David Barker along with his colleagues in Southampton, United Kingdom pioneered the concept of effects of intra-uterine environment on adult disease and put forward his ideas as the “foetal origins of adult disease” hypothesis. It stated that environmental factors, particularly intrauterine nutrition, act in early life to program future cardiovascular and metabolic diseases and premature death. Nick Hales and David Barker showed that men with a low birth weight were six times more likely to have diabetes than those with the highest birth weight. These findings formed the basis of the Thrifty Phenotype Hypothesis that was put forward by Nick Hales during his Banting Award Lecture in Dublin in 1991. This was an extension of the “Thrifty Genotype” put forward by Neel in 1932. He had hypothesized that during evolution, when food resources were not readily available or during times of famine, “thrifty genes” were selected. According to Neel, this resulted in a “Fast insulin trigger” leading to increased storage of fat especially visceral fat. This leads to a heightened risk of diabetes mellitus and cardiovascular disease.

Epidemiological and animal studies have been able to prove Barker’s hypothesis and have shown associations between growth patterns after birth and increased risks for obesity, hypertension, dyslipidemia, cardiovascular disease, inflammatory states, insulin resistance and
diabetes mellitus in adult life and are part of the metabolic syndrome.\textsuperscript{10} The foetal origin hypothesis was thus extended to include all these components of the metabolic syndrome and is now known as the “Developmental Origins of Health and Disease (DOHaD). These studies have suggested that when the foetal environment is poor, there is an adaptive response, which optimizes the growth of key body organs to the detriment of others and leads to an altered postnatal metabolism, which is designed to enhance postnatal survival under conditions of intermittent or poor nutrition. These adaptations or “programming” lead to changes at the cellular and physiological levels. These studies have primarily focused on the pancreas, kidney, cardiovascular system, the endocrine system and the adaptations thereof.

Nutrition is known to affect the reproductive success in a variety of animal species. Undernutrition is known to result in loss of body weight, delay the onset of puberty, increase the postpartum interval to conception, and interfere with the normal ovarian cyclicity by decreasing the gonadotropin secretion & increase infertility. In cows it has been observed that restricting energy intake during late gestation is known to increase the length of postpartum anoestrous and reduce subsequent pregnancy rate. It is also known to cause delayed uterine involution, delayed first oestrous after calving & increased incidence of cystic
Maternal undernutrition in sheep indicates that meiotic maturation of germ cells is delayed by food restriction. Few studies have focused on the adaptations in multigenerational undernutrition especially on the reproductive system. The present study evaluated the effects of multigenerational undernutrition on anthropometry, folliculogenesis, steroidogenesis, histological changes and haematological profile in the undernourished as well as the transition group rats.

**Histology:**

The undernourished group rats on histological examination after haematoxylin and eosin staining showed greater number of follicles (7-8) as compared to the control group or the transition group rats. This appeared to be like the necklace pattern with multiple follicles arranged around the periphery; a typical feature seen in polycystic ovarian syndrome (PCOS) patients. (Figures 15a, 15b, 15c & 15d)

The transition group rats showed a greater amount of fat deposition in the ovarian stroma as compared to the control and undernourished group rats. (Figures 16a, 16b & 16c)
Hormonal Assays

Estrogen:

Estrogen levels were greatly reduced in the undernourished group rats but were elevated, though not to the control values in the transition group rats after a standard pellet diet. (Figure 17)

Progesterone:

Progesterone levels were reduced in the undernourished group rats but were almost near normal levels in the transition group rats. A standard pellet diet thus caused restoration of these levels in the transition group rats. (Figure 17)

Luteinizing Hormone [LH]:

The luteinizing hormone levels were lower in the undernourished group rats as compared to control group rats but elevated slightly in the transition group rats. (Figure 17)

Follicle Stimulating Hormone [FSH]:

The levels in the undernourished group rats and control group rats were almost the same but were raised in the transition group rats. (Figure 17).

Steroidogenesis is regulated by the interplay between the pituitary gonadotropins like FSH and LH, the changes in the gene expression pattern and a variety of transcription factors like follistatin, activin,
Insulin like growth factor-I (IGF-I) etc. This study therefore evaluated the role of FSH, LH, the expression of genes regulating steroid synthesis (CYP17A1 and CYP19A1), growth and folliculogenesis (BMP-4 and BMP 15) and the receptor population of the pituitary gonadotropins and ovarian hormones (FSHr, LHR, Esr α, Pgr).

**Steroidogenesis:**

The classical definition of a hormone is a substance that travels from a special tissue where it is released into the blood stream, to distant responsive cells where the hormone exerts its characteristic effects. Hormones have always been thought of as a means of communications and control along with the nervous system. The initial thoughts of a simple journey is now a much vaunted voyage as new discoveries bring forth a better understanding of the complex effects of these hormones. These hormones along with their receptors have also been discovered in unicellular organisms. Similarly, startling discoveries of these in the most unexpected places such as gastrointestinal hormones in the brain, reproductive hormones in intestinal secretions and the ability of malignant tissues for hormone synthesis makes the puzzle all the more interesting. The specialized endocrine glands evolved with the multicellular organisms. Even more puzzling is the fact that hormones probably appeared during evolution before the separation of the plant and
animal kingdom. This is corroborated by the fact that many substances of plant origin are very similar to the hormones. (Chapter 2 pg 31)\textsuperscript{51}

All steroid hormones have more or less a similar structure but with minor differences leading to differences in biochemical or physiological activities. Each hormone has a primary perhydrocyclopentanephenanthrene molecule made of a benzene ring, two naphthalene rings, three phenantherene rings and a cyclopentane.

The sex steroids are divided into 3 main groups based on the number of carbon atoms. These include the 21-carbon series consisting of the pregnane nucleus and includes the corticoids and progestins, the 19 carbon series consisting of the androstane nucleus and consists of all the androgens and the 18 carbon series comprising the estrane nucleus of which the estrogens are the primary hormones (Chapter 2, pg 34)\textsuperscript{53}.

Cholesterol forms the primary building blocks of the steroids and all steroid producing organs including the ovary synthesize cholesterol with the exception of the placenta. However, the ovarian tissue cannot meet all the demands and blood cholesterol is the only available resource. It binds to the LDL receptor on the cell membrane to enter the cell (Chapter 2, pg 37)\textsuperscript{53}.

The steroidogenic pathway that is known today has been a result of the pioneering work of K.J.Ryan and his co workers\textsuperscript{62}. All steroid producing
pathways follow a fundamental pattern that is seen even with the ovary and produce all the 3 classes of sex steroids: estrogens, androgens and progestins. The ovary is fundamentally different from the test due to a differing set of critical enzymes and from the adrenal cortex due to its deficiency in 21-hydroxylase and 11β-hydroxylase. The glucocorticoids and mineralocorticoids are thus not produced in the ovary (Chapter 2, pg 37).  

Cytochrome P450 is a generic term that includes the oxidative enzymes and steroidogenic enzymes that are members of this cytochrome P450 group of oxidases. The term 450 is used to denote the pigment (450) absorbance shift on reduction. These enzymes are known to metabolize toxins and environmental pollutants. There are many distinct enzymes associated with the process of steroidogenesis but this study has concentrated on the genes that regulate two important enzymes viz, CYP17 (P450c17) regulated by the Cyp17A1 and CYP19 (P450arom) that is regulated by the CYP19A1 gene. 

In the humans the reactions involved in the conversion of pregnenolone and progesterone to their 17-hydroxylated products are mediated by a single enzyme, P450c17 bound to the smooth endoplasmic reticulum and regulated by the gene on chromosome 10 (Chapter 2, pg 42). In the rats it is present on chromosome 1.
The aromatase enzyme regulated by the CYP19A1 gene brings about the aromatization process i.e. the conversion of testosterone to estrogen. The gene in the humans is located in the chromosome 15q21.1 (Chapter 2, pg 42) and for the rat on chromosome 8. This process is regulated by various cytokines, cyclic nucleotides, gonadotropins, glucocorticoids and growth factors. (Table 6)

Cholesterol is the basic building block in the process of steroidogenesis and it can be synthesized from acetate by all the steroid producing organs except the placenta. Thus the female sex hormones, estrogen and progesterone along with androgens are synthesized within the ovary. There is however a limit to the amount of the sex steroids that can be formed within the ovary and as stated above, the blood cholesterol (liver) is the main source for the extra amount of cholesterol that is required.

The first step in the process of steroidogenesis involves the conversion of cholesterol which is a C27 steroid to the C21 product pregnenolone. All other steroids, ovarian, testicular and adrenocortical are formed from pregnenolone. This rate limiting step is regulated by the cytochrome P450 enzyme (P450scC) regulated by the CYP11A gene. (Fig 12a & 12b). This process occurs in the mitochondria. The pregnenolone thus synthesized can follow 3 different pathways for steroid formation. Within the ovary, 2 different pathways, D5 and D4 pathways are common (Fig 12a).
third pathway is seen within the adrenal cortex and is related to pregnenolone metabolism. In the D5 pathway, the conversion of pregnenolone to 17 hydroxy-pregnenolone is catalyzed by the P45017a enzyme by the process of hydroxylation. The 17- hydroxy-pregnenolone is converted to dehydroepiandrosterone (DHEA) and is catalyzed by the same enzyme by the removal of the acetyl group. Thus the enzyme, CYP17 (P45017a) shows 2 activities, hydroxylase and lyase. This enzyme is regulated by the Cyp17A1gene and is present exclusively in the thecal (interstitial cells). These thecal cells thus provide the androgens for further synthesis to the granulose cells. The granulose cells in turn complete the process of conversion of the androgens into the estrogens by the process of aromatization. This process is regulated by the enzyme CYP19 (p450 arom) and the CYP19A1 gene. This process occurs exclusively in the endoplasmic reticulum. The second route of metabolism is the conversion by 3β-HSD of pregnenolone to progesterone and is an irreversible process. The next step is conversion of progesterone to 17-hydroxy progesterone. This is then converted to androstenedione and further to testosterone. (Figure 12a & 12b)

A two-compartment system called as the “two cells two gonadotropin system” consisting of the thecal cells and the granulose cells is a feature of the ovaries and both have the ability to synthesize androgens,
progestins and estrogens. However, the thecal cells synthesize the androgens and the granulose cells form the estrogen because the aromatase activity in the granulose cells is far greater than that of the thecal cells. It has been observed that the LH receptors are present predominantly on the thecal cells and the FSH receptors are seen to be present on the granulose cells especially in the human pre-antral and antral follicles. LH stimulation of androgen production is observed in the theca cells and FSH mediated aromatization to produce estrogen in the granulose cells. (Figure 13 a & 13 b)\textsuperscript{53,115,116}

In vitro both the granulosa as well as the theca cells show the presence of an androgen aromatase activity. However, in vivo, during the follicular phase, granulosa layer activity is a few hundred times greater than that of the theca layer. The granulosa cell is thus the main source for the production of the estrogens. Thus the two cells two gonadotropin system shows the theca cells to produce more androgens and the granulosa cells to produce more estrogen by the aromatization of testosterone(Chapter 2 page 45).\textsuperscript{53}

**CYP17A1 gene:**

In this study, the reproductive hormone levels as measured by ELISA showed decreased levels of estrogen in the undernourished rats. This decrease in hormone levels was associated with an increase in the levels
DISCUSSION

of the estrogen receptor α. (figure 18a & 18 b). Thus a classical physiological response is observed, decreased hormone levels are associated with increased receptor levels. The decrease in the hormone levels was due to a reduction in the synthesis of the precursors for estrogen, viz, androstenedione and testosterone. The decrease in the levels of these precursors was observed because of the decreased availability of cholesterol for their synthesis. As discussed above, 50% of de novo synthesis of cholesterol occurs in the ovary and the rest comes from the blood (liver). As these animals receive a diet deficient in fat, cholesterol availability is reduced leading to decreased production of the precursors for estrogen. The decreased estrogen level caused an increase in the levels of estrogen receptor α and was reflected as increased transcript abundance (upregulation) of these receptors. The decreased estrogen levels were due to the decreased transcript abundance (downregulation) when measured by TaqMan assays of the CYP17A1 gene in the undernourished group rats as compared to the control rats. This is probably indicative of a hypogonadism like picture. Taken together, this clearly shows a decreased synthesis of the steroid hormones (estrogen) by the ovary causing a hypogonadism like picture. Similar observations have been reported by Bernal et al 48 and Shiyan Sui et al. 77
The Transition (recuperation) group rats showed increased levels of estrogen. This was due to the increased availability of cholesterol from the diet. This was clearly reflected in the increased deposition of fat in the ovary as seen histologically (Figure 16a, 16b & 16c). Restoration of the standard diet caused increased availability of cholesterol that in turn caused increased transcript abundance (up regulation) for the CYP17A1 gene. This was reflected in the increased formation of androstenedione and testosterone and therefore increased estrogen synthesis. This was accompanied by decreased transcript abundance (down regulation) of the estrogen receptor α. Thus again a classical physiological response is seen, increased hormone levels within the transition group rats caused decreased levels of the estrogen receptor α. This is clearly reflected in the decreased transcript number (down regulation) for the Estrogen receptor α gene. The ovarian steroid synthesis in the transition group rats has thus increased though it did not reach the levels in the control group rats.

A decreased nutrient supply i.e. fat led to the decreased synthetic ability of the ovary in the undernourished group rats. Restoration of the food supply in the transition group rats was reflected in increased production of the steroid hormones. The hypogonadism like picture seen in the undernourished group rats thus seems to be transient, a response to the
decreased nutrient availability that was partially restored with the standard diet and was not due to any abnormality within the ovary.

The increased synthesis of estrogen was not only due to the restoration of the standard rat chow. The recuperation group rats showed increased levels of Follicle Stimulating Hormone (FSH). The level of FSH in the undernourished group rats is not very different from the control group rats. The role of FSH is perhaps equally or more important in restoring or correcting the decreased hormone levels. This is because FSH is known to play a significant role in steroid hormone synthesis. FSH is known to cause growth and differentiation of granulosa cells (page 186, Chapter 6)\textsuperscript{53,68}, increased expression of LH receptors (page 44, Chapter 6)\textsuperscript{53,68} and induction of steroideogenesis\textsuperscript{68}. The LH receptor activation causes increase in transcription and protein levels of two very important enzymes involved in steroideogenesis, viz, cholesterol side-chain cleavage (P450scc) and aromatase (P450arom). Smyth CD showed that recombinant human FSH (rhFSH) causes increased CYP17 mRNA expression of thecal cells in the mature female rats. However this expression was undetectable in the immature females due to a lack of endogenous FSH but rhFSH, when injected in these immature females caused a marked increase in expression only in the theca cells and not the granulosa cells.\textsuperscript{117} Thus we see a very interesting scenario where FSH
receptors are present on the granulosa cells and CYP17A1 enzyme along with LH receptors on theca cells but CYP17 enzymes are regulated by FSH.\textsuperscript{52, 117}(Fig 13a & 13b)

Another interesting feature that can be seen in these undernourished group rats is the presence of a necklace like pattern in the ovary as seen histologically. This pattern is a feature of Polycystic Ovarian Syndrome (PCOS) where multiple follicles are seen in a necklace like pattern at the periphery of the ovary (Figure 15a, 15b, 15c & 15d). Such a picture could be due to the decreased estrogen levels in the undernourished group rats. This can be best understood with the scenario seen during the normal sexual (menstrual) cycle in humans. Normally in the humans, growth of only one dominant follicle is seen and occurs due to the increased secretion of estrogen within this follicle. This sets up a vicious cycle of increasing FSH stimulation causing increased estrogen levels in this follicle. Simultaneously; all other follicles are suppressed leading to their atresia. However, the picture in the undernourished group rats shows decreased estrogen synthesis. This decreased synthesis may not cause the dominance of one follicle and atresia of the others. This would make it difficult to set up the vicious cycle leading to the growth of only a dominant follicle. The growth of these multiple follicles gives the appearance of the necklace pattern and results in ovulation of more
number of follicles and therefore causes an increase in litter size. (Table 8).

The restoration of the diet in the transition rats causes increased FSH levels and therefore increased transcript abundance of the CYP17A1 gene. This in turn led to increased activity of CYP17 enzymes and increased production of testosterone and androstenedione. Thus the levels of these precursors were increased that led to increased synthesis of estrogen. The increase in estrogen probably restored the vicious cycle and led to less number of follicles that could grow and ovulate. This was clearly reflected in the decreased litter size similar to the levels in this control group rats. (Table 8).

The FSH levels in the undernourished group rats were very similar to the control group rats. Thus even though FSH levels are normal in the undernourished group rats they are not able to increase the transcript abundance of CYP17 enzymes because of the reduced dietary supply of fat. But in the transition group rats this supply of dietary fat is restored and coupled with increased FSH levels causes the increase in estrogen levels. Interestingly the levels of LH are almost the same in all the 3 group of animals. Thus we see that both FSH and LH levels are maintained in the undernourished group rats that have prevented the development of hypogonadism in these rats. Conversely, diet restoration
in the transition group rats caused increased FSH levels along with normal LH levels that led to increased steroidogenesis and partial correction in the estrogen levels. These studies were carried out in the sixth generation with standard diet of the transition group rats. Studies in the future generations would help us to understand if the estrogen levels and the epigenetic changes thereof can be completely corrected.

An interesting feature that is seen in the undernourished group rats is the tendency of the animal to preferentially store more fat for future use.\(^5\)\(^2\) Thus the undernourished group rats, inspite of being centrally obese i.e. having more fat stores, showed decreased steroid hormone synthesis. This could be due to the fat being directed to adipose tissue rather than the tissues like ovary. This could be true because LDL is known to cause uptake of free cholesterol from cell membranes especially in the steroid producing cells like the ovary. Hardikar et al have observed greatly reduced levels of LDL in the undernourished group rats that are greatly increased in the transition group rats.\(^5\)\(^2\) The undernourished group rats are unable to receive their normal supply of cholesterol from the blood due to a reduction in the LDL levels that play an important role in carrying the cholesterol to all the cells of the body (Chapter 2, pg 37).\(^5\)\(^3\) The correction of the LDL levels in the transition group rats causes an increased supply of cholesterol.\(^5\)\(^2\)
The increased FSH levels have thus corrected the decrease in the availability of testosterone by up regulating the levels of CYP17A1 gene leading to increased transcript abundance of the CYP17 enzyme. The restoration of the standard diet also caused increased FSH levels. The increased synthesis thus appears to be due to restoration of diet causing increased FSH levels and up regulation of the CYP17A1 gene.

**CYP19A1 Gene:**

CYP19A1 gene also called as p450arom regulates the CYP19 enzyme and is involved in the conversion of testosterone to estrogen by the process of aromatization. The C19 steroids, androstenedione and testosterone undergo the process of aromatization by the process of microsomal reactions. This involves 3 processes: initial hydroxylation of the angular 19-methyl group and then oxidation that involves the loss of the 19-carbon formaldehyde and lastly dehydrogenation or ring A aromatization (Chapter 2 page 42).\textsuperscript{53}

As discussed above, steroid synthesis is a two-step process (Figure 13a & 13b). The initial precursors, the C19 steroids, androstenedione and testosterone are formed in the theca cells and then enter the granulosa cells. Both, the theca as well as the granulosa cells exhibit aromatase activity in vitro but in vivo, this activity in the granulosa cells is about
100 fold greater than the theca cells (Chapter 2 page 45). The granulosa cells thus remain the principal source of estrogen in the growing follicle. However, this process of aromatization is dependent on the availability of the substrate i.e. testosterone or androstenedione from the theca cells. A reduction in this substrate can thus directly affect the formation of estrogen. This picture is seen in the undernourished group rats as CYP17A1 is under-expressed leading to decreased transcript abundance and therefore reduced enzyme levels inevitably causing reduced precursors and therefore decreased estrogen synthesis. Restoration of the diet in the transition group rats corrected this anomaly and increased estrogen levels were observed.

B. I. Castañon et al observed a 3.3 fold increase in the mRNA expression of the steroidogenic enzyme CYP19 on treatment of the granulosa cells with FSH. The transition group rats show increased levels of FSH that causes greater activation of CYP19A1 gene leading to increased transcript abundance. This increased activation of this gene leads to greater aromatization of the substrates leading to increased formation of estrogen in these rats. FSH levels were almost the same in the undernourished group rats and the control group rats. However, similar to the increased expression of the CYP19A1 gene in the transition group rats, CYP19A1 transcript abundance was also increased in the
undernourished group rats (Figure 18a). But because the undernourished group rats had a reduced substrate level due to a reduction in the CYP17A1 expression and the increased stimulation by FSH was also not available, the estrogen levels remained at a much lower level in the group rats.

Another important aspect in the formation of the precursors is the role of LH. The theca cells produces the androgens in response to secretion of LH. Thus, LH plays an equally important role in the formation of this precursor (chapter 2 page 43)\(^{53}\) and the estrogen so formed is due to the combined actions of both FSH and LH. LH levels were marginally lower in the undernourished group rats but marginally higher in the transition group rats. Thus LH levels appear to be almost the same in all the three colonies and nutrition does not seem to affect the LH levels. The receptor population is almost similar in the control and undernourished group rats but slightly higher in the transition group rats. Thus LH and its receptor do not seem to be affected by the reduction in the diet in the undernourished group rats nor by the increased diet in the transition group rats.

CYP19A1 levels were raised both in the undernourished as well as the transition group rats. Increased levels in the undernourished group rats may be significant as the estrogen levels would have been drastically
DISCUSSION

lower were it not for this increase. This may have lead to a possibility of classical hypogonadism and reproduction in this colony would have been hampered. This is especially important because CYP17A1 gene expression that regulates the levels of the precursors, testosterone and androstenedione is reduced. The increased expression of CYP19A1 thus has played a protective role by maintaining the estrogen albeit at lower levels and prevented reproductive failure. This may thus appear to be a compensatory increase to maintain estrogen levels.

CYP19A1 levels in the transition group rats are marginally higher than the undernourished group rats but higher as compared to the control group rats. However, the estrogen levels in the transition group rats are higher as compared to the undernourished group rats but lower as compared to the control group rats. This is interesting as the CYP19A1 levels are marginally higher in the transition group rats compared to the undernourished group rats. But the increase in estrogen levels in the transition group rats is much higher than the undernourished group rats. This would imply an increase in sensitivity of this machinery to a marginal increase in CYP19A1 levels that caused a comparatively greater increase in estrogen levels. The other possibility could be the increased availability of the substrate, testosterone due to up regulation of CYP17A1 gene making a greater amount available to be converted to
estrogen or possibly greater sensitivity to FSH. This can be confirmed only by measuring the actual levels of the enzymes regulated by both these genes by western blot analysis.

The increased FSH could also have played a distinct role in the increased formation of estrogen. FSH is known to regulate the aromatization process and increased FSH levels could have not only have caused stimulation of the CYP17A1 machinery but also the process of aromatization. Studies by B.I. Castanon et al have shown a 3.3 fold increase in CYP19A1 enzyme mRNA transcription after granulosa cells were treated with FSH. Thus FSH is a critical regulator of the process of aromatization. Increasing FSH levels causes increase in the activity of the steroidogenic enzymes regulated by both the CYP17A1 and CYP19A1 genes. This is critical because without this increase in FSH there would be failure not only of steroidogenesis but also the process of follicular maturation. This process of steroidogenesis involves other autocrine and paracrine peptides and growth factors like TGFβ, activins, inhibins and IGF-1. The exact role of each of these factors along with the likely role played by other genes would require further study.

The shift to a standard diet thus has lead to increased levels of FSH that in turn has rescued the steroidogenic apparatus causing an increase in the estrogen levels though they could not match the levels in the control
group rats. This transition group rats is the sixth generation to receive a standard diet but has been unable to completely recover the epigenetic changes due to 50 generations of multigenerational undernourishment. Future studies with later generations could provide an answer whether these changes can be completely reversed or not.

**BMP15 and BMP 4:**

Folliculogenesis is regulated by a variety of genes and some of these genes like Bone Morphometric Protein-4 \(^ {74}\) and Growth Differentiation Factor 9 \(^ {72, 73, 75}\) are also key regulators of cholesterol synthesis. It is well known that there is a two way communication between the oocyte and the surrounding cells\(^ {67}\). This is essential for the growth and maturation of the follicle. The granulosa cell provide the necessary nutrients for the growth of the oocyte and the oocyte in turn is known to affect all stages of growth of the follicles. There is thus a presence of a regulatory loop between the granulosa cells and the oocyte where complementary signaling and metabolic pathways help and regulate the growth and physiological activity of both these compartments. Similarly enzymes that are involved in the glycolytic pathways like Eno1, Aldoa, Pfkpetc are expressed in the cumulus cells than the granulosa cells. Oocyte derived
paracrine factors are known to regulate this expression in the cumulus cells.\textsuperscript{119,120} Oocytes show poor uptake of L-alanine and are unable to derive energy by glucose metabolism. The growth and metabolic activity of the oocyte requires these substrates or energy and are dependent on the cumulus cells for their supply.\textsuperscript{119,121,122,123} A bi-modal regulation between the granulosa cells and the oocyte is thus mutually beneficial due to the metabolic co-operation.\textsuperscript{124}

Across a variety of mammalian species that have been studied, two oocyte derived growth factors, GDF-9 and BMP15 have been found to play a critical role in follicular growth and ovulation. They are especially important in cholesterol biosynthesis and cumulus metabolism. You-Qiangsu et al \textsuperscript{67} showed that cumulus cells provide the oocyte the necessary products that are involved in cholesterol synthesis which are deficient in the oocyte.

Studies in both the undernourished and the recuperation animals showed deficiency of the steroid hormones. This was due to the deficiency in the necessary enzymatic machinery after cholesterol enters the cells. It is well known that the ovarian de novo synthesis of cholesterol is only about 50\% of the total requirement. The remaining demands are met by the supplies by the liver. The undernourished group rats probably receive a reduced cholesterol supply from blood (liver). If the ovarian de novo
synthesis was affected it would perhaps lead to a near absence of the reproductive hormones leading to reproductive failure. Interestingly BMP 15 transcript abundance was not different from the control colony indicating that the de novo synthesis was not affected (Figure 18a). BMP 15 is extremely important for follicular growth and decreased BMP15 transcript levels cause follicular atresia. This clearly shows the critical role played by BMP15 in not only rescuing the steroid hormone biosynthesis but also the process of folliculogenesis.

BMP 4 is known to cause induction of cell proliferation in the body. It is also known to cause the survival and the development of the primordial follicles in the ovaries. There was no change in the transcript abundance of this gene in either the undernourished group rats or the transition group rats indicating that the regulatory process of cell growth especially the survival of the ovarian follicles as controlled by this gene remains unaffected (Figure 18a). Thus as the BMP-4 levels are the same in all the three group of animals, it is clear that BMP-4 has maintained the growth of the follicles in the undernourished rats. However, the lack of nutrient availability prevents the overall growth of the various body structures and not the down regulation of this gene.

Taken together, increased FSH levels have partially restored the steroid hormone synthetic machinery, BMP15 has maintained the de novo
synthesis and along with BMP4 the folliculogenesis in the undernourished group rats failing which there would have been reproductive failure due to not only absence of the steroid hormones but also failure of follicular growth and maturation.

**Progesterone:**

A similar picture is seen with the progesterone hormone synthesis. The undernourished group rats showed a significant reduction in the progesterone levels along with slightly increased levels of its receptor as seen by the increased abundance of the progesterone receptor (Pgr) transcript number. The hormone levels along with its receptor were normalized in the Transition group rats due to restoration of a standard diet. Restoration of the diet not only helps to restore the D5 pathway but also the D4 pathway where there was increased conversion of pregnenolone to progesterone. Similar observations with the estrogen synthesis are indicative of the role of decreased diet causing decreased steroid synthesis that was normalized after a restoration of the standard diet (Table 7 & Figure 17).

Steroidogenesis thus seems to be very sensitive to the amount of fat in the diet. This is especially significant against the backdrop that the
undernourished group rats are centrally obese and the obesity worsens in the transition group rats with diet restoration. Thus, in spite of increased availability of the fat stores (central fat) in the undernourished group rats, steroidogenesis is directly affected by the amount of fat in the diet and not the fat stores.

This also has great significance from the evolution ecology point of view. The availability and use of energy resources has been characterized by Short and Adams based on their studies in beef cattle reproduction. The approximate use of the energy resources is prioritized by these animals as follows:

1) Basal metabolism, 2) activity, 3) growth, 4) energy reserves, 5) pregnancy, 6) lactation, 7) additional energy reserves, 8) oestrous cycles and initiation of pregnancy, and 9) excess energy reserves\textsuperscript{125}.

It can be seen from this list that the animals tend to provide maximum resources for the basal metabolic activity and other day to activities followed by pregnancy and lactation. Initiation of oestrous cycles and pregnancy is low on priority. Thus a small change in the quantity and quality of the diet may prevent initiation of pregnancy and oestrous cycles. Though pregnancy and oestrous cycle are low on priority, diet restoration as seen in the transition group rats causes the ovarian synthetic machinery to return to normal. Thus even with small increases in diet it is
DISCUSSION

easier to restore pregnancy and oestrous cycles to normal than basal metabolism. The amount of food required to restore basal metabolism would be far more than that required to restore pregnancy and oestrous cycles.

It is therefore much easier to restore pregnancy and oestrous cycles by keeping it on a low priority. Small decreases can prevent both but restoration of the nutritional environment can help to re-initiate the process of oestrous cycles and pregnancy. In this scenario the role of GnRH that is known to regulate reproductive activity could be critical.

GnRH has been identified in the brains of most vertebrate and even some non-vertebrate species. In most mammals, GnRH-I is known to be formed from the anterior portion of the medial ventral forebrain. This area projects to the portal system that affects the pituitary by the pulsatile secretion of GnRH. It has also been observed that vertebrates show two forms of GnRH. GnRH-II that is evolutionary conserved from bony fish to man is secreted from the mid brain and is responsible for sexual behaviour like mounting, tail wag or a receptive position like lordosis etc. It has been observed in musk shrews that even a 48 hour period of reduced nutrition alters the sexual behaviour of the females. However, after restoration of the nutrition GnRH II causes the activation of the sexual behaviour. Thus, both sexual activity and sexual behaviour are
affected by the reduced availability of food but are restored when the scarcity of food resources can be overcome.\textsuperscript{126, 127}

Undernutrition in the foetal life thus has pronounced effects on the reproductive ability of the female Wistar rats. A decrease in steroidogenesis causing decreased ovarian sex steroids estrogen and progesterone along with decrease in the pituitary gonadotropin FSH is seen and is indicative of a hypogonadism like picture in the undernourished thrifty jerry group of rats. However undernutrition does not affect the process of folliculogenesis due to absence of epigenetic changes in BMP-4 and BMP 15 genes. Restoration of the diet in the transition rats partially corrects the process of steroidogenesis as seen by the increased production of ovarian sex steroids estrogen and progesterone.

**Haematology:**

Undernutrition is also known to affect the haemopoietic environment\textsuperscript{102} and a variety of effects on the haemopoietic environment have been observed. The known effects include anaemia\textsuperscript{97,101}, altered white blood cell function\textsuperscript{98,99,100,103,105,106,110}, altered megakaryopoiesis\textsuperscript{114}, hypocellularity, necrosis and extracellular matrix modifications of the bone marrow\textsuperscript{104,108}. However the role of multigenerational undernutrition (50 generations) on the blood cell counts and the hematopoietic environment
have not been clearly delineated. This study evaluated the effects of multigenerational undernutrition on the complete blood cell counts in 4 week (28 day) pups and young adult rats at 8 weeks (56 days).

In 4 weeks old pups (Table 4 and Figures 10a & 10b), red blood cell count, haemoglobin and haematocrit are all reduced in the undernourished and the transition rats. This is accompanied by a decrease in mean corpuscular haemoglobin concentration but no change in the mean corpuscular volume. This appears to be indicative of iron deficiency anaemia as these animals receive a diet deficient in both vitamin B$_{12}$ and folate. Red blood cell distribution width (RDW) is higher in the undernourished group rats but shows no significant difference. However, it is very high in the transition group rats and is thus indicative of a mixed population of RBC’s where younger RBC’s tend to be larger in size. This is very commonly seen in iron deficiency anaemia. These animals thus seem to show a classical picture of iron deficiency anaemia at 4 weeks of life.

The transition and the undernourished group rats showed a decrease in the total white blood cell count (TLC) accompanied with a decreased lymphocyte %. However, the undernourished group rats showed a significant increase in the Granulocyte %. This count in transition group rats was only marginally higher as compared to the control group rats and
not significant statistically. The decrease in the TLC and lymphocyte% is a clear indication of the reduced immunity in these animals. But this is accompanied by an increased granulocyte count, a clear sign of an inflammatory process. Studies carried out by Hardikar et al \textsuperscript{52}; in the adult undernourished group rats have shown higher globulin & endotoxin levels confirming a state of inflammation in these rats. Thus the pups show not only a compromised immunity but also a state of inflammation. This picture is very similar to the Indian scenario where people of lower socio-economic strata in both urban and rural areas are constantly exposed to an infective environment due to unhygienic conditions.

The undernourished group rats show a decrease in the platelet count as well as a decrease in the plateletcrit (analogous to the Haematocrit). They also show a low mean platelet volume that again shows the presence of older platelets. Undernourishment thus leads to a decrease in the activity of the bone marrow, a hypoplastic bone marrow as seen by the decreased red blood cell, white blood cell and platelet counts. The underlying mechanisms in these undernourished group rats that cause the hypoplastic bone marrow have not been identified. Further studies that could identify the factors leading to bone marrow suppression like changes in the gene expression, the role various growth factors and changes in the
extracellular matrix would help to identify the cause of the bone marrow suppression.

The haematological profile in the young adult when measured at 8 weeks (56 days) was different from the pups (refer Table 5 & Figures 11a & 11b). Red blood cell count and haemoglobin values were similar in the three groups but haematocrit was significantly lower in the transition group rats. The pups at 4 weeks showed a different profile where the RBC count and haemoglobin were lower in the undernourished and transition group rats as compared to the control group rats. The exact reasons for these variations in the pups and adults have not been evaluated and need to be further explored.

The blood indices were again different in the adults. The undernourished group rats showed an increase in MCV as compared to both the control and transition group rats. This is along expected lines as the undernourished group rats are deficient in Vitamin B\textsubscript{12} and folate that cause macrocytosis restoration of the diet in the transition rats causes a reduction in the MCV that is not significantly different from the control animals. This clearly shows that when the diet is corrected for both B\textsubscript{12} and folate the macrocytosis is corrected. Interestingly, in spite of the correction in B\textsubscript{12} and folate, the hyperhomocystenemia as seen in the transition group rats is not completely rectified\textsuperscript{52}. MCHC was significantly
lower in the undernourished group rats as compared to the controls but interestingly was higher in the transition group rats.

The lower MCHC levels are indicative of iron deficiency anaemia in the undernourished group and are expected as these animals receive a deficient diet especially in proteins. However this study has not measured the iron levels in both the pups and the adult rats.

The RDW in the undernourished group rats is lower as compared to both the control and undernourished group rats at 8 weeks. This is exactly opposite to the pups where the distribution width was maximum. The pups thus show anisocytosis; a mixed population of small and large RBC’s but the young adult rats don’t show such a picture, in fact exhibit a uniformity in size and shape. The exact cause of these changes in the distribution width need to be further evaluated.

The platelet count and platelet distribution width (PDW) was significantly higher in both the undernourished and transition group rats. The plateletcrit was significantly higher in the undernourished group rats but not significant in the transition group rats as compared to the control group rats. The transition and undernourished group rats thus showed a picture of secondary thrombocytosis. It is a reactive process seen in a variety of conditions like infection, inflammation, iron deficiency, tissue damage, post-surgical trauma, cardiovascular pathology.
Plateletcrit is analogous to the haematocrit and is a reflection of the total the number of circulating platelets in a unit volume of blood. PDW is a specific activation marker for coagulation and is also known to be an indicator of platelet activation. The undernourished group rats show an active inflammatory process as seen by the increased granulocyte count as well as the increased globulin levels as reported by Hardikar et al. The secondary thrombocytosis thus seems to due to the active inflammatory process as seen in these young adults but the increased risk of atherogenecity due to increased risk of coagulation and therefore thrombosis.

Slow coronary flow is a phenomenon that is seen on coronary angiography as a delayed opacification of distal vessels. One of the factors that have been implicated in this process is platelet activation and also inflammation. Hardikar et al in their study have observed congestion of the coronary blood vessels and the presence of occasional infarcts in these animals. It is well known that undernutrition is associated with an inflammatory process. In fact it has been observed to be present even before birth and this is attributed to the immunoepigenome of the undernourished parents. Whether the increased platelet numbers along with platelet activation is due to the inflammatory process or undernutrition or both remains to be evaluated.
The increased activity of the platelets presents an increased risk for cardiovascular disease. This is clearly demonstrated by the increased blood pressures, congestion of blood vessels and abnormal ECG’s that show elevated Q and ST segments and P and T wave inversion in both the undernourished and the transition group. These observations along with the observed decrease in the estrogen hormone levels in both the undernourished and transition group rats deny the protection to these female rats against cardiovascular disease especially myocardial infarction.

An interesting observation in the transition group rats as well as the undernourished group rats is the inflammatory changes that are observed. Though these signify an active stage of infection, their role in reproduction also needs to the understood in context of the undernourishment. The inflammatory markers like prostaglandins are known to play an active role during the normal process of ovulation and are also known to be actively secreted in the semen. The undernourished group rats also show reduced levels of both estrogen and progesterone that could affect the reproduction in these animals. The increased inflammatory markers could be important to rescue the anovulation that may occur. Future studies with anti-inflammatory drugs could help to delineate the role of these inflammatory markers in reproduction in these
rats. Interestingly the transition group rats show increase in the ovarian hormones and a reduced litter size but an increase in the inflammatory markers.

The undernourished female rats thus show a decrease in haemopoiesis, an active infective process and inflammation and are at a greater risk of cardiovascular risk especially myocardial infarction. The decreased haemopoiesis was a clear reflection of the undernourished status in the undernourished group rats. Correction of the diet in the transition group rats corrected some of these abnormalities again clearly underlying the role of nutrition in these haematological changes. Future studies would help to identify the causative factors especially the epigenetic changes thereof. This is very important because even after six generations of allowing unrestricted access to an ad libitum diet, the metabolic abnormalities and the associated epigenetic changes, if any were not completely reversed. This holds great significance for a developing country like India where small for gestational age babies exposed to a rapid transition to an increased nutritional diet may exhibit an increased risk for adult cardiovascular disease and disturbances in reproductive functions.
Anthropometry

The undernourished group rats showed a lower body weight as compared to the control group rats but the transition group rats were heavier as compared to both the controls and undernourished group rats. Similarly the body length was less in the undernourished group rats but the transition group rats showed an increased body length. Thoracic circumference was significantly less in the undernourished group rats but was significantly higher in the transition group rats. This clearly showed that the undernourished group rats receive less nutrition and therefore show a lower body weight, body length and thoracic circumference. The transition group rats receive the normal standard diet and show significantly higher body weight, body length and thoracic circumference. Thus not only do the transition group rats grow longer but also become significantly heavier. A sizeable component of the increased body weight appears to be due to increased fat deposition.

The abdominal girth and skin fold thickness are regarded as an index for body fatness. Abdominal girth is said to provide a measure of abdominal or visceral fat and skin folds provide an index of subcutaneous fat. Abdominal girth and skin fold thickness were more in the undernourished group rats as compared to the control group rats but the transition group rats showed the highest abdominal girth and skin fold thicknesses. Thus
the undernourished group rats were centrally obese but central obesity was the highest in the transition group rats. These observations have been confirmed by MRI studies by Hardikar et al which shows increased central fat (visceral) deposition. Animals that are thus born small for gestational age have grown up in an environment where nutritional resources are scarce and show adaptations to this environment. The transition group rats have however been exposed to a standard diet. This leads to increased storage of central fat. Neel had put forth his theme of the thrifty genotype hypothesis which stated that thrifty genes were selected during periods of decreased food availability that led to increased fat deposition especially central fat. However, the genes responsible for this increased fat deposition have not yet been identified. However most epidemiological studies have shown the presence of abdominal obesity in adult life on exposure to an undernourished environment in utero. This is especially true in Indians where central obesity along with insulin and leptin resistance is present even at birth. This visceral obesity is a risk factor for cardiovascular disease in adult life and Hardikar et al have detailed the cardiovascular abnormalities like increased blood pressure and myocardial infarction in adult undernourished and transition group rats.
The undernourished group rats due to the central fat deposition are thus exposed to a greater risk of cardiovascular disease. The transition rats even after six generations of access to an ad libitum diet show no improvement but are in fact at an increased risk of cardiovascular risk. Future studies that would include future generations of undernourished group rats exposed to longer periods of unlimited access to a standard diet would help to identify whether these changes (epigenetic) can be reversed or remain permanent.

The head circumference in all the three groups remained unchanged. This is called as the brain sparing effect which states that individuals exposed to an undernourished environment in utero would not hamper the development of their brain, i.e. the brain would still get its normal quota of nutrition in spite of the reduced availability. An important aspect that needs to be understood is that the brain does not require insulin like other tissues for its glucose uptake. Insulin resistance is a feature in undernourished or small for gestational age babies and the brain escapes the increased requirement of insulin to push the necessary glucose into brain cells. The other tissues thus suffer the consequences of undernutrition and insulin resistance as reflected in reduced soft tissue growth. Thus brain sparing phenomenon is protective and allows unhindered growth of the brain in spite of scarcity of resources.
This study has thus shown the presence of increased central obesity in both the undernourished and the transition group rats. Increased adiposity is a known risk factor for cardiovascular disease as seen from many epidemiological and animal studies. Increased adiposity in females is associated with the polycystic ovarian syndrome (PCOS) and increased sterility. Another significant observation in animals with low birth weight is the early onset of puberty and menarche. Thus low birth weight and increased adiposity are risk factors not only cardiovascular disease and diabetes mellitus but also for reproductive functions.