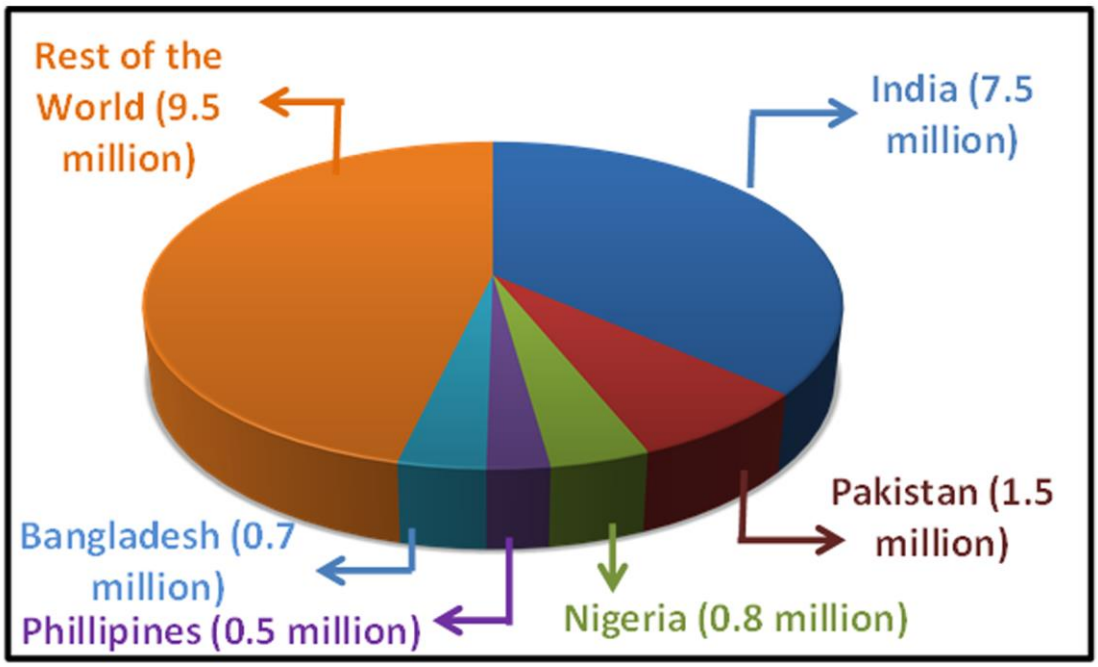
The World Health Organization (WHO) defines LBW as babies weighing <2,500g at term. Birth weight is considered as a potent indicator for both mortality and morbidity of the neonate and LBW is therefore a common feature in most neonatal deaths (Som et al. 2004).

The socio-economic development of any region or country is reflected by the proportion of babies with LBW (Som et al. 2004). Moreover, it has been observed that countries with the highest infant mortality rates are apparently those with the highest rates of LBW (WHO, 1992). This is of significance for India, which has recently been referred to as the world capital for LBW babies (Sukla et al. 2013).

3.5.1. Global Burden:

Globally, 20 million LBW infants are born every year, which is about 15-20% of all births (WHO, 2011). Reports indicate that in developing countries, the prevalence of LBW infants is 16.5%, as compared to 7% in the developed countries. Within the developing countries, around 70% of all LBW births occur in Asia with the greatest incidence in South-Central Asia (27%) followed by 13% to 15% in Africa (reviewed by Imdad and Bhutta, 2013). According to UNICEF Global Nutrition Database 2012, five countries including India, account for more than half of the global LBW burden (UNICEF, 2012) (Fig 21). In India, the highest prevalence of LBW was recorded in the north zone while lowest was recorded in the north-east zone (Bharati et al. 2011).

Fig 21: Global Burden of Low Birth Weight Babies



Source: *Global Nutrition Database 2012, based on MICS, DHS and other national surveys, 2007-2011, except for India.*

3.5.2. Risk Factors:

LBW is considered as having multifactorial causes involving genetic, placental, fetal and maternal factors (reviewed by Sharma and Mishra, 2013). The various maternal factors include maternal mental health, low prepregnancy weight or body mass index (BMI); inadequate energy intake and gestational weight gain; and specific complications of pregnancy, such as genital tract infections, pregnancy-

induced hypertension and incompetent cervix (reviewed by Valero et al. 2004; Kiess et al. 2009). Fetuses having certain genetic or chromosomal disorders are also considered at a greater risk for IUGR (reviewed by Valero et al. 2004). Further, it has been reported that malaria during pregnancy is associated with LBW (reviewed by Imdad and Bhutta, 2013).

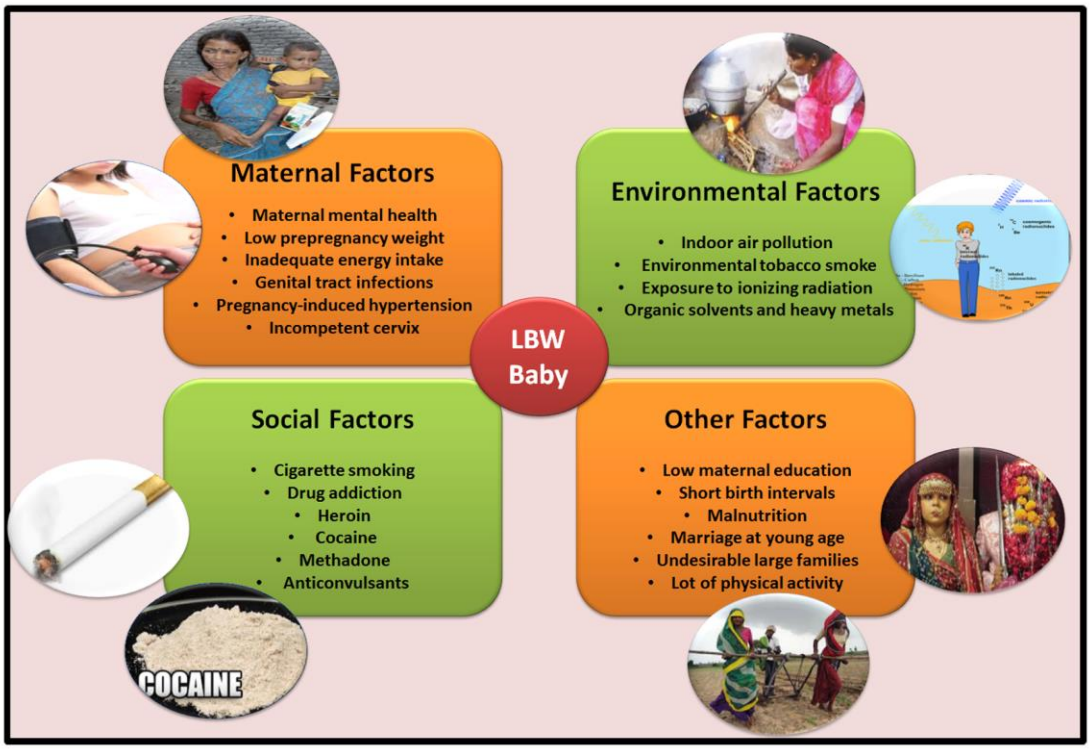
Women living in a developing country become pregnant in a context of gender inequality, inadequate educational opportunities, malnutrition, marriage and conception at a young age, short birth intervals and undesirably large families. They are also reported to suffer from chronic and communicable diseases, which further aggravate their already poor nutritional status (reviewed by Imdad and Bhutta, 2013). In addition to this, increased physical activity performed by women, such as farming or gathering water, is also reported to be associated with lower birth weights and smaller head and mid-arm circumferences in infants (reviewed by Shaw, 2003).

Drug abuse and cigarette smoking during pregnancy fall under the category of social factors. It has been reported that smoking in the last trimester of pregnancy reduces birth weight and the effect is proportional to the number of cigarettes smoked. Growth restriction is also reported in mothers addicted to heroin, cocaine, methadone, or other drugs like anticonvulsants (dilantin, phenobarbital and tegretol), antifolates (methotrexate), coumadin and prednisone (Uauy et al. 2013). Different environmental factors like indoor air pollution, environmental tobacco smoke, exposure to ionizing radiation (X-rays), organic solvents and heavy metals, especially mercury and cadmium are also considered as risk factors associated with malformation and compromised fetal growth. In addition to these, reports have indicated that human population living at higher altitude have diminished fetal growth due to lower ambient oxygen tension, less access to quality foods and less likely to have adequate sanitation (Kiess et al. 2009). Fig 22 shows the different risk factors involved in delivering low birth weight babies.

3.5.3. Clinical Consequences:

As described in section 1.2, LBW babies are at higher risk of early growth retardation, infectious disease, neurological dysfunction, developmental delay and death during infancy and childhood (WHO, 2011). They are also at increased risk for adult-onset hypertension, glucose intolerance, cardiovascular diseases and obesity (reviewed by Bhatia and Gates, 2013).

Fig 22: Risk Factors for Low Birth Weight Babies



Maternal nutrition plays an important role not only in determining the pregnancy outcome but also adversely affects the long term health of the offspring. The backbone of this concept was placed way back in 1986 by Late Prof. David Barker, Southhampton University, UK and was known as the "Fetal Origins Hypothesis". This concept was later termed as "Developmental Origins of Health and Disease (DOHaD)".

3.6. Developmental Origins of Health and Disease (DOHaD):

3.6.1. Epidemiological Studies:

Retrospective studies by Barker and Osmond resulted in the following observations: 1) low birth weight babies born in the year 1921-1925 predicted death rates from stroke and heart disease in 1968-1978 (Barker and Osmand, 1986) and 2) low birth weight and weight at one year were associated with an increased risk of death from cardiovascular disease in the county of Hertfordshire (Barker et al. 1989). Based on these studies, Prof. David Barker put forward the hypothesis, that adverse events *in-utero* induce compensatory responses in the fetus that results in an altered phenotype with adverse consequences in later life (reviewed by Lebenthal and Bier, 2007).

This hypothesis was a breakthrough in the field of science and was confirmed later by a number of studies in various parts of the world. These studies showed the association of poor intrauterine growth with hypertension (reviewed by Huxley et al. 2000), coronary heart disease (Stein et al. 1996), non-insulin dependent diabetes (reviewed by Newsome et al. 2003), stroke (Rich-Edwards et al. 2005), dislipidaemia (Roseboom et al. 2000) and elevated clotting factors (Henry et al. 1997).

The role of maternal nutrition and the risk for disease in later life was also highlighted by studies based on the Dutch Famine ('Hunger Winter') of 1944–1945 (Ravelli et al. 1976). Women living in the famine affected areas of the Netherlands had suboptimal nutrition during pregnancy. In a follow up study, it was observed that individuals exposed to famine in mid or late gestation had decreased glucose tolerance, in addition to other adverse effects like decreased birth weight, shorter body length and high head to birth weight ratio. Young men whose mothers were undernourished during early pregnancy had an increased risk of obesity and cardiovascular disease compared to men whose mothers lived in non-famine areas.

Subsequently, several studies across the world showed that poor intrauterine growth and nutrition are associated with reduced stature, lower physical work capacity and impaired cognitive function in adult life (reviewed by Victora et al. 2008). These studies led to a new branch of scientific research: the developmental origins of health and disease. Thus, nutrition during the perinatal period acts as a major environmental factor influencing the development of the fetus, causing adaptive and permanent changes in structure, physiology and metabolism of the fetus (reviewed by Fall, 2009).

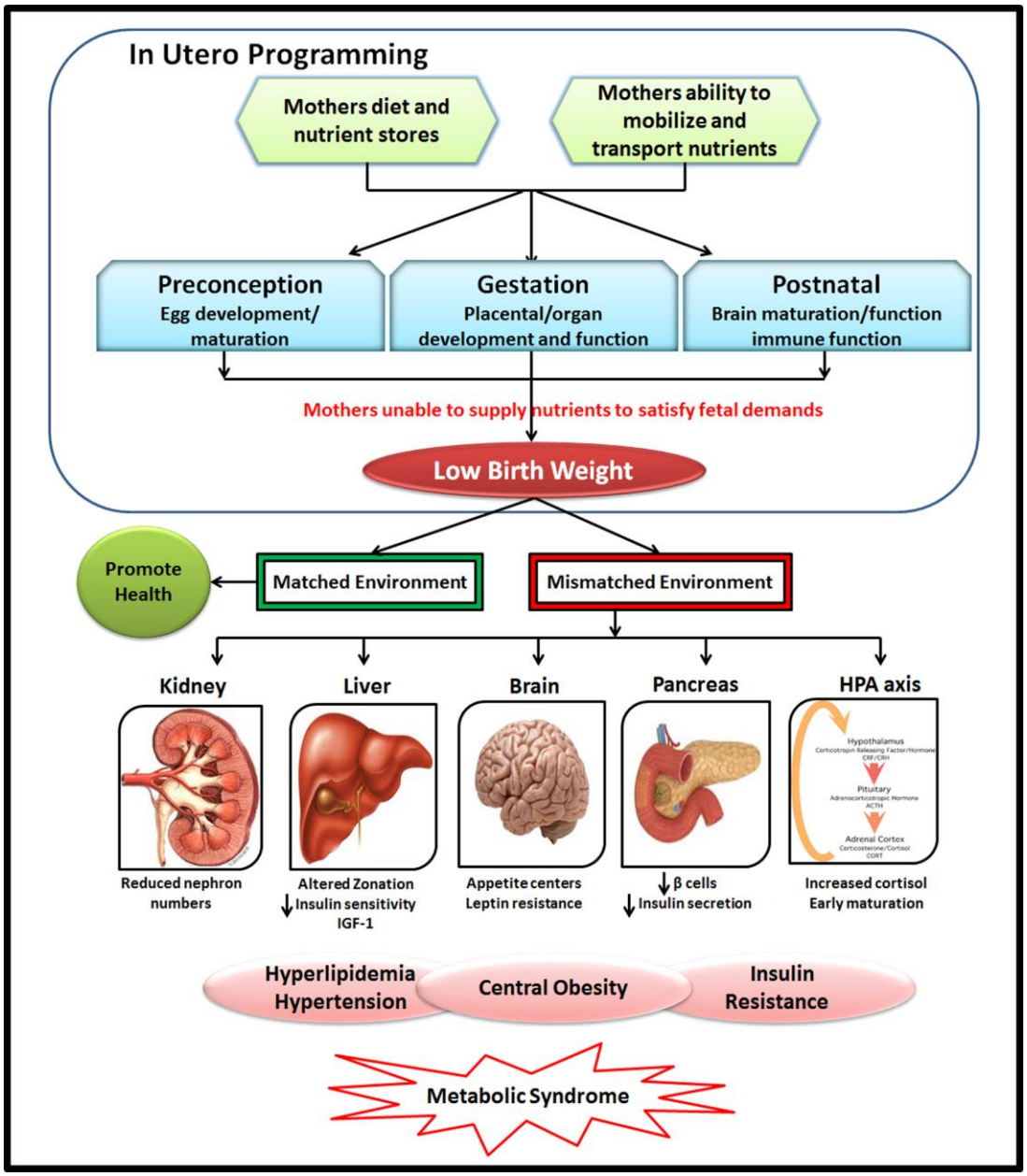
3.6.2. Thrifty Phenotype Hypothesis:

The 'thrifty phenotype' hypothesis proposed by Hales and Barker suggests that during the process of development, the fetus adjusts its developmental programme in response to environmental cues such that it will give an optimal chance of survival in the postnatal period (reviewed by Gluckman and Hanson, 2004). Thus, if the prediction made by the fetus is correct, then the fetus will be at low disease risk since the metabolism of the organism will be matched to the environment. However, if the organism's metabolism is mismatched to its environment, the individual would have an increased risk of metabolic disease (reviewed by Lillycrop and Burdge,

2012). The effects of the suboptimal *in-utero* nutritional environment on early growth and subsequent development of metabolic syndrome is shown in Fig 23.

Subsequently, the next step in the field of developmental programming explored the mechanisms by which the intrauterine environment affects fetal development. Several studies were carried out in animal models which further supported the concept of developmental programming.

Fig 23: The Thrifty Phenotype Hypothesis Illustrating the Suboptimal *In-Utero* Nutritional Environment on Early Growth and Subsequent Development of Metabolic Syndrome



***HPA**, Hypothalamus pituatory axis*
***Source:** Modified from reviewed by Fall, 2013, Nestlé Nutrition Workshop Series. 74:11-25; reviewed by Boersma et al. 2014, Journal of Neuroendocrinology. 26(9): 587-602.*

3.6.3. Animal Studies:

Different studies in animals have reported the effect of various nutritional exposures during pregnancy like global nutrient restriction, protein restriction, diets deficient in specific micronutrients and diets high in saturated fat or carbohydrate on the long term health of the offspring. The offspring of these deficient animals were seen to exhibit phenotypic outcomes consistent with one or more components of the metabolic syndrome, characterized by hypertension, obesity, diabetes insulin resistance, reduced leptin sensitivity, hepatic steatosis, elevated blood pressure and dyslipidaemia (Bieswal et al. 2006; Zambrano et al. 2006; Krechowec et al. 2006; Erhuma et al. 2007; Desai et al. 2007).

Several possible mechanisms have been proposed for the long-term programming of health and disease through nutrition. One of the mechanisms explains that inadequate substrate availability at critical periods of development is responsible for the inadequate growth and remodeling of tissues (reviewed by Reynolds and Caton, 2012). Reports indicate that the effects of 'programming' are mediated through epigenetic modifications. Maternal nutritional insult affects the gene expression by chemically modifying the DNA or chromatin, thereby activating or repressing the gene and involves either DNA methylation or histone acetylation (reviewed by Koletzko et al. 2012).

One of the key organ which is the target for the epigenetic modifications is the placenta. The role of placenta in programming of diseases is discussed below.

3.7. Placenta in Developmental Programming:

The placenta is considered as the first fetal organ to develop and is involved in several critical functions required for the fetal development (reviewed by Cross, 2006). It is responsible for the exchange of nutrients, vitamins, waste and growth factors between maternal and fetal circulations; and is therefore a major determinant of fetal growth (reviewed by Huppertz et al. 2014).

During placental development there is an increase in placental surface area for nutrient transfer, thinning of the trophoblast barrier, increase in fetal capillary surface area, increase in placental nutrient transporter abundance and modulation of placental blood flow (reviewed by Sandovici et al. 2012). These changes result from branching angiogenesis, non-branching angiogenesis, trophoblast differentiation and syncytium formation (reviewed by Myatt, 2006). The processes of angiogenesis and

vasculogenesis are known to be regulated by various growth factors like vascular endothelial growth factor (VEGF), placental growth factor (PlGF), transforming growth factor ß (TGF ß) family and angiopoietins (Ang) (reviewed by Basak et al. 2013). Thus, the development and establishment of the placenta and its circulatory system play a pivotal role in the successful maintenance of maternal health and also facilitates the development of the embryo and fetus (reviewed by Mistry and Williams, 2011). In addition, the placenta has several imprinted and non-imprinted genes, largely expressed in trophoblasts which regulate placental growth (reviewed by Maccani and Marsit, 2009). Altered expression of placental imprinted gene has been reported in the IUGR placenta (McMinn et al. 2006).

Several other factors like growth factors, mechanical stress during pregnancy, oxidative stress and nutrition affect the growth and development of the placenta (reviewed by Burton et al. 2009; reviewed by Belkacemi et al. 2010). The following section describes the role of maternal nutrition in placental development.

3.8. Maternal Nutrition and Placental Development:

Maternal nutrition plays a critical role in placental and fetal growth (reviewed by Igwebuike, 2010). Fetal growth is influenced by the nutrient transfer from the mother to the fetus through the placenta (reviewed by Lager and Powell, 2012), wherein alteration in placental development leads to disorders of pregnancy such as FGR and preeclampsia (reviewed by Kovo et al. 2013). Suboptimal maternal nutrition during pregnancy results in newborns suffering from IUGR, which is associated with increased perinatal morbidity, mortality and risk of developing diseases in later life (reviewed by Benz and Amann, 2010). It has been reviewed that maternal undernutrition alters placental weight, vascular development, diminishes angiogenic growth factor expression and reduces placental glucose, amino acid and lipid transport (reviewed by Belkacemi et al. 2010). Both maternal global food restriction and the deficiency of individual macro- or micro-nutrient can influence placental development.

3.8.1. Macronutrients and Placental Development:

Maternal protein restriction is known to alter placental vascular function by decreasing the expression of vascular endothelial adhesion molecules in the murine placenta (Rutland et al. 2007). Maternal undernutrition, in the form of protein or

calorie restriction, reduces placental weight, alters the development of trophoblast cells and reduces the length of placental labyrinthine vessels (Rutland et al. 2007; Coan et al. 2010). Nutrient restriction is also reported to affect placental vascularity in animals by decreasing cotyledonary capillary number and density (Vonnahme et al. 2007).

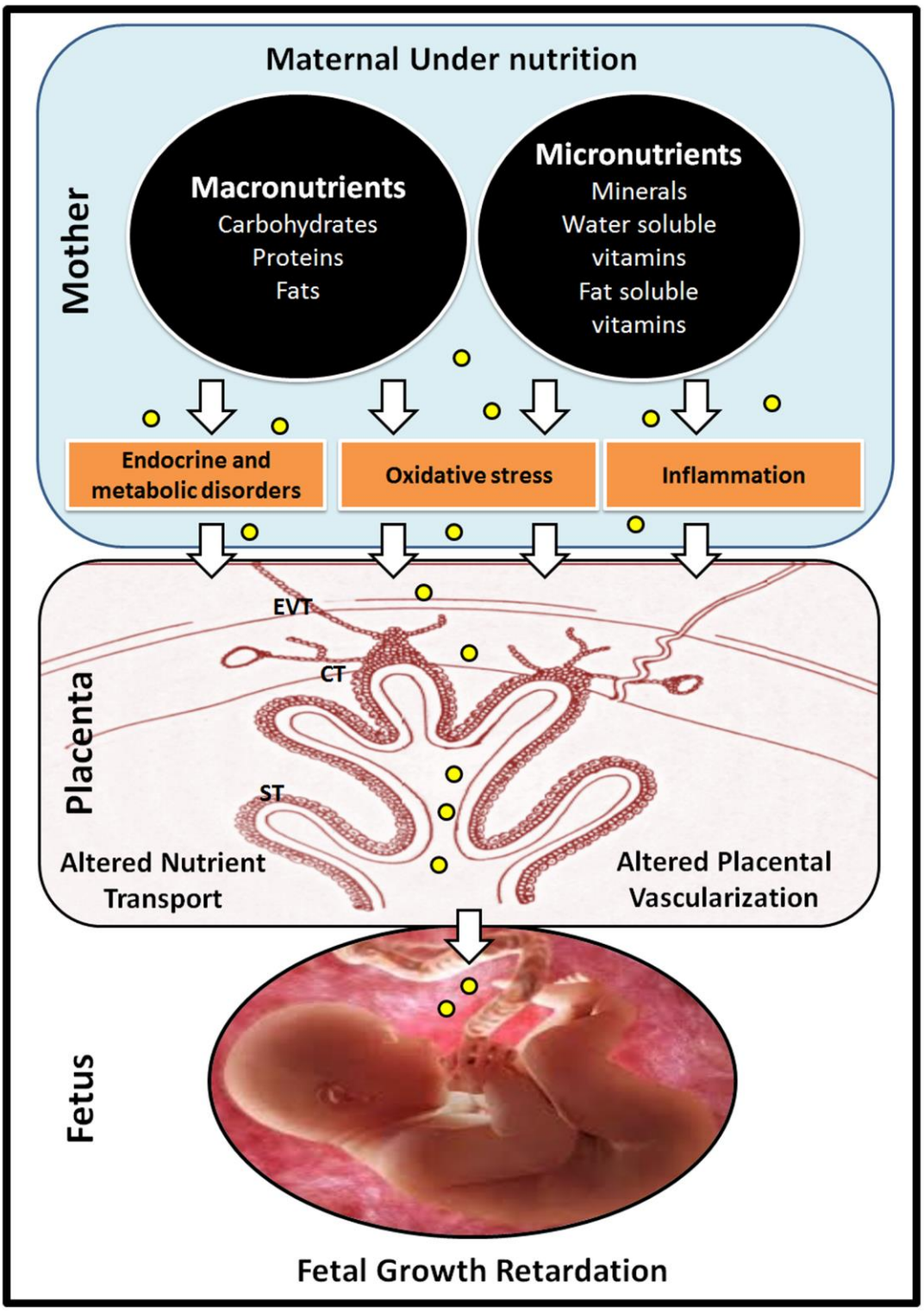
3.8.2. Micronutrients and Placental Development:

Reports indicate that micronutrients like vitamin D and folate are associated with placental development. Maternal vitamin D deficiency is associated with low PlGF levels in the placenta and increased risk of preeclampsia (Wei et al. 2013). Maternal iron restriction in animals before and during pregnancy is reported to alter placental vascularization, possibly contributing to fetal growth retardation (Lewis et al. 2001).

Folate is required for a number of crucial processes during placental development, like extravillous trophoblast invasion, angiogenesis and secretion of proteases (Williams et al. 2011). During pregnancy, exposure to folic acid antagonists can result in severe preeclampsia, placental abruption and IUGR (Wen et al. 2008). On the other hand, folic acid plus DHA supplementation is reported to stimulate proliferation of placental trophoblasts (Klingler et al. 2006). Similarly, maternal folate levels and first-trimester fetal size are reported to be positively associated with abundance of angiogenic growth factors during early pregnancy (Bouwland-Both et al. 2013). Further, choline insufficiency is known to alter the angiogenic expression profile, impair *in-vitro* angiogenesis and increase inflammation in cultured trophoblast cells (Jiang et al. 2014). The role of macro- as well as micro-nutrients on the placental development has been summarized in Fig 24.

In addition to the above nutrients, fatty acids are also considered as important regulators of placental development. Numerous reports indicate the association of altered LCPUFA levels with placental development and function (Kulkarni et al. 2011b; Dhobale et al. 2011; Wadhwani et al. 2014).

Fig 24: Effects of Maternal Undernutrition on Placental Development, resulting in Altered Placental Vascularization and Nutrient Transport from Placenta to Fetus, Ultimately Leading to Fetal Growth Retardation



EVT: *extravillous trophoblast;* ***CT:*** *Cytotrophoblasts;* ***ST:*** *syncytiotrophoblast*

3.8.3. Fatty acid and Placental Development:

LCPUFA act as building blocks for the growing placenta, induce placental angiogenesis, combat placental oxidative stress and regulate placental inflammation. They are required for the placental development right from early pregnancy. It has been reported that the endometrial gland delivers a secretion containing lipid droplets to the placenta that regulates trophoblast cell proliferation and differentiation from implantation till the onset of maternal blood flow in the placenta (Hempstock et al. 2004, reviewed by Burton et al. 2007).

A recent *in-vitro* study demonstrates that fatty acids like DHA, EPA, ARA and oleic acid (Ole) stimulate the expression of major angiogenic factors like VEGF and angiopoietin-like 4 (ANGPTL4) in the first trimester placental trophoblast cells. This study indicates that DHA is the most potent stimulator of angiogenesis (Basak and Duttaroy, 2013a). Further, DHA is shown to stimulate tube formation in the first trimester placental trophoblast cells by stimulating VEGF expression (Johnsen et al. 2011). Conjugated linolenic acid is also reported to increase the expression of ANGPTL4 and other pro-angiogenic factors such as fatty acid binding protein-4 (FABP-4) and cyclooxygenase-2 (COX-2) during early placentation (Basak and Duttaroy, 2013b). Further, it has been reported that there is involvement of prostaglandins synthesized from ARA during the process of angiogenesis and is associated with enhanced tubular network formation (Finetti et al. 2008).

Despite the importance of maternal nutrition in placental development, the underlying mechanisms still remain unclear. It is likely that maternal nutrition influences placental development by regulating the activity of various transcription factors. The following section describes the role of LCPUFA in regulating the transcription factors involved in placental development as well as in fatty acid metabolism.

3.9. Nuclear Transcription Factors:

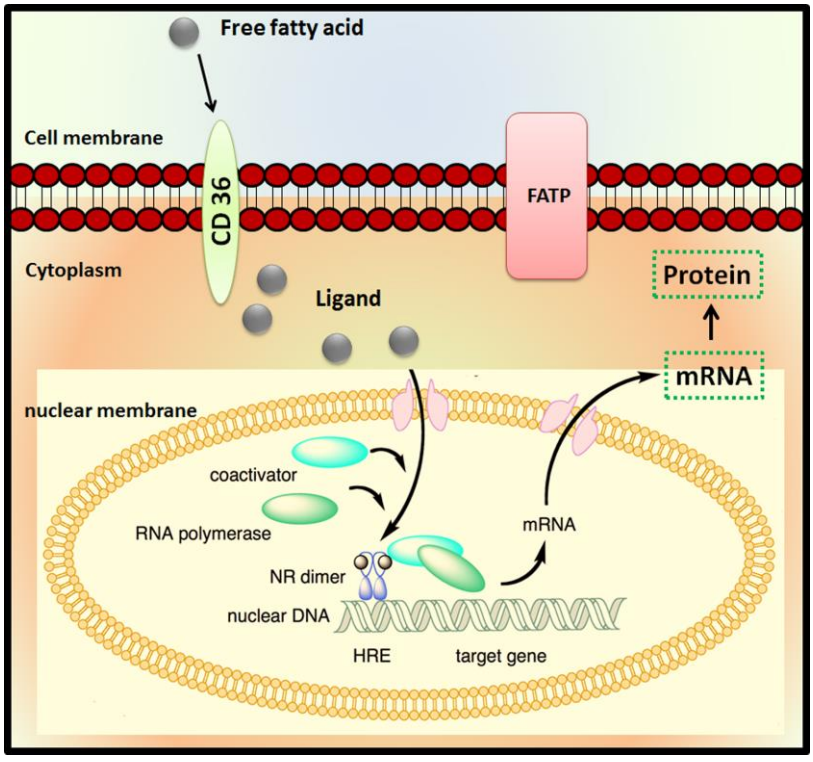
Nuclear transcription factors also known as nuclear receptors are proteins involved in the regulation of gene transcription from DNA to mRNA by binding specific DNA sequences (reviewed by Latchman et al. 1997). These nuclear receptors include nuclear hormone receptors, for which hormonal ligands have been identified; and orphan nuclear receptors for which ligands are unknown (reviewed by Olefsky, 2001). On the basis of function, these nuclear receptors are divided into two groups;

endocrine receptors and metabolic receptors. The endocrine receptors include androgen, mineralocorticoid, glucocorticoid and progesterone receptors. The metabolic receptors include peroxisome proliferator activated receptors (PPAR), retinoid X receptors (RXR), liver X receptors (LXR) and hepatic nuclear factor-4 alpha (HNF-4α) (Hughes, 2011).

Nuclear receptors have a characteristic structure consisting of central DNA binding domain (DBD), the ligand binding domain (LBD), variable N-terminal and C-terminal domains and variable length hinge region between the DBD and LBD. These nuclear receptors exist either as a homodimers (class 1 receptors) or heterodimers (class 2 receptors) working in a ligand dependent or independent manner (reviewed by Germain et al. 2006).

The ligands for these nuclear receptors are small hydrophobic molecules that include fatty acids, cholesterol derivatives, retinoids, thyroid hormone, prostaglandins, leukotrienes and xenobiotics (reviewed by Aranda and Pascual, 2001). Out of the different ligands, fatty acids are widely studied in regulating the activity of these transcription factors. The mechanism of activation of transcription factors by fatty acids is shown in Fig 25.

Fig 25: Mechanism of Activation of Transcription Factors by Fatty Acids



CD: *Cluster of Differentiation;* ***NR:*** *Nuclear receptor*
Source: *http://www.mn.uio.no/farmasi/english/research/groups/mures/research/background/*

Transcription factors regulate various processes in the body like cell proliferation, cell differentiation, organogenesis, metabolism and cell death. The role of four major transcription factors (PPAR, RXR, LXR, SREBP) which are interlinked with each other in regulating the fatty acid metabolism are discussed below.

3.9.1. Fatty Acids and Transcription Factors:

Peroxisome Proliferator Activated Receptors:

PPAR are nuclear transcription factors that regulate anti-inflammatory, metabolic and tissue developmental processes. In mammals, three PPAR isotypes; PPARα, PPARβ/δ and PPARγ have been identified. PPAR ligands include several naturally occurring LCPUFA, such as ARA, linoleic acid (LA), DHA and EPA. DHA is considered as the more preferred ligand since it fits into the long and large hydrophobic ligand binding pocket of PPAR (reviewed by Itoh and Yamamoto, 2008). After binding to its ligand, PPAR heterodimerizes with another nuclear transcription factor RXR. This heterodimer further binds to a specific DNA element, peroxisome proliferator response element (PPRE) of the target genes, thus allowing the transcription of target genes (reviewed by Pawlak et al. 2012).

Retinoid X Receptor:

RXR is a member of the steroid/thyroid hormone superfamily of nuclear receptors that functions as a homodimer or heterodimer with other transcription factors like PPAR, LXR and farnesoid X receptor (reviewed by Yamada and Kakuta, 2014). There are three isoforms of RXR; RXRα, RXRβ and RXRγ, encoded by the RXRα, RXRβ, RXRγ genes, respectively. These transcription factors are mostly involved in processes like cell differentiation, metabolism and apoptosis. 9-cis-retinoic acid is considered as a natural ligand for the activation of RXR. In addition to this, poly unsaturated fatty acids (PUFA) like DHA and a saturated metabolite of chlorophyll, phytanic acid significantly activate RXR molecules (reviewed by Dawson and Xia, 2012).

Liver X Receptor:

LXR are well known as the important regulators of cholesterol, fatty acid and glucose metabolism. The LXR subfamily consists of two isoforms, LXRα (NR1H3) and LXRβ (NR1H2). Ligand activated LXR, forms an obligate heterodimer with RXR

and further this LXR/RXR complex binds to LXR response elements (LXRE) in the promoter regions of the target genes. This LXRE consists of hexanucleotide sequence motifs separated by 4 bases called as direct repeat four (DR-4) (reviewed by Baranowski, 2008). PUFAs like ARA are reported to antagonize the binding of oxysterols (ligand for LXR), thereby inhibiting the action of LXR in cell lines (Ou et al. 2001).

Sterol Regulatory Binding Proteins (SREBP):

These are the transcription factors involved in cholesterol and lipid synthesis. Mammalian cells produce three isoforms of SREBPs: SREBP-1a and -1c are transcribed from the same gene locus and a separate gene encodes SREBP 2. SREBP-1c is known to enhance the transcription of genes involved in fatty acid, triglyceride and phospholipid synthesis while SREBP-1a and SREBP-2 activate genes involved in cholesterol synthesis (reviewed by Pegorier et al. 2004). PUFA are reported to selectively inhibit the transcription of SREBP-1 and not of SREBP-2, since the transcription of SREBP-1 is activated by LXRs, which are antagonized by PUFA (Ou et al. 2001).

3.9.2. Transcription Factors during Pregnancy:

The success of human pregnancy is dependent upon multiple regulatory mechanisms (e.g., immunologic, endocrine and metabolic) and disturbances in any of these processes can lead to fetal loss. Transcription factors are known to regulate several processes during pregnancy, since they are expressed in many of the reproductive tissues. One of the widely studied transcription factor during pregnancy is PPAR.

All the three isoforms of PPAR are reported to be expressed in the following tissues: ovary, uterus, mammary gland, pituitary gland and placenta (reviewed by Froment et al. 2006; Wang et al. 2002). It has been reported that PPARγ and PPARβ/δ are expressed in the preimplantation bovine and mouse embryos where PPARβ/δ is associated with embryo development (reviewed by Huang, 2008). During the first trimester of pregnancy, human placentation is known to undergo a high degree of trophoblastic invasion, during which PPARγ is expressed in the syncytiotrophoblast, and eventually in the villous and extravillous trophoblast (reviewed by Fournier et al. 2007; reviewed by Wieser et al. 2008). A series of animal and cell culture studies

have shown that inactivation of PPARγ leads to an irreversible arrest of trophoblast differentiation, early embryonic lethality and severe developmental placental damage thereby affecting the placental labyrinth zone (Kubota et al. 1999; Barak et al. 1999, Parast et al. 2009).

The heterodimeric partner of PPAR i.e. RXR is ubiquitously expressed early in development (reviewed by Maden, 2000). RXRα is known to play an important role in the morphogenesis of the embryo (reviewed by Mark et al. 2009). In addition, LXRα and LXRβ are reported to be expressed in the trophoblast cells of the placenta right from early gestation (Marceau et al. 2005). A recent report also demonstrates that LXR expression is downregulated in the syncytiotrophoblast of spontaneous abortion placentas (Knabl et al. 2013). LXR is suggested to be involved in the regulation of cholesterol transport from the maternal to the fetal circulation (reviewed by Bełtowski and Semczuk, 2010). Reports in animals have also indicated that any alteration in the expression of SREBP in both fetuses and dams during pregnancy, may lead to defects in lipid metabolism in the adult offspring (Mukai et al. 2013). Further, assisted reproductive technologies have also reported impaired the expression of SREBP in the placenta as well as in the fetus (Lou et al. 2014).

Thus, it is clear that the activity of transcription factors is essential for the normal progression of pregnancy. In general, the placenta and baby grow proportionately; and conditions that compromise the placental development may adversely affect fetal growth and birth weight (Longtine and Nelson, 2011).

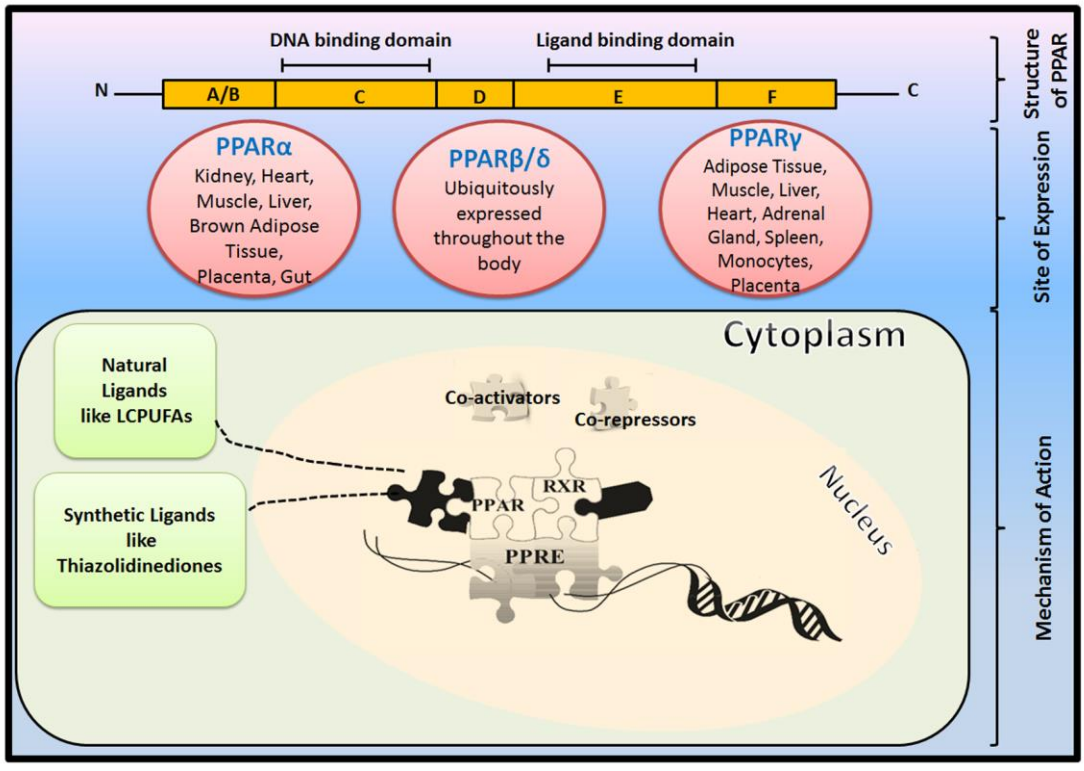
3.10. Transcription Factors in Placental Development:

The placenta requires several growth factors and signaling molecules to initiate complex biological processes of implantation and maternal-fetal exchange (reviewed by Gude et al. 2004). Different transcription factors located within the cell nucleus, are known to determine the placental cellular response (reviewed by Murthi et al. 2013). These transcription factors regulate the expression of their target genes within the cell and therefore play essential roles in cellular development and differentiation of a variety of cell types in the placenta. One of the widely studied transcription factor with respect to placental development is peroxisome proliferator activated receptors (PPARs).

3.10.1 Peroxisome Proliferator Activated Receptors:

PPARs belong to the steroid receptor superfamily of nuclear receptors that regulate metabolic, anti-inflammatory and developmental processes in a ligand-dependent manner (reviewed by Bensinger and Tontonoz, 2008). It was once classified as "sensors" or an "orphan receptor" rather than classic hormone receptors (reviewed by Evans and Mangelsdorf, 2014). In mammals, three PPAR isotypes, PPARα (NR1C1), PPARδ (PPARβ or NR1C3) and PPARγ (NUC1 or NR1C2) have been identified. These are encoded by a separate single-copy genes, each with distinct tissue distribution patterns and groups of target genes. PPARγ is further divided into four mRNA isotypes which encode the same protein: PPARγ1 and PPARγ2 are expressed predominantly in the adipose tissue; PPARγ3 is expressed in the colon, adipose tissue and monocytic leukemia, whereas nothing is known about PPARγ4 expression (reviewed by Toth et al. 2007). PPARα is expressed in high levels in the kidney, heart, muscle, liver, brown adipose tissue, placenta and gut, whereas PPARδ is ubiquitously expressed throughout the body (reviewed by Qi et al. 2000) (Fig 26).

Fig 26: Structure and Mechanism of PPAR



PPAR *– peroxisome proliferator activated receptors,* ***RXR*** *– retinoid X receptors,* ***PPRE*** *– peroxisome proliferator response element,* ***LCPUFA*** *– long chain poly unsaturated fatty acids*
Source: *modified from Bordoni et al, 2006, Genes and Nutrition. 1(2):95-106*

***Ligands of PPAR**:*

PPAR ligands include several naturally occurring LCPUFAs, such as ARA, LA, DHA and EPA. In addition to these, oxidized low-density lipoprotein compounds 9S-hydroxy-10E, 12S-octadecadienoic acid (9-HODE), 13S-hydroxy-9Z, 11E-octadecadienoic acid (13-HODE), 15-deoxy-delta prostaglandin J2 (15d-PGJ2) are also potent PPAR agonists (reviewed by Jawerbaum and Capobianco, 2011).

Structure of PPARs:

PPARs, like other members of the nuclear receptor superfamily, display amino-terminal modulatory domain, a DNA binding domain and a carboxyl-terminal ligand binding domain. The N-terminal portion of the receptor contains a variable A/B region, which contains a ligand independent transactivation function (AF-1). DNA-binding domain contains a highly conserved C region responsible for sequence specific DNA recognition and dimerization. The C-terminal half of PPAR is subdivided into D, E and F regions. The short D region facilitates the protein to bend or alter conformation by acting as a variable hinge region connecting the C and E domains. The E region is the LBD, also called as ligand dependent transactivation domain (AF-2 domain). The C-terminal F region may be involved in interacting with nuclear receptor coactivators (reviewed by Yu and Reddy, 2007) (Fig 26).

***Mechanism of Action**:*

In order to activate the transcription of the downstream genes, PPARs heterodimerize with another nuclear transcription factor RXR and bind to a specific DNA element, termed the PPRE. PPAR/RXR heterodimers are known to be activated by either selective RXR or PPAR ligands. Ligand-activated PPARs undergo a conformational change in protein structure thereby releasing the co-repressor proteins and recruiting the co-activators, thus allowing the transcription of target genes (reviewed by Pawlak et al. 2012) (Fig 26). In contrast, in the absence of the ligand, PPARγ forms a complex with co-repressor molecules such as nuclear receptor co-repressor (NCoR). This PPARγ/NCoR complex further prevents the transcription and expression of the target genes (reviewed by Li et al. 2010). On addition of PPARγ ligand, the formation of PPARγ/NCoR complex is blocked thereby activating the PPARγ transcriptional activity (Yu et al. 2005).

3.10.2. PPAR in Placental Development:

All PPAR isotypes are expressed in the placenta, with PPARγ and PPARβ playing key roles during implantation and placentation (reviewed by Fournier et al. 2007). A recent review suggested that PPARγ- and PPARβ-knockout mice are embryonic-lethal due to gross placental abnormalities and defects in placental morphogenesis, respectively, whereas PPARα-knockout mice are associated with increased rates of maternal abortion, although surviving pups develop normally (reviewed by Kadam et al. 2014). PPARγ and PPARβ proteins are present in preimplantation bovine and mouse embryos, with PPARβ functioning during embryo development (Huang et al. 2007b; reviewed by Huang, 2008). PPARβ is also known to regulate the production of COX2-derived prostacyclin I2, which enhances mouse blastocyst invasion (Huang et al. 2007b).

Human trophoblasts differentiate into either villous or extravillous cytotrophoblasts (VCTs or EVCTs, respectively). Aberrant trophoblast differentiation and/or VCT fusion are associated with placental pathology (Holdsworth-Carson et al. 2010; Ruebner et al. 2012). PPARγ is expressed in VCTs and EVCTs (Tarrade et al. 2001; Wang et al. 2002), and is required for the fusion of mono-nucleated VCTs into multi-nucleated syncytiotrophoblast (Ruebner et al. 2012). PPARγ activity affects trophoblast differentiation by regulating the functions of both VCTs and EVCTs in a differential manner (reviewed by Kadam et al. 2014). Indeed, PPARγ is involved in the differential modulation of human chorionic gonadotropin in two stages of trophoblast maturation; specifically, the genes encoding chorionic gonadotropin are believed to be PPARγ targets that help mediate the role of PPARγ in human cytotrophoblast differentiation and invasion (Handschuh et al. 2007, 2009). PPARγ also regulates trophoblast proliferation, invasion and labyrinthine differentiation by activating glial cells missing-1 (GCM1) (Parast et al. 2009); and controls spongiotrophoblast layer thickness, which influences maternal-fetal transport (Schaiff et al. 2007).

PPARs play an additionally important role in placental angiogenesis. Activation of PPARα and PPARγ can directly increase VEGF abundance, leading to endothelial tube formation in an endothelial-and-interstitial-cell co-culture assay (Biscetti et al. 2008). Pregnant rats treated with a PPARγ antagonist, however, showed reduced plasma VEGF and developed features of preeclampsia-like

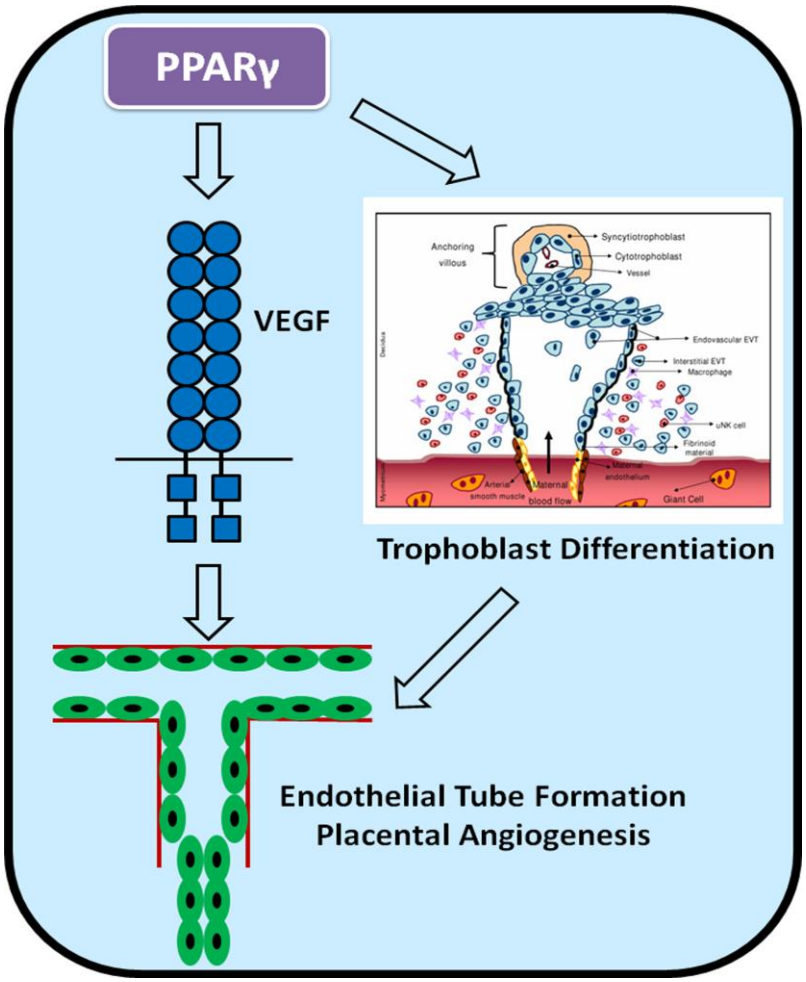
endothelial dysfunction, suggesting that PPARγ may play a vital role in the progression of a healthy pregnancy and may modulate the risk of preeclampsia (McCarthy et al. 2011). Animals with 11 beta-hydroxysteroid dehydrogenase 2 (Hsd11β2) gene-deleted placentas exhibited reduced fetal capillary development within the labyrinth zone accompanied with a decline in VEGF and PPARγ mRNA levels, which could lead to altered fetal development (Wyrwoll et al. 2009).

The placentas of PPARγ- null mutants possess altered anti-angiogenic prolactin 7d1/proliferin-related protein (Prl7d1) and pro-angiogenic prolactin 2c2/proliferin (Prl2c2) expression, suggesting that PPAR directly regulates these genes (Nadra et al. 2010). On the other hand, pregnant wild-type mice activated by PPAR agonist rosiglitazone (100 mg/kg) displayed disorganized placental layers, altered placental microvasculature and decreased expression of pro-angiogenic genes Prl2c2, VEGF and platelet endothelial cell adhesion molecule (Pecam1) although these phenotypes may be attributed to the high dose of agonist used (Nadra et al. 2010). Therefore, proper examination of the role of PPAR in placental angiogenesis requires the appropriate model. For example, PPARγ ligands are also reported to reduce the production of VEGF (Xin et al. 1999; Peeters et al. 2005). PPARγ ligand binding dose-dependently suppressed the interaction of specificity proteins 1 and 3 (SP1 and SP3) to the promoter of the gene encoding VEGF receptor 2 (KDR), thereby inhibiting its expression (Sassa et al. 2004). PPARα agonists also suppressed endothelial cell proliferation and VEGF production in a tumor cell line, revealing the utility of PPARα agonists beyond their current application as lipid-lowering drugs (Panigrahy et al. 2008). Thus, the role of PPAR in modulating angiogenesis in both normal and pathological states needs further investigation.

The indirect contributions of PPAR to angiogenesis, through its regulatory functions during placentation, may account for the reported contradictory effects of PPAR agonists on VEGF signaling. Within the placenta, PPARγ expression is restricted to the diploid trophoblast lineages, including the spongiotrophoblasts and the labyrinthine trophoblasts, but was not found in the fetal endothelium permeating the presumptive labyrinth. Abnormal establishment and maintenance of fetal and maternal vascular networks were reported in PPARγ-null placentas, resulting in embryonic lethality (Barak et al. 1999). This phenotype is consistent with the prominent role that PPARγ plays during trophoblast invasion and differentiation of labyrinthine trophoblast lineages, which, along with fetal endothelium, form the

vascular-exchange interface with maternal blood (Parast et al. 2009). Fig 27 shows the possible direct or indirect regulation of the placental angiogenesis through PPARγ.

Fig 27: Possible Direct or Indirect Regulation of the Placental Angiogenesis through PPARγ



PPAR *– Peroxisome proliferator activated receptors,* ***VEGF*** *– Vascular endothelial growth factor*

Other studies have similarly suggested that the genetic disruption of nuclear receptor-activating protein 250 (NRP250), a co-activator of PPAR, results in altered distribution of spongiotrophoblasts, which may thereby contribute to the altered-vasculature phenotype described in their placentas (Antonson et al. 2003). Mechanistically, both PPARα and PPARγ were shown to elevate heme oxygenase 1 (HMOX1) in both smooth muscle and endothelial cells, where they regulate vascular tone (Kronke et al. 2007). Induction of Hmox1 expression in rats is reported to ameliorate hypertension associated with placental ischemia/hypoxia and to shift the angiostatic balance ratio (sFLT-1-to-VEGF abundance) (George et al. 2011). Together, these reports implicate an indirect involvement of PPARs in placental

angiogenesis. The pro-angiogenic effect of tocopherol supplementation late in pregnancy has been further attributed to the PPARγ-mediated regulation of genes responsible for angiogenesis (Kasimanickam and Kasimanickam, 2011).

Finally, PPARs are also known to control a variety of target genes involved in lipid homeostasis. PPARγ participates in the normal function of the fetal-placental unit, including nutrient and hormone exchange from mother and fetus (reviewed by Toth et al. 2007). For example, PPAR regulates fatty acid β- and ω-oxidation, fatty acid synthesis, preferential lipid utilization (reviewed by Xu et al. 2007) and the expression of fatty acid transporters FATP1 and FATP4 in human trophoblast cells (Schaiff et al. 2005, 2007). The molecular regulation by which PPAR influences placental development, however, still needs to be elucidated. The next section describes the role of PPAR along with other transcription factors in the lipid as well as fatty acid metabolism in the liver.

3.11. Transcription Factors Involved in Hepatic Fatty Acid Metabolism:

The carbohydrate and lipid metabolism occurs in the liver. The hepatic lipid metabolism involves a balance between strong up-regulation of lipogenic gene expression and the expression of genes involved in fatty acid oxidation. These changes are regulated with the help of transcription factors like PPARα, LXRα and β, SREBPs and RXRα (reviewed by Jump, 2002).

3.11.1. Peroxisome Proliferator Activated Receptor α:

Studies carried out in PPARα null mice have revealed the role of PPARα in regulating the expression of different genes related to lipid metabolism in the liver. This includes genes involved in mitochondrial β oxidation, peroxisomal β oxidation, fatty acid uptake and binding (liver FABP, FASN) and lipoprotein assembly and transport (APOA1, APOA2 and APOA5) (reviewed by Kidani and Bensinger, 2012). During fasting, the liver plays a critical role in energy metabolism, where PPARα is required for the upregulation of the genes involved in fatty acid oxidation and ketogenesis (reviewed by Contreras et al. 2013).

The activity of PPARα in controlling the expression of genes involved in lipid metabolism is majorly regulated by different natural (fatty acids) and pharmacological

ligands (fibrates) (reviewed by Grygiel-Górniak, 2014). The next section focuses on the role of fatty acids in the hepatic gene regulation mediated through PPARα.

Fatty acids and PPARα:

Prior to 1992, it was basically thought that dietary fat exerted its effects on gene expression by affecting changes at the level of membrane phospholipids or through the production of signaling molecules such as eicosanoids. However, a study carried out by Gottlicher et al. in 1992 led to the identification of nuclear receptors capable of binding fatty acids and thus affecting gene transcription (Gottlicher et al. 1992). Since then several transcription factors including PPAR were identified as targets regulated by dietary fat for fatty acid regulation (reviewed by Pegorier, 2004). *In-vitro* and *in-vivo* studies have reported that hepatic PPARα responds to changes in exogenous (dietary) fat and newly synthesized fat (de-novo lipogenesis), thereby enabling fatty acids to act as endogenous ligands of PPAR (reviewed by Jump, 2008). PPAR are known to bind many fatty acids including saturated, monounsaturated and polyunsaturated fatty acids. However, unlike saturated and monounsaturated fatty acids, only dietary PUFAs are reported to uniquely modulate hepatic lipid metabolism (reviewed by Clarke, 2004).

Omega-3 fatty acids, they readily undergo oxidation and stimulate PPARα, thereby making them a stronger PPARα agonist (reviewed by Grygiel-Górniak, 2014). Reports have indicated that the 20 carbon EPA is a robust activator of PPAR, while the 22 carbon DHA and DPA require prior retroconversion to 20 carbon PUFA in order to activate PPAR (reviewed by Jump et al. 2008). In this context, it has been reported that challenging rat hepatocyte cells with EPA promoted a robust response in PPARα target genes, like cytochrome P450-4A (CYP4A) and cytosolic fatty acyl thioesterase-1 (CTE1), while no such effect was seen when other fatty acids like Ole were added (Pawar and Jump, 2003). Omega-3 fatty acids act as feed-forward activators of fatty acid oxidation to control mitochondrial and peroxisomal lipid metabolism. They act as feedback inhibitors of de novo lipogenesis, mono- and polyunsaturated fatty acid synthesis to control the production and cellular content of saturated, mono- and polyunsaturated fatty acids (reviewed by Jump et al. 2008). In addition to this, rodents fed fish oil containing diets (at <20% calories as fat) demonstrated that multiple hepatic genes were induced through PPARα (Wang et al. 2005).

Despite the well-established role of PPARα in lipid metabolism, it was observed that in PPARα null mice, there was PUFA induced suppression of lipogenic genes such as S14 and fatty acid synthase (Ren et al. 1997). This indicated that PUFA mediated these effects through another transcription factor, other than PPAR, which led to the identification of SREBPs as possible candidates (reviewed by Sampath and Ntambi, 2004).

3.11.2. Sterol Regulator Element Binding Proteins (SREBPs):

SREBP is a basic helix-loop-helix-leucine zipper membrane bound transcription factor involved in the transcription of genes related to cholesterol and fatty acid synthesis. SREBP were initially identified as factors which bind to sterol regulatory element in the low density lipoprotein receptor promoter, and confer sterol regulation to several genes involved with cholesterol synthesis (reviewed by Nakamura and Nara, 2003). Three isoforms of SREBPs are currently identified: SREBP-1a and -1c (also known as adipocyte determination and differentiation 1, ADD1) and SREBP-2. SREBP-1a and SREBP-1c are formed from a single gene through the use of alternate promoters that produce transcripts with different first exons while a separate gene encodes SREBP-2 (reviewed by Ye and DeBose-Boyd, 2011). Reports indicate that SREBP-1a and SREBP-1c cDNAs were cloned from human and mouse cells (Shimomura et al. 1997, Shimano et al. 1997). SREBP-1c is known to be highly expressed in the liver, adrenal gland, adipose and brain while SREBP-2 is predominantly expressed in the liver and adipose tissue. Studies have demonstrated that SREBP-2 is actively involved in the transcription of cholesterogenic enzymes while SREBP-1 can activate genes involved in fatty acid synthesis (reviewed by Ye and DeBose-Boyd, 2011. SREBP-1c constitutes 90% of the SREBP-1 found in-vivo and is involved in regulating hepatic fatty acid metabolism (reviewed by Clarke, 2004).

Structure of SREBP:

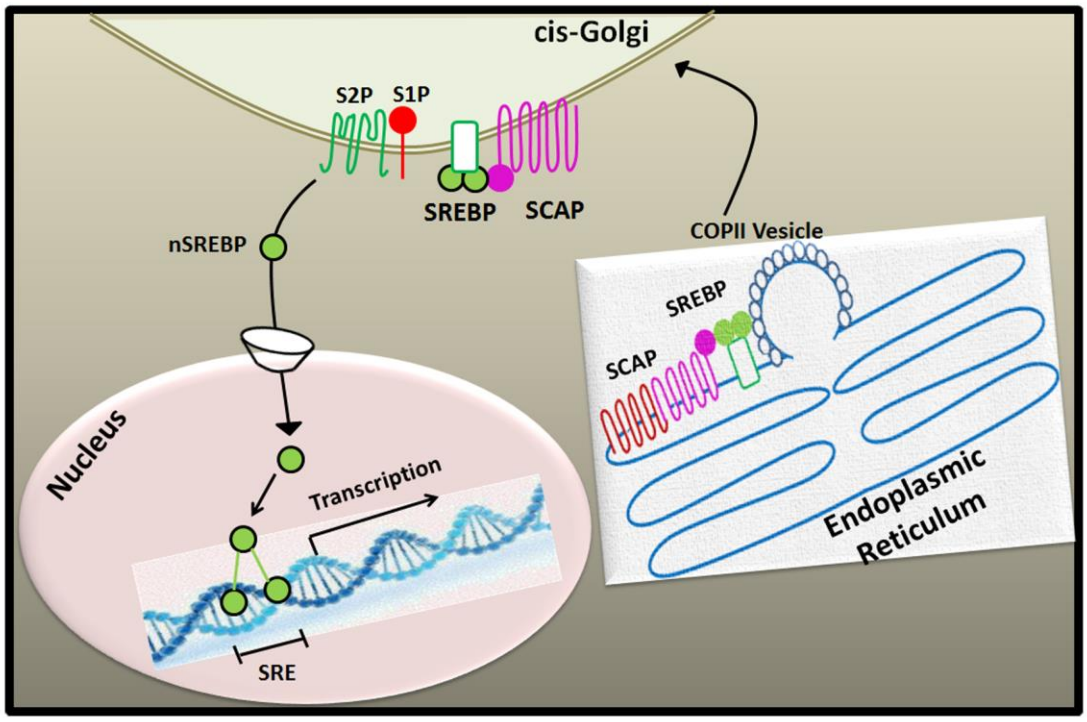
SREBPs are three domain proteins comprising of 1,150 amino acids bound to membranes of the endoplasmic reticulum (ER) and nuclear envelope in a hairpin orientation. The amino (NH_2-) terminal domain comprising of 480 amino acids and carboxy terminal domain (COOH-) comprising of 590 amino acids are projected into the cytosol (reviewed by Brown and Goldstein, 1997). The NH_2- terminal domains of

SREBPs are known to bind enhancer sequences called sterol response elements (SRE) situated in the promoter region of several lipogenic genes. A central domain (80 amino acids) through which SREBP is anchored to membranes is comprised of two membrane spanning sequences (182 kDa) separated by a short 31 amino acid loop which projects into the lumen of the endoplasmic reticulum and nuclear envelope (reviewed by Ye and DeBose-Boyd, 2011).

Mechanism of Action of SREBP:

SREBP proteins are synthesized in an immature form (125000 M_r) bound to the membranes of the ER, wherein they must undergo a two-step proteolytic cleavage in order to liberate their N-terminal domain constituting the mature transcription factor (68000 M_r) (reviewed by Vallim and Salter, 2010). Two proteins are essential for this cleavage: SREBP cleavage-activating protein (SCAP) and insulin-induced gene (INSIG) (reviewed by Ferré and Foufelle, 2010). Through the action of the SCAP protein, SREBP are transported to the Golgi in the form of SCAP-SREBP complex which then fuse with the Golgi (Radhakrishnan et al. 2007). In case of increased cholesterol concentration, there is a conformational change in the SCAP protein which inhibits their binding to SREBP and thus retains them in the ER. In the ER, SREBP binds to two ER anchor proteins called INSIG 1 and 2 (reviewed by Vallim and Salter, 2010). *In-vitro* studies have reported that both SREBP-1 and SREBP-2 display sensitivity to sterols, while *in-vivo* studies indicate SREBP-1 may not respond to sterol depletion (Sheng et al. 1995). In the Golgi, SREBP is cleaved with the proteases (site 1 protease and site 2 protease) to release a mature transcriptional form (nSREBP). This nSREBP travels to the nucleus where they bind to SRE promoters of specific genes (reviewed by Bordoni et al. 2006). Thus, SREBP activate several genes involved in de-novo lipogenesis and triacylglycerol synthesis, including ATP-citrate lyase, acetyl CoA- carboxylase, fatty acid synthase, stearoyl CoA desaturase 1 and glycerol phosphate acyl transferase (reviewed by Xu et al. 2013) (Fig 28).

Fig 28: Cellular Trafficking of SREBP



SCAP *– SREBP cleavage-activating protein,* ***COPII*** *– Coat protein complex,* ***SRE*** *– Sterol regulatory element,* ***S1P*** *– Sphingosine-1-phosphate,* ***S2P****- Site-2 protease*
Source: *http://flipper.diff.org/app/pathways/info/2768*

Fatty acids and SREBPs:

Fatty acids play a vital role in the human body since they form the building blocks for all cell membranes. However, excessive production of fatty acids can be toxic to cells and therefore there is a need to have regulatory mechanisms which control intracellular levels of these lipids. This regulatory feedback system involves SREBPs which sense the intracellular levels of fatty acids and thus regulates the transcription of genes encoding lipogenic enzymes. Fatty acids regulate the levels of SREBP in two ways; 1) by lowering the concentration of SREBP-1 precursor protein as well as inhibiting the proteolytic release of mature SREBP-1 2) by reducing the cellular content of precursor SREBP-1 protein and therefore the cellular abundance of SREBP-1 mRNA (Yahagi et al. 1999; reviewed by Georgiadi and Kersten, 2012). The process of the proteolytic release of mature SREBP is known to involve a stimulation of sphingomyelin hydrolysis and subsequent ceramide signaling while the amount of SREBP mRNA is lowered by accelerating SREBP-1 mRNA decay (Worgall et al. 2002).

The first mechanism involving proteolytic release is considered to be rapid (<60 min) while the second one involves an adaptative (about 48 hours) reduction in

the hepatic content of SREBP-1. PUFAs are known to reduce the half-life of SREBP-1c mRNA from 11 hours to <5hours (reviewed by Bordoni et al. 2006). Among the different types of fatty acids, it has been reported that unsaturated fatty acids selectively suppress hepatic levels of the mRNA encoding SREBP-1 (both 1a and 1c), but not SREBP-2 (Mater et al. 1999). DHA unlike any other fatty acids is reported to stimulate the removal of mature nuclear SREBP-1 via a mechanism dependent on 26S-proteosome and extracellular signal–regulated kinase (Botolin et al. 2006).

In view of the important role of SREBP-1c in the regulation of hepatic lipogenesis, numerous groups across the world were prompted to investigate the mechanism responsible for mediating the effect of PUFA in accelerating SREBP mRNA decay. These studies highlight the existence of another transcription factor LXR which is involved in mediating the effects of PUFA on SREBP (reviewed by Georgiadi and Kersten, 2012).

3.11.3. Liver X Receptor:

LXRs are ligand-activated transcription factors belonging to the class II nuclear receptor superfamily. These were first identified in 1994 by screening a rat liver cDNA library. Two isoforms of LXR subfamily are identified; LXRα (NR1H3) and LXRβ (NR1H2), both of which share ~78% identity of their amino acid sequences in DNA and ligand-binding domains (reviewed by Baranowski, 2008; reviewed by Ducheix et al. 2011). Reports indicate that LXRα is highly expressed in liver, spleen, adipose tissue, intestine, kidney and lung whereas LXRβ is ubiquitously expressed in all the tissues (reviewed by Patel et al. 2008). LXRs were first known to play a central role in cholesterol homeostasis, however, it is now well accepted that LXRs also exert major regulatory functions in hepatic fatty acid metabolism (reviewed by Hong and Tonotonoz, 2014). Among the two isoforms, LXRα is reported to be a stronger regulator of lipogenic gene expression pattern than LXRβ. LXR regulated several genes involved in the hepatic fatty acid metabolism such as hydroxymethyl glutaryl (HMG)-CoA synthase/reductase, farnesyl diphosphate synthase, squalene synthase, SREBP, stearoyl CoA desaturase (SCD)-1 and fatty acid synthase (reviewed by Steffensen and Gustafsson, 2004).

Ligands of LXR:

Due to the absence of natural ligands, LXRs were initially classified as orphan nuclear receptors. In the following years identification of several physiological ligands has "adopted" these receptors. Oxysterols, oxidized cholesterol derivatives, were first identified to be the potent LXR agonists (reviewed by Bełtowski, 2008). Other important ligands include 22(R)-hydroxycholesterol and 20(S)-hydroxycholesterol (intermediates in steroid hormone synthesis), 24(S)-hydroxycholesterol (produced in the brain, the major oxysterol of human plasma) and 24(S),25-epoxycholesterol. The synthetic ligands include compounds like T0901317, GW3965 and N,N-dimethyl-3b-hydroxycholenamide (reviewed by Liu et al. 2012). Several *in-vitro* and cell line studies have reported PUFAs to be the major antagonists of the LXRs (Ou et al. 2001; Svensson et al. 2003). These fatty acids inhibit the activation of LXR by competing with the activating ligands in the order ARA>EPA>DHA>ALA, whereas saturated and monounsaturated fatty acids have very little effect on the activation of LXR (Yoshikawa et al. 2002).

Structure of LXR:

LXRs are comprised of different functional domains; viz a DNA-binding domain, an N-terminal functional domain and a C-terminal functional domain. The amino-terminal domain (A/B) contains a ligand independent transactivation function (AF-1). Even in the absence of a ligand, the AF-1 stimulates a basal transcription. The highly conserved central domain or DBD interact with direct repeat-4 (DR-4) binding sites situated in the promoter region of the target genes. The carboxy terminal or LBD exhibits a ligand-dependent transactivation function (AF-2), which interacts with different co-regulators on binding of ligands (reviewed by Viennois et al. 2011).

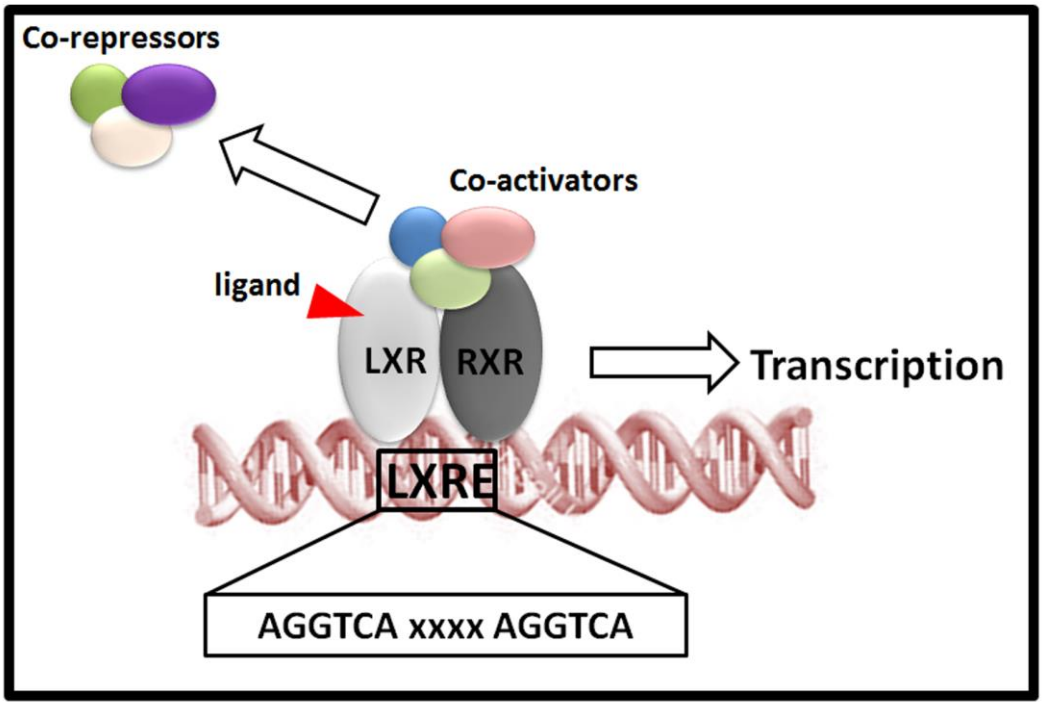
Mechanism of Action of LXR:

LXR-mediated gene regulation requires its heterodimerization with another nuclear receptor, the RXR. This LXR/RXR is a so called "permissive heterodimer" and can be activated by ligands for either partner in an independent manner (reviewed by Baranowski, 2008). On binding of a ligand to the LBD, LXR undergoes conformational modification which releases the co-repressors and recruits the co-activators on the helix of the LBD. The different co-activators are activating signal cointegrator- 2 (ASC-2) or receptor-interacting protein 140 (RIP140) (Freidman, 2006). This modification enables LXR to recruit the transcription machinery and

initiate the transcription. The LXRs then become acylated and bind to a liver X receptor response element (LXRE) of two AGGTCA repeats separated by four nucleotides in the target gene. Once the target gene is transcribed, LXR is deacetylated by NAD-dependent deacetylase sirtuin-1 (SIRT1). This deacetylation results in the ubiquitination of LXR, thereby degrading it by the proteasome (Li et al. 2007). The mechanism of transcriptional regulation mediated by LXRs is shown in Fig 29.

In the absence of the ligand, the heterodimer LXR/RXR interacts with corepressors such as NCoR or silencing mediator of retinoid and thyroid receptors (SMRT) (Hu et al. 2003). These co-repressors recruit proteins with histone deacetylases activity, thereby keeping the target gene in a non-transcription permissive state.

Fig 29: Mechanism of Transcriptional Regulation Mediated by LXRs



RXR *- Retinoid X receptor,* ***LXRE*** *- LXR response element*
Source: *Modified from Baranowski, 2008, Journal of Physiology and Pharmacology, 59(7): 31–55.*

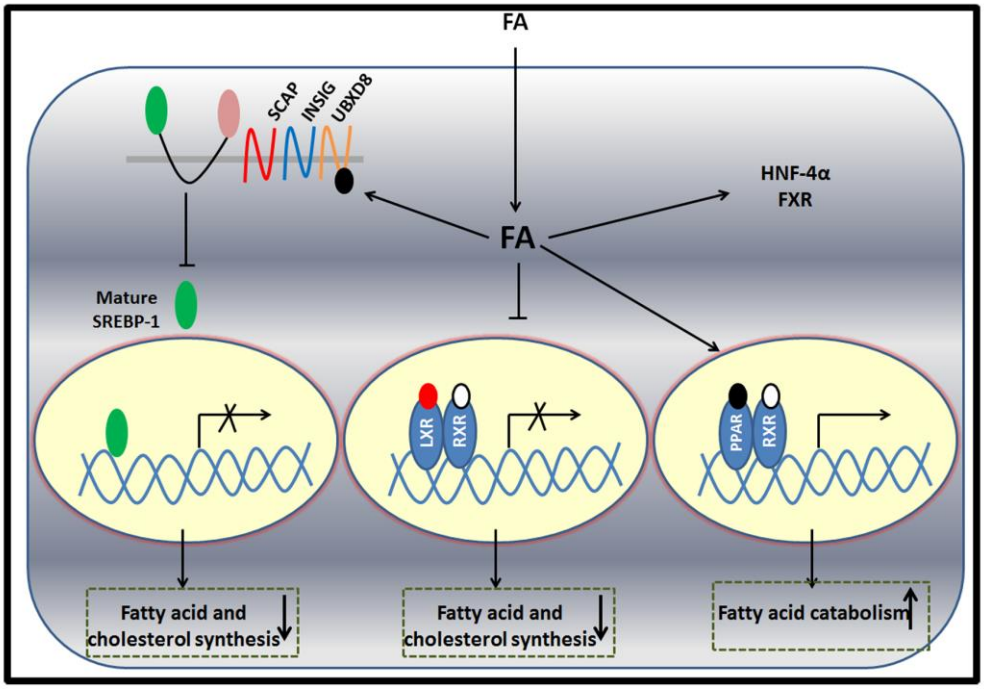
Fatty Acids and LXR:

Fatty acids are known to be the potential antagonist for LXRα wherein they inhibit LXRα binding of activating ligands, that is, oxysterols. Thus, inhibition of LXR activity by PUFA represents a feedback mechanism to prevent excessive fatty

acid synthesis when intracellular non-esterified fatty acids are high (reviewed by Vallim and Salter, 2010). Reports suggest that binding of PUFAs to LXRs results in the failure of LXR to induce transcription of SREBP-1c, causing a consequent decrease in lipogenesis. This suppression of SREBP involves the 'trapping' of LXR as a PPARα/LXR heterodimer by PUFAs (Ide et al. 2003). The formation of PPARα/LXR heterodimer is determined by the ratio of their ligands. When the ratio of PUFAs to oxysterol is low, the LXR/RXR heterodimer favors lipogenic gene expression whereas when the ratio of PUFA to oxysterol is high, the PPARα/LXR heterodimer favors fatty acid oxidation over fatty acid synthesis. Thus, the ratio of PUFAs to oxysterol acts as nutrient sensing mechanism that determines the partitioning of fatty acids between triglyceride synthesis and oxidation (reviewed by Clarke, 2004).

Finally, a recent study based on the *in-vivo* genome-wide map of hepatic binding of the nuclear receptors reports a surprising overlap between the PPAR and LXR binding sites; indicating a possible cross talk between PPAR, LXR and SREBP (Boergesen et al. 2012) that can be regulated positively as well as negatively by fatty acids (Fig 30).

Fig 30: Fatty Acid Regulation of Hepatic Transcription Factors



***FA**- Fatty acids, **SREBP**- sterol regulatory element binding protein, **PPARα** - peroxisome proliferator activated receptor α, **RXR**- retinoid X receptor, **LXR**- liver X receptor, **HNF-4α**- hepatic nuclear factor 4α, **FXR**- farsenoid X receptor, **SCAP**- SREBP cleavage-activating protein, **INSIG**- insulin-induced gene*

***Source:** Modified from Georgiadi and Kersten, 2012, Advances in Nutrition 3: 127-134.*

In addition to fatty acids, literature also indicates that some of the maternal nutrients influence the hepatic transcription factors, as enlisted below.

3.11.4. Maternal Nutrition and Hepatic Transcription Factors:

Studies carried out in human and animal models have highlighted the role of nutrition during pregnancy in influencing the lipid metabolism in the offspring. Most of the studies are focused on maternal protein restriction, which alters the expression of PPAR which is associated with impaired lipid homeostasis in the liver of the offspring (Lillycrop et al. 2005; Burdge et al. 2007). In addition to protein restriction, it has been reported that dams fed a low carbohydrate, high fat and high protein diet during pregnancy and lactation reduced hepatic triglyceride levels in association with increased levels of PPARα in the offspring (Zhang et al. 2005a).

Studies have also reported that several micronutrients mediate their effects in the offspring through changes in lipid metabolism. Maternal dietary iron restriction is reported to reduce triglyceride concentrations in the liver of the offspring with corresponding changes in the expression of SREBP-1c and its downstream genes (Zhang et al. 2005b). A recent study demonstrates that offspring born to mothers fed vitamin B_{12} deficient diets had differential hepatic expression of PPARα and PPARγ along with other proteins involved in the lipid metabolism (Ahmad et al. 2013). In addition to this, pregnant rats fed diets deficient in either folic acid or folic acid along with choline and methionine report no change in the expression of PPARα and SREBP-1c in the offspring liver (McNeil et al. 2009). However, there is a need to examine the effect of maternal micronutrient (folic acid and vitamin B_{12}) and omega-3 fatty acids on both the placental and hepatic transcription factors using an animal model.

3.12. Need for Animal Studies:

Several epidemiological studies carried out in large cohorts have demonstrated the association between the factors influencing intrauterine life and risk for non-communicable diseases in adulthood. Maternal nutrient supply during fetal development has been proposed as the major programming stimulus that determines risk of disease in adulthood (reviewed by Roseboom et al. 2011). However, most of these studies were carried out in retrospective cohorts, and raised serious issues regarding control for confounding factors, selection bias and measurement bias

(reviewed by Huxley et al. 2002). To overcome these issues, experimental animal studies were carried out to support the idea of maternal nutritional programming.

Research in animal models has contributed to the field of nutrition and metabolism, providing strong evidence of an association between early-life exposures and metabolic risk factors in later life (reviewed by McMullen and Mostyn, 2009). The studies in animal models were helpful in answering the key questions relating to exposures, mechanisms and outcomes of developmental programming (reviewed by Nathanielsz, 2006). Animal studies offer several advantages over human epidemiological studies. Animal studies are carried out in a relatively homogeneous group of animals instead of a heterogeneous group of patients. It is possible to control different environmental and confounding factors (reviewed by Hooijmans and Ritskes-Hoitinga, 2013). It is also possible to examine the toxicity of interventions or study the pathology and mechanisms of disease in animals (reviewed by Clancy et al. 2007). Invasive techniques like biopsies can be performed in animals (reviewed by Baker, 2008). Further, animal studies have made it possible to evaluate the effect of pre-pregnancy nutrition on the long term health of the offspring.

Animal studies are carried out either in large (e.g. sheep and pig) or small (e.g. mouse, rat and guinea-pig) animal models. Large animal models have two major advantages viz., firstly the genetic make-up and secondly the placental structure is similar to those observed in humans (reviewed by Rabadán- Diehl and Nathanielsz, 2013). However, their use in research have few major disadvantages due to their high maintenance cost, length of gestation and time required to design intergenerational studies (reviewed by McMullen and Mostyn, 2009). Small animals have the advantage of short generation times and lifespan. It is easy to carry out studies across the subsequent generations using the rodent model. They also require less space and housing costs are lower (reviewed by Carter, 2007). Further, the rodent diets can be manipulated easily in order to evaluate the effect of very specific changes in diet composition (reviewed by McMullen and Mostyn, 2009). The short cycle length and easy observation of cytological characteristics makes rat an ideal animal for investigation of changes occurring during the reproductive cycle (Martins et al. 2005).

Rodents are used extensively in DOHaD literature. However, some disadvantages have questioned their utility in this field and are as follows: Rats are born immature in comparison with humans and large animals (reviewed by Clancy et al. 2007). Human dietary patterns are different from the diets fed to rodents and

therefore they develop different disease profiles as in the human subjects. In addition to these, several challenges exist in translating animal research to humans. There are biological differences between species and strains; poor methodological quality of animal experiments; differences in the design of experimental animal studies and insufficient reporting of details of animals (reviewed by Hooijmans and Ritskes-Hoitinga, 2013). Nevertheless, rats have often been used in nutrition research and can be used to examine the effects of maternal nutrition on the reproductive performance.

ANIMAL STUDY

4.1. Materials and Methods:

The current study was carried out in strict accordance with the CPCSEA guidelines (Committee for the purpose of control and supervision of experimental animals) Government of India and was approved by the Bharati Vidyapeeth Animal Ethical Committee (2/2011/CPCSEA).

4.1.1. Animals:

Wistar albino rats (15 females, 10 males), weighing approximately 45 g, were obtained from the Animal House of the National Toxicology Centre (Pune, India). In the F1 generation, progeny were culled to maintain a litter size of eight pups per dam to reduce any litter size-related variability in the growth and development of pups during the postnatal period, thus increasing the sensitivity of the model for the detection of treatment-related effects (Agnish and Keller, 1997). Hence, instead of using the F0 generation in this study, it was thought appropriate to use their progeny (i.e. the F1 generation). All rats were maintained at 22^{o}C under a 12-h light–dark cycle with an appropriate ventilation system. Rats were marked with picric acid on the Head (H), Back (B), Tail (T), Head Back (HB), Back Tail (BT), Head Tail (HT) and Head Back Tail (HBT) for identification.

After these young adults (F0) had reached 200g, they were used for breeding. Males were housed individually before mating to acquire cage dominance. Virgin female rats were allowed to breed (sex ratio 1:3, male:female). Mating was confirmed the following morning on the basis of vaginal smears. Briefly, vaginal smears were taken on a clean microscope slide using a cotton bud dipped in saline. Slides were examined under a microscope at 10X magnification. Sperm-positive smears were considered the result of successful mating and indicated day 0 of gestation. Pregnant dams were housed individually [in polypropylene cages (29 X 22 X 14 cm) containing rice husk as bedding material] and allowed to deliver normally. Female pups that were born were separated from the dam on postnatal day 21 and were used in the study; male pups were not used in the study. Female pups (F1) were distributed randomly into one of five different groups (n=16 in each group) from birth and throughout pregnancy and lactation as follows: (1) a control group, which received normal dietary levels of folic acid and vitamin B_{12}; (2) a folic acid deficient (FD) group; (3) a vitamin B_{12} deficient (BD) group; (4) a folic acid deficient + omega-3 fatty acid supplemented (FDO) group; and (5) a vitamin B_{12} deficient + omega-3 fatty

acid supplemented (BDO) group. Rats fed with the vitamin B_{12} and folate deficient diets were kept in special cages to prevent coprophagy.

4.1.2. Diets:

The control and treatment diets (Table 1) were prepared as per the American Institute of Nutrition (AIN-93G) purified diets for laboratory rodents (Reeves et al. 1993). Both the control and treatment diets contained 18% protein. Five isocaloric treatment diets were formulated to investigate the effects of folic acid and vitamin B_{12} deficiency, as well as the effects of omega-3 fatty acid (DHA + EPA) supplementation to the micronutrient deficient groups.

Table 1: Composition of the Diets in Various Groups

Diets (g/kg)	Control	BD	FD	BDO	FDO
Corn Starch	398	398	398	398	398
Casein (>85% protein)	200	200	200	200	200
Dextrinized Starch	132	132	132	132	132
Sucrose	100	100	100	100	100
Soya Bean Oil	70	70	70	25	25
Omega-3 fatty acids	0	0	0	45	45
Fiber	50	50	50	50	50
Mineral mixture[a]	35	35	35	35	35
Vitamin mixture[b]	10	10	10	10	10
Folic acid (mg)	2	2	0	2	0
B_{12} (in 0.1% Mannitol) (µg)	2.5	0	2.5	0	2.5
Cystine	3	3	3	3	3
Choline Bitartarate	2.5	2.5	2.5	2.5	2.5
Tertiary Butyl Hydroquinone	0.014	0.014	0.014	0.014	0.014
Total Energy (kcal)	3766.0	3766.0	3766.0	3766.0	3766.0

***Control**, normal dietary levels of folic acid and vitamin B_{12}; **FD**, folic acid deficient group; **BD**, vitamin B_{12} deficient group **FDO**, omega-3 supplemented folic acid deficient group; **BDO**, omega-3 supplemented vitamin B_{12} deficient group.*

*[a]**The mineral mixture contained the following (in g kg^{-1} mixture): calcium carbonate** 357; **potassium phosphate** 196; **potassium citrate** 70.78; **sodium chloride** 78; **potassium sulfate** 46.6; **magnesium oxide** 24; **ferric citrate** 6.06; **zinc carbonate** 1.65; **manganous carbonate** 0.63; **cupric carbonate** 0.3; **potassium iodate** 0.01; **sodium selenate** 0.01; **ammonium paramolybdate** 0.007; **sodium metasilicate** 1.45; **chromium potassium sulfate** 0.275; **lithium chloride** 0.01; **boric acid** 0.08; **sodium fluoride** 0.06; **nickel carbonate** 0.03; **ammonium vanadate** 0.006; **sucrose** 221.02.*

*[b]**The vitamin mixture contained the following (in g kg^{-1} mixture): nicotinic acid** 3; **calcium pantothenate** 1.6; **pyridoxine HCl** 0.7; **thiamine HCl** 0.6; **riboflavin** 0.6; **D-biotin** 0.02; **vitamin B_{12} (in 0.1% mannitol)** 2.5; **vitamin E** 15; **vitamin A** 0.8; **vitamin D_3** 0.25; **vitamin K** 0.075; **folic acid** 0.2 **(control only); sucrose** 974.655 **to make up the total weight of the vitamin mixture to 1 kg.***

The omega-3 fatty acid supplementation used in the present study was fish oil (Maxepa; Merck, Goa, India) and was a combination of DHA (120 mg) and EPA (180 mg). Folic acid and vitamin B_{12} deficiencies were achieved exclusively through dietary means. Vitamin free casein was used for all treatment diets. The AIN-93G guidelines provide recommendations for both the folic acid and vitamin B_{12} content in rodent diets. Thus, the diets used for the micronutrient deficient groups in the present study were created by omitting these vitamins from the vitamin mixture and replacing it with sucrose. Diets were prepared on alternate days in the laboratory and were stored in the refrigerator. Rats were provided with fresh diet daily. The different vitamin mixtures and the omega-3 fatty acid supplement were stored at 4^{o}C. All diets contained tertiary butyl hydroquinone to prevent oxidation (Fritsche and Johnston, 1988; Gonzalez et al. 1992; Reeves et al. 1993). The ingredients were weighed on a Shimadzu (Kyoto, Japan) electronic balance (accurate to 0.001 g), mixed thoroughly and moulded into cylindrical pellets. To ensure that there was no loss of vitamins at high temperatures, the diets were dried in an oven at 50^{o}C. All rats had free access to food and water.

4.1.3. Monitoring of the Oestrous Cycle:

Young adult female (F1) rats were monitored for cyclicity for 15 days before breeding (i.e. ~ 8 weeks of age) according to the method described by Marcondes et al. (2002). Vaginal smears were collected every morning between 08.00 and 09.00 hours using a pipette filled with 20 μL normal saline (0.8% NaCl). The vaginal fluid was placed on a clean glass slide and the unstained slide was observed under a light microscope at 10X and 40X magnifications. Three types of cells were identified: leucocytes, epithelial and cornified cells. The proportions of these cells were used to determine the phase of the oestrous cycle, as described previously (Long and Evans, 1922).

4.1.4. Breeding and Death:

After adult female (F1) rats had reached 200 g weight (i.e. ~10 weeks of age), they were mated with male rats, with pregnancy confirmed as described above. The time taken for mating was different for female rats in different groups. In general, female rats in the control, BDO and FDO groups mated within 1 week. However, female rats in the BD and FD group took longer (~10 days) for mating. 8 dams/group

were delivered by cesarean section on gestational Day (GD) 20. Dam blood was collected in ethylene di amine tetra acetic acid (EDTA) containing tubes and plasma was separated by centrifugation at 1300 g for 15 min at 22^{o}C. Plasma samples of dams were stored at -80^{o}C until further use. The placenta, ovaries, mammary glands and liver were dissected out, snap frozen in liquid nitrogen and stored at -80^{o}C until further use.

Remaining 8 dams/group were continued on the same diet and allowed to deliver normally. Litter size was culled to eight (4 males and 4 females) to maintain nutritional adequacy. All the pups were dissected on the postnatal day 22. Pups were anesthetized with anesthetic ether and blood was drawn from the heart of the pups using the syringe (2 mL) and collected in the tubes containing EDTA. Pup plasma was separated and stored at -80^{o}C. Pup plasma samples were used for the folic acid and vitamin B_{12} estimations. Pup liver tissues were removed; snap frozen and stored at -80^{o}C until further use. The study design is shown in Fig 31.

Fig 31: Experimental Design of the Study

Control, *normal dietary levels of folic acid and vitamin* B_{12}***; FD,*** *folic acid deficient****; BD,*** *vitamin* B_{12} *deficient****; FDO,*** *folic acid deficient + omega-3 fatty acid supplemented****; BDO,*** *vitamin* B_{12} *deficient + omega-3 fatty acid supplemented****; GD20,*** *gestational Day 20.*

4.1.5. Observations Recorded:

Daily dietary intake of the dams was recorded before pregnancy, during pregnancy and during lactation. Before pregnancy, weight was recorded weekly, whereas during pregnancy, weight was recorded on GD0, GD7, GD14 and GD20 to determine weight gains. On GD20, litter weight, litter size and pup weight were recorded for each group. During lactation, daily feed intake of dams was recorded and weight of pups was recorded on postnatal day 7, 14 and 21.

4.1.6. Folate and Vitamin B_{12} Estimations:

Plasma folate and vitamin B_{12} concentrations were determined using chemiluminescent microparticle immunoassay technology (Abbott Diagnostics, Abbott Park, IL, USA). Folate and vitamin B_{12} concentrations were determined in 100 µL plasma using a two-step assay with automated sample pretreatment for determining the presence of folate and vitamin B_{12} in plasma. The reference range for plasma folate assay was 2.34-17.56 ng ml^{-1} and for plasma vitamin B_{12} assay was 187-883 pg ml^{-1}. Low plasma folate and vitamin B_{12} concentrations were defined as <10 ng ml^{-1} and <150 pg ml^{-1} respectively (Wadhwani et al. 2015).

4.1.7. Hormonal Levels:

Plasma oestradiol and progesterone concentrations were determined using commercially available kits based on microparticle enzyme immunoassay technology (Abbott AXSYM Systems, Abbott Park, IL, USA). Oestradiol concentrations were determined in 194 µL plasma samples, whereas progesterone concentrations were determined in 100 µL plasma samples.

4.1.8. Histology of Mammary Glands and Ovaries:

For histological assessment, mammary glands and ovaries from dams on GD20 were fixed in 10% formalin after dissection (n=3 per group). Tissues were then processed for routine paraffin embedding and cut at 4 mm prior to staining with haematoxylin and eosin. Slides were analyzed by an experienced histopathologist on a microscope (020–519.011DMLB 100S; Leica Microsystems, Wetzlar, Germany) with a camera attachment (WAT202B; Watec, Newburg, NY, USA). Images (10X and 40X magnification) were captured on an Intel Pentium III Cerelon computer (Intel, Santa Clara, CA, USA) using ASUS P3B-F software (ASUS, Taipei, Taiwan).

Morphometric analysis were performed using Image Drafter software (KEM Hospital and Research Center, Pune, India; developed using NET framework 2.0, New York, NY, USA), which enabled accurate, reproducible and standardisable analysis.

4.1.9. Dam Plasma, Erythrocyte, Placental and Liver Fatty Acid Levels:

The procedure for fatty acid analysis used in this study was revised from the original method of Manku et al. (Manku et al. 1983) and is also described in detail in our earlier departmental studies (Mehendale et al. 2008; Dangat et al. 2010). Briefly, the placenta and liver of the animals were homogenized using a Teflon glass homogenizer in chilled phosphate-buffered saline, with a pH of 7.5. The homogenate was then centrifuged at 10,000 rpm, 4°C for 20 min to separate the supernatant and cell membrane. The cell membrane fraction was used for the analysis of fatty acids. The cell membrane fraction was further reconstituted in 1 mL phosphate buffered saline and stored at -80°C until further use.

Transesterification of cell membrane fraction of placenta, liver, total plasma and total erythrocyte was carried out using a hydrochloric acid-methanol. Methyl esters were separated using a Perkin-Elmer gas chromatograph (SP 2330, 30-m capillary Supelco column; Perkin Elmer, Shelton, CT, USA). Peaks were identified by comparison with standard fatty acid methyl esters (Sigma-Aldrich). A total of 15 fatty acids were estimated which include: SFA like myristic acid (Myr), palmitic acid (Pal), stearic acid (Ste); MUFA like myristoleic acid (Myro), palmitoleic acid (Palo), Ole, nervonic acid (NA); omega-6 fatty acid like LA, GLA, DGLA, ARA, DPA; and omega-3 fatty acids like ALA, EPA and DHA. Fatty acids were expressed as g/100 g fatty acids.

4.1.10. Dam Plasma Malondialdehyde (MDA) Levels:

Malondialdehyde (MDA) levels were estimated from dam plasma using the Bioxytech MDA-586 kit (Oxis International, Beverly Hills, USA). The method has been described earlier (Roy et al. 2012). Briefly, thiobarbituric acid reacts with MDA to form a pink color and the absorbance was measured at 586 nm. Tetramethoxypropane was used as a standard. Plasma MDA concentration was expressed as nmol mL^{-1}.

4.1.11. RNA Isolation and cDNA Synthesis:

Total RNA was isolated from the placental and liver tissue using Trizol reagent (Invitrogen, Van Allen 150 Way, Carlsbad CA, USA) and was quantified using the Biophotometer (Eppendorf, Germany). RNA quality was assessed spectrophotometrically prior to analysis. The A260/A280 ratio within the range of 1.8-2.2 and A260/A230 ratio within the range of 2.0-2.2 was included in the analysis. Samples falling out of this range were repeated. Reverse transcription was carried out with oligo(dT) primer and SuperScript II reverse transcriptase from 1000 ng of total RNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA).

4.1.12. Tissue (Dam Liver, Placenta, Pup Liver) mRNA Levels of the Transcription Factors:

Quantitative real time polymerase chain reaction (qRT-PCR) for PPARγ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from the placenta; PPARα, PPARγ, HNF-4α, GAPDH from the dam liver and PPARγ, PPARα, SREBP-1c, LXRα, RXRα, GAPDH from pup liver was performed using the Applied Biosystems 7500 standard system. The relative expression level of the gene of interest was computed with respect to GAPDH mRNA to normalize for variation in the quality of RNA and the amount of input cDNA. qRT-PCR was performed with the TaqMan Universal PCR Master Mix (Applied Biosystems, USA) using cDNA equivalent to 100 ng total RNA. ΔCT (cycle threshold) values corresponded with the difference between the CT values of the GAPDH (internal control) and those of the target gene. Relative expression level of the gene was calculated and expressed as $2^{\Delta CT}$. The following TaqMan assays (Applied Biosystems, USA) were used in this study: GAPDH (Rn99999916_s1), PPARγ (Rn00440945_m1), PPARα (Rn00566193_m1), HNF-4α (Rn00573309_m1), SREBP-1c (Rn01495769_m1), LXRα (Rn00581185_m1) and RXRα (Rn00441185_m1).

4.1.13. Preparation of Placental Tissue Lysates:

Whole placental tissue was weighed and centrifuged twice with 1X PBS at 4°C. The supernatant was discarded and pellet was collected. The tissue pellet was lysed in chilled cell lysis buffer [50 mM TRIS HCl, 150 mM NaCl, 1 mM EDTA, 1 mM phenyl methane sulfonyl fluoride (PMSF), 10 mM Leupeptin, 0.1 mM Aprotinin]

for 30 min on ice with intermittent vortexing. The extract was then centrifuged at 13000 rpm for 10 min at 4°C. The clear supernatant (lysate) was then used for the assay. Total protein content of the lysates was estimated by Lowry method.

4.1.14. PPARγ Protein Levels in the Placenta:

PPARγ protein levels in the placenta were determined using the ELISA kit (USCN Life, Wuhan EIAab Science Co. Ltd, China). Briefly, placental lysates were added to microtiter plate pre-coated with an antibody specific to PPARγ. The plate is then incubated with biotin-conjugated polyclonal antibody preparation and avidin conjugated to horseradish peroxidase. After adding TMB substrate, the color change was measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The protein levels were expressed as ng/mL/gm of placenta.

4.1.15. IL-6 and TNF-α Levels in Placenta:

Interleukin-6 (IL-6) levels were estimated by the standard sandwich enzyme linked immunosorbent assay (Abnova, Walnut, CA, USA). Tumor necrosis factor-α (TNF-α) levels were also estimated by the *in-vitro* enzyme linked immunosorbent assay (Abcam, Cambridge, MA, USA). 100 µL of placental lysate was used for analysis of IL-6 and TNF-α. The detection limit for placental IL-6 levels was 62.5 - 4000 pg mL^{-1} and for placental TNF-α levels was 82.3 - 20000 pg mL^{-1}. These cytokines are expressed as pg/mL/mg of total protein.

4.1.16. Placental and Pup Liver Global DNA Methylation:

Genomic DNA extraction from placental and pup liver tissue was carried out with the Qiagen Blood and Tissue kit (Qiagen, Hilden, Germany, Catalog no- 69504). The method has been described earlier (Kulkarni et al. 2011c). Global DNA methylation was measured using the Methyl amp TM Global DNA Methylation Quantification Kit (Epigentek Group Inc., New York, NY, U.S.A, Catalog no. P - 1034). The kit yields accurate measures of methyl cytosine content as a percentage of total cytosine content. The methodology for estimation of global methylation levels used in this study takes into account methylation of all CpG's irrespective of their position in the genome (promoter and non-promoter CpG).

4.1.17. Statistical Analysis:

Data is represented as the mean ± SD. Data was analyzed using SPSS/PC+ (IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY). Mean values of various parameters were compared between the control and treatment groups using the one-way analysis of variance (ANOVA) and post hoc least significant difference (LSD) at conventional levels of significance ($p<0.05$, $p<0.01$).

4.2. Results:

Gestational Day 20

4.2.1. Feed Intake:

Feed intake was recorded before and during pregnancy. As indicated in Table 2, during pregnancy, the average intake of rats in the FD, BD and FDO groups was comparable with the control group. Rats in the BDO group had a higher ($p<0.01$ for both) average intake than rats in the control as well as in the BD group. The feed conversion ratios (mass of the food eaten divided by the body mass gain, all over a specified period) for the different groups were as follows: control 0.142; FD 0.142; BD 0.137; FDO 0.118; and BDO 0.163).

Table 2: Intakes of Animals in Different Groups during Pre-Pregnancy and Pregnancy Period

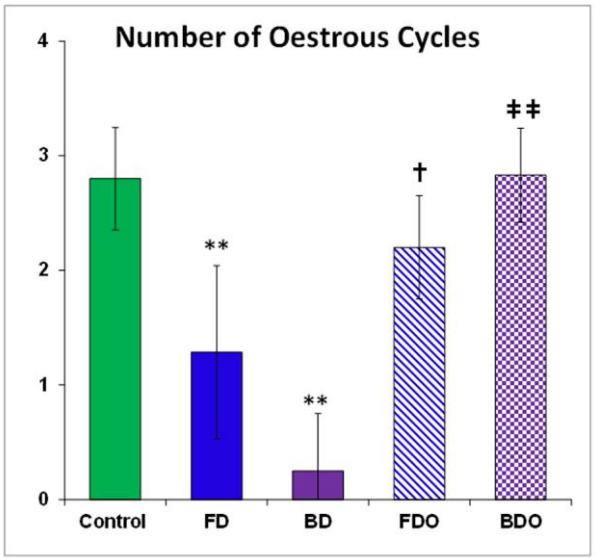
Group	Pre-pregnancy	Pregnancy Week 1	Pregnancy Week 2	Pregnancy Week 3	Average Intake during Pregnancy
Control	9.9 ± 2.3	12.2 ± 4.6	14.9 ± 4.4	16.0 ± 5.7	14.3 ± 2.9
FD	9.8 ± 2.5	14.0 ± 2.7*	15.0 ± 3.9	17.2 ± 3.8	15.4 ± 1.3
BD	11.7 ± 3.0**	12.4 ± 3.1	15.2 ± 3.6	15.6 ± 4.0	14.4 ± 1.7
FDO	10.7 ± 3.6$^{** \dagger\dagger}$	14.4 ± 5.1**	15.2 ± 4.4	18.0 ± 3.7*	15.8 ± 1.0
BDO	10.1 ± 4.2‡‡	17.5 ± 4.4**‡‡	18.3 ± 2.9**‡‡	16.4 ± 5.0	17.3 ± 1.2**‡‡

Data are expressed as mean ± SD. $^{*}p<0.05$, $^{**}p<0.01$ compared with control; $^{\dagger\dagger}p<0.01$ compared with FD group; $^{\ddagger\ddagger}p<0.01$ compared with the BD group.
***Control**, normal dietary levels of folic acid and vitamin B_{12}; **FD**, folic acid deficient group; **BD**, vitamin B_{12} deficient group **FDO**, omega-3 supplemented folic acid deficient group; **BDO**, omega-3 supplemented vitamin B_{12} deficient group.*

4.2.2. Oestrous Cycle:

The number of oestrous cycles observed during the 15 days period prior to breeding was significantly reduced in both the FD and BD groups compared to the control group (1.40 ± 0.55 and 0.25 ± 0.50 vs 2.80 ± 0.45, respectively; $p<0.01$ for both). Supplementation with omega-3 fatty acid improved the number of oestrous cycles to 2.20 ± 0.44 in the FDO group ($p<0.05$ vs the FD group) and 2.83 ± 0.41 in the BDO group ($p<0.01$ vs the BD group) (Fig 32).

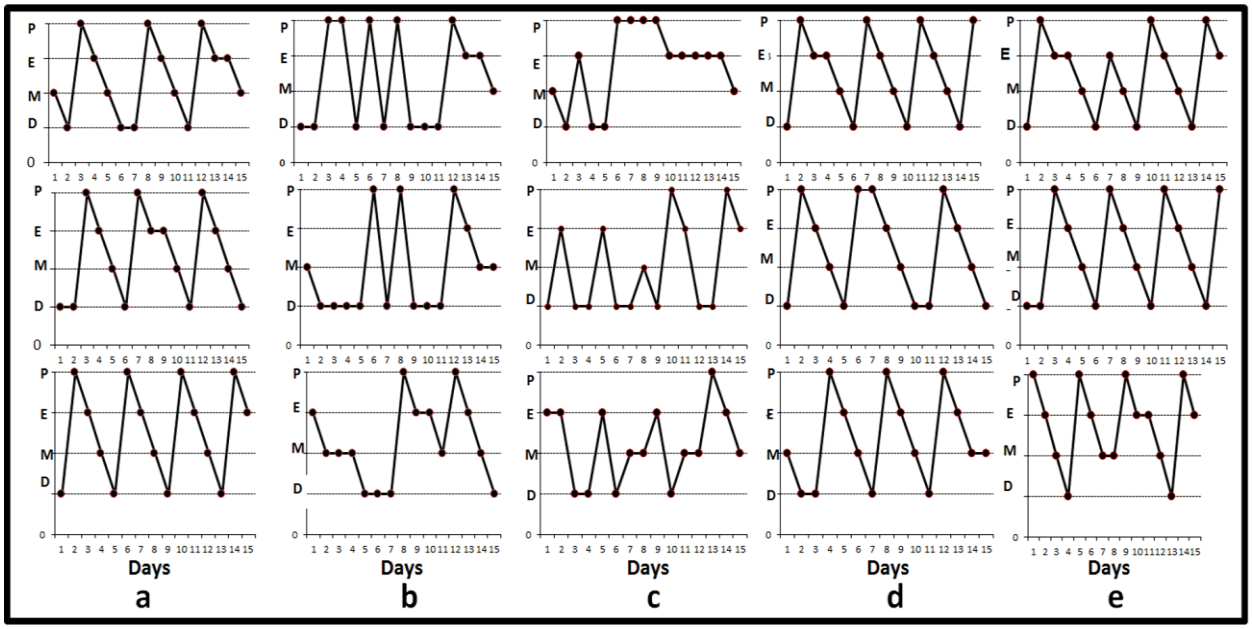
Fig 32: Number of Oestrous Cycle in Rats in Each of the Different Groups over a 15-Day Period



Data are expressed as mean ± SD ********$p<0.01$ *compared with the control group;* $^{\dagger}p<0.05$ *compared with the FD group;* $^{\ddagger\ddagger}p<0.01$ *compared with the BD group.*
Control*, normal dietary levels of folic acid and vitamin* B_{12}*;* ***FD****, folic acid deficient;* ***BD****, vitamin* B_{12} *deficient;* ***FDO****, folic acid deficient + omega-3 fatty acid supplemented (FDO);* ***BDO****, vitamin* B_{12} *deficient + omega-3 fatty acid supplemented.*

Changes in the stages of the oestrous cycle were also seen in the BD and FD groups compared with control, whereas supplementation with omega-3 fatty acid resulted in a return to normal oestrous cycles (Fig 33).

Fig 33: Variations in Phases of the Oestrous Cycle in Each of the Different Groups over the 15-Day Period Prior to Breeding

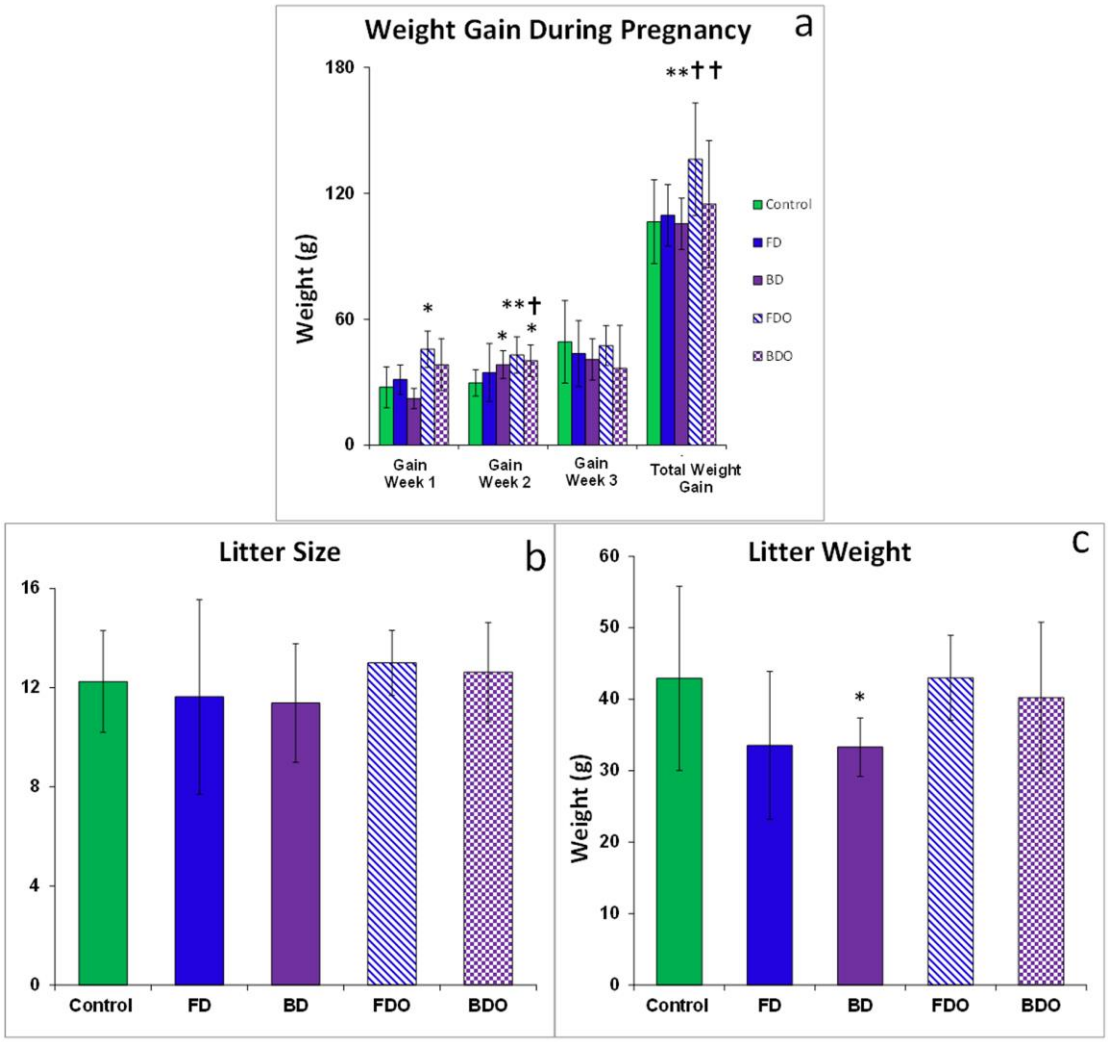


(a) ***control*** *(normal dietary levels of folic acid and vitamin B_{12}), (b) folic acid deficient, (c) vitamin B_{12} deficient, (d) folic acid deficient + omega-3 fatty acid supplemented and (e) vitamin B_{12} deficient + omega-3 fatty acid supplemented groups.*
P*, pro-oestrus;* ***E****, oestrus;* ***M****, metoestrus;* ***D****, dioestrus.*

4.2.3. Reproductive Performance:

Dams in the FDO group exhibited a significantly greater weight gain during pregnancy compared with dams in the FD and control groups (136.25 ± 16.50 g vs 109.50 ± 14.63 g and 106.50 ± 19.97 g, respectively; $p<0.01$ for both). The weight gain during pregnancy in the BD and BDO groups (105.50 ± 12.27 g and 114.87 ± 30.24 g, respectively) was comparable to that in the control group (Fig 34a). There were no significant differences in litter size among any of the groups (Fig 34b). The litter weight of the FD group was comparable to that of the control group (33.53 ± 10.35 g vs 42.93 ± 12.90 g, respectively), whereas that in the BD group was significantly lower (33.29 ± 4.10 g; $p<0.05$). Omega-3 fatty acid supplementation tended to improve litter weight in both the FDO and BDO groups (43.00 ± 5.93 g and 40.21 ± 10.58 g, respectively), although the differences failed to reach statistical significance (Fig 34c).

Fig 34: Reproductive Performance of Dams



Data are expressed as mean ± SD. ***p<0.05*, ****p<0.01 compared with control;* †*p<0.05,* ††*p<0.01 compared with the FD group.*
Control*, normal dietary levels of folic acid and vitamin* B_{12}*;* ***FD****, folic acid deficient group;* ***BD****, vitamin* B_{12} *deficient group* ***FDO****, omega-3 supplemented folic acid deficient group;* ***BDO****, omega-3 supplemented vitamin* B_{12} *deficient group.*

4.2.4. Weight of Dam Ovary, Mammary Gland, Placenta and Liver on GD20:

The weight of the left ovary was similar in the FD, BD and control groups (0.06 ± 0.01 g, 0.06 ± 0.01 g and 0.07 ± 0.02 g, respectively). Omega-3 fatty acid supplementation increased ($p<0.01$ for both) the weight of the left ovary in FDO group (0.1 ± 0.02 g) compared to both the FD and control groups. Similarly, omega-3 fatty acid supplementation increased ($p<0.05$) the weight of the left ovary in the BDO group (0.08 ± 0.02 g) as compared to the the BD group. There were no significant differences in the weight of the right ovary and mammary glands among any of the groups.

There was no difference in the placental weights of the FD (0.37 ± 0.07 g) and BD groups (0.37 ± 0.06 g) as compared to the control group (0.36 ± 0.06 g). Omega-3 fatty acid supplementation increased the weight of the placenta in the FDO group (0.39 ± 0.05 g) as compared to the control ($p<0.01$) and FD groups ($p<0.05$). However, there was no difference in the BDO group (0.37 ± 0.05 g).

The absolute dam liver weights of animals in the FD (11.09 ± 1.75 g) and BD groups (11.14 ± 1.77 g) were similar as compared to the control group (10.36 ± 1.23 g). The absolute liver weights of animals in FDO group (12.15 ± 0.83 g) and BDO group (12.18 ± 1.26 g) was significantly higher ($p<0.05$ for both) as compared to control group. There was no change in the relative dam liver weights of animals in all the treatment groups i.e FD (3.42 ± 0.46), BD (3.53 ± 0.41), FDO (3.54 ± 0.16), BDO (3.64 ± 0.24) as compared to control group (3.34 ± 0.42).

4.2.5. Folate and Vitamin B_{12} Concentrations in Dams at GD20:

Plasma folate concentrations decreased significantly in the FD, BD and FDO groups compared with control (6.68 ± 7.73 ng mL^{-1}, 16.13 ± 3.26 ng mL^{-1}, 3.15 ± 1.07 and 26.00 ± 14.48 ng mL^{-1}, respectively; $p<0.01$, $p<0.05$ and $p<0.01$ vs control, respectively). There was a tendency for decreased plasma folic acid concentrations in the BDO group (19.65 ± 2.60 ng mL^{-1}) compared with the control group, but the differences failed to reach statistical significance.

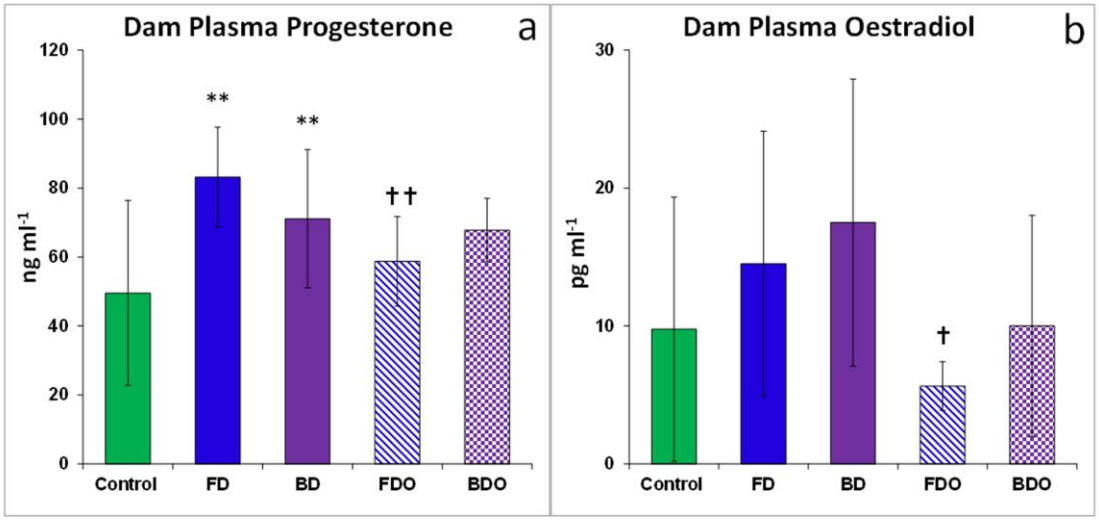
Significant decreases in plasma vitamin B_{12} concentrations were found in the FD and FDO groups compared with control (228.50 ± 82.28 pg mL^{-1} and 220.87 ± 19.39 pg mL^{-1} vs 287.62 ± 56.32 pg mL^{-1}, respectively; $p<0.05$ and $p<0.01$ vs control, respectively). Plasma vitamin B_{12} concentrations were below the limit of detection in both the BD and BDO groups (i.e. <83 pg mL^{-1}).

4.2.6. Hormone Analysis in Dams at GD20:

Plasma progesterone levels were significantly higher in the FD and BD groups as compared to the control (83.15 ± 14.50 ng mL^{-1} and 71.07 ± 20.05 ng mL^{-1} vs 49.53 ± 26.87 ng mL^{-1}, respectively; $p<0.01$ vs control, respectively). Omega-3 fatty acid supplementation significantly decreased progesterone concentrations in the FDO group (58.71 ± 13.00 ng mL^{-1}; $p<0.01$ vs the FD group). However, progesterone levels in the BDO group (67.77 ± 9.21 ng mL^{-1}) were similar to those in the BD group, regardless of omega-3 fatty acid supplementation (Fig 35a).

Plasma oestradiol levels were similar in the FD and BD groups as compared to the control (14.50 ± 9.61 pg mL^{-1} and 17.50 ± 10.41 pg mL^{-1} vs 9.75 ± 9.59 pg mL^{-1}, respectively). Omega-3 fatty acid supplementation significantly decreased oestradiol concentrations in the FDO group (5.63 ± 1.77 pg mL^{-1}; $p<0.05$ vs the FD group), but had no significant effect on oestradiol levels in the BDO group (10.00 ± 8.02 pg mL^{-1}; $p>0.05$ vs the BD group) (Fig 35b).

Fig 35: Plasma Progesterone and Oestradiol Levels in Different Groups

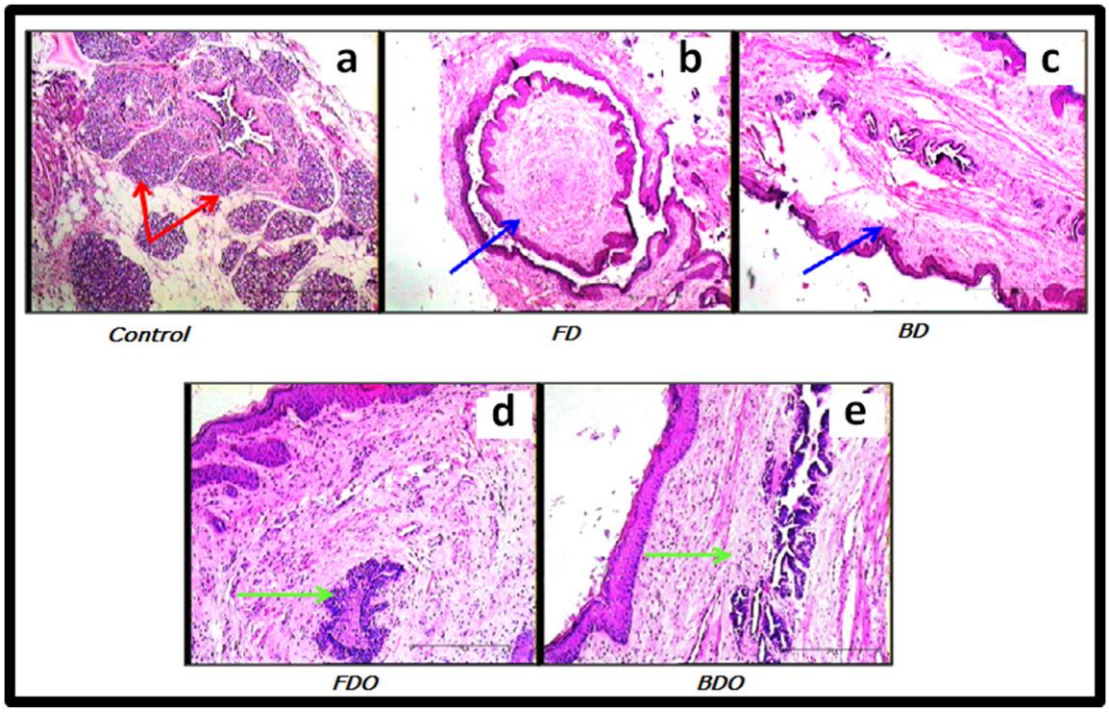


Data are expressed as mean ± SD. ********$p<0.01$ *compared with control;* $^{\dagger}p<0.01$, $^{\dagger\dagger}p<0.01$ *compared with the FD group.*
Control*, normal dietary levels of folic acid and vitamin* B_{12}*;* ***FD****, folic acid deficient group;* ***BD****, vitamin* B_{12} *deficient group* ***FDO****, omega-3 supplemented folic acid deficient group;* ***BDO****, omega-3 supplemented vitamin* B_{12} *deficient group.*

4.2.7. Histological Analysis of Mammary Glands at GD20:

Qualitative analysis of the morphology of mammary glands revealed the presence of acini, forming lactating ducts, in rats from the control group (Fig 36a). These acini were absent in mammary glands from both the FD and BD groups (Fig 36b, c). Thus, there was absence of lactating ducts in these groups. Following omega-3 fatty acid supplementation, there was a small increase in the number of acini in the mammary glands from the FDO and BDO groups (Fig 36d, e).

Fig 36: Representative Images Showing the Morphology of the Mammary Gland in Various Groups

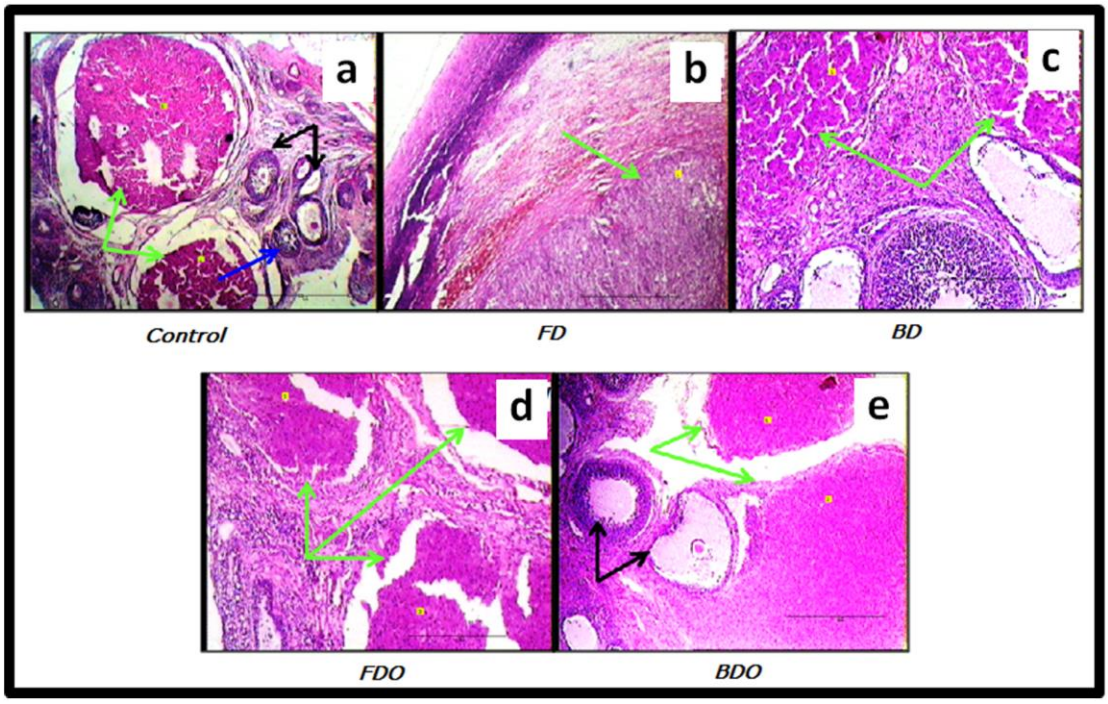


***Control**, normal dietary levels of folic acid and vitamin B_{12}; **FD**, folic acid deficient group; **BD**, vitamin B_{12} deficient group **FDO**, omega-3 supplemented folic acid deficient group; **BDO**, omega-3 supplemented vitamin B_{12} deficient group.*
*(a) In the control group, many acini are visible (red arrows) that will develop into lactating ducts. (b, c) In the FD (b) and BD (c) groups, there were no acini (blue arrows) and, hence, an absence of lactating ducts. (d, e) In the FDO (d) and BDO (e) groups, there was a slight increase in the number of acini (green arrows) as compared to that seen in the unsupplemented groups. **Scale bar** = 200 mm.*

4.2.8. Histological Analysis of Ovaries at GD20:

Qualitative analysis of the morphology of ovaries from the control group revealed healthy ovaries, with primordial follicles, corpora lutea and Graafian follicles (Fig 37a). There was a decrease in the number of corpora lutea in the FD group (Fig 37b) compared with control, but not in the BD group (Fig 37c). Following omega-3 fatty acid supplementation, the number of corpora lutea increased in both the FDO and BDO groups to values greater than in the control group (Fig 37d, e).

Fig 37: Representative Images Showing the Morphology of Ovaries in Various Groups



***Control**, normal dietary levels of folic acid and vitamin B_{12}; **FD**, folic acid deficient group; **BD**, vitamin B_{12} deficient group **FDO**, omega-3 supplemented folic acid deficient group; **BDO**, omega-3 supplemented vitamin B_{12} deficient group.*
*(a) In the control group, Graafian follicle (blue arrows), primordial follicles (black arrows) and corpora lutea (CL; green arrows) are seen. (b–e) Green arrows indicate the presence of CL in the FD (b), BD (c), FDO (d) and BDO (e) groups. **Scale bar** = 200 mm.*

4.2.9. Dam Plasma Fatty Acids at GD20:

Table 3 shows the fatty acid composition in the dam plasma from different groups. DHA levels were similar in both FD and BD groups as compared to the control group. Supplementation of omega-3 fatty acids to these micronutrient deficient diets increased ($p<0.01$ for all) the levels of DHA in the FDO and BDO groups as compared to the FD and BD groups, respectively, as well as when compared to the control group. ARA levels in the FD and BD groups were similar to that of the control group. Omega-3 fatty acid supplementation reduced the levels of ARA both in the FDO group and the BDO group as compared to their respective deficiency groups ($p<0.01$ for both) as well as when compared to the control group ($p<0.05$ for both).

Table 3: Dam Plasma Fatty Acid Levels (g/100g Fatty Acids) in Different Groups

	Control	FD	BD	FDO	BDO
(g/100g fatty acid)					
MYR	0.01 ± 0.01	0.49 ± 0.06**	0.54 ± 0.11**	0.73 ± 0.18**††	0.62 ± 0.25**
MYRO	0.02 ± 0.01	0.04 ± 0.01**	0.07 ± 0.09*	0.06 ± 0.02**	0.04 ± 0.02**
PAL	22.23 ± 1.69	22.00 ± 1.08	21.04 ± 1.39	23.63 ± 2.38	22.21 ± 2.79
PALO	0.14 ± 0.08	1.12 ± 0.38**	1.21 ± 0.37**	2.24 ± 0.80**††	1.52 ± 0.39**
STE	10.97 ± 2.43	8.39 ± 2.29*	7.60 ± 0.95**	7.50 ± 1.23**	8.97 ± 2.17*
OLE	17.82 ± 3.11	17.45 ± 3.12	18.82 ± 2.42	16.24 ± 3.25	14.02 ± 2.24*‡‡
LA	25.29 ± 3.87	23.81 ± 4.93	25.07 ± 2.36	11.89 ± 1.31**††	11.72 ±1.09**‡‡
GLA	0.05 ± 0.03	0.30 ± 0.52*	0.07 ± 0.04	0.01 ± 0.01**†	0.01 ± 0.01**
ALA	1.34 ± 0.68	1.20 ± 0.59	1.35 ± 0.53	0.40 ± 0.10**††	0.40 ± 0.05**‡‡
DGLA	0.07± 0.04	0.05 ± 0.03*	0.14 ± 0.24	0.25 ± 0.43††	0.22 ± 0.10**‡
ARA	13.34 ± 3.74	17.45 ± 6.06	15.68 ± 1.84	9.43 ± 5.00*††	8.74 ± 3.08*‡‡
EPA	0.09 ± 0.10	0.45 ± 0.27**	0.46 ± 0.21**	8.77 ± 2.19**††	9.47 ± 3.79**‡‡
NA	3.16 ± 0.79	1.89 ± 0.78**	1.82 ± 0.48**	0.11 ± 0.10**††	0.24 ± 0.36**‡‡
DPA (n-6)	0.22 ± 0.12	0.58 ± 0.27**	0.50 ± 0.14**	1.99 ± 0.30**††	2.07 ±0.70**‡‡
DHA	4.03 ± 0.98	3.23 ± 1.23	3.47 ± 0.94	13.83 ± 1.67**††	18.36 ± 2.88**‡‡
Omega-3	5.47 ± 0.98	4.87 ± 1.57	5.27 ± 1.02	23.00 ±3.30**††	28.23 ± 5.02**‡‡
Omega-6	38.97 ± 2.59	42.19 ± 2.47	41.45 ± 2.39	23.57± 4.45**††	22.76 ± 3.30**‡‡
MUFA	21.14 ± 2.53	20.49 ± 3.17	21.92 ± 2.18**	18.64 ± 3.88	15.82 ± 2.52**‡‡
SFA	33.21 ± 2.59	30.88 ± 1.57	29.18 ± 1.49**	31.86 ± 3.15	31.80 ± 3.59**

Data are expressed as mean ± SD. $^{*}p<0.05$, $^{**}p<0.01$ *compared with the control group;* $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ *compared with the FD group;* $^{\ddagger}p<0.05$, $^{\ddagger\ddagger}p<0.01$ *compared with the BD group*

***Control**, normal dietary levels of folic acid and vitamin B_{12}; **FD**, folic acid deficient; **BD**, vitamin B_{12} deficient; **FDO**, folic acid deficient + omega-3 fatty acid supplementation; **BDO**, vitamin B_{12} deficient + omega-3 fatty acid supplementation.*

***MYR** - Myristic acid; **MYRO** - Myristoleic acid; **PAL** - Palmitic acid; **PALO** - Palmitoleic acid; **STE** - Stearic acid; **OLE** - Oleic acid; **LA** - Linoleic acid; **GLA** - Gamma linolenic acid; **ALA** - Alpha linolenic acid; **DGLA** - Dihomo gamma linolenic acid; **ARA** - Arachidonic acid; **EPA** - Eicosapentaenoic acid; **NA** - Nervonic acid; **DPA**, omega-6 - Docosapentaenoic acid; **DHA** - Docosahexaenoic acid; Saturated fatty acids **(SFA)**: (MYR + PAL + STE); Monounsaturated fatty acids **(MUFA)**: (MYRO + PALO + OLE + NA); **Omega-3**: (ALA + EPA + DHA); **Omega-6**: (LA + GLA + DGLA + ARA + DPA); **n-6** – omega-6.*

4.2.10. Dam Erythrocytes Fatty Acids at GD20:

Table 4 shows the fatty acid composition in the dam erythrocytes from different groups. DHA levels were lower in both FD ($p<0.05$) and BD ($p<0.01$) groups as compared to the control group. Supplementation of omega-3 fatty acids to these micronutrient deficient diets increased ($p<0.01$ for all) the levels of DHA in the FDO and BDO groups as compared to the FD and BD groups, respectively, as well as when compared to the control group. ARA levels in the FD and BD groups were

lower than that of the control group; however, they did not reach the level of statistical significance. Omega-3 fatty acid supplementation reduced ($p<0.01$ for all) the levels of ARA both in the FDO and the BDO group compared with their respective deficiency groups as well as when compared with the control group.

Table 4: Dam Erythrocytes Fatty Acid Levels (g/100g Fatty Acids) in Different Groups

	Control	FD	BD	FDO	BDO
(g/100g fatty acid)					
MYR	0.30 ± 0.13	0.32 ± 0.10	0.39 ± 0.19	0.40 ± 0.12	0.45 ± 0.13*
MYRO	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.03
PAL	25.91 ± 1.74	27.20 ± 0.68	28.78 ± 2.81**	27.14 ± 1.64	26.01± 2.20‡‡
PALO	0.35 ± 0.12	0.47 ± 0.30	0.46 ± 0.38	0.78 ± 0.31**†	0.69 ± 0.30*
STE	16.77 ± 1.39	16.31 ± 0.96	16.51 ± 2.34	15.02 ± 1.07*	15.39 ± 1.71
OLE	5.28 ± 1.14	5.71 ± 1.06	6.12 ± 1.42	5.61 ± 0.73	5.83 ± 1.02
LA	8.50 ± 1.14	8.88 ± 0.73	8.57 ± 1.78	6.71±0.61**††	6.48±0.44**‡‡
GLA	0.09 ± 0.02	0.11 ± 0.08	0.09 ± 0.05	0.05±0.03††	0.04±0.02*‡‡
ALA	0.24 ± 0.42	0.09 ± 0.03	0.08 ± 0.04	0.08 ± 0.05	0.05 ± 0.03*
DGLA	0.20 ± 0.05	0.25 ± 0.05	0.21 ± 0.04	0.37±0.04**††	0.42±0.11**‡‡
ARA	22.41 ± 1.99	21.68 ± 1.10	20.66 ± 3.31	15.03±0.7**††	13.77±1.52**‡‡
EPA	0.23 ± 0.10	0.35 ± 0.43	0.73 ± 0.85	4.92±0.79**††	5.87±1.48**‡‡
NA	0.71 ± 0.07	0.82 ± 0.09	1.03 ± 0.42**	1.16±0.19**††	1.08 ± 0.12**
DPA (n-6)	0.58 ± 0.20	0.78 ± 0.22	0.59 ± 0.21	3.02±0.22**††	3.00±0.46**‡‡
DHA	2.83 ± 0.37	2.40 ± 0.37*	2.21 ± 0.42**	7.06±0.34**††	7.32±1.12**‡‡
Omega-3	3.31 ± 0.63	2.85 ± 0.63	3.01 ± 0.86	12.06±0.82**††	13.23±2.4**‡‡
Omega-6	31.78 ± 1.95	31.70 ± 1.29	30.12 ± 4.37	25.18±0.93**††	23.71±1.4**‡‡
MUFA	6.36 ± 1.19	7.01 ± 1.30	7.63 ± 1.96*	7.56 ± 0.93*	7.63 ± 1.20*
SFA	42.98 ± 2.41	43.83 ± 0.94	45.69 ± 4.35*	42.56 ± 1.90	41.86 ± 3.11‡

*Data are expressed as mean ± SD. *$p<0.05$, **$p<0.01$ compared with the control group; $^{†}p<0.05$, $^{††}p<0.01$ compared with the FD group; $^{‡}p<0.05$, $^{‡‡}p<0.01$ compared with the BD group*

***Control**, normal dietary levels of folic acid and vitamin B_{12}; **FD**, folic acid deficient; **BD**, vitamin B_{12} deficient; **FDO**, folic acid deficient + omega-3 fatty acid supplementation; **BDO**, vitamin B_{12} deficient + omega-3 fatty acid supplementation.*

***MYR** - Myristic acid; **MYRO** - Myristoleic acid; **PAL** - Palmitic acid; **PALO** - Palmitoleic acid; **STE** - Stearic acid; **OLE** - Oleic acid; **LA** - Linoleic acid; **GLA** - Gamma linolenic acid; **ALA** - Alpha linolenic acid; **DGLA** - Dihomo gamma linolenic acid; **ARA** - Arachidonic acid; **EPA** - Eicosapentaenoic acid; **NA** - Nervonic acid; **DPA**, omega-6 - Docosapentaenoic acid; **DHA** - Docosahexaenoic acid; Saturated fatty acids **(SFA)**: (MYR + PAL + STE); Monounsaturated fatty acids **(MUFA)**: (MYRO + PALO + OLE + NA); **Omega-3**: (ALA + EPA + DHA); **Omega-6**: (LA + GLA + DGLA + ARA + DPA); **n-6** – omega-6.*

4.2.11. Placental Fatty Acids:

Table 5 shows the fatty acid composition of placenta in different groups. The levels of DHA in both the FD and BD groups were lower ($p<0.01$ for both) as compared to the control group. Omega-3 fatty acid supplementation to these micronutrient deficient diets increased ($p<0.01$ for all) the levels of DHA in the FDO and the BDO groups.

Table 5: Placental Fatty Acid Levels (g/100g fatty acids) in Different Groups

	Control	FD	BD	FDO	BDO
(g/100g fatty acid)					
MYR	0.16 ± 0.07	0.37 ± 0.19**	0.39 ± 0.13**	0.51 ± 0.10**†	0.55 ± 0.08**‡
MYRO	0.10 ± 0.04	0.01 ± 0.01**	0.01 ± 0.01**	0.01 ± 0.01**	0.01 ± 0.01**
PAL	19.21 ± 3.83	24.21 ± 1.38**	24.00 ± 2.16**	26.85 ± 1.13**†	25.79 ± 0.98**
PALO	0.38 ± 0.31	0.38 ± 0.04	0.48 ± 0.34	0.80 ± 0.19**††	0.77 ± 0.14**‡
STE	21.59 ± 1.74	19.97 ± 1.37	19.93 ± 2.35*	18.63 ± 0.65**	19.93 ± 1.38*
OLE	8.89 ± 0.48	9.80 ± 1.07	11.92 ± 2.70**	12.12±1.20**††	12.09 ± 1.17**
LA	12.71 ± 1.06	13.34 ± 1.31	14.31 ± 1.50**	9.44 ± 0.55**††	10.05±0.85**‡‡
GLA	0.01 ± 0.02	0.01 ± 0.01	0.05 ± 0.11	0.01 ± 0.01	0.01 ± 0.01
ALA	0.68 ± 0.12	0.56 ± 0.23**	0.48 ± 0.08**	0.33 ± 0.04**††	0.37 ± 0.05**‡‡
DGLA	0.56 ± 0.23	0.38 ± 0.16	0.42 ± 0.13	0.63 ± 0.14†	0.79 ± 0.23*‡‡
ARA	18.53 ± 1.68	18.69 ± 1.93	16.80 ± 1.86*	11.03±0.97**††	10.61±1.16**‡‡
EPA	0.09 ± 0.03	0.41 ± 0.64**	0.43 ± 0.28**	2.54 ± 0.35**††	2.36 ± 0.64**‡‡
NA	3.99 ± 1.05	2.49 ± 0.98**	1.70 ± 0.49**	0.31 ± 0.07**††	0.43 ± 0.32**‡‡
DPA (n-6)	0.52 ± 0.17	0.55 ± 0.15	0.57 ± 0.08	2.39 ± 0.09**††	2.31 ± 0.48**‡‡
DHA	3.91 ± 0.83	2.08 ± 0.49**	2.03 ± 0.32**	7.98 ± 1.21**††	7.55 ± 0.86**‡‡
Omega-3	4.67 ± 0.88	3.05 ± 0.77**	2.94 ± 0.28**	10.86±1.27**††	10.28±1.23**‡‡
Omega-6	32.33 ± 2.70	32.97 ± 1.73	32.15 ± 1.70	23.50±1.07**††	23.77±1.81**‡‡
MUFA	13.36 ± 0.95	12.69 ± 1.51	14.10 ± 3.12	13.24 ± 1.26	13.30 ± 1.16
SFA	40.95 ± 4.82	44.55 ± 1.40*	44.33 ± 3.99*	45.99 ± 1.31**	46.27 ± 2.08**

*Data are expressed as mean ± SD. *p<0.05, **p<0.01 compared with the control group; $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ compared with the FD group; $^{\ddagger}p<0.05$, $^{\ddagger\ddagger}p<0.01$ compared with the BD group.*

***Control**, normal dietary levels of folic acid and vitamin B_{12}; **FD**, folic acid deficient; **BD**, vitamin B_{12} deficient; **FDO**, folic acid deficient + omega-3 fatty acid supplementation; **BDO**, vitamin B_{12} deficient + omega-3 fatty acid supplementation.*

***MYR** - Myristic acid; **MYRO** - Myristoleic acid; **PAL** - Palmitic acid; **PALO** - Palmitoleic acid; **STE** - Stearic acid; **OLE** - Oleic acid; **LA** - Linoleic acid; **GLA** - Gamma linolenic acid; **ALA** - Alpha linolenic acid; **DGLA** - Dihomo gamma linolenic acid; **ARA** - Arachidonic acid; **EPA** - Eicosapentaenoic acid; **NA** - Nervonic acid; **DPA**, omega-6 - Docosapentaenoic acid; **DHA** - Docosahexaenoic acid; Saturated fatty acids **(SFA)**: (MYR + PAL + STE); Monounsaturated fatty acids **(MUFA)**: (MYRO + PALO + OLE + NA); **Omega-3**: (ALA + EPA + DHA); **Omega-6**: (LA + GLA + DGLA + ARA + DPA); **n-6** – omega-6.*

The ARA levels were significantly lower ($p<0.05$) in the BD group, whereas there was no significant difference observed in the FD group as compared to the control group. However, omega-3 fatty acid supplementation significantly reduced ($p<0.01$ for all) the levels of ARA in both the FDO and BDO groups as compared to the control group as well as their respective deficiency groups.

4.2.12. Dam Liver Fatty Acids at GD20:

The levels of 15 different fatty acids in the dam liver are as described in table 6.

Table 6: Dam Liver Fatty Acid Levels (g/100g fatty acids) in Different Groups

	Control	FD	BD	FDO	BDO
(g/100g fatty acid)					
MYR	0.28 ± 0.08	0.37 ± 0.08	0.52 ± 0.21**	0.47 ± 0.17*	0.40 ± 0.11*
MYRO	0.09 ± 0.04	0.11 ± 0.03	0.12 ± 0.02	0.15 ± 0.06**†	0.16 ± 0.03**‡
PAL	20.53 ± 1.55	19.19 ± 0.86	19.51 ± 1.13	22.90 ± 1.20**††	20.67 ± 2.04
PALO	0.78 ± 0.33	0.97 ± 0.22	1.28 ± 0.51*	1.86 ± 0.77**††	1.24 ± 0.29**
STE	18.02 ± 3.19	16.99 ± 2.75	15.31 ± 2.60*	16.24 ± 2.19	17.15 ± 2.06
OLE	8.23 ± 2.44	7.73 ± 1.56	11.20 ± 3.13*	7.90 ± 3.18	6.72 ± 1.45‡‡
LA	15.11 ± 2.96	16.04 ± 2.43	18.79 ± 2.45**	9.07 ± 1.10**††	8.57 ± 1.31**‡‡
GLA	0.36 ± 0.08	0.35 ± 0.10	0.36 ± 0.11	0.09 ± 0.03**††	0.06 ± 0.02**‡‡
ALA	0.63 ± 0.19	0.95 ± 0.31*	1.15 ± 0.40**	0.31 ± 0.13*††	0.30 ± 0.11*‡‡
DGLA	0.14 ± 0.03	0.14 ± 0.04	0.17 ± 0.06	0.27 ± 0.06**††	0.35 ± 0.12**‡‡
ARA	17.61 ± 1.76	17.21 ± 1.75	15.29 ± 1.96**	9.08 ± 1.40**††	9.04 ± 1.04**‡‡
EPA	0.33 ± 0.22	1.12 ± 0.85**	0.45 ± 0.28	5.56 ± 0.62**††	5.94 ± 2.06**‡‡
NA	0.60 ± 0.21	0.75 ± 0.26	0.70 ± 0.22	2.95 ± 0.42**††	2.64 ± 0.76**‡‡
DPA (n-6)	0.89 ± 0.31	0.77 ± 0.38	1.30 ± 1.01	0.24 ± 0.16*††	0.11 ± 0.04**‡‡
DHA	8.10 ± 2.30	6.43 ± 1.17*	6.34 ± 1.46*	19.19 ± 2.59**††	20.62 ± 2.14**‡‡
Omega-3	9.06 ± 2.41	8.49 ± 0.76	7.95 ± 1.25	25.06 ± 2.08**††	26.85 ± 2.59**‡‡
Omega-6	33.82 ± 1.73	34.48 ± 2.44	35.30 ± 2.97	21.47 ± 1.03**††	20.66 ± 1.38**‡‡
MUFA	9.99 ± 2.95	9.57 ± 1.77	13.89 ± 3.41*	10.15 ± 4.01	8.24 ± 1.71‡‡
SFA	38.83 ±1.89	36.55 ± 2.75	35.33 ± 3.16**	39.61 ± 2.17†	38.22 ± 2.55‡

*Data are expressed as mean ± SD. *p<0.05, **p<0.01 compared with the control group; †p<0.05, ††p<0.01 compared with the FD group; ‡p<0.05, ‡‡p<0.01 compared with the BD group.*

***Control**, normal dietary levels of folic acid and vitamin B_{12}; **FD**, folic acid deficient; **BD**, vitamin B_{12} deficient; **FDO**, folic acid deficient + omega-3 fatty acid supplementation; **BDO**, vitamin B_{12} deficient + omega-3 fatty acid supplementation.*

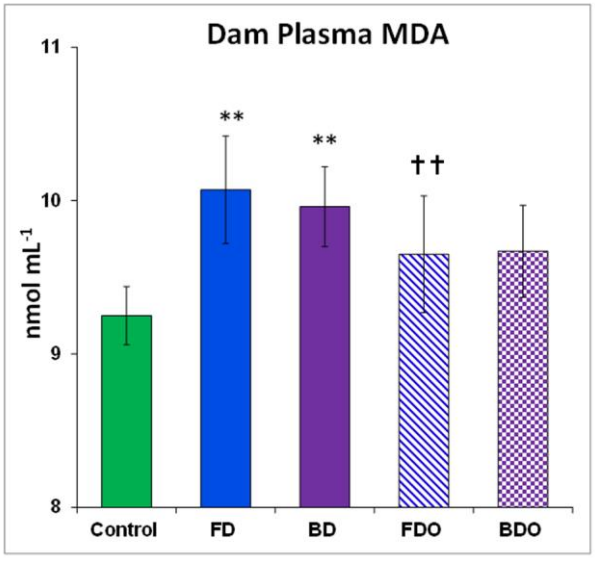
***MYR** - Myristic acid; **MYRO** - Myristoleic acid; **PAL** - Palmitic acid; **PALO** - Palmitoleic acid; **STE** - Stearic acid; **OLE** - Oleic acid; **LA** - Linoleic acid; **GLA** - Gamma linolenic acid; **ALA** - Alpha linolenic acid; **DGLA** - Dihomo gamma linolenic acid; **ARA** - Arachidonic acid; **EPA** - Eicosapentaenoic acid; **NA** - Nervonic acid; **DPA**, omega-6 - Docosapentaenoic acid; **DHA** - Docosahexaenoic acid; Saturated fatty acids **(SFA)**: (MYR + PAL + STE); Monounsaturated fatty acids **(MUFA)**: (MYRO + PALO + OLE + NA); **Omega-3**: (ALA + EPA + DHA); **Omega-6**: (LA + GLA + DGLA + ARA + DPA); **n-6** – omega-6.*

The levels of DHA were lower ($p<0.05$ for both) in the BD and FD group as compared to the control. Omega-3 fatty acid supplementation to the micronutrient deficient groups i.e FDO and BDO increased ($p<0.01$ for all) the levels of DHA as compared to their respective deficiency groups as well as compared to control. The levels of ARA were lower ($p<0.01$) in BD group as compared to control. These levels were also lower in FD group as compared to the control, although the difference was not statistically significant. Omega-3 fatty acid supplementation to the micronutrient deficient groups i.e FDO and BDO decreased ($p<0.01$ for all) the levels of ARA as compared to their respective deficiency groups as well as compared to control.

4.2.13. Dam Plasma MDA Levels at GD20:

Dam plasma MDA levels in both the micronutrient deficient groups, i.e., FD (10.07 ± 0.34 nmol mL^{-1}) and BD (9.96 ± 0.38 nmol mL^{-1}), were higher ($p<0.01$ for both) as compared to the control group (9.25 ± 0.19 nmol mL^{-1}). Omega-3 fatty acid supplementation reduced ($p<0.01$) the MDA levels in the FDO group (9.65 ± 0.25 nmol mL^{-1}) as compared to the FD group. Supplementation of omega-3 fatty acids to vitamin B_{12} deficient (BDO) (9.68 ± 0.30 nmol mL^{-1}) diet reduced the MDA levels, although the reduction was not statistically significant ($p<0.075$) (Fig 38).

Fig 38: Dam Plasma MDA Levels in Different Groups



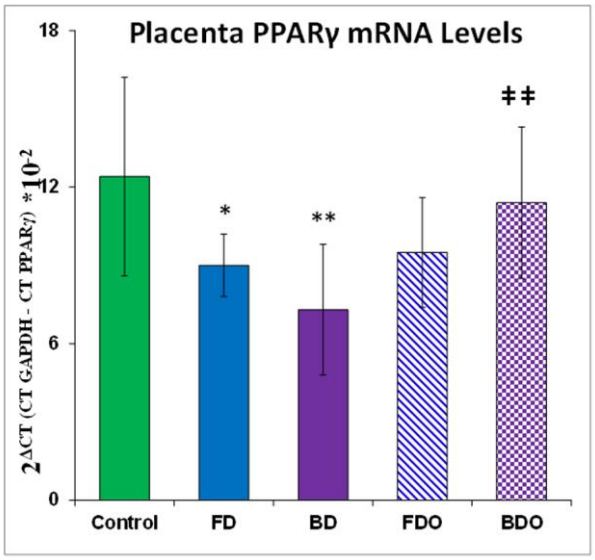
Data are expressed as mean ± SD. $^{**}p<0.01$ *compared with control;* $^{††}p<0.01$ *compared with the FD group.*

***MDA**, malondialdehyde; **control**, normal dietary levels of folic acid and normal vitamin B_{12}; **FD**, folic acid deficient; **BD**, vitamin B_{12} deficient; **FDO**, folic acid deficient + omega-3 fatty acid supplementation; and **BDO**, vitamin B_{12} deficient + omega-3 fatty acid supplementation.*

4.2.14. PPARγ mRNA Levels in the Placenta:

The mRNA levels of the PPARγ gene in the FD ($p<0.05$) and BD ($p<0.01$) groups were lower as compared to the control group. Omega-3 fatty acid supplementation increased the mRNA levels of PPARγ in the BDO group ($p<0.01$) as compared to the BD group (Fig 39).

Fig 39: Placental PPARγ mRNA Levels in Different Groups



Data are expressed as mean ± SD. ***** *$p<0.05$ compared with the control group;* ‡ *$p<0.05$ compared with the BD group.*
***PPARγ**, peroxisome proliferator activated receptor gamma; **GAPDH**, glyceraldehyde-3-phosphate dehydrogenase; **control**, normal dietary levels of folic acid and vitamin B_{12}; **FD**, folic acid deficient; **BD**, vitamin B_{12} deficient; **FDO**, folic acid deficient + omega-3 fatty acid supplementation; and **BDO**, vitamin B_{12} deficient + omega-3 fatty acid supplementation.*

4.2.15. PPARγ Protein Levels in the Placenta:

There was no change observed in the protein levels of PPARγ across the treatment groups (control, 564.97 ± 182.47 ng/mL/gm of placenta; FD, 437.32 ± 133.87 ng/mL/gm of placenta; BD, 573.47 ± 232.95 ng/mL/gm of placenta; FDO, 498.32 ± 167.18 ng/mL/gm of placenta; and BDO, 518.72 ± 110.48 ng/mL/gm of placenta).

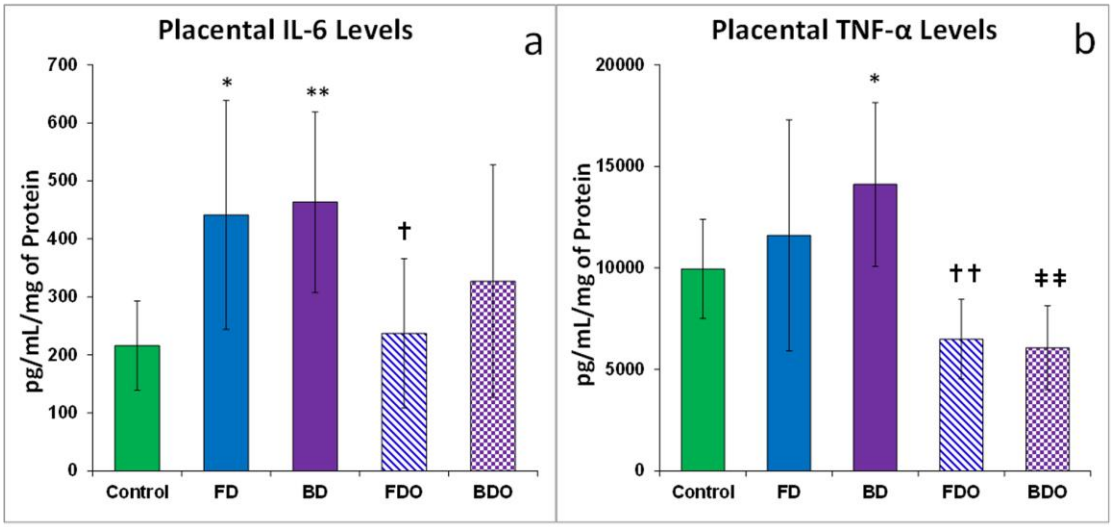
4.2.16. IL-6 and TNF-α Levels in the Placenta:

The IL-6 levels in the placenta in the FD (441.26 ± 197.22 pg mg^{-1} of protein) ($p<0.05$) and the BD groups (463.33 ± 155.63 pg mg^{-1} of protein) ($p<0.01$) were higher as compared to the control group (216.06 ± 77.13 pg mg^{-1} of protein). Supplementation of omega-3 fatty acid reduced ($p<0.05$) the levels of IL-6 in the FDO group (237.07 ± 128.54 pg mg^{-1} of protein) as compared to the FD group. The

levels of IL-6 in the BDO group (326.96 ± 200.52 pg mg^{-1} of protein) were also lower compared with BD group, although the difference was not significant (Fig 40a).

TNF-α levels in the placenta of the BD group (14111.83 ± 4035.77 pg mg^{-1} of protein) were higher ($p<0.05$) as compared to the control group (9953.99 ± 2444.72 pg mg^{-1} of protein), whereas in the FD group (11600.03 ± 5689.43 pg mg^{-1} of protein) the levels were comparable to the control group. Supplementation of omega-3 fatty acids reduced ($p<0.01$) the levels of TNF-α in the BDO group (6067.56 ± 2066.40 pg mg^{-1} of protein) as compared to the BD group. The levels of TNF-α in the FDO group (6487.03 ± 1963.06 pg mg^{-1} of protein) were also lower ($p<0.01$) as compared to the FD group (Fig 40b).

Fig 40: IL-6 and TNF-α Levels in Placenta of Different Groups

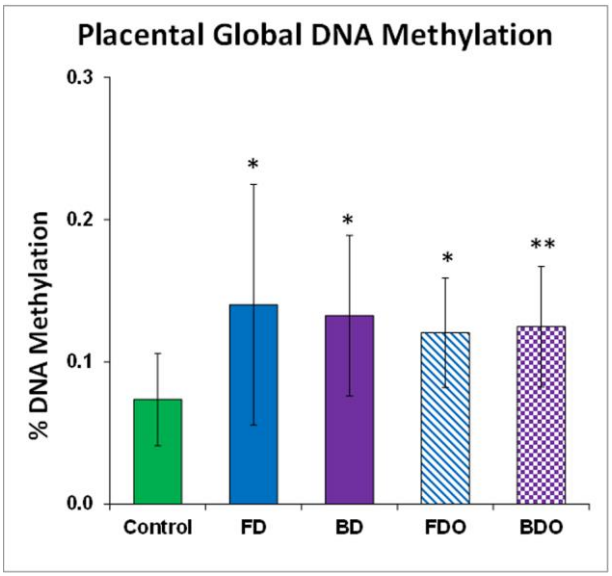


*Data are expressed as mean ± SD. *$p<0.05$, **$p<0.01$ compared with the control group; †$p<0.05$, ††$p<0.01$ compared with the FD group; ‡‡$p<0.01$ compared with the BD group.*
***IL-6**, Interleukin-6; **TNF-a**, tumor necrosis factor-α; **control**, normal dietary levels of folic acid and vitamin B_{12}; **FD**, folic acid deficient; **BD**, vitamin B_{12} deficient; **FDO**, folic acid deficient + omega-3 fatty acid supplementation; and **BDO**, vitamin B_{12} deficient + omega-3 fatty acid supplementation.*

4.2.17. Global DNA Methylation Levels in the Placenta:

In case of placental global DNA methylation, the levels were higher ($p<0.05$ for both) in both the micronutrient deficient groups (FD and BD) as well as the omega-3 fatty acid supplemented groups [FDO ($p<0.05$) and BDO ($p<0.01$)] as compared to the control group (Fig 41).

Fig 41: Placental Global DNA Methylation in Different Groups



*Data are expressed as mean ± SD. ***p<0.05, **p<0.01** compared with the control group.*
***Control**, normal dietary levels of folic acid and vitamin B_{12}; **FD**, folic acid deficient; **BD**, vitamin B_{12} deficient; **FDO**, folic acid deficient + omega-3 fatty acid supplementation; and **BDO**, vitamin B_{12} deficient + omega-3 fatty acid supplementation.*

4.2.18. Dam Liver mRNA Levels of the Transcription Factors at GD20:

Dam liver PPARα mRNA levels had a lower trend in the FD group (p=0.078) as compared to the control group. The mRNA levels were lower in case of BD group also, although it did not reach the level of significance. Omega-3 fatty acid supplementation to the folic acid deficient diet (FDO group) increased (p<0.05) the mRNA levels of PPARα in the dam liver tissue as compared to the FD group. However, in case of BDO group, there was no difference in the mRNA levels of PPARα as compared to BD group (Fig 42a).

Dam liver PPARγ mRNA levels were lower (p<0.05 for both) in both FD and BD group as compared to control group. Omega-3 fatty acid supplementation to the micronutrient deficient diet in case of FDO group increased (p<0.01) the mRNA levels of PPARγ as compared to the FD group. However, in case of BDO group, there was no difference in the mRNA levels of PPARγ as compared to the BD group (Fig 42b).

No change was observed in the mRNA levels of HNF-4α in the treatment groups as compared to the control group (Fig 42c).

Fig 42: Dam Liver mRNA Levels of the Transcription Factors in Different Groups

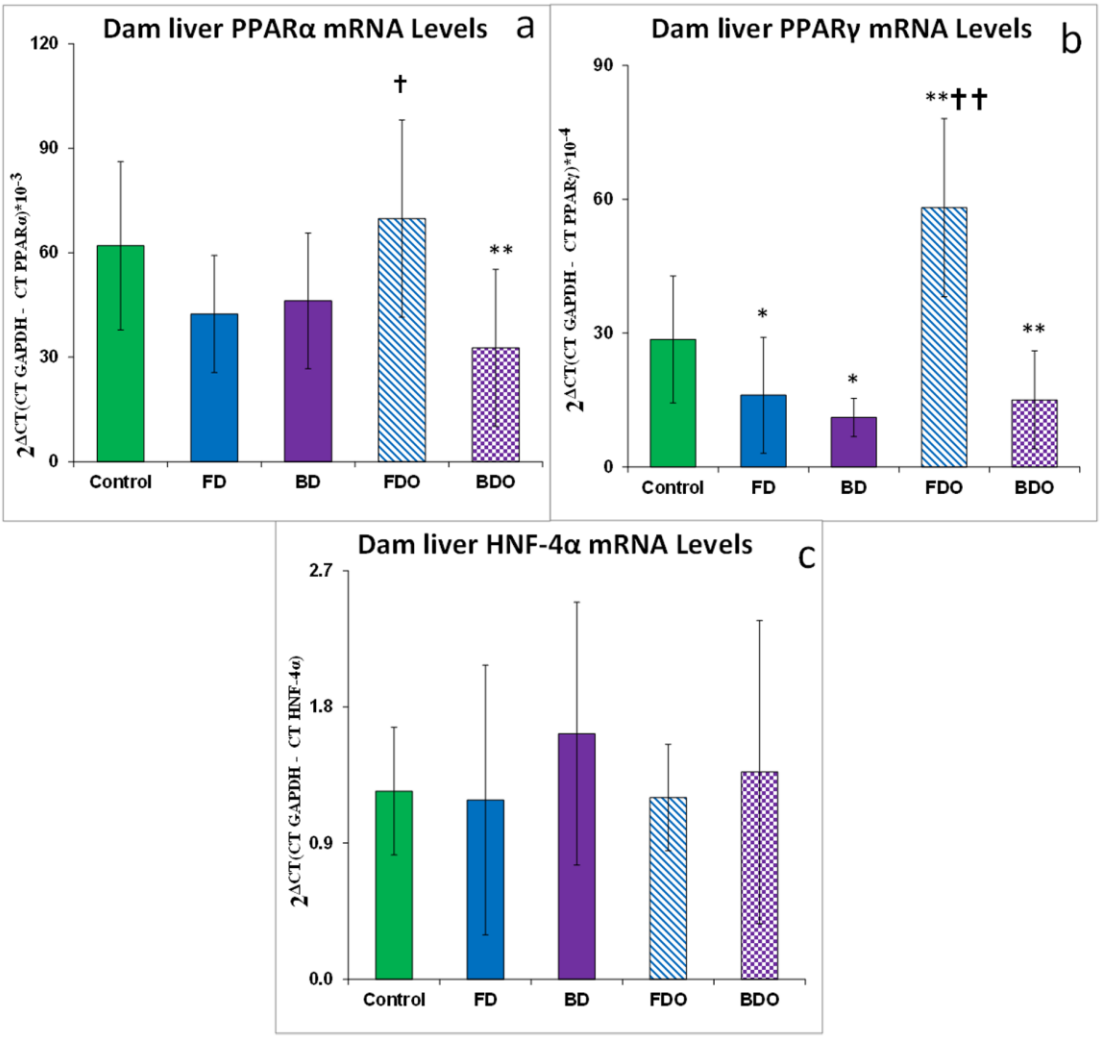


Fig 42a- *PPARα* ***Fig 42b***- *PPARγ* ***Fig 42c***- *HNF-4α.*
Data are expressed as mean ± SD. *******$p<0.05$, ********$p<0.01$ *compared with the control group;* $^{††}p<0.01$ *compared with the FD group.*
Control*, normal dietary levels of folic acid and vitamin* B_{12}*;* ***FD****, folic acid deficient;* ***BD****, vitamin* B_{12} *deficient;* ***FDO****, folic acid deficient + omega-3 fatty acid supplementation; and* ***BDO****, vitamin* B_{12} *deficient + omega-3 fatty acid supplementation.*

End of Lactation (Postnatal Day 22)

4.2.19. Feed Intake:

The weekly feed intakes (g) of the dams during lactation are described in table 7. The average intake in FD, BD and BDO group was comparable with the control group. The average intake of dams during lactation was significantly lower ($p<0.05$ for both) in the FDO group as compared to control as well as compared to FD group.

Table 7: Intakes (g) of Animals in Different Groups during the Period of Lactation

Group	Week 1 (g)	Week 2 (g)	Week 3 (g)	Average intake (g)
Control	22.08 ± 3.09	32.12 ± 7.08	45.87 ± 12.17	33.35 ± 6.68
FD	21.40 ± 4.66	34.67 ± 3.85	44.01 ± 7.38	33.36 ± 4.33
BD	21.46 ± 4.43	28.29 ± 8.39	36.72 ± 13.58	28.83 ± 7.83
FDO	16.53 ± 4.67**††	29.26 ± 4.18	35.92 ± 6.43	27.24 ± 3.84*†
BDO	20.44 ± 2.04	32.34 ± 6.83	41.22 ± 10.05	31.33 ± 5.82

*Data are expressed as mean ± SD. *****p<0.05, ******p<0.01 compared with the control group; †p<0.05, ††p<0.01 compared with the FD group.*
***Control,** normal dietary levels of folic acid and vitamin B_{12}; **BD**, vitamin B_{12} deficient group; **FDO**, omega-3 supplemented folic acid deficient group; **BDO**, omega-3 supplemented vitamin B_{12} deficient group.*

4.2.20. Average Pup Weights during the Period of Lactation:

Average pup weights at birth in all the treatment groups were lower ($p<0.05$ for all) than control. At day 21 of lactation, the average pup weight in the BD, FDO as well as BDO groups was lower ($p<0.01$ for all) as compared to the control group. Pup weight in the FDO group was lower ($p<0.01$) as compared to the FD group while it was higher ($p<0.05$) in the BDO group as compared to the BD group (Table 8).

Table 8: Average Pup Weight (g) in Different Groups during Lactation

Group	At birth (g)	Day 7 (g)	Day 14 (g)	Day 21 (g)
Control	7.94 ± 1.10	16.61 ± 1.84	32.33 ± 4.46	48.77 ± 5.12
FD	6.67 ± 0.43**	15.07 ± 1.66	32.06 ± 4.23	46.50 ± 7.51
BD	6.30 ± 0.81**	13.40 ± 2.09**	25.73 ± 4.17**	36.82 ± 6.97**
FDO	6.97 ± 0.59*	14.79 ± 2.06	28.85 ± 3.99	41.57 ± 7.08**††
BDO	6.57 ± 0.58**	15.01 ± 1.74	27.90 ± 4.42*	39.73 ± 8.13**‡

*Data are expressed as mean ± SD. *****p<0.05, ******p<0.01 compared with the control group; ††p<0.01 compared with the FD group; ‡p<0.05 compared with the BD group.*
***Control**, normal dietary levels of folic acid and vitamin B_{12}; **FD**, folic acid deficient; **BD** vitamin B_{12} deficient; **FDO**, folic acid deficient + omega-3 fatty acid supplemented; **BDO**, vitamin B_{12} deficient + omega-3 fatty acid supplemented.*

4.2.21. Absolute and Relative Liver Weights at Postnatal Day 22:

The absolute liver weights of animals in BD group (1.24 ± 0.33 g) was lower ($p<0.01$ for both) as compared to the control group (1.84 ± 0.26 g) as well as BDO group (1.50 ± 0.37 g). Also, the absolute liver weights of animals in FD (1.61 ± 0.32 g), FDO (1.65 ± 0.33 g) and BDO groups were lower ($p<0.01$ for all) as compared to the control group. The relative liver weight of animals in both the micronutrient

deficient groups i.e FD (3.46 ± 0.31) and BD (3.36 ± 0.51) were lower ($p<0.01$ for both) as compared to control group (3.79 ± 0.51). Further omega-3 fatty acid supplementation i.e both FDO (3.98 ± 0.63) and BDO (3.77 ± 0.52) groups increased ($p<0.01$ for both) the relative liver weight of animals as compared to their respective micronutrient deficient groups.

4.2.22. Pup Plasma Folate and Vitamin B_{12} Levels at Postnatal Day 22:

Pup plasma folic acid levels were lower ($p<0.01$) in the FD group (10.13 ± 3.30 ng mL^{-1}) as compared to the control group (43.94 ± 19.94 ng mL^{-1}). Further, these levels were lower ($p<0.01$ for both) in the FDO group (4.86 ± 2.28 ng mL^{-1}) as compared to the control group. The plasma folic acid levels in the BD group (18.20 ± 4.36 ng mL^{-1}) and BDO group (19.46 ± 1.07 ng mL^{-1}) were also lower ($p<0.01$ for both) as compared to the control group.

Pup plasma vitamin B_{12} levels were lower ($p<0.01$ for all) in all the treatment groups i.e. FD (504.75 ± 96.01 pg mL^{-1}), BD (104.25 ± 28.72 pg mL^{-1}), FDO (622.00 ± 103.87 pg mL^{-1}) and BDO (91.88 ± 13.46 pg mL^{-1}) as compared to the control group (846.63 ± 208.10 pg mL^{-1}).

4.2.23. Pup Plasma Fatty Acids at Postnatal Day 22:

The pup plasma fatty acid levels in different groups are described in table 9. Pup plasma DHA levels were comparable in the both FD and BD groups as compared to the control. Omega-3 fatty acid supplementation to the micronutrient deficient groups i.e FDO and BDO increased ($p<0.01$ for all) the levels of DHA as compared to their respective deficiency group as well as compared to the control group. Similarly, the levels of ARA were comparable in both the micronutrient deficient groups i.e. FD and BD as compared to control group. Omega-3 fatty acid supplementation to the micronutrient deficient groups i.e FDO and BDO decreased ($p<0.01$ for all) the levels of ARA as compared to their respective deficiency groups as well as compared to control.

Table 9: Pup Plasma Fatty Acid Levels (g/100g fatty acids) in Different Groups

	Control	FD	BD	FDO	BDO
(g/100g fatty acids)					
MYR	1.02 ± 0.24	1.69 ± 0.35*	1.25 ± 0.26	2.61 ± 0.85**††	2.78 ± 0.71**‡‡
MYRO	0.07 ± 0.03	0.08 ± 0.03	0.08 ± 0.03	0.07 ± 0.02	0.08 ± 0.02
PAL	20.18 ± 3.68	19.93 ± 1. 83	19.97 ± 0.81	25.03 ± 2.42**††	24.71±1.65**‡‡
PALO	0.92 ± 0.36	0. 83 ± 0.24	0.79 ± 0.20	1.90 ± 0.74**††	1.99 ± 0.46**‡‡
STE	10.15 ± 1.61	10.84 ± 1.26	11.50 ± 0.42	9.57 ± 1.49	9.95 ± 1.63‡
OLE	11.59 ± 3.05	9.52 ± 2.49	9.77 ± 1.32	11.34 ± 3.35	10.60 ± 2.45
LA	23.26 ± 2.03	21.18 ± 3.13	21.88 ± 1.06	15.21 ± 1.76**††	15.28±1.17**‡‡
GLA	0.41 ± 0.11	0.38 ± 0.07	0.32 ± 0.09*	0.14 ± 0.05**††	0.15 ± 0.04**‡‡
ALA	0.83 ± 0.17	0.73 ± 0.25	0.61 ± 0.15*	0.33 ± 0.08**††	0.31 ± 0.08**‡‡
DGLA	0.45 ± 0.08	0.49 ± 0.09	0.61 ± 0.13*	0.60 ± 0.18*	0.68 ± 0.20**
ARA	20.57 ± 5.30	21.93 ± 2.14	23.02 ± 1.73	6.68 ± 2.34**††	7.45±2.29**‡‡
EPA	0.92 ± 0.29	1.16 ± 1.09	0.55 ± 0.28	7.72 ± 1.90**††	8.18 ± 1.36**‡‡
NA	0.88 ± 0.17	0.56 ± 0.13**	0.49 ± 0.07**	0.11 ± 0.03**††	0.08 ± 0.02**‡‡
DPA (n-6)	0.78 ± 0.24	0.66 ± 0.22	0.73 ± 0.19	2.51 ± 0.62**††	2.41±0.48**‡‡
DHA	2.57 ± 0.55	2.48 ± 0.14	2.72 ± 0.26	9.87 ± 2.47**††	10.18±0.98**‡‡
Omega-3	4.32 ± 0.47	4.37 ± 0.90	3.88 ± 0.78	17.91 ± 4.10**††	18.67±1.80**‡‡
Omega-6	45.46 ± 0.47	44.65 ± 1.91	4.57 ± 1.98	25.14 ± 4.19**††	25.98±2.41**‡‡
MUFA	13.46 ± 3.25	10.99 ± 2.77	11.12 ± 1.52	13.42 ± 4.05	12.75 ± 2.84
SFA	31.34 ± 2.52	32.46 ± 2.39	32.72 ± 1.21	37.21 ± 2.28**††	37.45±1.52**‡‡

Data are expressed as mean ± SD. ***p<0.05,* ****p<0.01 compared with the control group;* †*p<0.05,* ††*p<0.01 compared with the FD group;* ‡*p<0.05,* ‡‡*p<0.01 compared with the BD group.*
***Control**, normal dietary levels of folic acid and vitamin B_{12}; **FD**, folic acid deficient; **BD**, vitamin B_{12} deficient; **FDO**, folic acid deficient + omega-3 fatty acid supplementation; **BDO**, vitamin B_{12} deficient + omega-3 fatty acid supplementation.*
***MYR** - Myristic acid; **MYRO** - Myristoleic acid; **PAL** - Palmitic acid; **PALO** - Palmitoleic acid; **STE** - Stearic acid; **OLE** - Oleic acid; **LA** - Linoleic acid; **GLA** - Gamma linolenic acid; **ALA** - Alpha linolenic acid; **DGLA** - Dihomo gamma linolenic acid; **ARA** - Arachidonic acid; **EPA** - Eicosapentaenoic acid; **NA** - Nervonic acid; **DPA**, omega-6 - Docosapentaenoic acid; **DHA** - Docosahexaenoic acid; Saturated fatty acids **(SFA)**: (MYR + PAL + STE); Monounsaturated fatty acids **(MUFA)**: (MYRO + PALO + OLE + NA); **Omega-3**: (ALA + EPA + DHA); **Omega-6**: (LA + GLA + DGLA + ARA + DPA); **n-6** – omega-6.*

4.2.24. Pup Liver Fatty Acids at Postnatal Day 22:

The pup liver fatty acid levels in different groups are described in table 10. Pup liver DHA levels were lower ($p<0.05$ for both) in the FD and BD groups as compared to the control group. Omega-3 fatty acid supplementation to the micronutrient deficient groups i.e FDO and BDO increased ($p<0.01$ for all) the levels of DHA as compared to their respective deficiency groups as well as compared to control. The levels of ARA were lower ($p<0.01$ for both) in both the micronutrient

deficient groups i.e. FD and BD as compared to the control group. Omega-3 fatty acid supplementation to the micronutrient deficient groups i.e FDO and BDO decreased ($p<0.01$ for all) the levels of ARA as compared to their respective deficiency groups as well as compared to the control group.

Table 10: Pup Liver Fatty Acid Levels (g/100g Fatty Acids) in Different Groups

	Control	FD	BD	FDO	BDO
g/100g fatty acids					
MYR	1.15 ± 0.53	1.84 ± 0.44**	1.40 ± 0.52	1.34 ± 0.52†	1.15 ± 0.26
MYRO	0.04 ± 0.01	0.08 ± 0.06	0.07 ± 0.06	0.03 ± 0.01†	0.04 ± 0.03
PAL	18.42 ± 0.98	17.38 ± 1.16	17.45 ± 0.95	21.88±1.42**††	20.94±2.3**‡‡
PALO	0.52 ± 0.14	0.62 ± 0.26	0.62 ± 0.28	0.59 ± 0.12	0.66 ± 0.12
STE	17.49 ± 1.44	14.70 ±2.22**	14.94 ± 2.24*	15.46 ± 2.58*	14.74±0.68**
OLE	7.80 ± 1.04	9.36 ± 1.83	8.70 ± 2.26	5.20 ± 1.10**††	5.62±0.77**‡‡
LA	18.67 ± 1.23	22.94 ±2.53**	21.11 ± 2.54*	10.01±0.65**††	10.04±1.6**‡‡
GLA	0.21 ± 0.12	0.38 ± 0.26*	0.21 ± 0.10	0.06 ± 0.04*††	0.06±0.03*‡
ALA	0.63 ± 0.13	0.90 ± 0.29**	0.74 ± 0.20	0.25 ± 0.11**††	0.28±0.10**‡‡
DGLA	0.46 ± 0.08	0.57 ± 0.13	0.74 ± 0.25**	0.74 ± 0.14**†	0.74 ± 0.11**
ARA	21.39 ± 3.65	17.86 ±1.71**	18.42 ± 1.10**	7.66 ± 1.82**††	7.74±0.95**‡‡
EPA	0.30 ± 0.14	0.39 ± 0.16	0.96 ± 1.34	5.37 ± 0.72**††	5.57±1.13**‡‡
NA	1.47 ± 0.32	1.03 ± 0.17	1.45 ± 1.35	0.16 ± 0.04**††	0.13±0.05**‡‡
DPA (n-6)	1.17 ± 0.30	1.06 ± 0.29	1.26 ± 0.30	5.12 ± 1.64**††	4.60±1.04**‡‡
DHA	6.21 ± 0.95	5.41 ± 0.72*	5.47 ± 0.88*	22.37±1.55**††	21.69±1.3**‡‡
Omega-3	7.14 ± 0.79	6.71 ± 0.48	7.16 ± 1.56	28.00±1.81**††	27.54±2.3**‡‡
Omega-6	41.91 ± 3.86	42.80 ± 1.62	41.74 ± 2.44	23.58±1.40**††	23.17±1.6**‡‡
MUFA	9.82 ± 1.10	11.08 ± 2.06	10.83 ± 1.83	5.98 ± 1.25**††	6.44±0.81**‡‡
SFA	37.06 ± 1.75	33.91 ±2.52**	33.79 ± 1.95**	38.68 ± 1.99††	36.83±2.43‡‡

*Data are expressed as mean ± SD. ***** $p<0.05$, ****** $p<0.01$ compared with the control group; **†** $p<0.05$, **††** $p<0.01$ compared with the FD group; **‡** $p<0.05$, **‡‡** $p<0.01$ compared with the BD group.*

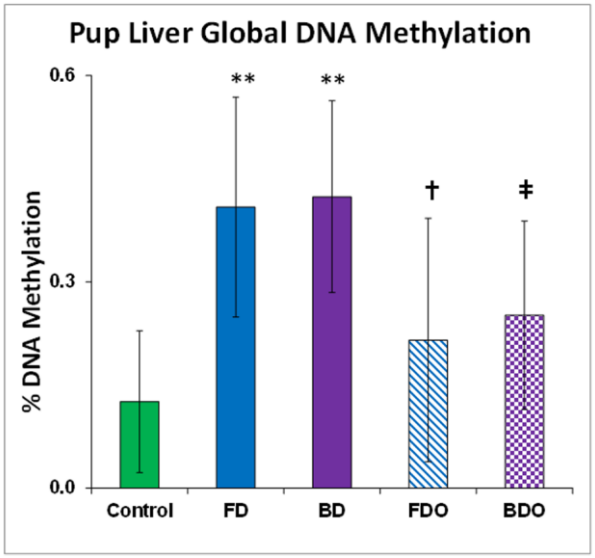
***Control**, normal dietary levels of folic acid and vitamin B_{12}; **FD**, folic acid deficient; **BD**, vitamin B_{12} deficient; **FDO**, folic acid deficient + omega-3 fatty acid supplementation; **BDO**, vitamin B_{12} deficient + omega-3 fatty acid supplementation.*

***MYR** - Myristic acid; **MYRO** - Myristoleic acid; **PAL** - Palmitic acid; **PALO** - Palmitoleic acid; **STE** - Stearic acid; **OLE** - Oleic acid; **LA** - Linoleic acid; **GLA** - Gamma linolenic acid; **ALA** - Alpha linolenic acid; **DGLA** - Dihomo gamma linolenic acid; **ARA** - Arachidonic acid; **EPA** - Eicosapentaenoic acid; **NA** - Nervonic acid; **DPA**, omega-6 - Docosapentaenoic acid; **DHA** - Docosahexaenoic acid; Saturated fatty acids **(SFA)**: (MYR + PAL + STE); Monounsaturated fatty acids **(MUFA)**: (MYRO + PALO + OLE + NA); **Omega-3**: (ALA + EPA + DHA); **Omega-6**: (LA + GLA + DGLA + ARA + DPA); **n-6** – omega-6.*

4.2.25. Pup Liver Global DNA Methylation at Postnatal Day 22:

In case of pup liver global DNA methylation, the levels were higher ($p<0.01$ for both) in both the micronutrient deficient groups (FD and BD) as compared to the control group. Omega-3 fatty acid supplementation to these groups (FDO and BDO) reduced ($p<0.05$ for both) the levels of global DNA methylation as compared to their respective micronutrient deficient groups (Fig 43).

Fig 43: Pup Liver Global DNA Methylation in Different Groups



Data are expressed as mean ± SD. ***p<0.01 compared with the control group;* †*p<0.05 compared with the FD group;* ‡*p<0.05 compared with the BD group.*
***Control**, normal dietary levels of folic acid and vitamin B_{12}; **FD**, folic acid deficient; **BD**, vitamin B_{12} deficient; **FDO**, folic acid deficient + omega-3 fatty acid supplementation; and **BDO**, vitamin B_{12} deficient + omega-3 fatty acid supplementation.*

4.2.26. Pup Liver mRNA Levels of the Transcription Factors at Postnatal Day 22:

Pup liver PPARα mRNA levels were lower ($p<0.05$ for both) in the BD and FDO groups as compared to the control group. Omega-3 fatty acid supplementation to the vitamin B_{12} deficient diet (BDO group) increased ($p<0.01$) the mRNA levels of PPARα in the pup liver tissue as compared to the BD group. However, in case of FDO group, there was no difference in the mRNA levels of PPARα as compared to the FD group (Fig 44a).

Pup liver PPARγ mRNA levels were lower ($p<0.01$) in the BD group as compared to the control group. Omega-3 fatty acid supplementation to the micronutrient deficient diet [FDO ($p<0.01$) and BDO group ($p<0.05$)] increased the mRNA levels of PPARγ as compared to their respective deficiency groups (Fig 44b).

Fig 44: Pup Liver mRNA Levels of the Transcription Factors in Different Groups

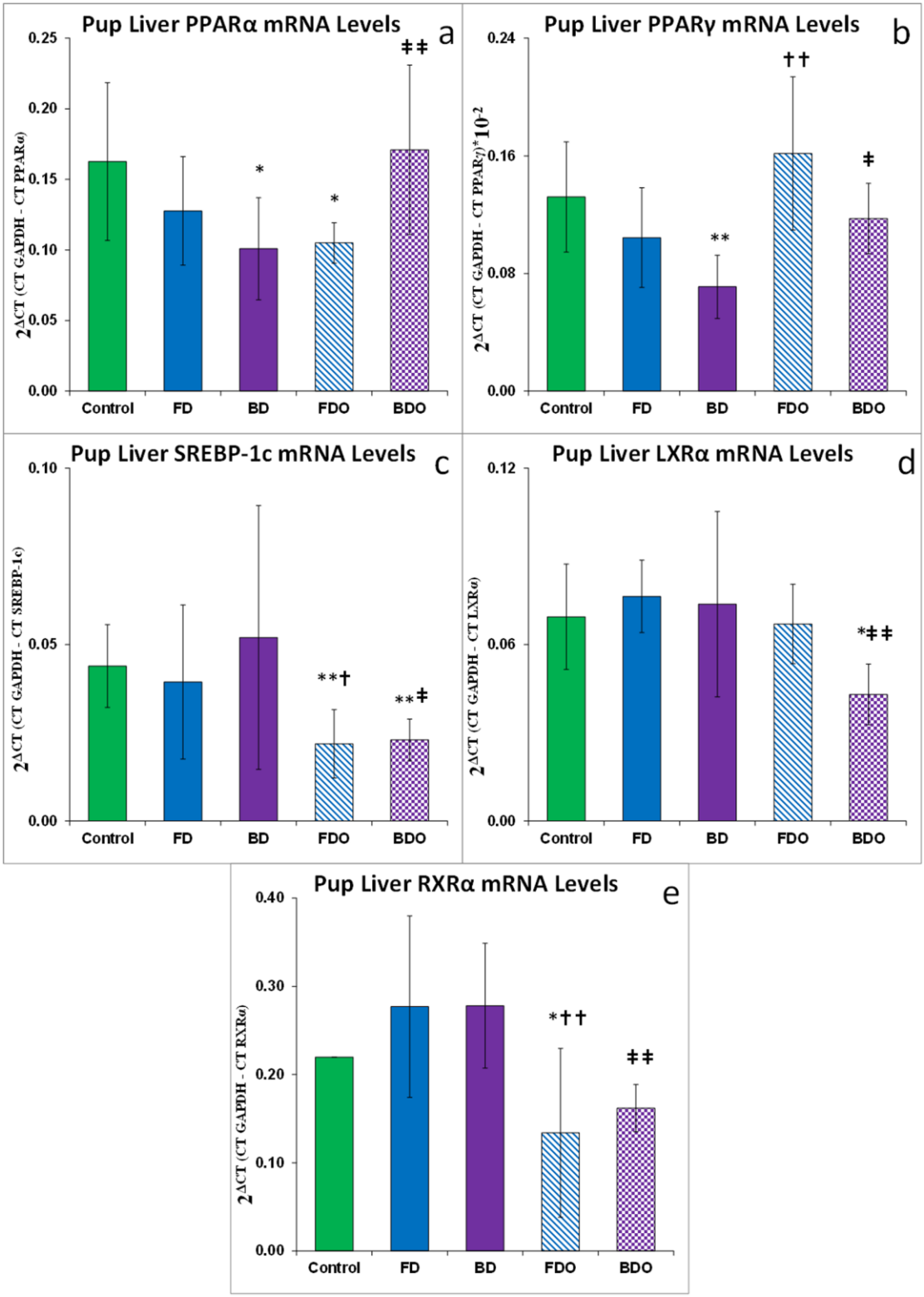


Fig 44a- *PPARα*, ***Fig 44b***- *PPAR-γ*, ***Fig 44c***- *SREBP-1c*, ***Fig 44d***- *LXR-α*, ***Fig 44e***- *RXR-α.*
Data are expressed as mean ± SD. ***** *p<0.05,* ****** *p<0.01 compared with the control group;* † *p<0.05,* †† *p<0.01 compared with the FD group;* ‡ *p<0.05,* ‡‡ *p<0.01 compared with the BD group.*
Control*, normal dietary levels of folic acid and vitamin* B_{12}*;* ***FD****, folic acid deficient;* ***BD****, vitamin* B_{12} *deficient;* ***FDO****, folic acid deficient + omega-3 fatty acid supplementation; and* ***BDO****, vitamin* B_{12} *deficient + omega-3 fatty acid supplementation.*

There was no difference observed in the pup liver SREBP-1c mRNA levels between the micronutrient deficiency groups (FD and BD) and the control group. However, omega-3 fatty acid supplementation to the micronutrient deficient diet (FDO and BDO group) decreased the mRNA levels of SREBP-1c as compared to their respective deficiency groups ($p<0.05$ for both) as well as compared to the control group ($p<0.01$ for both) (Fig 44c).

Pup liver LXRα and RXRα mRNA levels were similar in both the micronutrient deficient groups (FD and BD), as compared to the control group. Omega-3 fatty acid supplementation decreased the mRNA levels of LXRα significantly in BDO group as compared to the control ($p<0.05$) as well as compared to the BD group ($p<0.01$). No difference was observed in case of FDO group as compared to the FD group (Fig 44d).

In case of RXRα, omega-3 fatty acid supplementation to the micronutrient deficient diet (FDO and BDO group) decreased ($p<0.01$ for both) the mRNA levels of RXRα as compared to their respective deficiency groups as well as compared to the control ($p<0.05$) in case of FDO group (Fig 44e).

4.3. Discussion:

To the best of our knowledge, the present study is the first to have examined the effects of pre-conceptional folic acid and vitamin B_{12} deficiency on the oestrous cycle and reproductive performance, placental gene expression of the transcription factors and dam as well as pup liver gene expression of the transcription factors involved in the fatty acid metabolism. Further, the study has also examined the effects of omega-3 fatty acid supplementation to the micronutrient deficient diets on these parameters.

The current study indicates that the pre-conceptional folic acid and vitamin B_{12} deficiency resulted in (i) reduced number of oestrous cycles and altered the phases of the oestrous cycle, as well as abnormal plasma hormone levels, (ii) altered morphology of both the mammary glands and ovaries, (iii) lower levels of DHA in the dam erythrocytes and placenta, (iv) higher levels of MDA in dam plasma, (v) lower levels of ARA in placenta, (vi) lower expression of PPARγ in the placenta, (vii) higher levels of pro-inflammatory cytokines such as IL-6 and TNF-α in the placenta, (viii) higher levels of placental global DNA methylation, (ix) lower levels of DHA in the dam liver, (x) lower expression of

PPARγ genes in the dam liver, (xi) no change in the expression of PPARα and HNF-4α in the dam liver, (xii) lower levels of DHA in the pup liver, (xiii) lower expression of PPARα, PPARγ genes in the pup liver, (xiv) no change in the expression of SREBP-1c, LXRα, RXRα in the pup liver, and (xv) increased levels of pup liver global DNA methylation. Omega-3 fatty acid supplementation to the micronutrient deficient diets ameliorated most of the above effects of folic acid and vitamin B_{12} deficiency.

4.3.1. Oestrous Cycle and Reproductive Performance Data:

4.3.1.1. Oestrous Cycle:

The reproductive cycle of female rats is called as oestrous cycle, having four stages viz pro-oestrus, oestrus, metoestrus and dioestrus (Long and Evans, 1922). In order to determine the stages of the oestrous cycle, vaginal smear is viewed at low microscopic magnification (reviewed by Freeman, 1994). These phases are usually identified according to cell types observed in vaginal smears. Each phase of oestrous cycle is characterized based on the proportion among three types of cells observed in the vaginal smear: epithelial cells, cornified cells and leukocytes (reviewed by Marcondes et al. 2002). The various phases of the oestrous cycle are briefly described below.

Pro-oestrus Phase:

The pro-oestrus phase lasts for about 12-14 hours and is characterized by round nucleated cells of uniform size, reduction in the leukocytes and the presence of keratinization (Long and Evans, 1922; Freeman, 1994). During this stage, there is a presence of 9-12 layers of cells on the vaginal epithelium, with the mature cells at the surface.

Oestrus Phase:

The next stage, oestrus, lasts for about 25-27 hours. This stage is characterized by the abundant keratinization of the epithelium, absence of leukocytes and the appearance of irregularly shaped, un-nucleated cornified cells (Long and Evans, 1922; Freeman 1994). Physiologically, during this phase there is maximum estrogenic stimulation within the tissue.

Metoestrus Phase:

Metoestrus phase lasts for about 6-8 hours and is characterized by the diminishing keratinization and the appearance of nuclei cells and leukocytes. The leukocytes infiltrate (113-fold greater as compared to pro-oestrus) the thinned vaginal epithelium owing to a decline in estrogen secretion and pass into the vaginal canal (Montes and Luque, 1988). Vaginal secretions taken during this phase make the smear appear white and opaque (Long and Evans, 1922).

Dioestrus Phase:

The dioestrus phase lasts for about 55-57 hours and is marked by the predominance of leukocyte infiltration (16 fold greater as compared with pro-oestrus) and nucleated cells (Freeman, 1994). During this phase, the epithelium reaches its thinnest point (4-7 layers), followed by the cessation of degenerating epithelium and the height of the epithelium increases again because of mitosis (Long and Evans, 1922).

The irregularity of the oestrous cycle is characterized by being in the same phase over a period of 4–5 days. Cycles that do not follow the sequence pro-oestrus, oestrous, metoestrus and dioestrus are considered irregular (Marcondes et al. 2002). In the present study, rats in the vitamin B_{12} deficient group were found to be in the pro-oestrous stage for 4 days, as determined by vaginal smears. Other studies have reported a similar alteration of the oestrous cycle in rats following food restriction (Tropp and Markus, 2001). Early studies by Guilbert and Goss (1931) suggested that protein restriction causes the cessation of oestrus or results in a long and irregular oestrous cycle in rats. Furthermore, energy restriction in addition to intense physical training has been reported to alter the oestrous cycle in rats (Dos Santos et al. 2011). In rhesus monkeys, irregular menstrual cycles are observed when the monkeys consume a folate-restricted diet (Mohanty and Das, 1982). In humans, prolonged vitamin B_{12} deficiency has been reported to result in infertility by possibly changing ovulation or the development of the ovum, or as a result of changes that lead to defective implantation (Bennett, 2001).

The results of the present study have shown that folic acid and vitamin B_{12} deficiencies can both reduce the number of oestrous cycles and alter the phases of the oestrous cycle in rats. A decrease in the reproductive cycle has been reported to reduce the ovulatory period, which may eventually lead to a reduction in fertility in

mice (Nah et al. 2011). Thus, negative effects on the oestrous cycle are suggestive of negative effects on the reproductive health of animals (Oluyemi et al. 2007). The results of the present study, indicating poor reproductive performance in terms of reduced litter weight in the BD group, could be attributed to a disturbance of the oestrous cycle in that group.

Omega-3 fatty acid supplementation of the vitamin deficient groups in the present study restored the oestrous cycle to that seen in control rats. This effect may be due to EPA, an omega-3 fatty acid. This fatty acid is a precursor for eicosanoids such as prostaglandins, thromboxanes, leukotrienes and hydroxy fatty acids, which are important regulators of reproductive processes (reviewed by Sales and Jabbour, 2003). Prostaglandin injections to cows have been shown to synchronise the oestrous cycle by regressing the corpus luteum (Cavalieri et al. 2005). In addition, omega-3 fatty acid supplementation is known to improve reproductive function and fertility in ewes and cows (Burke et al. 1996; Staples et al. 1998).

Studies from our department have reported that folate, vitamin B_{12} and omega-3 fatty acid are interlinked in the one carbon cycle in both humans and Wistar rats. Specifically, we have demonstrated adverse effects of vitamin B_{12} deficiency in terms of increased oxidative stress, reduced gastric milk fatty acids and reduced placental global methylation levels can be ameliorated by omega-3 fatty acid supplementation (Kulkarni et al. 2011c; Dangat et al. 2011; Roy et al. 2012). The results of the present study further demonstrate the beneficial effects of omega-3 fatty acids in improving the oestrous cycle in the absence of maternal micronutrients, such as folic acid and vitamin B_{12}.

4.3.1.2. Histology of Mammary Gland:

During pregnancy, DHA and ARA cross the placenta to the fetus, whereas postnatally these fatty acids are supplied through breast milk (reviewed by Crawford, 2000). The mammary gland is the organ that delivers essential nutrients, in the form of rich proteinaceous and lipid fluid termed ‘milk’, to the newborn offspring. The initial formation of the mammary bud and primitive mammary epithelial tree occurs during the fetal life. The development of these structures appears dependent on the oestrogenic environment of pregnancy (Anbazhagan et al. 1991). In the mammary gland, the major development during puberty is the formation of ducts that will eventually convey milk to the nipple (Prall et al. 1998). With each oestrous cycle,

cyclic proliferation and involution occurs, as a result of which there is side-branching of the ducts and development of alveoli (reviewed by Brisken and O'Maley, 2010).

Our department has previously reported that maternal micronutrients like folate and vitamin B_{12} play a key role in determining both the quantity and quality of milk (Dangat et al. 2011). Breast development is a multistep process characterized by complex mesenchymal-epithelial interactions. In the present study the absence of lactating ducts in mammary glands in the BD group indicates that vitamin B_{12} deficiency may result in altered breast development. This alteration in morphology may have contributed to the reduced litter weight of the pups in the BD group.

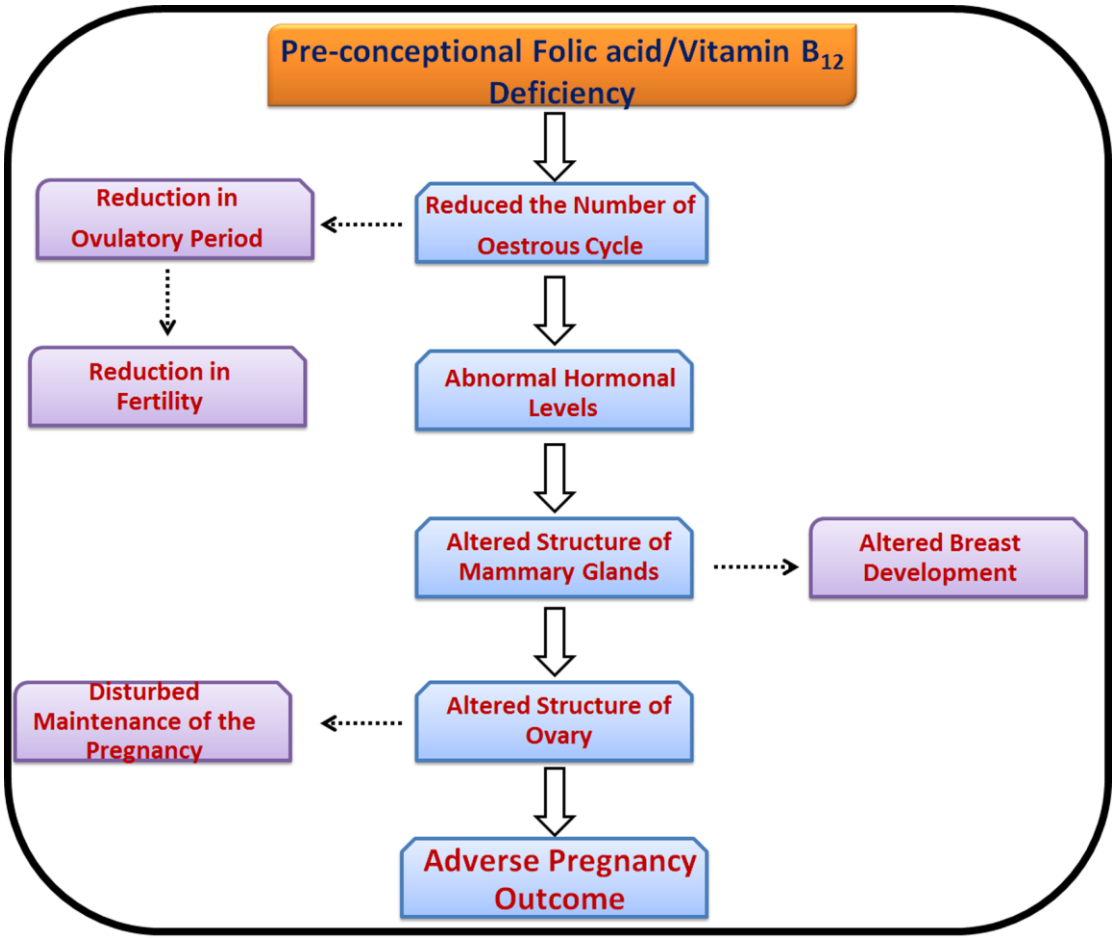
4.3.1.3. Histology of Ovary:

In the present study, folic acid deficiency resulted in changes to the structure of the ovary. The ovarian follicle plays an important role in the maturation and release of a fertilizable oocyte, and promotes and maintains embryo implantation (reviewed by Channing et al. 1978). The corpus luteum plays a central role in the regulation of the oestrous cycle, as well as in the maintenance of pregnancy, largely via progesterone (reviewed by Stocco et al. 2007). In the present study, the number of corpora lutea was reduced and progesterone levels were increased in the FD group. The increased progesterone levels are consistent with the findings reported by Kechrid et al. (2006), who found increased progesterone levels after feeding rats a zinc-deficient diet during pregnancy. In the present study, omega-3 fatty acid supplementation to the folic acid deficient diet increased the number of corpora lutea in the rats fed this diet (FDO group). In cattle, fish meal supplementation has been shown to increase the luteal omega-3 fatty acid content and reduce the available ARA content, the precursor for prostaglandin F2a, resulting in increased fertility (White et al. 2012).

Some of the limitations of the present study include the fact that differences in the vaginal opening and/or sexual maturity were not taken into account and that the degree of fertility and ductal branching was not evaluated. In addition, quantitative analysis regarding the morphology of mammary glands and ovaries was not undertaken. The results of the present study are qualitative in nature and further studies are ongoing in the laboratory to gain an understanding of the mechanisms involved. Future studies are also planned to undertake quantitative assessment of

morphological changes. Fig 45 shows the effects of pre-conceptional folic acid or vitamin B_{12} deficiency on the reproductive performance in rats.

Fig 45: Effects of Pre-Conceptional Folic Acid or Vitamin B_{12} Deficiency on the Reproductive Performance in Rats



4.3.2. Fatty Acids and Transcription Factors in Placenta:

4.3.2.1. Placental Fatty Acid levels:

In this study, the levels of DHA in the placenta were lower in the folic acid and vitamin B_{12} deficient groups. Our departmental studies demonstrate that the folic acid, vitamin B_{12} and omega-3 fatty acids are interlinked in the one carbon cycle and a deficiency of maternal vitamin B_{12} exclusively during pregnancy leads to a reduction of placental DHA levels (Dangat et al. 2011; Kulkarni et al. 2011c; Wadhwani et al. 2012; Sable et al. 2012; Roy et al. 2012; Wadhwani et al. 2015). In our earlier reports, we extensively discussed the possible mechanisms through which an imbalance or deficiency of micronutrients leads to increased oxidative stress that can be mediated through increased homocysteine (Roy et al. 2012). This increased oxidative stress is

known to result in degradation of LCPUFA (reviewed by Jaeschke et al. 2002; reviewed by Martindale and Holbrook, 2002; reviewed by Videla et al. 2004). Alternatively, the reduced DHA can be a result of reduced expression of the phosphatidyl ethanolamine methyl transferase gene that catalyzes the conversion of phosphatidyl ethanolamine to phosphatidyl choline as previously reported by our department (Kale et al. 2010; Kulkarni et al. 2011c).

Further, we also observed decreased levels of ARA in the omega-3 fatty acid supplemented groups in both dam erythrocytes and placenta. This may be because an increase in one fatty acid (i.e., DHA) leads to a decrease in the levels of other fatty acids such as ARA as these fatty acids balance each other in membrane phospholipids (Nevigato et al. 2012).

4.3.2.2. Placental PPARγ Gene Expression:

LCPUFA and their metabolites such as ARA, EPA and prostaglandins are some of the ligands that activate PPAR (reviewed by Sampath and Ntambi, 2004). Reports indicate that DHA is the more preferred ligand as it is bulkier and fits into the long and large hydrophobic ligand binding pocket of PPAR (reviewed by Itoh and Yamamoto, 2008). It is known that in the brain DHA binds to retinoid X receptors (a transcription factor) that heterodimerize with PPARs and influences retinoid X receptor mediated transcription (Lengqvist et al. 2004). Further, maternal protein restriction during pregnancy in rats has also been shown to alter the expression of PPARγ (Burdge et al. 2004; Lillycrop et al. 2005).

In the current study, we found that maternal vitamin B_{12} reduces the expression of PPAR in the placenta. A recent report indicates that dams subjected to folate and vitamin B_{12} deficiency during gestation and lactation decrease PPARα expression in the myocardium of weaning rats (Garcia et al. 2011). In contrast, dams fed a diet deficient in folic acid and associated methyl donors during the periconception and early preimplantation periods did not alter the levels of PPARα (Maloney et al. 2013). Further, in the current study, supplementation of omega-3 fatty acids to the micronutrient deficient diet increased the expression of PPARγ in the vitamin B_{12} deficient groups. These results are similar to an earlier study where adipocytes when incubated with DHA increased the cellular adiponectin possibly by a mechanism that involved PPARγ regulation (Oster et al. 2010).

4.3.2.3. Placental PPARγ Protein Levels:

There was no change in the PPARγ protein levels across the different groups. It has been reported that there is a separate and possibly independent regulation of protein translation and stability (Murphy et al. 2004). Reports also suggest that RNA and protein have differences in synthesis time (Pérez-Sepúlveda et al. 2013). Similar variations in the RNA and protein levels have been reported by others for hypoxia inducible factor-1α, vascular endothelial growth factor and superoxide dismutase genes (Bruells et al. 2013; Poisson et al. 2013). In contrast, others have reported maternal DHA supplementation at normal protein levels in the intrauterine growth retarded rat model increases the levels of PPAR mRNA as well as protein in the neonatal rat lung (Joss-Moore et al. 2010).

4.3.2.4. Dam Plasma MDA Levels at GD20:

In the current study, micronutrient deficiency increased the levels of MDA, which is the end product of lipid peroxidation. Omega-3 fatty acid supplementation decreased the levels of lipid peroxidation in the folic acid deficient group. It is well demonstrated that a membrane rich in DHA should be exceptionally fluid and a DHA deficient diet results in a brush border membrane with decreased fluidity (reviewed by Stillwell and Wassall, 2003). It is known that omega-3 fatty acids in membrane lipids make the double bonds less available for free radical attack (Applegate and Glomset, 1986). Further, omega-3 fatty acids upregulate gene expression of antioxidant enzymes and downregulate genes associated with production of reactive oxygen species (Takahashi et al. 2002). Recent studies in rats have also shown that maternal omega-3 PUFA supplementation reduces isoprostanes, a marker of oxidative damage; and enhances placental and fetal growth (Jones et al. 2013a). Maternal omega-3 PUFA supplementation also increases the labyrinth zone mRNA expression of antioxidant enzymes such as catalase and superoxide dismutase (Jones et al. 2013b). Thus, the increased maternal plasma oxidative stress results in reduced placental DHA levels that alter PPAR regulation.

4.3.2.5. Placental IL-6 and TNF-α Levels:

In this study, deficiency of micronutrients such as folic acid and vitamin B_{12} from preconception through pregnancy results in increased levels of placental IL-6 and TNF-α. Therefore, we propose that the increased maternal oxidative stress results in the reduced DHA levels in the placenta, further affecting the PPARγ regulation in

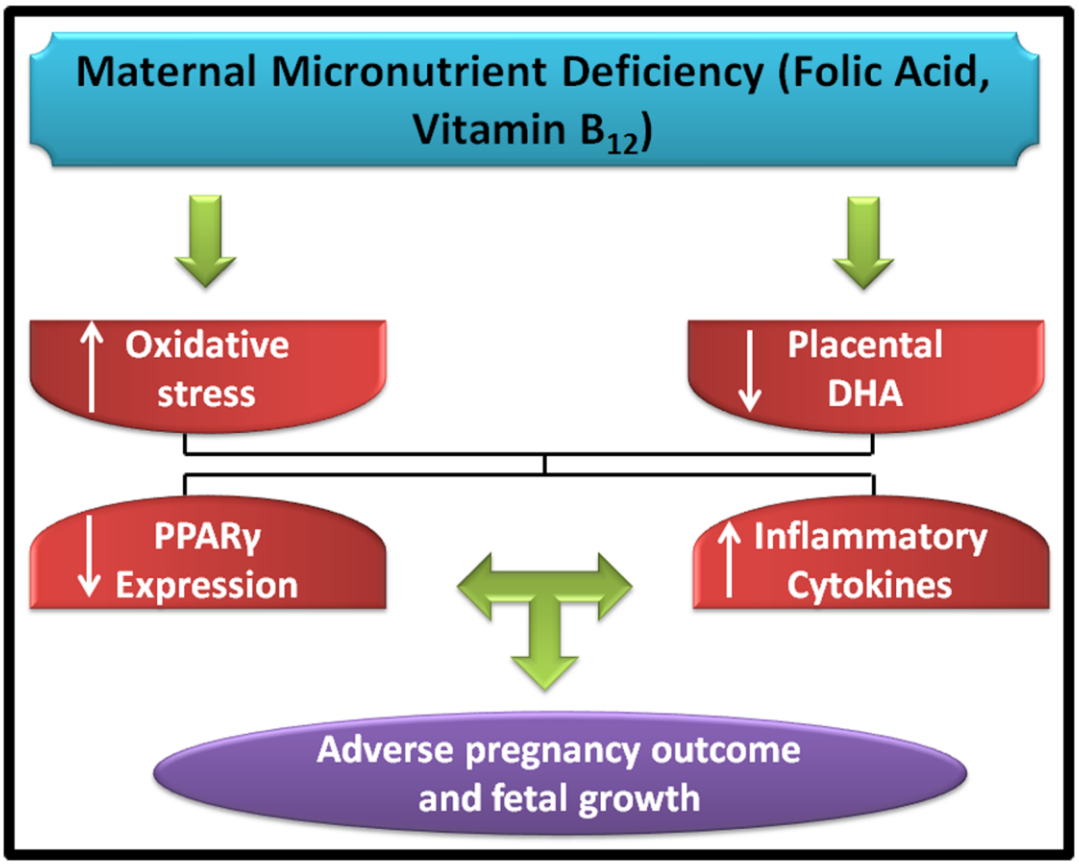
the placenta and increasing the levels of pro-inflammatory cytokines. A recent report indicates that in myeloid cell lines, inhibition of PPAR upregulates different pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α (Wu et al. 2012). Studies in mice fed choline deficient diets during pregnancy have been shown to be associated with adverse reproductive outcomes because of a decrease in maternal PPARα expression in the liver and an increase in inflammatory cytokines (Mikael et al. 2012).

The current study examined how maternal micronutrient deficiency affects the pregnancy outcome and, therefore, measured the oxidative stress and inflammatory markers that is representative of abnormal physiological changes in the placenta. Further, reports indicate that there is no transfer of proinflammatory cytokines, TNF-α, IL-1β and IL-6 across the placenta in either the maternal or fetal direction (Aaltonen et al. 2005). Therefore, it is likely that the levels of inflammatory markers in the placenta are possibly of placental origin. Nevertheless, future studies need to examine TNF-α and IL-6 mRNA levels from total placental RNA.

Additionally, supplementation with omega-3 fatty acids was beneficial in bringing the IL-6 and TNF-α levels back to normalcy. DHA as well as PPAR are extensively studied for their anti-inflammatory activity and proresolving mechanisms in animals (reviewed by Wang and Wan, 2008; Rogers et al. 2011; Jones et al. 2013b). In the current study, although there was an increase in both pro-inflammatory cytokines and MDA, the magnitude of change was different. It may be possible that as MDA represents the oxidative stress contributed by lipid peroxidation and the inflammatory markers (TNF-α, IL-6) are the markers of systemic inflammation, their magnitude of change may not correlate with each other. Thus, in the present study, an adequate amount of DHA may have led to the activation of PPARγ in the omega-3 fatty acid supplemented groups which in turn decreased the levels of inflammatory cytokines such as IL-6 and TNF-α (Fig 46).

The current study was carried out in rats which, like human beings, exhibit a highly invasive type of placental development and is considered an appropriate model for studying the mechanisms of placentation (reviewed by Fonseca et al. 2012). However, structurally the human and rat placenta differ in the number of trophoblastic layers that separate maternal and fetal endothelium (Ramsey, 1982). Further, others suggest that the rat is not a good model to study placental blood flow in humans as it has lower number of spiral arteries (Teklenburg et al. 2010).

Fig 46: Possible Mechanism of Maternal Micronutrient Deficiency Leading to Reduced Placental PPARγ Expression



***PPAR**, Peroxisome proliferator activated receptor; **DHA**, Docosahexanoic acid*

One limitation of this study is that the data for placenta were reported for whole placenta with no distinction between the placental zones. Therefore, future studies need to be carried out to examine the effect of maternal micronutrient deficiency and omega-3 fatty acid supplementation on the expression of PPAR in the different zones of placenta and the subsequent implications in the fetal development.

4.3.3. Transcription Factors in Dam Liver at GD20:

4.3.3.1. PPAR Expression in Dam Liver at GD20:

In the current study, the expression of PPAR was lower in the dam liver in the micronutrient deficient groups. The possible reason for decrease in the PPAR expression in the micronutrient deficient groups may be due to lower levels of DHA and ARA in these groups. DHA is known to act as a natural ligand for activating nuclear transcription factors like PPARs (reviewed by Itoh and Yamamotto, 2008). Dietary folate and vitamin B_{12} are known to provide methyl groups to the liver. These

methyl groups are essential for the release of DHA stored in the liver. Thus the dietary deficiency of folate and vitamin B_{12} will affect the levels of DHA in the liver (Umhau et al. 2006). Further, we have reported that alterations in the maternal micronutrients like folic acid and vitamin B_{12} exclusively during pregnancy reduce levels of LCPUFA and alter the activity and expression of fatty acid desaturases in the dam liver at end of pregnancy (Wadhwani et al. 2012). Similar relationship has been described in one of the recent study where plasma DHA was lowest in rats consuming the B vitamin (folate, vitamin B_{12} and vitamin B_6) poor diet (van Wijk et al. 2012). Also, some of the earlier studies have shown that rats fed 6 weeks on a low folate diet had decreased concentrations of DHA in plasma and platelets (Durand et al. 1996) and dietary folate deficiency has been found to cause the depletion of DHA in rat nervous tissue (Hirono and Wada, 1978).

During late gestation, it is known that maternal liver plays a central role in whole body lipid homeostasis (Ruyle et al. 1990) and also have major consequences for fetal growth (Napoli et al. 1997). Further, PPAR is known to be a master transcriptional regulator of lipid and carbohydrate metabolism in liver (reviewed by Lefebvre et al. 2006). They are also known to regulate the expression of several genes involved in metabolic processes that are potentially linked to the development of some diseases such as hyperlipidemia, diabetes and obesity (reviewed by Contreras et al. 2013). Also, there are reports where protein restriction during pregnancy leads to changes in maternal lipid metabolism which may negatively impact normal fetal brain development (Torres et al. 2010). In the current study, the birth weight of the offspring was lower in the micronutrient deficient groups. Thus, the observed lower expression of PPARs in the maternal liver may affect the lipid metabolism in the dam which may be responsible for adverse birth outcome and the subsequent risk for metabolic disorders and cardiovascular disease in the adults.

Further, omega-3 fatty supplementation to these micronutrient deficient diets increased the expression of PPARs in the dam liver. Both DHA and EPA levels were found to be increased in both the omega-3 fatty acid supplemented groups. Reports have shown that EPA rich oil as well as purified EPA activated human PPARα and PPARγ (Gillies et al. 2012). Further, dietary supplementation with omega-3 fatty acids was also shown to trigger the activation of PPARα in the rat model for liver ischemia-reperfusion injury (Zúñiga et al. 2011). The activity of PPARα is also reported to be increased by DHA in HepG2 cells (Kuang et al. 2012).

4.3.3.2. HNF-4α Expression in Dam Liver at GD20:

Another nuclear transcription factor i.e. HNF-4α was found to be similar in all the treatment groups. HNF-4α, PPARα and PPARγ are known to play a fundamental role in the coordinated expression of genes involved in fatty acid transport and metabolism (Martinez-Jimenez et al. 2010). Our results are in contrast with earlier reported study where combined deficiency of folate and vitamin B_{12} in the mice during pregnancy and lactation leads to lower protein expression of HNF-4α. This study suggests the link between methyl donor deficiency and epigenomic deregulation of energy metabolism due to impaired mitochondrial fatty acid oxidation through PPAR-α, estrogen-related receptor alpha (ERR-α) and HNF-4α (Pooya et al. 2012). In cultured mammalian cells, previously it was shown that saturated fatty acyl CoAs act as agonistic ligands for HNF-4α, while unsaturated fatty acyl CoAs act as antagonistic ligands (Hertz et al. 1998). However, a recent review by Georgiadi and Kersten (2012) suggest that the binding and activation of HNF-4α by fatty acids or acyl-CoAs remains controversial (reviewed by Georgiadi and Kersten, 2012). Our results are consistent with the earlier reported study where no induction of HNF-4α targets by unsaturated fatty acids was observed in a human colon cancer cell line (Yuan et al. 2009).

4.3.4. Fatty Acids and Transcription Factors in Pup Liver at Postnatal Day 22:

4.3.4.1. Pup Liver Gene Expression at Postnatal Day 22:

In the current study, the expression of PPARα and PPARγ genes in the pup liver was significantly lower as a consequence of maternal micronutrient deficiency. Studies have reported that dietary manipulation in pregnant rats alters the hepatic PPARα gene expression in the offspring (Lillycrop et al. 2005; Burdge et al. 2007; Mikael et al. 2012). It is well known that dietary PUFAs regulate the abundance of these nuclear transcription factors (reviewed by Bordoni et al. 2006). Thus, it may be possible that the decrease in the levels of DHA and ARA may be associated with the lower expression of PPARs in the pup liver. Fatty acid metabolism can be altered by desaturation or elongation reactions, which mainly occur in the liver (Wang et al. 2005). Our earlier departmental study reported that maternal micronutrients like folic acid and vitamin B_{12} altered the levels of LCPUFA, activity and expression of fatty acid desaturases in the dam liver at end of pregnancy (Wadhwani et al. 2012). Thus,

one of the possible reasons for the decrease in the DHA and ARA levels is the insufficient conversion of ALA to DHA and LA to ARA, since we have observed increased ALA and LA in both the micronutrient deficient groups.

Omega-3 fatty acid supplementation to the micronutrient deficient diet normalized the expression of PPARγ in both BD and FD groups while PPARα in BD group. These results are consistent with the earlier report which suggests that dietary LCPUFAs activate PPARα and PPARγ by increasing lipid oxidation (reviewed by Rodrı´guez-Cruz et al. 2005). Maternal DHA supplementation has also been reported to normalize intrauterine growth restriction induced visceral PPARγ expression in the male rats (Bagley et al. 2013). This interaction of PPARs with their ligands at the time of fetal development is important for the adaptation of long term lipid metabolism (reviewed by Rees et al. 2008).

There was no change in the expression of pup liver SREBP-1c, LXRα and RXRα in the micronutrient deficient groups while omega-3 fatty acid supplementation decreased the expression of these transcription factors. This may be due to the fact that the omega-3 and omega-6 LCPUFAs are feed-forward activators of PPARs, while these same fatty acids are feedback inhibitors of LXRs and SREBPs (reviewed by Jump, 2002). These transcription factors are interdependent and the amount of these nuclear receptors in the nucleus and the concentrations of their ligands are the important factors in determining the overall effect (reviewed by Benatti et al. 2004). Cell line studies have demonstrated that hepatic PPARα activation suppresses the LXR mediated expression of SREBP-1c and this inhibitory effect is enhanced by the addition of PPAR ligands (Yoshikawa et al. 2001). Similar effect was also seen due to decreased amount of LXR/RXR heterodimers and enhanced binding of PPARα to RXR (Yoshikawa et al. 2003). Thus, the coordinated relationship between these transcription factors is critical for the regulation of downstream genes involved in the fatty acid metabolism.

4.3.4.2. Average Pup Weight at Postnatal Day 22:

In the current study, omega-3 fatty acid supplemented group had a lower birth weight. This may be because these omega-3 fatty acids are known to increase the lean mass of the body (Murphy et al. 2011; reviewed by Murphy et al. 2012; McDonald et al. 2013). Further, omega-3 polyunsaturated fatty acids have also been reported to elicit a number of effects such as improvements in circulation which might facilitate

nutrient delivery to skeletal muscles and changes in gene expression which shift metabolism towards increased accretion of lean tissue, enhanced fat oxidation and energy expenditure and reduced fat deposition (reviewed by Buckley and Howe, 2010).

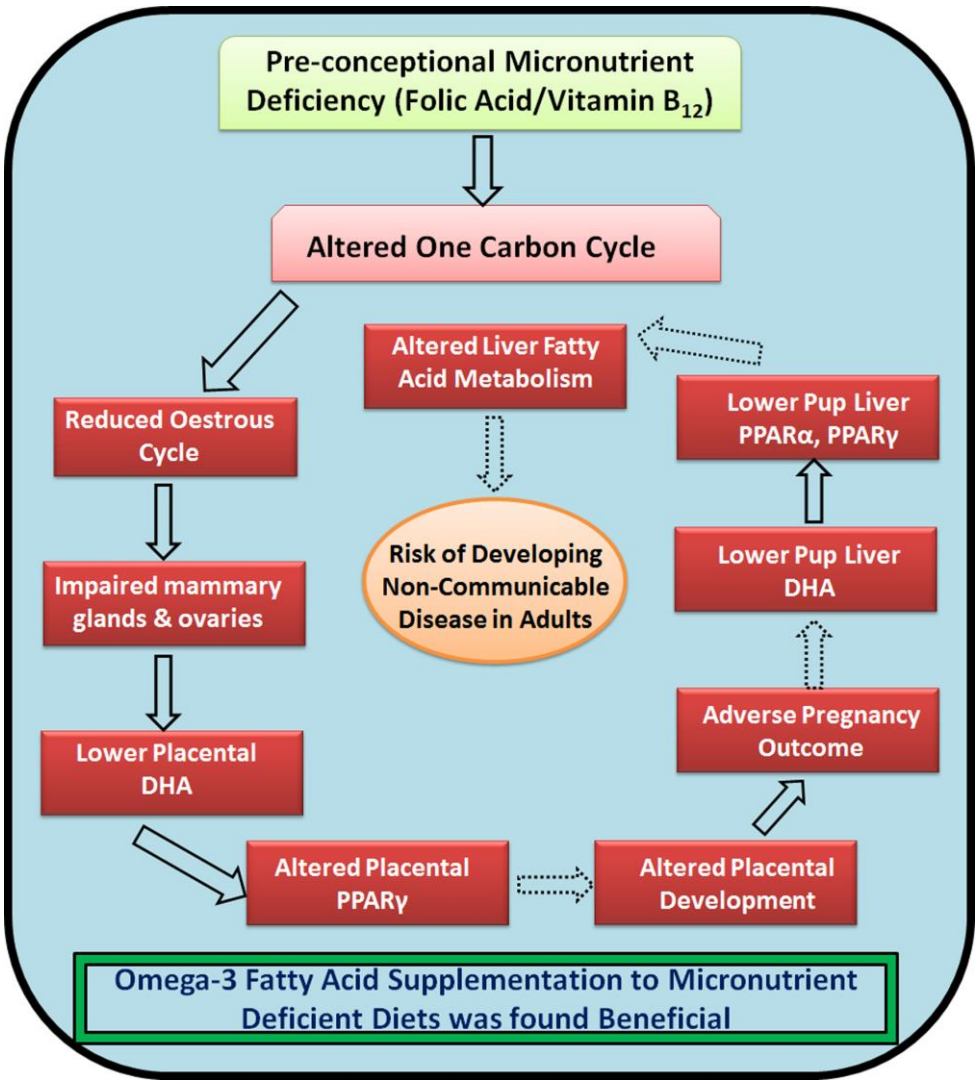
4.3.4.3. Pup Liver Global DNA Methylation Levels at Postnatal Day 22:

A recent report indicates that changes in maternal dietary patterns influence the metabolic homeostasis in the liver and thereby play a central role in fetal programming (Maloney et al. 2013). In the current study, we observed higher global DNA methylation levels in the pup liver in both the micronutrient deficient groups. The levels of methylation were similar to those reported by us earlier where we have shown that altered micronutrients like folic acid and vitamin B_{12} during pregnancy cause global hypo DNA methylation in the placenta (Kulkarni et al. 2011c). Folate and vitamin B_{12} are known to regulate the formation of SAM molecule which is the universal methyl donor for most of the reactions and any disturbance in the one carbon cycle affects cell proliferation and gene expression (reviewed by Kalhan and Marczewski, 2012). Reports indicate that altered methyl nutrition during peri-conception and early preimplantation development modifies the patterns of DNA methylation in adult tissues (reviewed by Waterland and Michels, 2007; reviewed by Sinclair et al. 2007). Further, there are contradictory reports regarding the effect of methyl donor (methionine, folate, vitamin B_{12} and choline) deficiency on the global DNA methylation status. Some reports suggest that these deficiencies lead to global DNA hypomethylation in various tissues like brain, liver, leukocyte (Rampersaud et al. 2000; reviewed by Niculescu and Zeisel, 2002; Kim et al. 2013) while others suggest that folic acid deficiency during pregnancy has no change in the global DNA methylation in the rat offspring (Maloney et al. 2007).

A recent study has also shown that maternal omega-3 fatty acid availability during pregnancy and lactation alters the epigenetic status of fatty acid desaturases 2 (Fads2) in both mother and its offspring (Niculescu et al. 2013). Thus altered expression of PPAR, SREBP-1c, LXRα and RXRα genes in the present study may possibly be due to low levels of pup liver DHA and altered promoter DNA methylation of these genes. Further, studies are being carried out in our laboratory to examine gene specific methylation changes in these transcription factors. The current study has some limitations like the interaction between the gender and the diet has not

been studied. In this study, the degree of fertility in various groups was not evaluated. Nevertheless, we have demonstrated that, the adverse effects of micronutrient deficiencies resulted in abnormal oestrous cycle which could be normalized by omega-3 fatty acid supplementation. There is also a need to understand the effects of maternal micronutrient deficiency and omega-3 fatty acid supplementation on other transcription factors like hepatic nuclear factor-4α, nuclear factor kβ, glucocorticoid receptor involved in the hepatic fatty acid, cholesterol, carbohydrate and bile acid metabolism in the offspring. This will provide an insight into the role of one carbon cycle and its interaction with omega-3 fatty acids in the fetal lipid metabolism and the subsequent risk for the metabolic syndromes in the adults. The results of this animal study are summarized in Fig 47.

Fig 47: Summary of the Effects of Pre-Conceptional Maternal Folic Acid or Vitamin B_{12} Deficiency in the Placenta and Offspring Liver



PPAR, *Peroxisome proliferrator activated receptor;* ***DHA,*** *Docosahexanoic acid.*

4.3.5. Conclusion:

The results of the present study have demonstrated that maternal vitamin B_{12} and folate deficiency alters the oestrous cycle. Supplementation with omega-3 fatty acid to the deficient diets restored the number and phases of the oestrous cycle. These findings are of significance because the nutritional intake of women of child-bearing age has been suggested to be inadequate, especially during the preconceptional period (de Weerd et al. 2003; Mouratidou et al. 2006; Ronnenberg et al. 2007). Therefore, supplementation with omega-3 fatty acids in addition to micronutrients may help regulate the menstrual cycle, ultimately leading to improved fertility. However, there is a need for future studies to examine the molecular and epigenetic changes as a consequence of omega-3 fatty acid supplementation to these micronutrient deficient diets to delineate the time-dependent and/or prospective molecular mechanisms.

Our findings demonstrate that maternal micronutrient deficiency leads to increased oxidative stress in the mother and results in lower levels of DHA, a lower expression of PPAR and an increase of inflammatory cytokines. This study also demonstrates that maternal folic acid or vitamin B_{12} deficiency alters the expression of hepatic transcription factors in the dam as well as pup liver. The current animal study indicates that maternal omega-3 fatty supplementation to a micronutrient deficient diet was able to ameliorate the negative effects of a micronutrient deficient diet.

We and others have demonstrated that oxidative stress and placental inflammation can lead to preterm or low birth weight babies (Joshi et al. 2008; Raunig et al. 2011; reviewed by Dhobale and Joshi, 2012). Our findings are of significance as the development of placenta plays a key role in maternal-fetal transfer and thereby determines fetal growth. This may have implications in countries such as India where the majority of the population is vitamin B_{12} deficient due to vegetarian food habits and mothers are most likely to deliver low birth weight babies that are at a higher risk of developing non-communicable diseases such as diabetes in later life (Yajnik et al. 2008). Further, studies examining the epigenetic regulation of PPAR genes in the human placenta will throw more light into the mechanism leading to fetal programming of adult diseases.

Therefore, on the basis of the above study it is clear that omega-3 fatty acids before and during pregnancy are the important determinants of fetal growth. In view of this, the next section describes a human study which examines the association of maternal fatty acids from early pregnancy with cord fatty acids and birth outcome. Further, the levels of placental fatty acids as well as the placental gene expression of the transcription factors are also examined.

HUMAN STUDY

5.1. Materials and Methods:

The current study is a longitudinal study where pregnant women were enrolled from the Department of Obstetrics and Gynecology, Bharati hospital Pune, India. This study was approved by the Bharati Vidyapeeth Medical College Institutional Ethical Committee (Ref No.: BVDU/MC/02) and written consent was taken from each subject at the time of enrollment.

5.1.1. Study Design:

The current study is a part of an ongoing prospective departmental study which recruits all healthy women at 16^{th}-20^{th} week of gestation and follows them throughout pregnancy. The site of recruitment of women was different from the site of experimental procedures carried out on the samples collected. Subjects included in this study were only those women with singleton pregnancy, delivering at term (total gestation $\geq$ 37 weeks) and having no medical or obstetric complications. At delivery, these women were divided into 2 groups; those delivering at term a baby weighing >2.5kg [Normal birth weight (NBW) group] and those delivering at term a baby weighing <2.5kg [Low birth weight (LBW) group].

The first sample was obtained between 16^{th}- 20^{th} weeks of gestation (T1), the second between 26^{th}- 30^{th} weeks of gestation (T2) and the third sample was taken just before the delivery (T3). Umbilical cord blood and placenta was also collected. The study includes data on 60 women delivering at term NBW babies and 51 women delivering at term LBW babies.

All study participants neither consumed alcohol nor smoked. All women were routinely given iron (60 mg) and folic acid (500 µg) tablets during the first trimester of pregnancy as per the National Anemia Prophylaxis Program. The gestational age in the current study was determined by last menstrual period and ultrasound examination. It is suggested that ultrasound is an accurate and useful modality for the assessment of gestational age in the first and second trimester of pregnancy (Kalish et al. 2004). Cases with a discrepancy of more than a week between clinical and ultrasound readings were excluded from the study.

5.1.2. Demographic and Anthropometric Measures*:*

Maternal characteristics such as maternal age and income were recorded at the time of recruitment. Clinical information like BMI, systolic and diastolic blood

pressure and gestation were recorded at various time points i.e. T1 (16^{th}-20^{th} week of gestation), subsequently at T2 (26^{th}-30^{th} week of gestation) and at the time of delivery. Placental weight was also recorded after delivery.

5.1.3. Inclusion Criteria:

All women with singleton pregnancy between 18-35 years of age were included in the study.

5.1.4. Exclusion Criteria:

Pregnant women with medical problems like multiple gestation, chronic hypertension, type I or type II diabetes mellitus, seizure disorder, preeclampsia, gestational diabetes, renal or liver disease and anemia were excluded from the study. Alcohol or drug abuse in the pregnant women was also considered an exclusion criterion.

5.1.5. Dietary Assessments:

Pregnant women were administered a food frequency questionnaire during T1, T2 and at delivery to estimate the frequency of consumption of foods rich in omega-3 fatty acids. All pregnant women had to indicate the frequency of each food consumed during the last one month for which scores were calculated. For example, an item consumed once a week has a score of 4 while that consumed daily has a score of 30. These foods were identified using "Nutritive Values of Indian Foods" (Gopalan, 2003). The food frequency questionnaire has been used by the department in a number of studies on pregnant women (Kilari et al. 2009; Kilari et al. 2010; Kulkarni et al. 2011a).

5.1.6. Statistical Power of the Study:

The statistical power was calculated using the Power and Sample Size (PS) software. There is no data available on fatty acids examined across gestation in women delivering LBW babies. The power for this study was calculated based on our earlier departmental study (Kilari et al. 2011) which showed significant group differences in the levels of maternal erythrocyte DHA at the end of pregnancy from 29 women delivering LBW babies and 75 women delivering NBW babies ($p<0.05$). The current study included 60 women delivering NBW babies and 51 women

delivering LBW babies and will give an 80% probability of detecting a difference at an alpha of 0.05.

The statistical power for the placental fatty acids was based on our earlier departmental study (Wadhwani et al. 2014) which showed significant group differences in fatty acid levels between 84 women with normotensive pregnancy and 39 women with preeclampsia. Thus, with such an effect size, placental fatty acid levels were analyzed from 38 women delivering NBW babies and 36 women delivering LBW babies and will give an 80% probability of detecting a difference at an alpha of 0.05.

5.1.7. Sample Collection and Processing:

The subjects as well as the investigator were blinded to the samples being processed.

Maternal and Cord Blood

Blood sample (10mL) was collected from the subjects into EDTA tubes. All blood samples were immediately layered on histopaque (Sigma-Aldrich, St Louis, MO, USA) and centrifuged at 2000 rpm for 30 min to separate the plasma and erythrocytes. The erythrocyte fraction was washed 3 times with normal saline. Plasma and erythrocytes were stored at -80°C until further analysis.

Placental Tissues

Placental tissues were collected after delivery of the neonate. Fetal membranes of the placenta were trimmed off and small pieces were randomly cut out from different regions of the placental cotyledon. The tissue pieces were rinsed in phosphate buffer saline (PBS) to wash off maternal and fetal blood. Placental tissues were stored at -80ºC until assayed.

5.1.8. Fetal Growth Measures:

Baby weight, length, head and chest circumference were recorded within half an hour after birth. Birth weight was recorded using a digital weighing scale (Zeal medical private limited, India) with an accuracy of 10gm. The length was measured to the nearest 0.1cm using a portable infantometer. The head and chest circumference was measured using a fiber glass measuring tape which was placed around the head, just above the eyebrows anteriorly and around the most prominent bulge posteriorly.

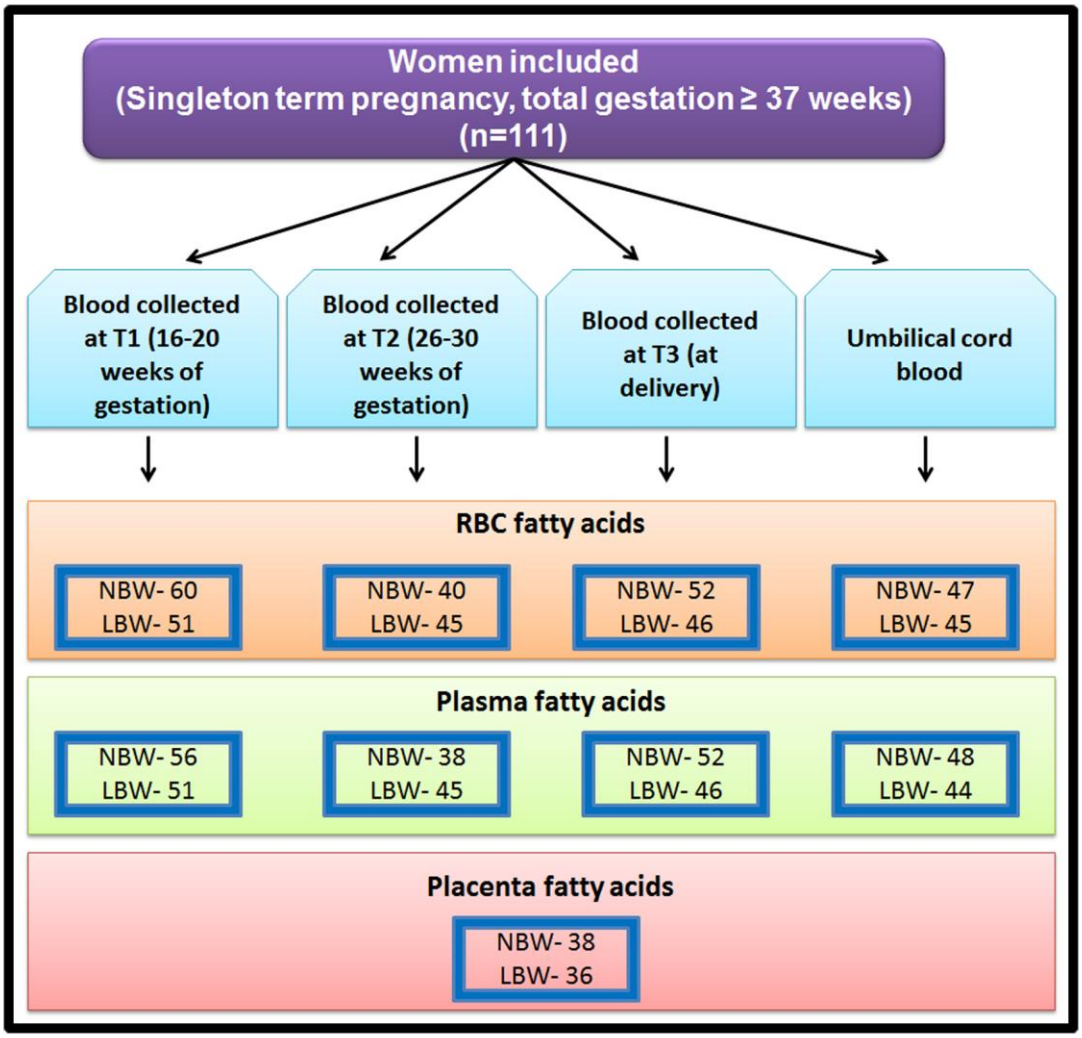
The chest circumference was measured using fiber glass measuring tape which was placed around the lower chest. These procedures have been described in our earlier study (Sundrani et al. 2013).

5.1.9. Plasma, Erythrocyte and Placental Fatty Acid Analysis:

Fatty acids were analyzed from the total plasma, total erythrocyte and placental samples using gas chromatography as described in the chapter 4 (section 4.1.9). The method is also described by us earlier (Mehendale et al. 2008; Kilari et al. 2009; Dangat et al. 2010; Kale et al. 2010; Kulkarni et al. 2011a).

The number of maternal and cord plasma, erythrocyte and placental samples analyzed for fatty acid levels at various time points during pregnancy is shown in Fig 48.

Fig 48: Flow Chart Showing Number of Maternal and Cord Plasma, Erythrocyte and Placental Samples Analyzed for Fatty Acid Levels at Various Time Points



NBW: *Normal Birth Weight;* ***LBW:*** *Low Birth Weight*

5.1.10. RNA Isolation and cDNA Synthesis:

The RNA isolation from the placenta tissue and cDNA synthesis was carried out as described in chapter 4 (section 4.1.11).

5.1.11. Placental mRNA Levels of Transcription Factors:

qRT-PCR for placental PPARα, PPARγ, SREBP-1c, LXRα, RXRα, RXRγ and GAPDH genes were performed as described in chapter 4 (section 4.1.12). The following TaqMan® Gene Expression Assays (Applied Biosystems) were used in this study: GAPDH (Hs99999905_m1); PPARα (Hs00947536_m1), PPARγ (Hs01115513_m1), SREBP-1c (Hs01088691_m1), LXRα (Hs00172885_m1), RXRα (Hs01067640_m1), RXRγ (Hs00199455_m1).

5.1.12. Statistical Analysis:

The data were analyzed using the SPSS/PC+ package (Version 20.0, Chicago, IL, USA). Values are reported as mean ± SD for biochemical analysis like fatty acid estimation and mean ± SE for mRNA levels of the transcription factors. Normal distribution of the data was checked by testing for skewness and kurtosis. Skewed variables were transformed to normality using the log to the base 10. Mean values of various parameters between the NBW and LBW group were compared using independent t test. Mean values of the various parameters within the NBW and LBW group across gestation were compared using one way analysis of variance (ANOVA) and the post-hoc least significant difference (LSD) test. Partial correlation analysis was used to study the association of fatty acids (plasma, erythrocyte and placental) and placental mRNA levels of transcription factors with birth outcome after adjusting for age, BMI and gestational age at the time of blood sampling. The association of cord fatty acids with maternal fatty acids was studied using partial correlation analysis after adjusting for age, BMI and gestational age. Pearson's correlation analysis was used to study the association between the placental fatty acids and placental mRNA levels of transcription factors. Chi-square test was used for comparison of categorical variables. To compare two proportions, Z test of proportions was used.

The variable sample number in different measures was either due to loss of follow up at various time points across gestation or insufficient sample volume available. Results corresponding to p-values lower than 0.05 or 0.01 are described as significant and reported. Statistical analysis was carried out on two sets of data. First

set of data includes all the women who have participated in the study. Second set of data was analyzed in women from whom the parameters were analyzed for all time points as well as in cord. The results of the first set of data have been shown in the figures. However, similar results and trends were observed for the second set of data (data not shown).

5.2. Results:

5.2.1. Maternal and Neonatal Characteristics:

Table 11 shows the demographic characteristics of normotensive mothers and their neonates.

Table 11: Maternal and Neonatal Characteristics

Maternal Characteristics		
	NBW (n=60)	**LBW (n=51)**
Age (yr)	23.82 ± 3.92	22.80 ± 3.19
Income (INR)	13223 ± 13703	8294 ± 6623*
BMI (Kg/m^2)		
T1	21.79 ± 3.55	20.40 ± 3.16*
T2	23.71 ± 3.69	22.25 ± 2.97
T3	25.40 ± 3.70	23.53 ± 3.21*
Gestation (wks)		
T1	19.69 ± 2.59	19.09 ± 2.20
T2	29.67 ± 2.61	29.03 ± 2.11
T3	39.00 ± 1.01	38.51 ± 1.44
Sys BP (mmHg)		
T1	115.82 ± 8.75	116.6 ± 7.60
T2	116.76 ± 7.83	117.49 ± 5.89
T3	119.12 ± 7.53	120.87 ± 8.39
Dias BP (mmHg)		
T1	74.25 ± 5.31	73.15 ± 6.86
T2	73.51 ± 6.33	75.74 ± 6.09
T3	76.96 ± 6.14	76.78 ± 4.78
Placenta Weight (g)	490.08 ± 106.51	410.80 ± 79.08**
Neonatal characteristics		
Baby weight (kg)	2.97 ± 0.29	2.31 ± 0.17**
Baby length (cm)	48.89 ± 2.81	46.01 ± 2.73**
Baby HC (cm)	33.99 ± 1.40	32.35 ± 1.84 **
Baby CC (cm)	32.43 ± 1.74	30.68 ± 1.77**

*Data are expressed as mean ± SD. *p<0.05; **p<0.01 as compared with NBW group.* ***T1*** *= 16th–20th week,* ***T2*** *= 26th–30th week,* ***T3*** *= at delivery.* ***NBW*** *- normal birth weight;* ***LBW*** *- low birth weight;* ***BMI*** *- body mass index;* ***Sys BP*** *- Systolic blood pressure;* ***Dias BP*** *- diastolic blood pressure;* ***HC*** *- head circumference;* ***CC*** *- chest circumference;* ***n*** *- Number of subjects.*

Maternal BMI at T1 and T3 was lower ($p<0.05$ for both) in the LBW group as compared to the NBW group. All the neonatal characteristics including baby weight, length, head and chest circumference were lower ($p<0.01$ for all) in the LBW group as compared to the NBW group.

5.2.2. Frequency of Consumption of ALA, DHA and Total Omega-3 Fatty Acid Rich Foods:

The percent women consuming total omega-3 fatty acid, DHA and ALA rich foods in both NBW and LBW groups is shown in Table 12. In our cohort, the ALA rich foods consumed by the women include pulses (e.g., cowpea, rajma) and green leafy vegetables (e.g., fenugreek, spinach, colocasia leaves, amaranth, ambat chukka) while fish consumption was considered as a DHA rich food.

Table 12: Frequency of Consumption of Foods Rich in ALA, DHA and Total Omega-3 Fatty Acids at Three Time Points during Pregnancy

	T1			T2			T3		
Food Group n (%)	**NBW (n=51)**	**LBW (n=44)**	**p**	**NBW (n=30)**	**LBW (n=37)**	**p**	**NBW (n=46)**	**LBW (n=40)**	**p**
ALA Rich Foods									
Weekly twice	19(37.3)	23(52.3)	0.14	10(33.3)	21(56.8)	0.06	16(34.8)	15(37.5)	0.79
Weekly 3-6 times	24(47.1)	15(34.1)	0.20	16(53.3)	9(24.3)	0.01	16(34.8)	16(40.0)	0.62
Weekly > 6 times	8(15.7)	6(13.6)	0.78	4(13.3)	7(18.9)	0.53	14(30.4)	9(22.5)	0.40
DHA Rich Food									
Never	33(64.7)	28(63.6)	0.91	20(66.7)	24(64.9)	0.88	26(56.5)	25(62.5)	0.57
Weekly once	8(15.7)	12(27.3)	0.17	3(10.0)	8(21.6)	0.20	7(15.2)	10(25.0)	0.26
More than once	10(19.6)	4(9.1)	0.15	7(23.3)	5(13.5)	0.30	13(28.3)	5(12.5)	0.07
Total Omega-3 Fatty Acid Rich Foods									
Weekly twice	16(31.4)	21(47.7)	0.10	10(33.3)	19(51.4)	0.14	13(28.3)	15(37.5)	0.36
Weekly 3-6 times	24(47.1)	16(36.4)	0.29	12(40.0)	10(27.0)	0.26	17(37.0)	14(35.0)	0.85
Weekly > 6 times	11(21.6)	7(15.9)	0.48	8(26.7)	8(21.6)	0.63	16(34.8)	11(27.5)	0.47

***NBW** - normal birth weight; **LBW** - low birth weight; **T1** = 16th-20th week, **T2** = 26th-30th week, **T3** = at delivery. p reported in the table is calculated by Z test.*
***ALA** - alpha linolenic acid, **DHA** - docosahexaenoic acid, **Total Omega-3**: [ALA + eicosapentanoic acid (EPA) + DHA].*

The frequency of consumption of total omega-3 fatty acid and DHA rich foods was similar in both the groups at T1 ($p=0.264$, $p=0.194$ respectively), T2 ($p=0.322$,

p=0.323 respectively) and T3 (p=0.623, p=0.157 respectively). Similarly, the frequency of consumption of ALA rich foods was similar in both the groups at T1 (p=0.326) and T3 (p=0.703). At T2, the frequency of consumption of ALA rich foods was different (p=0.049) between both NBW and LBW groups.

5.2.3. Maternal and Cord Erythrocyte Fatty Acids Levels at Different Time Points in the NBW and LBW Groups:

The maternal fatty acids and cord fatty levels at different time points in the NBW and LBW groups is shown in table 13 and table 14 respectively.

Table 13: Maternal Erythrocyte Fatty Acid Levels (g/100g fatty acids) at Three Time Points during Pregnancy

Maternal Erythrocyte Fatty Acids (g/100g fatty acids)						
	NBW	**LBW**	**NBW**	**LBW**	**NBW**	**LBW**
	T1 (n=60)	**T1 (n=51)**	**T2 (n=40)**	**T2 (n=45)**	**T3 (n=52)**	**T3 (n=46)**
MYR	0.32 ± 0.12	0.31 ± 0.13	0.31 ± 0.12	0.29 ± 0.09	0.34 ± 0.17	0.33 ± 0.13
MYRO	0.07 ± 0.11	0.07 ± 0.16	0.07 ± 0.10	0.05 ± 0.08	0.08 ± 0.14	0.04 ± 0.08
PAL	22.33 ± 1.62	22.33 ± 1.99	22.36 ± 1.66	22.78 ± 1.30	23.44 ± 3.75	24.89 ± 2.24*
PALO	0.19 ± 0.10	0.21 ± 0.19	0.21 ± 0.12	0.21 ± 0.13	0.30 ± 0.18	0.32 ± 0.31
STE	14.98 ± 0.95	15.49 ± 1.40*	14.79 ± 0.72	15.12 ± 1.49	15.04 ± 1.38	15.85 ± 2.23*
OLE	9.37 ± 0.90	9.11 ± 1.02	9.33 ± 0.74	8.93 ± 1.09*	9.63 ± 2.10	9.48 ± 1.47
LA	10.75 ± 1.42	10.79 ± 1.20	10.66 ± 1.52	11.11 ± 2.43	9.80 ± 3.41	10.70 ± 4.07
GLA	0.11 ± 0.10	0.07 ± 0.10*	0.11 ± 0.24	0.05 ± 0.04	0.07 ± 0.07	0.10 ± 0.10
ALA	0.23 ± 0.13	0.24 ± 0.10	0.23 ± 0.15	0.29 ± 0.22	0.24 ± 0.15	0.25 ± 0.12
DGLA	1.63 ± 0.38	1.54 ± 0.40	1.68 ± 0.32	1.65 ± 0.37	1.65 ± 0.47	1.63 ± 0.42
ARA	13.27 ± 1.19	13.82 ± 2.14	12.81 ± 1.07	13.48 ± 1.45*	12.12 ± 2.43	12.59 ± 2.75
EPA	0.50 ± 0.79	0.62 ± 0.84	0.60 ± 0.73	0.65 ± 0.86	0.76 ± 0.92	0.51 ± 0.90*
NA	0.92 ± 0.44	0.95 ± 0.30	1.11 ± 0.43	1.20 ± 0.45	1.25 ± 0.72	1.27 ± 0.66
DPA (n-6)	0.82 ± 0.28	0.72 ± 0.25 *	0.79 ± 0.34	0.69 ± 0.26	0.65 ± 0.35	0.58 ± 0.27
DHA	2.65 ± 0.85	2.41 ± 0.63	2.67 ± 0.80	2.69 ± 0.77	2.55 ± 0.87	2.38 ± 0.82
Omega-3	3.38 ± 1.04	3.27 ± 0.94	3.50 ± 1.01	3.63 ± 1.15	3.55 ± 1.09	3.15 ± 1.15*
Omega-6	26.58 ± 1.87	26.95 ± 2.59	26.05 ± 1.44	26.99 ± 2.13*	24.29 ± 3.16	25.59 ± 3.34*
SFA	37.63 ± 2.05	38.13 ± 3.27	37.46 ± 2.03	38.19 ± 2.30	38.83 ± 4.42	41.07 ±3.54**
MUFA	10.55 ± 1.02	10.33 ± 1.17	10.72 ± 0.94	10.39 ± 1.20	11.26 ± 1.93	11.11 ± 1.49

*Data are expressed as mean ± SD. *p<0.05, **p<0.01 as compared with NBW; **T1**=16th-20th week; **T2**= 26th-30th week; **T3**= at delivery. **NBW** – Normal birth weight; **LBW** – Low birth weight; **n** - Number of subjects.*

***MYR** - Myristic acid; **MYRO** - Myristoleic acid; **PAL** - Palmitic acid; **PALO** - Palmitoleic acid; **STE** - Stearic acid; **OLE** - Oleic acid; **LA** - Linoleic acid; **GLA** - Gamma linolenic acid; **ALA** - Alpha linolenic acid; **DGLA** - Dihomo gamma linolenic acid; **ARA** - Arachidonic acid; **EPA** - Eicosapentaenoic acid; **NA** - Nervonic acid; **DPA**, omega-6 - Docosapentaenoic acid; **DHA** - Docosahexaenoic acid; Saturated fatty acids **(SFA)**: (MYR + PAL + STE); Monounsaturated fatty acids **(MUFA)**: (MYRO + PALO + OLE + NA); O**mega-3**: (ALA + EPA + DHA); O**mega-6**: (LA + GLA + DGLA + ARA + DPA); **n-6** – omega-6.*

At T1, there was no change in the levels of SFA, MUFA, total omega-3 and omega-6 fatty acids and ARA between the two groups. There was a trend towards reduction in the levels of DHA in the LBW group as compared to the NBW group, although it was not statistically significant (Table 13).

At T2, the levels of SFA, MUFA, total omega-3 fatty acids and DHA were similar between the two groups. However, the total omega-6 fatty acids and ARA levels at T2 were higher ($p<0.05$ for both) in the LBW group as compared to the NBW group (Table 13).

Table 14: Cord Erythrocyte Fatty Acid levels (g/100g fatty acids)

Cord Erythrocyte Fatty Acid levels (g/100g fatty acids)		
	NBW (n=47)	**LBW (n=45)**
MYR	0.40 ± 0.38	0.32 ± 0.09
MYRO	0.06 ± 0.07	0.09 ± 0.14
PAL	24.16 ± 2.00	24.57 ± 2.52
PALO	0.38 ± 0.15	0.37 ± 0.17
STE	16.71 ± 1.62	16.95 ± 1.63
OLE	7.60 ± 1.33	7.65 ± 1.21
LA	4.69 ± 2.38	4.65 ± 2.30
GLA	0.07 ± 0.06	0.09 ± 0.11
ALA	0.38 ± 0.11	0.38 ± 0.11
DGLA	2.19 ± 0.63	2.28 ± 1.20
ARA	15.48 ± 1.81	15.48 ± 2.33
EPA	0.53 ± 0.69	0.54 ± 0.89
NA	2.10 ± 0.71	1.96 ± 0.56
DPA (n-6)	0.31 ± 0.29	0.30 ± 0.20
DHA	3.03 ± 0.89	3.01 ± 1.05
Omega-3	3.94 ± 1.02	3.93 ± 1.23
Omega-6	22.74 ± 2.36	22.81 ± 2.54
SFA	41.27 ± 3.19	41.85 ± 3.96
MUFA	10.13 ± 1.43	10.07 ± 1.18

*Data are expressed as mean ± SD. *p<0.05, **p<0.01 as compared with NBW.* ***NBW*** *– Normal birth weight;* ***LBW*** *– Low birth weight;* ***n*** *- Number of subjects*
MYR *- Myristic acid;* ***MYRO*** *- Myristoleic acid;* ***PAL*** *- Palmitic acid;* ***PALO*** *- Palmitoleic acid;* ***STE*** *- Stearic acid;* ***OLE*** *- Oleic acid;* ***LA*** *- Linoleic acid;* ***GLA*** *- Gamma linolenic acid;* ***ALA*** *- Alpha linolenic acid;* ***DGLA*** *- Dihomo gamma linolenic acid;* ***ARA*** *- Arachidonic acid;* ***EPA*** *- Eicosapentaenoic acid;* ***NA*** *- Nervonic acid;* ***DPA****, omega-6 - Docosapentaenoic acid;* ***DHA*** *- Docosahexaenoic acid; Saturated fatty acids* ***(SFA)****: (MYR + PAL + STE); Monounsaturated fatty acids* ***(MUFA)****: (MYRO + PALO + OLE + NA);* ***Omega-3****: (ALA + EPA + DHA);* ***Omega-6****: (LA + GLA + DGLA + ARA + DPA).* ***n-6 –*** *omega-6.*

At the time of delivery (T3), SFA levels were higher ($p<0.01$) in the LBW group as compared to NBW group although the levels of MUFA were similar

between the two groups. The total omega-3 fatty acid levels were lower while the total omega-6 fatty acid levels were higher ($p<0.05$ for both) in the LBW group as compared to the NBW group. However, there was no change in the levels of DHA and ARA in between the two groups (Table 13).

The levels of SFA, MUFA, total omega-3 and omega-6 fatty acids, DHA and ARA in the cord were similar between the two groups (Table 14).

5.2.4. Comparison of Erythrocyte Fatty Acid Levels at Different Time Points within NBW and LBW Groups:

In both the NBW and LBW groups, there was no difference in the levels of SFA, MUFA, total omega-3 fatty acids, total omega-6 fatty acids, DHA and ARA at T2 as compared to the T1 (Fig 49, 50).

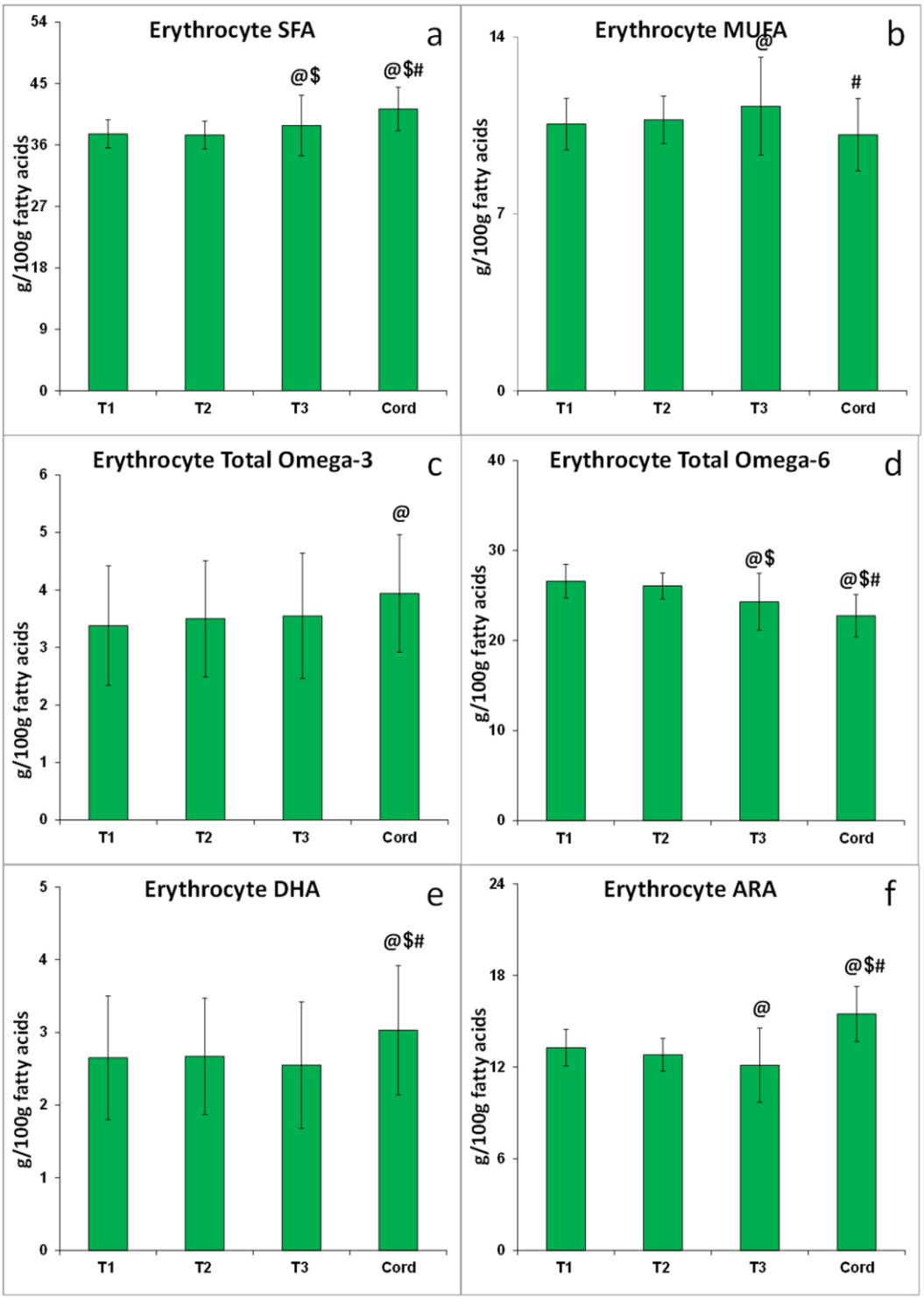
In both NBW and LBW groups, the levels of SFA and MUFA were higher ($p<0.05$ for both) while the levels of total omega-6 fatty acids and ARA were lower ($p<0.05$ for both) at T3 as compared to the T1. There was no difference in the levels of omega-3 fatty acids and DHA at T3 as compared to the T1 (Fig 49, 50).

In the NBW group, the levels of SFA were higher ($p<0.05$) while that of total omega-6 fatty acids were lower ($p<0.05$) at T3 as compared to the T2 (Fig 49). Similarly, in the LBW group, the levels of MUFA were higher ($p<0.05$) while total omega-6 and total omega-3 fatty acids were lower ($p<0.05$ for both) at T3 as compared to the T2. There was no change in the levels of DHA and ARA at T3 as compared to the T2 in both the groups (Fig 50).

In the NBW group, the cord levels of SFA, DHA and ARA were higher and total omega-6 fatty acids were lower ($p<0.05$ for all) as compared to maternal levels at all the time points. The cord MUFA levels were lower ($p<0.05$) as compared to maternal MUFA levels at T3. Cord total omega-3 fatty acid levels were higher ($p<0.05$) as compared to maternal levels at T1 (Fig 49).

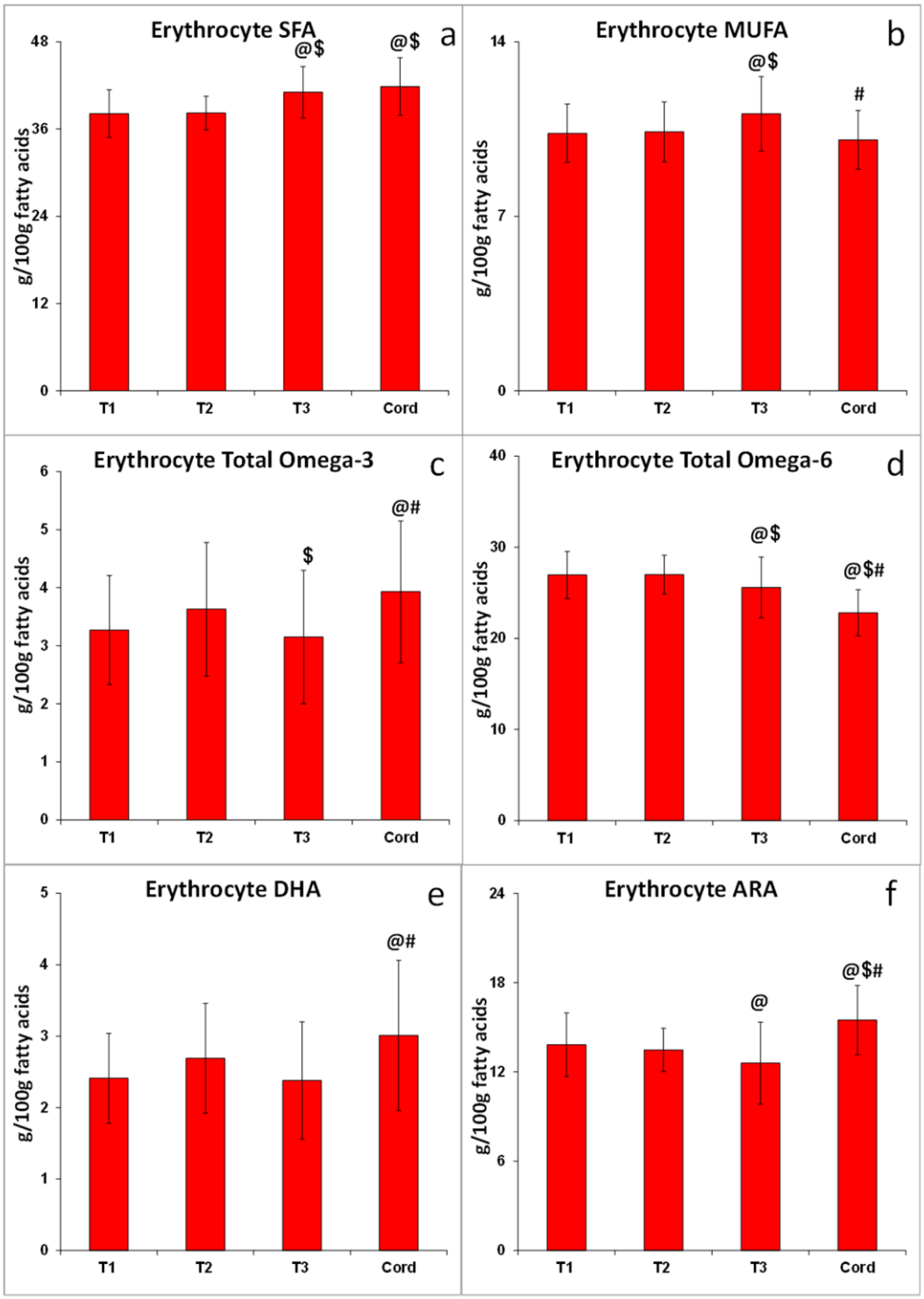
In the LBW group, the cord levels of SFA were higher ($p<0.05$ for both) as compared with maternal levels at T1 and T2. The cord MUFA levels were lower ($p<0.05$) as compared to maternal MUFA levels at T3. Cord total omega-3 fatty acids and DHA levels were higher ($p<0.05$ for both) as compared to maternal levels at T1 and T3. The cord levels of total omega-6 fatty acid were lower and ARA were higher ($p<0.05$ for both) as compared to maternal levels at all the time points (Fig 50).

Fig 49: Comparison of Erythrocyte Fatty Acid Levels at Different Time Points within NBW Group



Data are expressed as mean ± SD. $^{@}p<0.05$, $^{@@}p<0.01$ *as compared with T1,* $^{\$}p<0.05$, $^{\$\$}p<0.01$ *as compared with T2;* $^{\#}p<0.05$, $^{\#\#}p<0.01$*as compared with T3;* ***T1*** *=* 16^{th}*-*20^{th} *week,* ***T2*** *=* 26^{th}*-*30^{th} *week,* ***T3*** *= at delivery.* ***ARA*** *- Arachidonic acid,* ***DHA*** *- Docosahexaenoic acid, Saturated fatty acids* ***(SFA)****: (Myristic acid + Palmitic acid + Stearic acid), Monounsaturated fatty acids* ***(MUFA)****: (Myristoleic acid + Palmitoleic acid + Oleic acid + Nervonic acid),* ***Total omega-3****: (α linolenic acid + Eicosapentaenoic acid + DHA),* ***Total omega-6****: (Linoleic acid + γ linolenic acid + Dihomo γ linolenic acid + ARA + Docosapentaenoic acid).*

Fig 50: Comparison of Erythrocyte Fatty Acid Levels at Different Time Points within LBW Group



Data are expressed as mean ± SD. $^{@}p<0.05$, $^{@@}p<0.01$ *as compared with T1,* $^{\$}p<0.05$, $^{\$\$}p<0.01$ *as compared with T2;* $^{\#}p<0.05$, $^{\#\#}p<0.01$*as compared with T3;* ***T1*** *=* 16^{th}*-*20^{th} *week,* ***T2*** *=* 26^{th}*-*30^{th} *week,* ***T3*** *= at delivery.* ***ARA*** *- Arachidonic acid,* ***DHA*** *- Docosahexaenoic acid, Saturated fatty acids* ***(SFA)****: (Myristic acid + Palmitic acid + Stearic acid), Monounsaturated fatty acids* ***(MUFA)****: (Myristoleic acid + Palmitoleic acid + Oleic acid + Nervonic acid),* ***Total omega-3****: (α linolenic acid + Eicosapentaenoic acid + DHA),* ***Total omega-6****: (Linoleic acid + γ linolenic acid + Dihomo γ linolenic acid + ARA + Docosapentaenoic acid).*

5.2.5. Maternal and Cord Plasma Fatty Acids Levels at Different Time Points in the NBW and LBW Groups:

There was no change in the levels of SFA, MUFA, total omega-3 fatty acids, total omega-6 fatty acids and DHA between the two groups across the gestation. In contrast, maternal ARA levels at T2 were higher ($p<0.05$) in the LBW group as compared to the NBW group (Table 15).

Table 15: Maternal Plasma Fatty Acid Levels (g/100g fatty acids) at Three Time Points during Pregnancy

Maternal Plasma Fatty Acids (g/100g fatty acids)						
	NBW	**LBW**	**NBW**	**LBW**	**NBW**	**LBW**
	T1 (n=56)	**T1 (n=51)**	**T2 (n=38)**	**T2 (n=45)**	**T3 (n=52)**	**T3 (n=46)**
MYR	1.03 ± 0.43	0.91 ± 0.46	1.12 ± 0.46	1.01 ± 0.40	0.99 ± 0.42	0.87 ± 0.37
MYRO	0.07 ± 0.06	0.08 ± 0.10	0.08 ± 0.05	0.06 ± 0.06*	0.06 ± 0.05	0.09 ± 0.12
PAL	25.31 ± 2.68	24.33 ± 2.30*	26.73 ± 2.34	26.32 ± 2.25	27.42 ± 2.52	26.72 ± 2.34
PALO	1.28 ± 0.68	1.03 ± 0.60*	1.61 ± 0.82	1.25 ± 0.54*	2.00 ± 0.88	1.75 ± 0.87
STE	6.03 ± 0.74	6.14 ± 0.74	5.64 ± 0.71	5.60 ± 0.82	6.14 ± 1.62	6.05 ± 1.78
OLE	14.65 ± 5.07	14.35 ± 1.72	15.18 ± 1.80	15.11 ± 2.00	15.61 ± 1.86	15.63 ± 3.48
LA	34.55 ± 5.92	35.96 ± 3.78	34.10 ± 3.86	35.35 ± 3.97	30.79 ± 7.59	30.86 ± 7.55
GLA	0.20 ± 0.15	0.20 ± 0.18	0.18 ± 0.13	0.16 ± 0.09	0.15 ± 0.12	0.18 ± 0.11
ALA	0.41 ± 0.29	0.43 ± 0.22	0.42 ± 0.31	0.49 ± 0.28	0.48 ± 0.26	0.44 ± 0.23
DGLA	1.53 ± 0.31	1.30 ± 0.54**	1.42 ± 0.27	1.27 ± 0.43*	1.49 ± 0.51	1.47 ± 0.51
ARA	6.28 ± 1.26	6.67 ± 1.34	5.35 ± 0.96	5.85 ± 1.23*	6.22 ± 3.02	6.58 ± 2.82
EPA	0.32 ± 0.51	0.42 ± 0.48*	0.34 ± 0.57	0.39 ± 0.53	0.34 ± 0.59	0.54 ± 0.81
NA	0.50 ± 0.18	0.54 ± 0.20	0.50 ± 0.18	0.50 ± 0.16	0.61 ± 0.39	0.59 ± 0.30
DPA (n-6)	0.16 ± 0.09	0.16 ± 0.07	0.13 ± 0.07	0.16 ± 0.10	0.13 ± 0.06	0.15 ± 0.10
DHA	1.20 ± 0.38	1.12 ± 0.41	1.03 ± 0.37	1.10 ± 0.37	1.09 ± 0.44	1.10 ± 0.42
Omega-3	1.93 ± 0.71	1.97 ± 0.71	1.79 ± 0.82	1.99 ± 0.80	1.90 ± 0.67	2.07 ± 1.02
Omega-6	42.73 ± 5.94	44.30 ± 4.16	41.18 ± 3.71	42.79 ± 4.31	38.78 ± 5.01	39.23 ± 5.30
SFA	32.37 ± 3.11	31.39 ± 2.65	33.49 ± 2.58	32.93 ± 2.62	34.55 ± 3.09	33.65 ± 3.07
MUFA	16.50 ± 5.16	16.00 ± 1.80	17.36 ± 2.04	16.91 ± 2.06	18.29 ± 2.40	18.05 ± 3.79

*Data are expressed as mean ± SD. *p<0.05, **p<0.01 as compared with NBW **T1**=16th-20th week; **T2**= 26th-30th week; **T3**= at delivery.*
***NBW** – Normal birth weight; **LBW** – Low birth weight; **n** - Number of subjects.*
***MYR** - Myristic acid; **MYRO** - Myristoleic acid; **PAL** - Palmitic acid; **PALO** - Palmitoleic acid; **STE** - Stearic acid; **OLE** - Oleic acid; **LA** - Linoleic acid; **GLA** - Gamma linolenic acid; **ALA** - Alpha linolenic acid; **DGLA** - Dihomo gamma linolenic acid; **ARA** - Arachidonic acid; **EPA** - Eicosapentaenoic acid; **NA** - Nervonic acid; **DPA**, omega-6 - Docosapentaenoic acid; **DHA** - Docosahexaenoic acid; Saturated fatty acids **(SFA)**: (MYR + PAL + STE); Monounsaturated fatty acids **(MUFA)**: (MYRO + PALO + OLE + NA); **Omega-3**: (ALA + EPA + DHA), **Omega-6**: (LA + GLA + DGLA + ARA + DPA); **n-6** – omega-6.*

The levels of SFA, MUFA, total omega-3 fatty acids, total omega-6 fatty acids, DHA and ARA in the cord plasma were similar between the two groups (Table 16).

Table 16: Cord Plasma Fatty Acid Levels (g/100g fatty acids)

Cord Plasma Fatty Acid levels (g/100g fatty acids)		
	NBW (n=48)	**LBW (n=44)**
MYR	0.78 ± 0.24	0.84 ± 0.44
MYRO	0.09 ± 0.09	0.09 ± 0.12
PAL	27.76 ± 1.37	27.43 ± 2.76
PALO	2.97 ± 1.03	2.76 ± 1.14
STE	10.03 ± 2.04	9.77 ± 2.22
OLE	15.37 ± 1.63	15.11 ± 2.43
LA	14.33 ± 7.13	15.83 ± 8.39
GLA	0.28 ± 0.17	0.24 ± 0.16
ALA	0.33 ± 0.16	0.35 ± 0.14
DGLA	2.49 ± 0.68	2.23 ± 0.54*
ARA	12.69 ± 2.79	12.48 ± 3.48
EPA	0.34 ± 0.35	0.62 ± 0.78*
NA	1.14 ± 0.39	1.02 ± 0.40
DPA (n-6)	0.12 ± 0.08	0.12 ± 0.09
DHA	1.70 ± 0.60	1.77 ± 0.84
Omega-3	2.36 ± 0.72	2.74 ± 1.16
Omega-6	29.91 ± 4.62	30.91 ± 5.36
SFA	38.56 ± 2.15	38.04 ± 3.93
MUFA	19.57 ± 1.98	18.97 ± 3.00

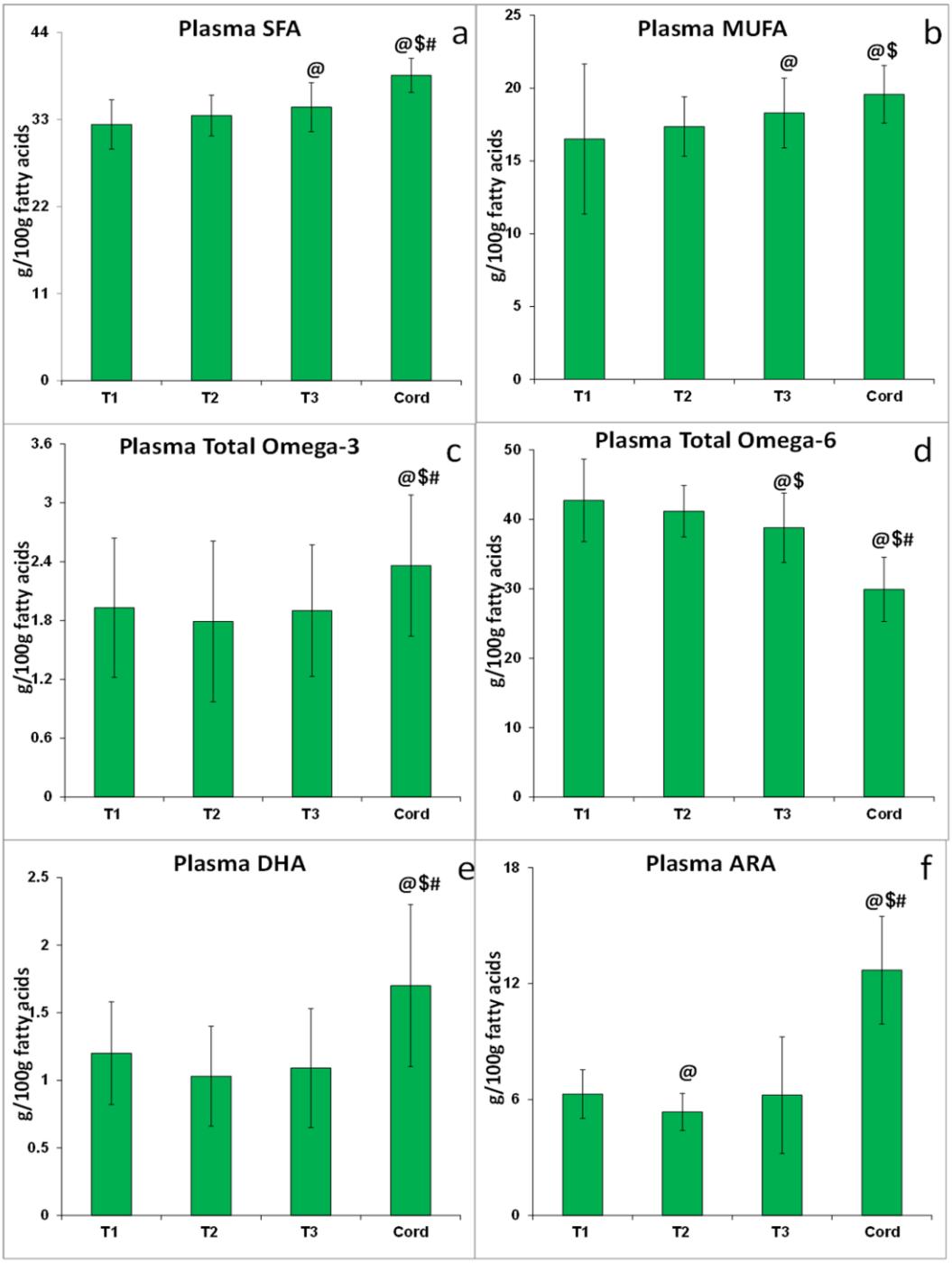
*Data are expressed as mean ± SD. *p<0.05 as compared with NBW.*
***NBW** – Normal birth weight; **LBW** – Low birth weight; **n** - Number of subjects.*
***MYR** - Myristic acid; **MYRO** - Myristoleic acid; **PAL** - Palmitic acid; **PALO** - Palmitoleic acid; **STE** - Stearic acid; **OLE** - Oleic acid; **LA** - Linoleic acid; **GLA** - Gamma linolenic acid; **ALA** - Alpha linolenic acid; **DGLA** - Dihomo gamma linolenic acid; **ARA** - Arachidonic acid; **EPA** - Eicosapentaenoic acid; **NA** - Nervonic acid; **DPA**, omega-6 - Docosapentaenoic acid; **DHA** - Docosahexaenoic acid; Saturated fatty acids **(SFA)**: (MYR + PAL + STE); Monounsaturated fatty acids **(MUFA)**: (MYRO + PALO + OLE + NA); **Omega-3**: (ALA + EPA + DHA), **Omega-6**: (LA + GLA + DGLA + ARA + DPA); **n-6 –** omega-6.*

5.2.6. Comparison of Plasma Fatty Acid Levels at Different Time Points within NBW and LBW Groups:

In the NBW group, there was no change in the levels of SFA, MUFA, total omega-3 fatty acids, total omega-6 fatty acids and DHA at T2 as compared to the T1. The levels of ARA were lower ($p<0.05$) at T2 as compared to the T1 (Fig 51). In the LBW group, the levels of SFA were higher ($p<0.05$) at T2 as compared to the T1. The

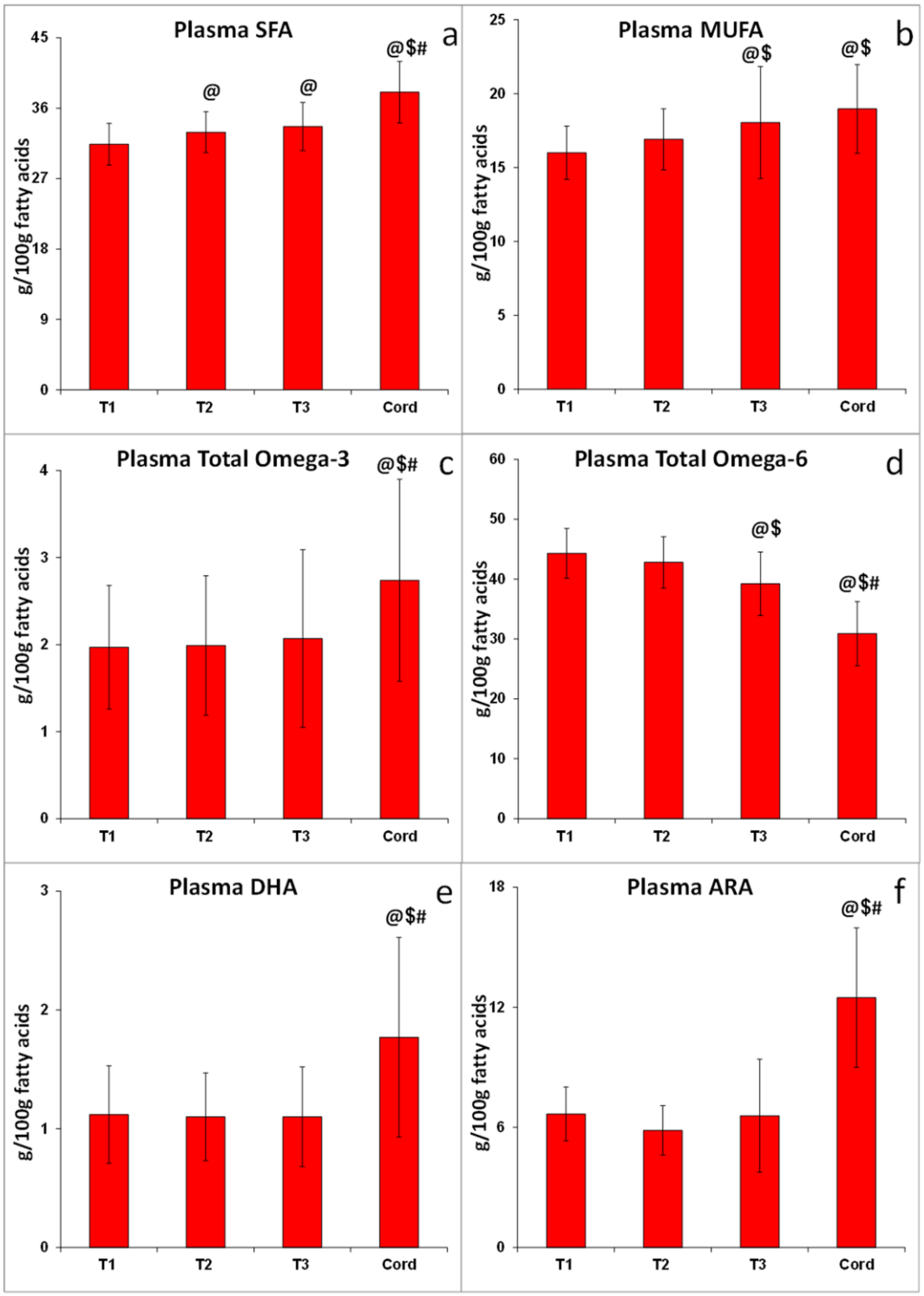
levels of MUFA, total omega-3 fatty acids, total omega-6 fatty acids, DHA and ARA were similar at T2 as compared to the T1 (Fig 52).

Fig 51: Comparison of Plasma Fatty Acid Levels at Different Time Points within NBW Group



Data are expressed as mean ± SD. $^{@}p<0.05$, $^{@@}p<0.01$ *as compared with T1,* $^{\$}p<0.05$, $^{\$\$}p<0.01$ *as compared with T2;* $^{\#}p<0.05$, $^{\#\#}p<0.01$*as compared with T3;* ***T1*** *=* 16^{th}*-*20^{th} *week,* ***T2*** *=* 26^{th}*-*30^{th} *week,* ***T3*** *= at delivery.* ***ARA*** *- Arachidonic acid,* ***DHA*** *- Docosahexaenoic acid, Saturated fatty acids* ***(SFA)****: (Myristic acid + Palmitic acid + Stearic acid), Monounsaturated fatty acids* ***(MUFA)****: (Myristoleic acid + Palmitoleic acid + Oleic acid + Nervonic acid),* ***Total omega-3****: (α linolenic acid + Eicosapentaenoic acid + DHA),* ***Total omega-6****: (Linoleic acid + γ linolenic acid + Dihomo γ linolenic acid + ARA + Docosapentaenoic acid).*

Fig 52: Comparison of Plasma Fatty Acid Levels at Different Time Points within LBW Group



Data are expressed as mean ± SD. $^{@}p<0.05$, $^{@@}p<0.01$ *as compared with T1,* $^{\$}p<0.05$, $^{\$\$}p<0.01$ *as compared with T2;* $^{\#}p<0.05$, $^{\#\#}p<0.01$ *as compared with T3;* ***T1*** *=* 16^{th}*-*20^{th} *week,* ***T2*** *=* 26^{th}*-*30^{th} *week,* ***T3*** *= at delivery.* ***ARA*** *- Arachidonic acid,* ***DHA*** *- Docosahexaenoic acid, Saturated fatty acids* ***(SFA)****: (Myristic acid + Palmitic acid + Stearic acid), Monounsaturated fatty acids* ***(MUFA)****: (Myristoleic acid + Palmitoleic acid + Oleic acid + Nervonic acid),* ***Total omega-3****: (α linolenic acid + Eicosapentaenoic acid + DHA),* ***Total omega-6****: (Linoleic acid + γ linolenic acid + Dihomo γ linolenic acid + ARA + Docosapentaenoic acid).*

In both the NBW and LBW groups, the levels of SFA and MUFA were higher ($p<0.05$ for both) while the levels of total omega-6 fatty acids were lower ($p<0.05$) at T3 as compared to the T1. There was no change in the levels of total omega-3 fatty acids, DHA and ARA at T3 as compared to the T1 (Fig 51, 52).

In both the NBW and LBW groups, there was no change in the levels of SFA, total omega-3 fatty acids, DHA and ARA at T3 as compared to the T2. The levels of total omega-6 fatty acids were lower ($p<0.05$) at T3 as compared to the T2 (Fig 51, 52). In the LBW group, the levels of MUFA were higher ($p<0.05$) at T3 as compared to the T2 (Fig 52).

In both the NBW and LBW groups, the cord levels of SFA, total omega-3 fatty acids, DHA and ARA were higher ($p<0.05$ for all) as compared to maternal levels at all the time points across gestation. The cord MUFA levels were higher ($p<0.05$ for both) as compared to the maternal MUFA levels at T1 and T2. The cord total omega-6 fatty acid levels were lower ($p<0.05$ for all) as compared to maternal levels at all the time points across gestation (Fig 51, 52).

5.2.7. Associations of Maternal and Cord Fatty Acids with Birth Outcome:

At T1, the maternal erythrocyte fatty acids like SFA, MUFA, total omega-3 fatty acids and ARA were not associated with any of the birth outcome parameters. In contrast, maternal erythrocyte DHA levels were positively associated with baby weight ($n = 105$, $r = 0.222$, $p = 0.025$). Further, there was positive association between maternal erythrocyte total omega-6 fatty acids with baby length ($n = 97$, $r = 0.223$, $p = 0.031$). There was no association between maternal plasma fatty acids like SFA, MUFA, total omega-3 fatty acids, total omega-6 fatty acids, DHA and ARA with any of the birth outcome parameters.

At T2, the maternal erythrocyte fatty acids like SFA, MUFA, total omega-3 fatty acids, total omega-6 fatty acids, DHA and ARA were not associated with any of the birth outcome parameters. Maternal plasma SFA levels were positively associated with baby head circumference ($n = 69$, $r = 0.257$, $p = 0.037$). There was a negative association between maternal plasma omega-6 fatty acids with baby weight ($n = 78$, $r = -0.255$, $p = 0.027$) and baby head circumference ($n = 69$, $r = -0.267$, $p = 0.030$).

At T3, the maternal erythrocyte fatty acids like SFA, total omega-3 fatty acids and ARA were not associated with any of the birth outcome parameters. There was a

negative association of MUFA (n = 87, r = -0.288, p = 0.008) and total omega-6 fatty acid (n = 87, r = -0.388, p = 0.000) levels with baby head circumference. In contrast, there was a positive association of DHA levels (n = 87, r = 0.241, p = 0.027) with baby head circumference. Maternal plasma total omega-6 fatty acid levels were negatively associated (n = 86, r = -0.304, p = 0.005) and ARA levels were positively associated (n = 86, r = 0.262, p = 0.071) with baby chest circumference.

Cord erythrocyte and plasma SFA, MUFA, total omega-3 fatty acids, total omega-6 fatty acids, DHA and ARA levels were not associated with any of the birth outcome parameters.

5.2.8. Associations between Cord Erythrocyte Fatty Acids and Maternal Erythrocyte Fatty Acids at T1, T2 and T3:

The SFA levels in the cord were not associated with maternal SFA levels at any time point during gestation. Cord MUFA and total omega-3 fatty acid levels were positively associated with maternal MUFA (n = 87, r = 0.284, p = 0.008) and total omega-3 fatty acid (n = 87, r = 0.392, p = 0.000) respectively at T1. Cord total omega-6 fatty acid levels were not associated with maternal total omega-6 fatty acid levels at any time point during gestation. Cord DHA was positively associated with maternal DHA at all the three time points (T1: n = 87, r = 0.493, p = 0.000; T2: n = 67, r = 0.565, p = 0.000; T3: n = 82, r = 0.243, p = 0.029). Cord ARA was negatively associated with maternal ARA at T1 and T3 (T1: n = 87, r = -0.229, p = 0.034; T3: n = 82, r = -0.428, p = 0.000) (Table 17).

5.2.9. Associations between Cord Plasma Fatty Acids and Maternal Plasma Fatty Acids at T1, T2 and T3:

Cord SFA levels were not associated with maternal SFA levels at any time point during gestation. Cord MUFA levels were positively associated with maternal MUFA at T2 and T3 (T2: n = 67, r = 0.288, p = 0.019; T3: n = 82, r = 0.276, p = 0.013). Cord total omega-3 fatty acids were positively associated with maternal total omega-3 fatty acids at all three time points during gestation (T1: n = 86, r = 0.422, p = 0.000; T2: n = 67, r = 0.336, p = 0.006; T3: n = 82, r = 0.322, p = 0.003). Cord total omega-6 fatty acids was negatively associated with maternal total omega-6 fatty acids at T3 (n = 82, r = -0.294, p = 0.008). Cord DHA was positively associated with maternal DHA at all the three time points (T1: n = 86, r = 0.396, p = 0.000; T2: n =

67, r = 0.456, p = 0.000; T3: n = 82, r = 0.335, p = 0.002). Cord ARA was negatively associated with maternal ARA at T3 (n = 82, r = -0.584, p = 0.000) (Table 17).

Table 17: Associations of Cord Fatty Acids with Maternal Fatty Acids at T1, T2 and T3

Fatty Acids (g/100g)	Erythrocyte			Plasma		
	n	**r**	**p**	**n**	**r**	**p**
Cord DHA						
Maternal DHA						
T1 (16^{th}-20^{th} week)	87	0.493	0.000	86	0.396	0.000
T2 (26^{th}-30^{th} week)	67	0.565	0.000	67	0.456	0.000
T3 (at delivery)	82	0.243	0.029	82	0.335	0.002
Cord ARA						
Maternal ARA						
T1 (16^{th}-20^{th} week)	87	-0.229	0.034	86	0.009	0.934
T2 (26^{th}-30^{th} week)	67	-0.227	0.066	67	0.121	0.335
T3 (at delivery)	82	-0.428	0.000	82	-0.584	0.000
Cord Total Omega-3 Fatty Acids						
Maternal Total Omega-3 Fatty Acids						
T1 (16^{th}-20^{th} week)	87	0.392	0.000	86	0.422	0.000
T2 (26^{th}-30^{th} week)	67	0.200	0.108	67	0.336	0.006
T3 (at delivery)	82	0.203	0.070	82	0.322	0.003
Cord Total Omega-6 Fatty Acids						
Maternal Total Omega-6 Fatty Acids						
T1 (16^{th}-20^{th} week)	87	-0.178	0.102	86	0.159	0.147
T2 (26^{th}-30^{th} week)	67	0.019	0.878	67	0.096	0.441
T3 (at delivery)	82	-0.091	0.421	82	-0.294	0.008
Cord SFA						
Maternal SFA						
T1 (16^{th}-20^{th} week)	87	0.078	0.477	86	0.174	0.112
T2 (26^{th}-30^{th} week)	67	0.115	0.359	67	0.027	0.828
T3 (at delivery)	82	0.176	0.115	82	-0.113	0.316
Cord MUFA						
Maternal MUFA						
T1 (16^{th}-20^{th} week)	87	0.284	0.008	86	0.172	0.115
T2 (26^{th}-30^{th} week)	67	0.238	0.054	67	0.288	0.019
T3 (at delivery)	82	0.032	0.779	82	0.276	0.013

T1*=16^{th}–20^{th} week,* ***T2****=26^{th}–30^{th} week,* ***T3****=at delivery. For Pearson's correlation coefficients, p<0.05.*
n *- Number of subjects;* ***r*** *- Correlation coefficient;* ***p*** *- Significance;* ***ARA*** *– Arachidonic acid,* ***DHA*** *– Docosahexaenoic acid,* *Saturated fatty acids* ***(SFA)****: (Myristic acid + Palmitic acid + Stearic acid), Monounsaturated fatty acids* ***(MUFA)****: (Myristoleic acid + Palmitoleic acid + Oleic acid + Nervonic acid),* ***Total omega-3****: (α linolenic acid + Eicosapentaenoic acid + DHA),* ***Total omega-6****: (Linoleic acid + γ linolenic acid + Dihomo γ linolenic acid + ARA + Docosapentaenoic acid).*

5.2.10. Placental Fatty Acid Levels:

Placental DHA levels were significantly lower (p<0.05) in the LBW group as compared to the NBW group. There was no significant difference in any other fatty acids between the NBW and LBW groups (Table 18).

Table 18: Placental Fatty Acid Levels (g/100g fatty acids)

Placental Fatty Acids (g/100g fatty acids)		
	NBW (n=38)	**LBW (n=36)**
MYR	0.48 ± 0.29	0.45 ± 0.18
MYRO	0.28 ± 0.97	0.17 ± 0.23
PAL	24.86 ± 2.46	25.68 ± 2.97
PALO	0.30 ± 0.12	0.33 ± 0.17
STE	13.32 ± 1.46	13.14 ± 1.87
OLE	5.90 ± 1.56	6.31 ± 1.30
LA	10.68 ± 1.42	10.61 ± 1.26
GLA	0.09 ± 0.04	0.10 ± 0.06
ALA	0.20 ± 0.10	0.21 ± 0.12
DGLA	3.98 ± 1.15	3.80 ± 0.70
ARA	19.39 ± 2.89	19.81 ± 4.52
EPA	0.75 ± 0.73	0.72 ± 0.75
NA	1.44 ± 0.53	1.42 ± 0.57
DPA (n-6)	0.38 ± 0.14	0.34 ± 0.16
DHA	2.53 ± 0.78	2.18 ± 0.56*
Omega-3	3.48 ± 1.09	3.11 ± 0.89
Omega-6	34.53 ± 3.78	34.66 ± 4.92
SFA	38.65 ± 3.65	39.26 ± 4.54
MUFA	7.92 ± 1.77	8.24 ± 1.49

*Data are expressed as mean ± SD. *p<0.05 as compared with NBW.*
***NBW** - normal birth weight; **LBW** - low birth weight;*
***MYR** – Myristic acid, **MYRO** – Myristoleic acid, **PAL** - Palmitic acid, **PALO** - Palmitoleic acid, **STE** – Stearic acid, **OLE** - Oleic acid, **LA** - Linoleic acid, **GLA** - Gamma linolenic acid, **ALA** - Alpha linolenic acid, **DGLA** – Di homo gamma linolenic acid, **ARA** - Arachidonic acid, **EPA** - Eicosapentaenoic acid, **NA** – Nervonic acid, **DPA**,omega-6 - Docosapentaenoic acid, **DHA** - Docosahexaenoic acid, Saturated fatty acids **(SFA)**: (MYR + PAL + STE), Monounsaturated fatty acids **(MUFA)**: (MYRO + PALO + OLE + NA), **total omega-3**: (ALA + EPA + DHA), **total omega-6**: (LA + GLA + DGLA + ARA + DPA); **n-6 -** omega-6.*

5.2.11. mRNA Levels of the Placental Transcription Factors:

The mRNA levels of placental PPARα in the LBW group were lower as compared to the NBW group; however it was not statistically significant (Fig 53a). The mRNA levels of placental PPARγ were significantly lower ($p<0.05$) in the LBW group as compared to the NBW group (Fig 53b). Similarly, mRNA levels of placental LXRα were also significantly lower ($p<0.05$) in the LBW group as compared to the NBW group (Fig 53d). The mRNA levels of placental SREBP-1c (Fig 53c), RXRα (Fig 53e) and RXRγ (Fig 53f) were lower although not statistically significant.

Fig 53: mRNA Levels of the Placental Transcription Factors

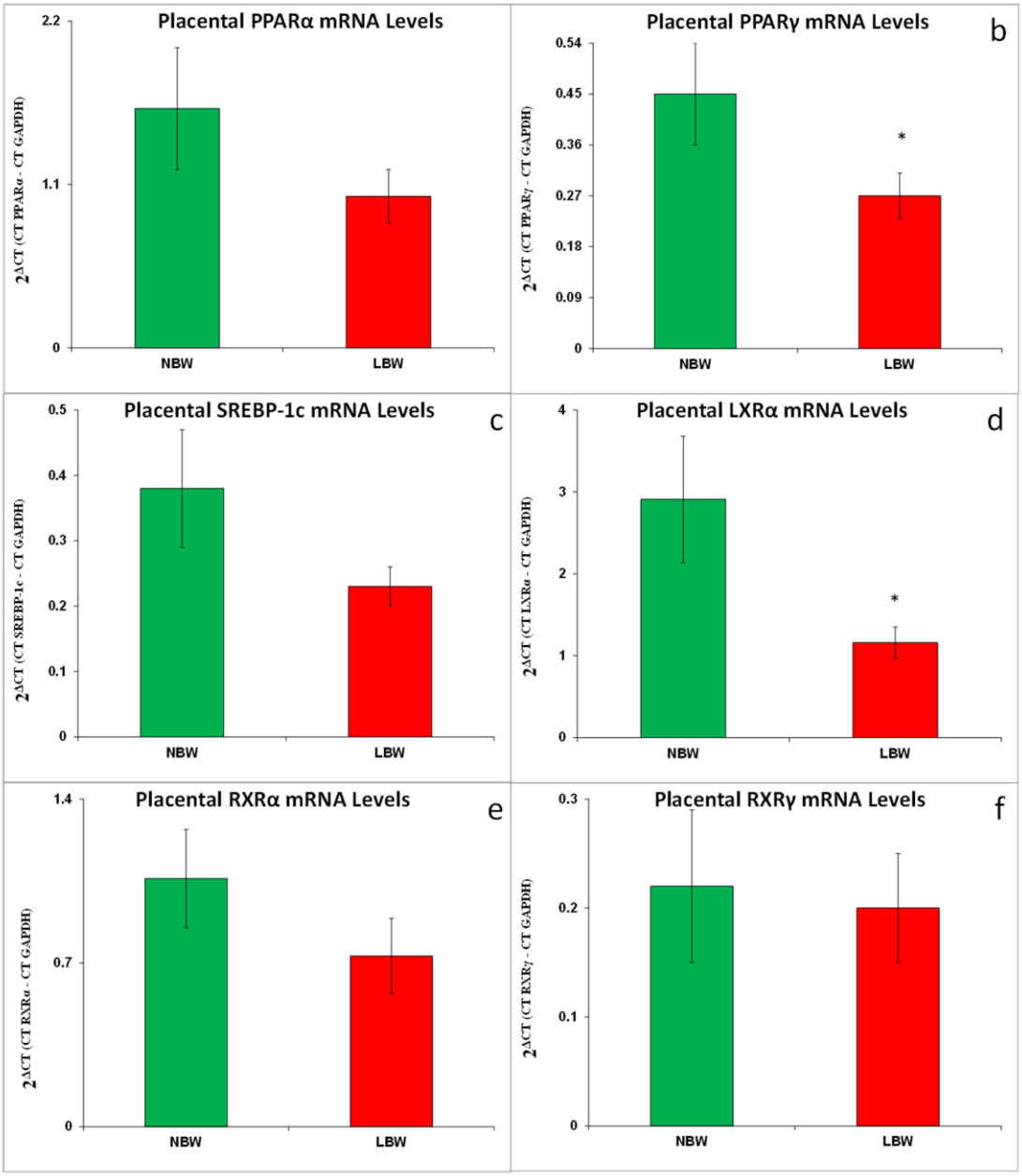


***Fig 53a**: PPARα **Fig 53b**: PPARγ **Fig 53c**: SREBP-1c **Fig 53d**: LXRα **Fig 53e**: RXRα **Fig 53f**: RXRγ. Data are expressed as mean ± SE. *p<0.05 as compared with NBW; **NBW** - normal birth weight; **LBW** - low birth weight.*

5.2.12. Associations of Placental Fatty Acids with Birth Outcome:

There was a positive association of placental DHA levels with baby weight (n = 64, r = 0.325, p = 0.011) and baby length (n = 64, r =0.250, p = 0.052). However, there was no association of any other placental fatty acids with birth outcome measures.

5.2.13. Associations of mRNA Levels of Placental Transcription Factors with Birth Outcome:

There was a positive association between the placental PPARγ mRNA levels and baby weight (n = 50, r = 0.307, p = 0.036). There was no association of other transcription factors with birth outcome measures.

5.3. Discussion:

To the best of our knowledge this is the first report which has examined the levels of plasma as well as erythrocyte LCPUFA at 3 different time points during pregnancy and examined their association with cord blood LCPUFA levels and birth outcome. Further, this study also examines placental fatty acid levels and mRNA levels of placental transcription factors in mothers delivering LBW and NBW babies.

Our results indicate the following i) Higher maternal erythrocyte total omega-6 fatty acids and ARA levels at 26^{th}-30^{th} week of pregnancy in women delivering LBW babies, ii) Lower maternal erythrocyte omega-3 fatty acid, higher omega-6 fatty acid and higher SFA levels at delivery in women delivering LBW babies, iii) Maternal erythrocyte DHA at T1 was positively associated with baby weight, iv) Cord plasma and erythrocyte DHA levels were positively associated with maternal DHA levels at all the time points across the gestation; while cord erythrocyte ARA levels were negatively associated with maternal ARA levels at T1 and T3 v) Lower levels of placental DHA in women delivering LBW babies, vi) Lower mRNA levels of placental PPARγ and LXRα in women delivering LBW babies, vii) A positive association of both placental DHA and mRNA levels of placental PPARγ with baby weight.

5.3.1. Maternal Plasma and Erythrocyte Fatty Acids:

A number of cross sectional human studies carried out in our department have extensively demonstrated lower LCPUFA levels in pregnancy complications like preeclampsia (Mehendale et al. 2008; Dangat et al. 2010; Kulkarni et al. 2011a). Further, in normotensive pregnancies we and others have also reported lower maternal LCPUFA levels at the time of delivery in mothers delivering LBW babies (Vilbergsson et al. 1991; Matorras et al. 1994; Alvino et al. 2008; Ortega-Senovilla et al. 2009; Kilari et al. 2011; Bobiński et al. 2013). However, since these studies were

carried out at the end of pregnancy; it is not clear whether the differences in the LCPUFA proportions were present early in pregnancy.

There are limited studies which report the plasma fatty acid profile of women only in early pregnancy and their association with birth weight (Rump et al. 2001; van Eijsden et al. 2008; Smits et al. 2013). However, it is likely that the results may be influenced by changes in fatty acid status in late gestation. Similarly other studies have examined only maternal erythrocyte fatty acid levels across the gestation (Muthayya et al. 2009) or only maternal plasma fatty acid levels across the gestation (Dirix et al. 2009). Therefore, it is necessary to examine both plasma and erythrocyte fatty acid levels, in order to determine the entire fatty acid status reflecting the recent as well as long term intake. Reports indicate that, the amount of umbilical cord fatty acids are correlated with the amount in maternal blood, and are critical for later health outcomes of children (Lattka et al. 2013). Thus, it is vital to examine the association of maternal fatty acids with cord fatty acids for better understanding of the effects of maternal LCPUFA levels leading to LBW babies.

5.3.1.1. Higher Maternal Omega-6 fatty acid and ARA levels:

In the current study, maternal erythrocyte omega-6 fatty acid and ARA levels were higher at T2 as well as at delivery in mothers delivering LBW babies. Our findings are similar to studies in Amsterdam Born Children and their Development cohort, where they observed the association between higher levels of ARA and reduced birth weight in early pregnancy (van Eijsden et al. 2008; Smits et al. 2013). Similarly, other study in Maastricht Essential Fatty Acid Birth cohort also report a negative association between higher ARA levels during late pregnancy and at the time of delivery with decreased birth weight (Dirix et al. 2009).

5.3.1.2. Lower Maternal Erythrocyte Omega-3 Fatty Acid:

In the current study, we observed lower levels of maternal erythrocyte total omega-3 fatty acids at delivery in women delivering LBW babies. Importance of omega-3 fatty acid in fetal development during pregnancy is well established (Smuts et al. 2003a). A study carried out on south Indian subjects reports the association between the lower intake of fish during the third trimester of pregnancy and higher risk of delivering LBW babies (Muthayya et al. 2009). During the third trimester of pregnancy, there is greatest accretion of LCPUFA by the fetus (reviewed by Innis, 2011). Omega-3 fatty acids are known to improve membrane fluidity and increase

flow mediated vasodilation, thereby improving the membrane receptivity for various biologically active ligands. This may further lead to reduction in the blood viscosity and increase in the placental blood flow, thereby improving fetal growth (reviewed by Calder, 2011). Thus, the lower levels of omega-3 fatty acids might reflect the mother's inability to supply adequate amounts of LCPUFA for optimal fetal development.

5.3.1.3. Higher Erythrocyte SFA Levels:

In the current study, we observed higher levels of maternal erythrocyte SFA at delivery in women delivering LBW babies. The imbalanced maternal levels of saturated fatty acids have adverse effects on the developing fetus (reviewed by Cetin et al. 2009). The higher levels of saturated fatty acids in women delivering LBW babies can possibly be attributed to the inadequate transfer of these fatty acids through placenta, contributing to inadequate fetal growth.

5.3.1.4. Higher Cord Fatty Acid Levels:

In the current study, the cord fatty acid levels i.e. DHA as well as ARA were higher as compared to maternal levels in both the NBW and LBW groups. There are several mechanisms in the placenta involving the action of lipases and fatty acid binding proteins for the preferential transfer of the critical LCPUFA to the fetal circulation (reviewed by Haggarty, 2014). This phenomenon is well known as biomagnification, where the fetus increases the LCPUFA percentage in fetal blood in order to support central nervous system development (reviewed by Duttaroy, 2009). This supports the increased cord fatty acid levels observed in the current study.

5.3.1.5. Negative Association of the Cord ARA Levels with the Maternal ARA Levels:

In the current study, there was a negative association of the cord ARA levels with the maternal ARA levels at T1 as well as at the time of delivery. These results are similar to our earlier reported study in normotensive pregnancy (Wadhwani et al. 2015). One possible explanation for this association may be that the fetus is less dependent on the maternal ARA supply as compared to the DHA (reviewed by Haggarty, 2010).

5.3.1.6. Positive Association between Maternal Erythrocyte DHA Levels and Birth Weight:

In the current study, maternal erythrocyte DHA levels at T1 were positively associated with birth weight, suggesting the potential benefits of DHA in influencing birth weight. This is supported by earlier studies which indicate the beneficial effects of DHA supplementation during pregnancy in increasing birth size (Ramakrishna et al. 2010; Carlson et al. 2013). Further, it is also suggested that dietary adaptation to adequate maternal fatty acid status helps in preventing fetal growth restriction which may improve health in later life (van Eijsden et al. 2008). This may have implications in improving neurodevelopment of the offspring. In addition, there was also a positive association of the both erythrocyte and plasma cord DHA levels with the maternal DHA levels right from the early pregnancy.

There could be several possible mechanisms leading to altered levels of LCPUFA in mothers delivering LBW babies as compared with mothers delivering NBW babies, despite of the observed similarity between the dietary intakes. 1) It is well known that enzymes like desaturases synthesize the omega-3 and omega-6 LCPUFA from their essential shorter chain precursors (reviewed by Cetin et al. 2009). Thus, any alteration in the levels of these enzymes can affect the levels of fatty acids in the mother's circulation. 2) There may be genetic variations from the single nucleotide polymorphism of the FADS1 and FADS2, which may influence the maternal plasma and erythrocyte phospholipid levels of omega-6 and omega-3 fatty acids during pregnancy (Xie and Innis, 2008).

5.3.2. Placental Fatty Acids and Gene Expression:

5.3.2.1. Lower Placental DHA:

In the current study, the levels of DHA were lower in the placenta of the mothers delivering LBW babies as compared to the mothers delivering NBW babies. LCPUFA especially DHA is known to influence placental and fetal development by carrying out several physiological functions (metabolic, energetic and structural) in the body (reviewed by Cetin et al. 2009). DHA is involved in several process of placental development like placental fatty acid transport, eicosanoid production, placental angiogenesis, placental oxidative stress and regulation of transcription factors (reviewed by Jones et al. 2014). Thus, the lower levels of DHA in the placenta observed in this study may have implications in placental development and function.

5.3.2.2. Lower Placental PPARγ mRNA levels:

In the current study, we observed lower mRNA levels of PPARγ in the placenta of women delivering LBW babies as compared with those delivering NBW babies. Despite the critical importance of transcription factors in the placental development, their role in women delivering LBW babies is not completely understood. Studies have examined the expression of transcription factors like PPAR or LXR in SGA, IUGR, preeclampsia, preterm, gestational diabetes and miscarriage. However these reports are variable and inconclusive with some showing lower, higher or no change in the mRNA levels of transcription factors (Jawerbaum et al. 2004; Rodie et al. 2005; Holdsworth-Carson et al. 2009, Holdsworth-Carson et al. 2010; Castell et al. 2012; Knabl et al. 2013).

Our results are similar to a recent study which reports decreased placental PPARγ expression in SGA (Díaz et al. 2012). DHA is known to act as natural ligand for activating PPAR (reviewed by Bordoni et al. 2006) and the role of LCPUFA in regulating the expression of PPAR is widely accepted and extensively reviewed earlier (reviewed by Jump, 2002, 2008; reviewed by Jump et al. 2005, 2008, 2013). Thus, the observed lower mRNA levels of placental PPAR in this study may be attributed to the observed lower DHA levels in the placenta of the women delivering LBW babies. Alternatively reports also indicate that the activity of mammalian target of rapamycin (mTOR) a member of the phosphatidylinositol kinase–related kinase family of Ser/Thr kinases regulates the expression and activity of PPARγ (Kim and Chen, 2004; Cho et al. 2004; reviewed by Laplante and Sabatini, 2009). mTOR signaling is reported to be reduced in placenta from pregnancies complicated by IUGR (Roos et al. 2007; Yung et al. 2008). Further, *in-vitro* studies have demonstrated that the expression of the PPARγ gene is inhibited by methylation of its promoter (Fujiki et al. 2009; Pancione et al. 2010). In a recent review we have discussed that altered methylation could be one of the mechanism regulating the activity of PPAR in the placenta (reviewed by Meher et al. 2015). In addition, our earlier animal studies have also shown that maternal DHA supplementation influences the global methylation patterns in the placenta (Kulkarni et al. 2011c). Thus, the reduced expression of PPARγ could be attributed to the altered epigenetic mechanisms in the placenta. Future studies need to examine these issues.

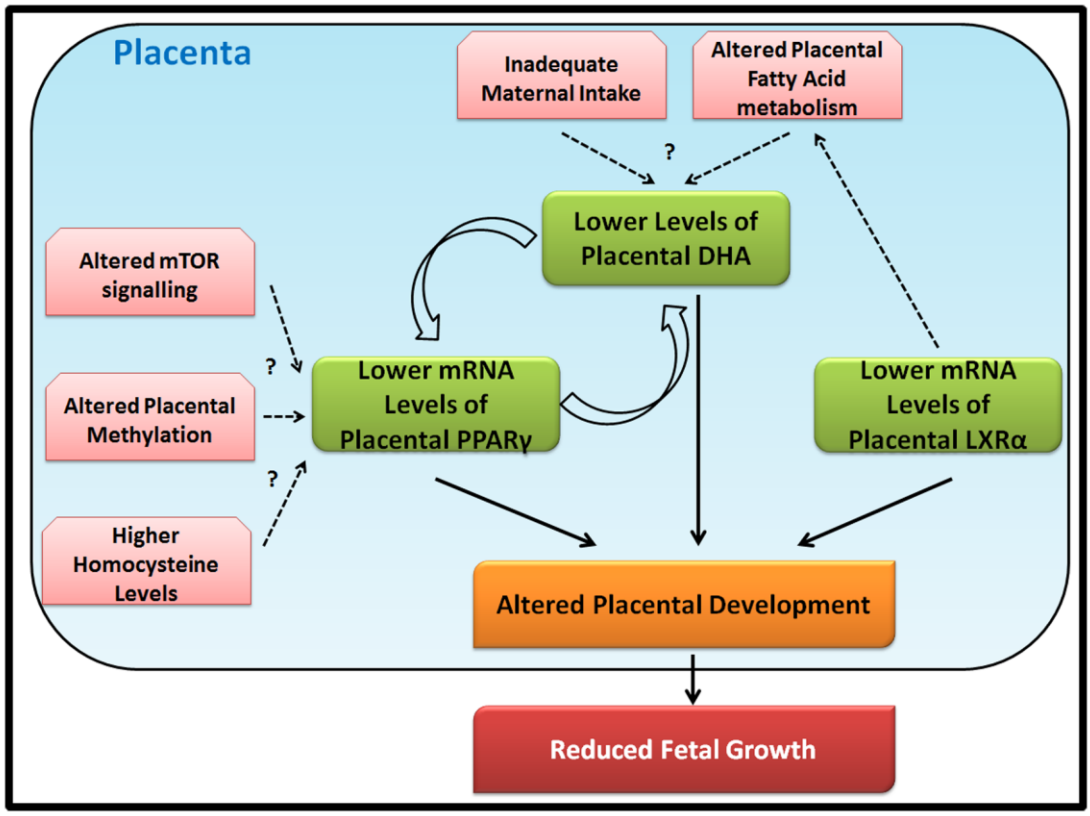
PPARγ is widely studied for its role in placental fatty acid and lipid metabolism (reviewed by Schaiff et al. 2006). Several fatty acid binding proteins

(FABPs) as well as fatty acid transport proteins (FATPs) are involved in the process of uptake of fatty acids from the maternal circulation and transported to fetus through placenta (reviewed by Lager and Powell, 2012). Reports indicate that DHA is highly preferred by fatty acid binding proteins as compared to other fatty acids (Campbell and Duttaroy, 1995; Campbell et al. 1997, 1998a, 1998b). It has been reported that genes encoding several fatty acid transport proteins have PPRE in their promoter regions (Frohnert et al. 1999; Schachtrup et al. 2004). Studies carried out in human trophoblast cells report that activation of PPARγ results in elevated mRNA expression of transport proteins like FATP-1 and FATP-4. Further, another study also demonstrates that *in*-vivo activation of PPARγ results in an increase in the placental expression of FABPpm and fatty acid translocase (FAT/CD36) (Schaiff et al. 2005, 2007). It has been speculated that PPAR plays a role in regulating LCPUFA specific placental uptake. It is likely that the lower levels of DHA and mRNA levels of PPARγ may regulate the activity of different placental fatty acid transport proteins that have a specific affinity for DHA. However, future studies need to investigate the mechanisms through which fatty acids regulate PPAR in the placenta.

5.3.2.3. Lower Placental LXRα mRNA Levels:

In the current study, the mRNA levels of LXRα were found to be lower in the placenta of mothers delivering LBW babies. A down regulation in the expression of LXR in decidua has been reported in spontaneous and recurrent miscarriages (Knabl et al. 2013). LXR has been identified as a key player in human placentation (Weedon-Fekjaer et al. 2005) and feto-placental lipid transport and metabolism (Pavan et al. 2004). Reports have indicated that LXR regulates the expression of fatty acid synthase, long chain acyl-CoA synthetase 3 (ACSL3) and SREBP involved in lipogenesis (Repa et al. 2000; Joseph et al. 2002; Weedon-Fekjaer et al. 2010). It has also been reported that fatty acid transporter protein (FAT/CD36) is a direct LXR target gene (Zhou et al. 2008). Our data suggests that in addition to PPARγ, LXRα may also influence birth outcome and needs further research (Fig 54).

Fig 54: Altered Placental DHA, PPAR and LXR in Women Delivering LBW Babies



DHA, *Docosahexanoic Acid;* ***PPAR,*** *Peroxisome Proliferator Activated Receptor;* ***LXR,*** *Liver X Receptor;* ***mTOR,*** *Mammalian Target of Rapamycin.*

5.3.2.4. Placental SREBP-1c levels:

In the current study, we did not observe any change in the expression of SREBP-1c and RXR genes. LXR is well known to act as a key regulator of the sterol metabolism in the placenta. It has been reported that exposure of trophoblast cells to a synthetic LXR agonist results in an increase in the amount of SREBP-1 and fatty acid synthase activity (Weedon-Fekjaer et al. 2005). To the best of our knowledge, there are no reports investigating the placental expression of SREBP in adverse pregnancy outcomes. Results from the current study demonstrating lower placental LXR mRNA levels in the LBW group, but no change in the mRNA levels of SREBP-1c needs further investigation. In addition to its role in placental fatty acid uptake, SREBP-1 is known to be involved in the process of *de-novo* synthesis of long-chain saturated and monounsaturated fatty acids (reviewed by Duttaroy et al. 2009). However, in this study, the levels of long-chain saturated and monounsaturated fatty acids were similar between the groups.

5.3.2.5. Positive Association of both Placental DHA and mRNA Levels of Placental PPARγ with Baby Weight:

In the current study, we observed a positive association of both placental DHA and mRNA levels of placental PPARγ with baby weight. Several studies have shown an association of maternal DHA status during pregnancy with gestation and birth outcome (Smuts et al. 2003b; Szajewska et al. 2006; van Eijsden et al. 2008; Muthayya et al. 2009; Salvig and Lamont, 2011; Smits et al. 2013). It is well established that PPARγ influences placental and fetal growth (Barak et al. 1999; Parast et al. 2009). Thus, the observed lower placental DHA levels and mRNA levels of PPARγ in this study may contribute to altered placental development thereby affecting fetal growth.

5.3.3. Conclusion:

To summarize, our data demonstrates a positive association of maternal DHA levels in early pregnancy with birth weight and therefore, suggests the potential benefits of the supplementation of DHA in improving pregnancy outcome. There is now a considerable body of evidence which suggests that early life environment of the fetus affects future disease risk. Therefore, the current study has implications for the same.

In addition, our study demonstrates that fatty acids especially DHA and transcription factors like PPAR and LXR are altered in the placenta of women delivering LBW babies. This study has implications for countries like India which is referred to as the world capital of LBW babies, who are at a higher risk of developing non-communicable diseases in later life.

SUMMARY

SUMMARY:

The present thesis entitled "**Effect of maternal nutrition during the pre/peri conceptional period on the transcription factors involved in the fatty acid metabolism**" comprises of an animal and a human study. The animal study was carried out to determine the effects of maternal micronutrient (folic acid and vitamin B_{12}) deficiency starting from the pre-conception period on the reproductive performance. The effect of supplementation of omega-3 fatty acids to these diets was also examined. The human study is a prospective study carried out on 111 women delivering normal birth weight (NBW) and low birth weight (LBW) babies at term. These women were followed from 16^{th}-20^{th} weeks of gestation until delivery to examine the levels of long chain polyunsaturated fatty acids (LCPUFA) at various time points and their association with birth outcome. Placental mRNA levels of various transcription factors like peroxisome proliferator activated receptor (PPAR), sterol regulatory binding proteins (SREBP), liver X receptor (LXR) and retinoid X receptor (RXR) were examined to understand their association with fatty acid metabolism both in the animal and human study.

Periconceptional maternal nutrition is known to be an important determinant of embryonic, placental as well as fetal growth and development. It has been reported that the Indian population is deficient in micronutrients like folic acid and vitamin B_{12} during pregnancy. These micronutrients are well recognized for their importance in pregnancy and fetal development. These micronutrients along with LCPUFA are known to regulate several processes involved in placental development such as extravillous trophoblast invasion and angiogenesis. Impaired placental development and function has been reported to be associated with low birth weight in babies.

Several animal and human studies carried out in our department have demonstrated that docosahexanoic acid (DHA) is interlinked with micronutrients like folic acid and vitamin B_{12} in the one carbon cycle. Our earlier departmental studies in humans have demonstrated lower maternal LCPUFA levels in preeclampsia and preterm pregnancy. LCPUFA especially DHA are known to regulate the expression of several transcription factors like PPAR, SREBP, LXR and RXR. These transcription factors are expressed in the human placenta and play a critical role in the placental development and fatty acid metabolism.

Few studies in animals have shown that maternal nutrition (folate, vitamin B_{12}, methyl deficient diets, high multivitamin diet) affects the expression of transcription factors (PPARα, PPARγ, SREBP) during pregnancy and lactation. Reports have also indicated that the effects of maternal nutrition on transcription factors are possibly mediated through promoter hypermethylation or hypomethylation. Animal studies in our department suggest that maternal folic acid, vitamin B_{12} and omega-3 fatty acids influence placental methylation levels. It is possible that these micronutrients regulate the activity of placental transcription factors and thereby influence development and ultimately fetal growth. However, despite of the importance of transcription factors in the placental development, their role in adverse pregnancy outcome like LBW is not completely understood. Further, the association of fatty acids and transcription factors in the human placenta remains to be established.

In view of this, it is important to undertake studies to examine the role for altered maternal micronutrient metabolism affecting the expression of transcription factors involved in the placental development.

HYPOTHESIS:

"Maternal folic acid, vitamin B_{12} and DHA modulate the activity of key transcription factors involved in the placental development thereby affecting the fetal growth".

Animal Study:

Female rats (n=16 per group) were randomly assigned to a control and 4 treatment groups. The compositions of control and treatment diets were as per AIN-93 guidelines for the laboratory rodent. Both the control and treatment diets contained 18% protein. Briefly, the treatment diets were as follows: folic acid deficient (FD), vitamin B_{12} deficient (BD), folic acid deficient + omega-3 fatty acid supplemented (FDO) and vitamin B_{12} deficient + omega-3 fatty acid supplemented (BDO). The omega-3 fatty acid supplementation used in the present study was fish oil (Maxepa; Merck, Goa, India) and was a combination of DHA (120 mg) and EPA (180 mg). Folic acid and vitamin B_{12} deficiencies were achieved exclusively through dietary means. 8 dams/group were delivered by caesarean section on GD20. Remaining 8 dams/group continued on the same diet and were allowed to deliver normally. The dam and offspring were dissected at two time points (GD20 and postnatal day 22) to

collect the blood, placenta and liver tissue for various biochemical and molecular estimations.

The novel findings from the animal study are:

Maternal micronutrient (Folic acid and Vitamin B_{12}) deficiency resulted in

1. Reduced number of oestrous cycles and altered the phases of the oestrous cycle, which may influence the reproductive health.
2. Abnormal levels of progesterone, which is required for the maintenance of pregnancy.
3. Altered morphology of the mammary gland and ovary.
4. Increased MDA levels in the dam plasma at GD20 which indicate increased maternal oxidative stress.
5. Lower levels of DHA in the placenta which may be a result of reduced expression of the phosphatidyl ethanolamine methyl transferase gene that catalyzes the conversion of phosphatidyl ethanolamine to phosphatidyl choline. Alternatively it may be due to altered expression of fatty acid desaturases.
6. Reduced expression of PPARγ in the placenta which may be attributed to the observed lower levels of DHA in the placenta.
7. Increased inflammatory cytokines like IL-6 and TNF-α in the placenta.
8. Increased levels of placental global DNA methylation. Folate and vitamin B_{12} are known to regulate the formation of S-adenosyl methionine molecule which is the universal methyl donor for most of the reactions and any disturbance in the one carbon cycle can affect DNA methylation levels.
9. Lower levels of DHA in dam liver at GD20.
10. Lower expression of PPARγ in the dam liver at GD20. PPAR is known to be a master transcriptional regulator of lipid metabolism in liver. Thus, the lower expression of PPARs in the maternal liver may affect the lipid metabolism in the dam which may be responsible for adverse birth outcome.
11. Lower levels of DHA in pup liver.
12. Lower expression of PPARα and PPARγ genes in the pup liver. Dietary PUFAs regulate the abundance of these nuclear transcription factors and thus, it may be possible that the decrease in the levels of DHA and ARA is associated with the lower expression of PPARs.

13. No change in the expression of SREBP-1c, LXRα, RXRα in the pup liver.
14. Increased pup liver global DNA methylation levels.

The results of the present study for the first time demonstrate that maternal vitamin B_{12} and folate deficiency alters the oestrous cycle in the pre-conception period. During pregnancy, it also reduces fatty acid levels as well as expression of transcription factors in the dam liver, placenta as well as in the offspring liver on postnatal day 22. Omega-3 fatty acid supplementation was found to be beneficial in improving the reproductive performance as well as the mRNA levels of transcription factors involved in the fatty acid metabolism.

Human Study:

Pregnant women were enrolled for this longitudinal study from Department of Obstetrics and Gynaecology, Bharati hospital Pune, India. The study was approved by the Bharati Vidyapeeth Medical College Institutional Ethical Committee and written consent was taken from each subject at the time of enrollment. The current study is a part of ongoing prospective departmental study which recruits all healthy women at 16^{th}-20^{th} weeks of gestation and follows them throughout pregnancy. At delivery, these women were divided into 2 groups; those delivering at term a baby weighing >2.5kg [Normal birth weight (NBW) group] and those delivering at term a baby weighing <2.5kg [Low birth weight (LBW) group]. Blood was collected at three different time points; i.e. between 16^{th} - 20^{th} weeks of gestation (T1), between 26^{th} - 30^{th} weeks of gestation (T2) and just before going to the labor room (T3). Umbilical cord blood and placenta was also collected. This study includes data on 60 women delivering NBW babies at term and 51 women delivering LBW babies at term. Subjects included in this study were only those women with singleton pregnancy and having no medical or obstetrical complications. Maternal demographic characteristics and clinical information such as maternal age, body mass index (BMI), systolic and diastolic blood pressure, gestation, parity, placental weight, income and education were recorded. Neonatal measurements like baby weight, length, head circumference and chest circumference were recorded by standard techniques.

The novel findings from the human study are:

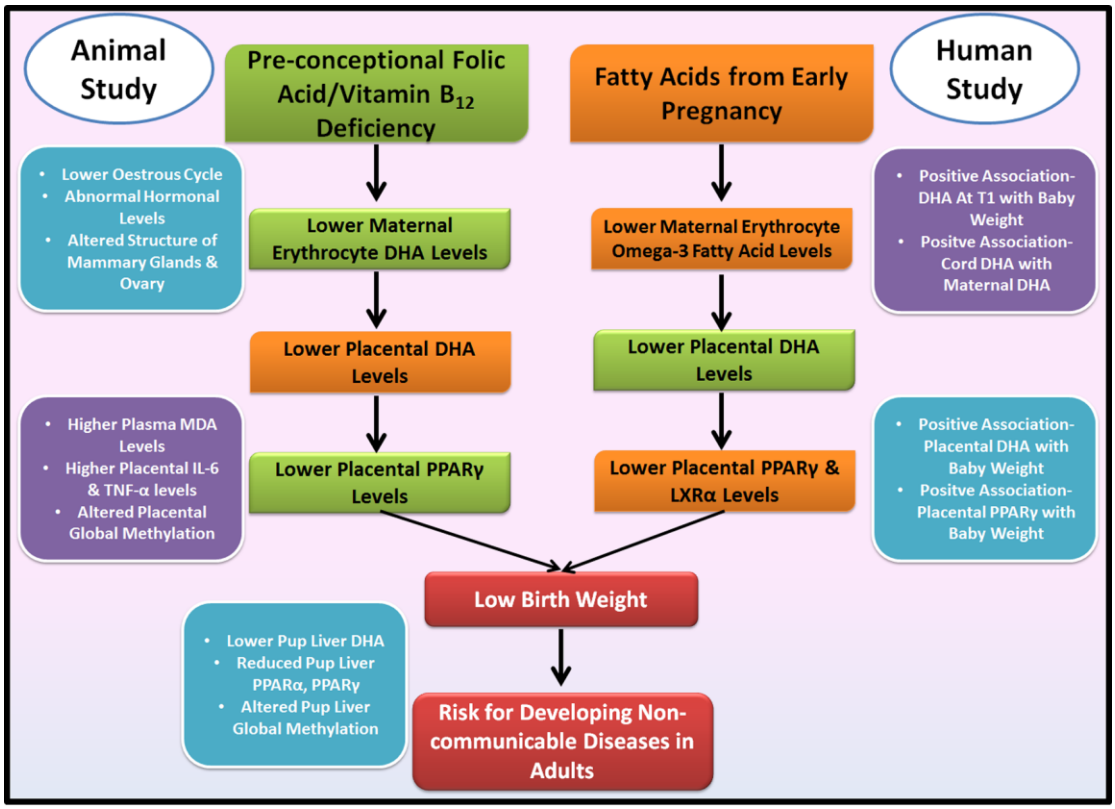
1. Higher maternal erythrocyte total omega-6 fatty acids and ARA levels at 26^{th}-30^{th} weeks of gestation in women delivering LBW babies. Our findings are

similar to earlier studies which report a negative association between higher ARA during pregnancy with birth weight.

2. Lower maternal erythrocyte total omega-3 fatty acids at delivery in women delivering LBW babies, might reflect the mother's inability to supply adequate amounts of LCPUFA for optimal fetal development.
3. Higher levels of maternal erythrocyte SFA at delivery in women delivering LBW babies can possibly be attributed to the inadequate transfer of these fatty acids through placenta, contributing to inadequate fetal growth.
4. Higher cord fatty acid levels as compared to maternal levels in both NBW and LBW groups suggest that fetus increases the LCPUFA percentage in the fetal blood in order to support central nervous system development.
5. Maternal erythrocyte DHA at T1 was positively associated with baby weight. Further, a positive association between cord DHA levels and maternal DHA levels at all the time points across the gestation suggests the possible benefits of DHA supplementation in early pregnancy.
6. Lower levels of placental DHA in women delivering LBW babies. DHA is involved in several processes of placental development like placental fatty acid transport, eicosanoid production, placental angiogenesis, placental oxidative stress and regulation of transcription factors. Thus, the lower levels of placental DHA may have implications in placental development and function. This result is supported with the observed positive association between placental DHA levels and baby weight.
7. Lower mRNA levels of placental PPARγ in women delivering LBW babies. These results may be attributed to the observed lower DHA levels in the placenta of the women delivering LBW babies. Alternatively, altered epigenetic mechanisms in the placenta could result into lower levels of placental PPARγ.
8. Lower mRNA levels of placental LXRα in women delivering LBW babies. LXR is a key player in human placentation and feto-placental lipid transport and metabolism. Thus, the lower levels of placental LXRα may influence birth outcome.
9. A positive association of mRNA levels of placental PPARγ with baby weight is consistent with the fact that PPARγ influences placental and fetal growth.

The entire summary of results of animal and human study is shown in Fig 55.

Fig 55: Summary of the Animal and Human Study



DHA, *Docosahexanoic acid;* ***PPAR,*** *Peroxisome proliferator activated receptor;* ***LXR,*** *Liver X receptor;* ***IL-6,*** *Interleukin-6;* ***TNF-α,*** *Tumor necrosis factor-α;* ***MDA,*** *Malondialdehyde;* ***T1-*** *16th-20th week of pregnancy.*

Implications:

Epidemiological studies have established the association between maternal nutrition and the risk for non-communicable diseases in the offspring in later life. The animal study presented in this thesis has demonstrated that omega-3 fatty acid supplementation right from the pre-conception period and continuing through pregnancy is beneficial. In addition, the human study demonstrates a positive association of maternal DHA in early pregnancy with birth weight and therefore, suggests the potential benefits of supplementing DHA from early pregnancy. The current study for the first time establishes an association of maternal one carbon cycle with placental transcription factors which may influence fetal growth.

Limitations:

In the animal study, we did not evaluate the degree of fertility and the interaction between gender and the diet was also not studied. In the human study, we

did not evaluate the levels of folic acid and vitamin B_{12} across the gestation. The study needs to be carried out on larger sample size. In addition, future studies examining the gene specific methylation of PPARγ and LXRα genes in the human placenta will throw light into the mechanism leading to fetal programming of adult diseases.

Societal Relevance:

The current work helps in understanding the mechanisms through which maternal nutrition influences the regulation of transcription factors during pregnancy. In India, the majority of the population consumes a vegetarian diet which is deficient in both vitamin B_{12} and DHA. This study provides clues for nutritional interventions for a better pregnancy outcome. Further, it is well established that children born low birth weight are at increased risk of developing non-communicable diseases; therefore improving maternal nutrition will be helpful in reducing later life diseases.

Future Prospects:

The role of the endogenous ligands as well as the activation of transcription factors depends on different factors, including maternal nutritional status and methylation potential. More research is needed to identify the different maternal environmental factors as well as the concentration of ligands that influence the activation of transcription factors. In addition, there is a need to examine the involvement of transcription factors in preeclampsia and preterm birth.

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PUBLICATIONS

PUBLICATIONS (TOTAL NO: 5)

Total Impact Factor (10.5)

Average Impact Factor (2.625)

1. **Meher AP**, Sundrani DP, Joshi SR. "Maternal Nutrition Influences Angiogenesis in the Placenta through Peroxisome Proliferator Activated Receptors" **Molecular Reproduction and Development.** 2015;82(10):726-34. **(Impact Factor- 2.527).**

2. **Meher AP**, Joshi AA, Joshi SR. "Differential Regulation of Hepatic Transcription Factors in the Wistar Rat Offspring Born to Dams Fed Folic Acid, Vitamin B_{12} Deficient Diets and Supplemented with Omega-3 Fatty Acids" **PLoS One**. 2014;9(2):e90209. **(Impact Factor- 3.234).**

3. **Meher AP**, Joshi AA, Joshi SR. "Maternal Micronutrients, Omega-3 Fatty Acids, and Placental PPARγ Expression" **Applied Physiology Nutrition and Metabolism.** 2014;39(7):793-800. **(Impact Factor- 2.339).**

4. **Meher AP**, Joshi AA, Joshi SR. **"**Preconceptional Omega-3 Fatty Acid Supplementation on a Micronutrient-Deficient Diet Improves the Reproductive Cycle in Wistar Rats**" Reproduction Fertility and Development.** 2013;25(7):1085-94. (**Impact Factor- 2.400)**.

Book Chapter:

1. Rani A, **Meher A,** Wadhwani N, Joshi S. 2015. "Role of Maternal Long-Chain Polyunsaturated Fatty Acids in Placental Development and Function". In the book "**Human Placental Trophoblast: Impacts on Maternal Nutrition**" (Eds Duttaroy A and Basak S), pp 120-129, Taylor and Francis Group Ltd.

Submitted:

1. **Meher AP**, Randhir K, Mehendale S, Wagh G, Joshi SR. "Maternal Fatty Acids and Their Association with Birth Outcome: A Prospective Study" communicated to **Journal PLoS One. (Impact Factor- 3.234).**
2. **Meher AP**, Wadhwani N, Randhir K, Mehendale S, Wagh G, Joshi SR. "Lower mRNA Levels of Key Placental Transcription Factors in Women Delivering Low Birth Weight Babies" communicated to **Journal of Clinical Lipidology. (Impact Factor- 3.904).**

Molecular Reproduction and Development 2015, 82(10):726-34.

Molecular Reproduction and Development

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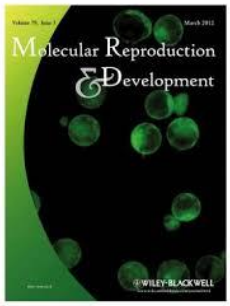

Maternal Nutrition Influences Angiogenesis in the Placenta Through Peroxisome Proliferator Activated Receptors: A Novel Hypothesis

Akshaya P. Meher, Deepali P. Sundrani, Sadhana R. Joshi

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ABSTRACT

Placental angiogenesis is critical to maintain adequate blood flow during gestation, and any alterations in this process can result in an adverse pregnancy. Growing evidence indicates that suboptimal maternal nutrition can alter placental development. Although the underlying mechanisms are not clear, maternal nutrition likely influences the expression of genes involved in placental development through regulation of various transcription factors such as peroxisome proliferator-activated receptors (PPARs), which can be activated by ligands including long-chain polyunsaturated fatty acids. Indeed, several studies demonstrated a role for PPAR in implantation, trophoblast differentiation, and angiogenesis. Alterations in maternal nutrition during pregnancy can affect the expression of PPARs via epigenetic mechanisms or through homocysteine, which is known to compete for PPARs. This review discusses the role of maternal nutrition particularly micronutrients like folate, vitamin B_{12}, and omega-3 fatty acids in modulating the activity of PPARs during placentation and angiogenesis, which affects placental and fetal growth. Additional animal and human studies need to be undertaken to elucidate the molecular mechanisms through which maternal nutrition regulates PPARs, specifically to determine whether PPARs affect placental angiogenesis directly through angiogenic factors or indirectly by modulating trophoblast differentiation.

PLoS One, 2014, 9(2):e90209

Differential Regulation of Hepatic Transcription Factors in the Wistar Rat Offspring Born to Dams Fed Folic Acid, Vitamin B_{12} Deficient Diets and Supplemented with Omega-3 Fatty Acids

Akshaya P. Meher, Asmita A. Joshi, Sadhana R. Joshi

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ABSTRACT

Nutritional status of the mother is known to influence various metabolic adaptations required for optimal fetal development. These may be mediated by transcription factors like peroxisome proliferator activated receptors (PPARs), which are activated by long chain polyunsaturated fatty acids. The objective of the current study was to examine the expression of different hepatic transcription factors and the levels of global methylation in the liver of offspring born to dams fed micronutrient deficient (folic acid and vitamin B_{12}) diets and supplemented with omega-3 fatty acids. Female rats were divided into five groups (n = 8/group) as follows; control, folic acid deficient (FD), vitamin B_{12} deficient (BD) and omega-3 fatty acid supplemented groups (FDO and BDO). Diets were given starting from pre-conception and continued throughout pregnancy and lactation. Pups were dissected at the end of lactation. Liver tissues were removed; snap frozen and stored at -80^{o}C. Maternal micronutrients deficiency resulted in lower ($p<0.05$) levels of pup liver docosahexaenoic acid (DHA) and arachidonic acid (ARA) as compared to the control group. Pup liver PPARα and PPARγ expression was lower ($p<0.05$) in the BD group although there were no differences in the expression of SREBP-1c, LXRα and RXRα expression. Omega-3 fatty acids supplementation to this group normalized ($p<0.05$) levels of both PPARα and PPARγ but reduced ($p<0.05$) SREBP-1c, LXRα and RXRα expression. There was no change in any of the transcription factors in the pup liver in the FD group. Omega-3 fatty acids supplementation to this group reduced ($p<0.05$) PPARα, SREBP-1c and RXRα expression Pup liver global methylation levels were higher ($p<0.01$) in both the micronutrients deficient groups and could be normalized ($p<0.05$) by omega-3 fatty acid supplementation. Our novel findings suggest a role for omega-3 fatty acids in the one carbon cycle in influencing the hepatic expression of transcription factors in the offspring.

Applied Physiology Nutrition and Metabolism, 2014, 39(7):793-800

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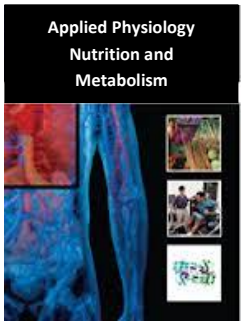

Maternal Micronutrients, Omega-3 Fatty Acids, and Placental PPARγ Expression

Akshaya P. Meher, Asmita A. Joshi, Sadhana R. Joshi

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ABSTRACT

An altered one-carbon cycle is known to influence placental and fetal development. We hypothesize that deficiency of maternal micronutrients such as folic acid and vitamin B_{12} will lead to increased oxidative stress, reduced long-chain polyunsaturated fatty acids, and altered expression of peroxisome proliferator activated receptor (PPARγ) in the placenta, and omega-3 fatty acid supplementation to these diets will increase the expression of PPARγ. Female rats were divided into 5 groups: control, folic acid deficient, vitamin B_{12} deficient, folic acid deficient + omega-3 fatty acid supplemented, and vitamin B_{12} deficient + omega-3 fatty acid supplemented. Dams were dissected on gestational day 20. Maternal micronutrient deficiency leads to lower ($p<0.05$) levels of placental docosahexaenoic acid, arachidonic acid, PPARγ expression and higher ($p<0.05$) levels of plasma malonidialdehyde, placental IL-6, and TNF-α. Omega-3 fatty acid supplementation to a vitamin B_{12} deficient diet normalized the expression of PPARγ and lowered the levels of placental TNF-α. In the case of supplementation to a folic acid deficient diet it lowered the levels of malonidialdehyde and placental IL-6 and TNF-α. This study has implications for fetal growth as oxidative stress, inflammation, and PPARγ are known to play a key role in the placental development.

Reproduction Fertility and Development, 2013; 25(7):1085-94

Reproduction Fertility and Development

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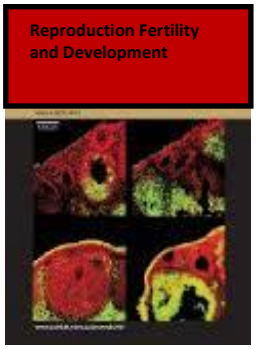

Preconceptional Omega-3 Fatty Acid Supplementation on a Micronutrient-Deficient Diet Improves the Reproductive Cycle in Wistar Rats

Akshaya P. Meher, Asmita A. Joshi, Sadhana R. Joshi

Interactive Research School for Health Affairs, Bharati Vidyapeeth University, Pune 411043, India

ABSTRACT

Folic acid and vitamin B_{12} deficiencies are associated with high reproductive risks ranging from infertility to fetal structural defects. The aim of the present study was to examine the effects of preconceptional omega-3 fatty acid supplementation (eicosapentaenoic acid and docosahexaenoic acid) to a micronutrient-deficient diet on the reproductive cycle in Wistar rats. Female rats were divided into five groups from birth and throughout pregnancy: a control group, a folic acid-deficient (FD) group, a vitamin B_{12}-deficient (BD) group, a folic acid-deficient + omega-3 fatty acid-supplemented (FDO) group and a vitamin B_{12} deficient + omega-3 fatty acid-supplemented (BDO) group. Dams were killed on gestation Day 20 and their ovaries and mammary glands were dissected out and subjected to histological examination. Maternal micronutrient deficiency (FD and BD groups) resulted in an abnormal oestrous cycle ($p<0.001$), whereas omega-3 fatty acid supplementation (FDO and BDO groups) restored the oestrous cycle to normal. There were fewer corpora lutea in the ovaries of FD rats compared with controls. In addition, rats in both the FD and BD groups exhibited an absence of lactating ducts in their mammary glands compared with controls. The findings of the present study indicate, for the first time, that maternal micronutrient deficiency affects the oestrous cycle and morphology of the ovary and mammary glands. Omega-3 fatty acid supplementation ameliorated these effects. This may have implications for infertility and pregnancy outcomes.

Human Placental Trophoblast: Impacts on Maternal Nutrition

Role of Maternal Long-Chain Polyunsaturated Fatty Acids in Placental Development and Function

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ABSTRACT

During pregnancy, maternal long-chain polyunsaturated fatty acids (LCPUFAs) are preferentially transferred by the placenta to meet the requirements of the developing fetus. These LCPUFAs are essentially required by the trophoblast cells right from early gestation for several physiological processes involved in optimum placental growth and activity. They are structural constituents of the cell membrane, stimulate angiogenesis, and are metabolized into various eicosanoids, which modulate inflammation in the placenta. LCPUFAs and their metabolites also act as ligands for transcription factors like peroxisome proliferator-activated receptors, which regulate the expression of various physiologically important genes. Several pregnancy complications like preeclampsia are associated with increased placental inflammation and oxidative stress, and omega-3 LCPUFAs are known to reduce excess inflammation and oxidative damage in the trophoblast cells. This chapter describes the multiple roles of maternal LCPUFAs in placental development and function, reducing the risk of adverse pregnancy outcomes.

AWARDS

AWARDS

Total Number - 4

1. **"Travel Award of 2000 SGD"** from the **"8th World Congress on Developmental Origins of Health and Disease"** for the paper **"Preconceptional Omega-3 Fatty Acid Supplementation on a Micronutrient Deficient Diet Alters Hepatic Transcription Factors Expression in Wistar Rats"** held at SUNTEC Singapore on Nov 26-29, 2013.

2. **"Best Oral Presentation Award"** at "**International Conference on Food and Nutrition Technology for Public Health Care**" held at New Delhi for the paper "**Effect of Pre-Conceptional Maternal Micronutrients and Omega-3 Fatty Acids on Dam Placental Fatty Acid Profile in Wistar Rats**" on May 4-5, 2012; pg-89.

3. **"Best Oral Presentation Award"** at "**43rd National Conference of Nutrition Society of India**" held at Hyderabad for the paper "**Preconception Maternal Micronutrient Deficiency: Reproductive Cycles and Breast Development in Wistar Rats**" on Nov 11-12, 2011; pg-73.

4. **"Junior and Senior Research Fellowship"** for pursuing PhD by **"Department of Science and Technology (DST)"** under the **"INSPIRE"** programme.

INTERNATIONAL CONFERENCES

INTERNATIONAL CONFERENCES

Total Number - 5

1. Mini Oral presentation at "**8th World Congress on Developmental Origins of Health and Disease**" held at SUNTEC Singapore of the paper "**Preconceptional Omega-3 Fatty Acid Supplementation on a Micronutrient Deficient Diet Alters Hepatic Transcription Factors Expression in Wistar Rats** " on Nov 17-20, 2013; Pg- S239.

2. Oral presentation at "**First International and Third National Conference on Biotechnology, Bioinformatics and Bioengineering**" held at Tirupati, Andhra Pradesh of the paper "**Effect of Omega-3 Fatty Acid Supplementation to a Micronutrient Deficient Diet on the Placental PPARγ Expression in Wistar Rats**" on June 28-29, 2013; Pg- 121.

3. Poster presentation at **17th Society of Natal effects on health in adults** meeting held at Mysore of the paper "**Omega-3 Fatty Acids, Folic Acid, Vitamin B_{12} and Placental PPARγ Expression"** on Feb 1-3, 2013; pg-39.

4. Oral presentation at **"International Conference on Food and Nutrition Technology for Public Health Care"** held at New Delhi for the paper **"Effect of Pre-Conceptional Maternal Micronutrients and Omega -3 Fatty Acids on Dam Placental Fatty Acid Profile in Wistar Rats"** on May 4-5, 2012; pg-89.

5. Poster presentation at **16th Society of Natal effects on health in adults** meeting held at CCMB Hyderabad of the paper "**Preconceptional Micronutrient Deficiency Leads to Abnormal Estrous Cyclicity while Omega-3 Fatty Acid Supplementation Results in Normalcy in Wistar Rats"** on Feb 3-5, 2012; pg-25.

NATIONAL CONFERENCES

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Total Number - 3

1. Oral presentation at "**46th National Conference of Nutrition Society of India**" held at Ludhiana for the paper "**Maternal One Carbon Cycle and Placental Transcription Factors (LXRα) in Women Delivering Low Birth Weight Babies**" on Nov 6-8, 2014; pg-55.
2. Oral presentation at "**44th National Conference of Nutrition Society of India**" held at Tirupati for the paper "**Preconception Omega-3 Fatty Acid Supplementation to a Micronutrient Deficient Diet Improves Placental PPARγ Expression**" on Nov 16-17, 2012; pg-69.
3. Oral presentation at "**43rd National Conference of Nutrition Society of India**" held at Hyderabad for the paper "**Preconception Maternal Micronutrient Deficiency: Reproductive Cycles and Breast Development in Wistar Rats**" on Nov 11-12, 2011; pg-73.