SUMMARY AND CONCLUSIONS

SUMMARY

David Barker’s hypothesis of the foetal origins of adult disease laid the foundation for epidemiological studies that showed a variety of adaptations of the foetus to the undernourished environment in utero. These cellular, endocrine and molecular adaptations also called as “programming” are usually reflected as epigenetic changes at birth and in later life. A variety of studies, both human and animal have shown the adaptations of the cardiovascular, renal, endocrine and other systems to the undernourished foetal life. The present study evaluated the role of foetal undernutrition on the reproductive system in a multigenerationally undernourished Wistar rat colony. Additionally, the effects of providing a standard rat feed (recuperation diet) to the undernourished group rats also called as the Transition group rats were evaluated. The present study was thus planned as very few studies have looked at the role of multigenerational undernutrition on the reproductive function. Similarly, the present study also evaluated the role of providing a standard diet (recuperation) to the undernourished group rats and its effect on reproductive function.
Anthropometry:

The growth of undernourished group female rats was compromised and was reflected in a lower birth weight, a reduced body length and thoracic circumference but with an increased abdominal circumference indicating the presence of central obesity. There was no change in the head circumference in all the three colonies indicating the preservation of brain growth called as brain sparing effect.

The transition group rats in response to the normalization of the diet showed not only an increased body weight along with the thoracic circumference but interestingly also showed an increased body length. The increase in the abdominal circumference was significantly more than both the control and the undernourished rats. The transition animals, in response to the increased nutrition, thus show a significant tendency to preserve central body fat as a store for future use. This increased body weight especially the central body fat in both the undernourished as well as the transition rat colony is not only a risk factor for cardiovascular disease but also has reproductive consequences. A variety of epidemiological as well as animal studies have shown increased risk of PCOS as well as early onset of puberty, menarche and menopause in the obese population.
Haematology:

The complete blood count was estimated in the undernourished and the transition group rats at 4 weeks and 8 weeks of age. Undernourished rats at 4 weeks showed a decreased RBC count, Haemoglobin and Haematocrit indicative of an anaemic state. The total leukocyte count (TLC) was reduced but the granulocyte count was increased. The increased granulocyte count was indicative of an inflammatory state in these animals. The platelet count and plateletcrit were also reduced in the undernourished animals. The decrease in the RBC, TLC and platelet count is indicative of a hypoplastic bone marrow, a reflection of the undernourished diet. Thus hypoplasia and inflammation seem to be the feature of this colony at 4 weeks. The recuperation rat colony showed a similar picture like the undernourished colony where a state of anaemia and decreased TLC count was seen associated with an inflammatory state. However, these pups showed an increase in the platelet count (secondary thrombocytosis) that was significantly higher and was indicative of a state of hypercoagulability and therefore an increased risk of thrombosis.

The picture when estimated at 8 weeks in the undernourished group rats was more or less similar to the pups at 4 weeks but with some significant differences. The animals showed a state of anaemia as seen by decreased
haemoglobin and haematocrit counts. The adults, however also showed macrocytic anaemia that was not seen at 4 weeks. This is expected as the undernourished group rats receive a diet deficient in vitamin B$_{12}$. The TLC was lower and these animals also showed an inflammatory state as seen by the increased granulocyte count. Platelet count was significantly higher in the undernourished group rats indicative of the risk of thrombosis. The transition group rats showed a picture of anaemia but it was due to the reduction in the haemoglobin and haematocrit levels. The macrocytosis as seen in undernourished group rats was corrected as these animals received a sufficient dietary source of Vitamin B$_{12}$. The TLC was lower and granulocyte count was higher, an indication of an inflammatory state. The platelet count was however very high in the transition animals indicating a state of secondary thrombocytosis. Platelet count is known to increase in a variety of states, inflammation being one of them. These animals are thus at a very high risk of cardiovascular disease. However, the increased inflammatory markers could perhaps also play a vital role in ovulation as the process of ovulation requires the presence of inflammatory mediators like prostaglandins. Whether this process of ovulation in the undernourished group rats is rescued by the increase in inflammatory markers can be identified with future studies.
Hormonal levels, Histology and Gene Expression:

The undernourished group rats showed significantly decreased levels of the ovarian hormones estrogens and progesterone. However, the levels of the pituitary gonadotropins, LH and FSH were nearly the same as in the control rats. The decreased ovarian hormones is clearly indicative of decreased steroidogenesis in these animals. This is possible as these animals receive a diet that has very little or no fat. The ovary is dependent for 50% of its source of its cholesterol on the liver. A reduction in the supply of cholesterol to the ovaries is probably due to the decreased LDL levels in these animals and remains a distinct possibility. The reduction in the hormone synthesis is also due to the decreased transcript abundance (downregulation) of the CYP17A1 gene that plays an important role in the synthesis of the 2 substrates, androstenedione and testosterone from which estrogen is synthesized. However, increased transcript abundance of CYP19A1 gene was also seen. This causes the process of aromatization that allows the conversion of testosterone to estrogen. The decreased levels of hormones appears to be a picture similar to hypogonadism but the fertility in these animals is not affected. This is because there is no change in the transcript abundance (expression) of BMP 15 gene that plays a very important role in folliculogenesis. This coupled with no changes in the levels of LH allow not only growth of the
follicle but also ovulation thus preventing infertility. These animals in fact showed increased folliculogenesis as seen histologically by the increased number of follicles, a necklace pattern, and an arrangement of the follicles at the periphery of the cell. This picture is due to multiple stimulation of follicles and is seen in PCOS.

The transition group rats on the other hand received a diet that had sufficient amount of fat. This helped these animals to rectify the process of steroidogenesis and was clearly reflected not only in the increased synthesis of both estrogen and progesterone but also increased deposition of fat in the ovarian stroma when seen histologically. This was a reflection of the increased transcript abundance (upregulation) of the CYP17A1 gene that allowed the increased enzymes to cause increased synthesis of the substrates, androstenedione and testosterone. Coupled with this, an increase in the transcript abundance of the CYP19A1 gene was seen that caused increased conversion of testosterone to estrogen. BMP15 levels were also not reduced. Thus providing a standard diet to these animals causes the steroidogenic machinery to increase synthesis though it could not match the normal levels. The epigenetic changes of 50 generations of undernutrition cannot be completely reversed by normalization of the diet in the 6 generation.
The other interesting finding in the transition group rats was the reduction in the litter size after normalization of the diet. The undernourished animals had an increased litter size of 11-13 litters/female as compared to about 5-6 in the control group rats. The increased litter size was in response to the undernutrition. As seen with both epidemiological and animal studies, undernutrition during foetal life is known to affect the quality of the offspring and make it vulnerable to disease. The same picture is seen in this undernourished group rats. Increased susceptibility to disease could hamper the development of the animal and increase both morbidity and mortality. This could affect the propagation of the species because of increased mortality. To counter act this, the species responds by increasing the litter size with the assumption that at least a few will survive to cause future propagation of the species. This is the classical ecological and environmental trade-off between quality and quantity of the species. A reduced number enables good parental care. Normalization of the diet in the Transition group rats allows the litters to get a normal source of food supply and therefore removes the chances of this trade off. This restores the litter size close to control values.
Social Relevance:

These studies bear a great relevance to the Indian scenario as the Indian phenotype resembles this undernourished group rats that is at an increased risk for cardiovascular disease and diabetes mellitus. This study has also highlighted the fact that such a scenario could hamper the development of the reproductive functions as well. The quality of the offspring thus produced is itself poor as these epigenetic adaptations or programming is in response to the foetal undernourishment in utero. These animals are therefore at a risk of early puberty and menarche as well as early menopause. There could also be the risk of PCOS in adult life. These animals also show a reduced ovarian reserve due to exhaustion of the follicles at an early life. Thus the reproductive years in the females could be compromised.

The restoration of diet in the transition group rats did not correct the epigenetic changes even after 6th generations of normalization of the diet, though the litter size was normalized. Whether this programming can be reversed after restoration for many more generations or is irreversible can only be determined by future studies. Indians show central adiposity at birth but poor soft tissue growth. The acceptance of the affluent western lifestyle is a mismatch to the “programming” of the fetus that has led to increased incidences of morbidity and mortality due to cardiovascular
diseases, diabetes mellitus and cancers. A diet that would include both macro and micronutrients would thus be advisable.

This study provides the necessary background for the need for counselling not only during pregnancy but also pre and peri-conceptual period and through adulthood in both the undernourished as well as the affluent population. This is a requirement because both these populations show increased obesity and this and other studies have shown that the epigenetic changes thereof persist in the future generations as well as affecting the quality of the ovum and the future life of the progeny. Counselling at the right time would thus help to reduce obesity and its effects like incidence of early menarche, menopause and also a higher probability of increased incidence of PCOS especially in the Indian scenario.
CONCLUSIONS

This study evaluated the role of multigenerational undernutrition on reproductive functions in an undernourished group of rats. This study also evaluated the effect of providing a normal diet to the undernourished rat called as the transition group of rats. Multigenerational undernutrition causes epigenetic changes in the undernourished group rats and these are not reversed by 6 generations of normal nourishment.

The present study observed the presence of central obesity in both undernourished group rats and transition group rats at 4 and 8 weeks of age. Restoration of the diet in the transition group rats caused preferential storage of fat leading to the worsening of the central obesity in this colony.

Transition group rats show increased (catch up) growth that could be a risk factor for cardiovascular disease, diabetes mellitus and also reproductive function.

Undernourished group rats show bone marrow suppression at young age as seen by the decrease in RBC, TLC platelet count. However, at 8 weeks the anaemia and leucopenia persisted but these animals showed the presence of secondary thrombocytosis probably due to an inflammatory state. They also showed macroytic anemia that was due to a deficient vitamin B_{12} diet. The transition group rats at 4 weeks also showed the
presence of anaemia and leucopenia but significantly higher platelet count. This picture persisted at 8 weeks of life and was in fact associated with a further increase in platelet count. These animals are therefore at an increased risk of thrombosis and therefore myocardial infarction. The macrocytic anaemia was however corrected as they received a diet with a normal Vitamin $\text{B}_{12}$ content.

Undernourished group rats received a diet that was deficient in fats that affected the synthesis of the ovarian steroid hormones, estrogen and progesterone. A significant reduction was thus observed in both estrogen and progesterone synthesis that was indicative of a hypogonadism like picture. This was due to epigenetic modifications in the genes like $\text{CYP19A1, CYP17A1 BMP4 and BMP15}$ that caused a decrease in the enzymes responsible for the synthesis of the substrates, androstenedione and testosterone that are then converted to estrogen. Histologically these animals showed a necklace like pattern i.e. arrangement of the follicles at the periphery as seen in PCOS, probably due to the activation of multiple follicles. The transition group rats received a normal diet and showed increased synthesis of the ovarian hormones due to not only an increased supply of fats from the diet but also due to the increased stimulation of FSH. This was seen as increased deposition of fat on histological
examination in the ovary. However, the changes observed in these rats could not reach the normal levels.

Transition group rats thus show a partial reversal at F6 generation indicating that the epigenetic modifications are not completely reversed. This study would help to explain the need for proper counselling and advise a standard diet that would be inclusive of macro and micro nutrients to avoid the deleterious effects and reduce the incidence of not only cardiovascular disease and diabetes but also the reproductive functions in later life which are important to maintain the quality of the future progeny.