

# Intergenerational protein deficiency and adolescent reproductive function of subsequent female generations (F<sub>1</sub> and F<sub>2</sub>) in rat model

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## ABSTRACT

**Background:** Efficient reproductive function is an important characteristic that has evolved through natural selection. Nutrition can modulate reproductive activities at different levels, and its effect on reproduction is deemed complex and less predictable.

**Objective:** This study aims at investigating the underlying effect of persistent dietary protein deficiency during early life on reproductive parameters of subsequent (F<sub>1</sub> and F<sub>2</sub>) generations.

**Method:** Rats in group of four (4) were fed daily, different ration of protein diet (PD) formulated as: 21% protein diet, 10% protein diet, 5% protein diet and control diet (rat chow, containing 16–18% protein). They were fed *ad libitum* before mating, throughout gestation and lactation, and next generations were weaned to the maternal diet. Reproductive function analysis (which include; gestation and pubertal hormonal profiling, onset of puberty, oestrus cyclicity, sexual response) and morphometric analysis of the ovarian structure were carried out to assess associated consequences.

**Results:** There was significant reduction in the fertility index (Control; 85.8%, 21%PD; 88.43%, as compared to 10%PD; 65.9%, 5%PD; 35.78%,) at F<sub>1</sub>, also recurring in F<sub>2</sub> respectively as a consequence of altered reproductive function in the protein deficient models at P ≤ 0.05. Low protein diet posed suboptimal intrauterine condition, which was linked to increased prenatal morbidity and mortality (control; 11.3%, 21%PD; 3.3%, 10%PD; 27.4%, 5%PD; 32.9%), low birthweight (control; 5.29, 4.9 g., 21%PD; 5.5, 5.06 g., 10%PD; 4.05, 3.86 g., 5%PD; 2.7, 2.5 g) at F<sub>1</sub> and F<sub>2</sub> respectively, delayed onset of puberty (with average pubertal age set at: control; PND 36, 21%PD; PND 38 while 10%PD; PND 62., and 5%PD; PND 67), followed by induced cycle irregularity, altered follicular maturation and endocrine dysfunction, more severe in 5%PD.

**Conclusion:** Reproductive status of a female organism depends on the maintenance of ovarian structure and function that has been associated with the hypothalamic pituitary-gonadal axis, hormonal events and sexual maturity. There is therefore an association between persistent early life protein deficiency and reproductive response which mechanistically involves life-long changes in key ovarian cytoarchitecture and function.

## 1. Introduction

Reproductive health encompasses the reproductive processes, functions and system at all stages of life. It is an important component of general health (WHO, 2008). Nutrients when detected by the cellular sensor systems as dietary signals can influence certain phenotypic changes which may alter activities and homeostatic control processes (Elizondo et al., 2019). Maternal malnutrition during gestation and lactation impairs embryonic fetal development which results in

deleterious outcomes and imprints. With the growing human population, approximately one billion people suffer from protein deficiency one-third of which are stunted under five, because the global food system is currently failing to meet its nutritional needs (Ritchie et al., 2018).

Undernutrition occurs in adolescent pregnancy because of the competition that exists between fetus and mother for nutrients (Nguyen et al., 2017). Early-life exposures trigger processes that set individuals ready for particular circumstances that are anticipated in the postnatal

**Abbreviations:** E<sub>2</sub>, Estradiol/Estrogen; PROG, Progesterone; LH, Leutenizing Hormone; FSH, Follicle Stimulating Hormone; (↓), Concentration decrease; (↑), Concentration Increase; IUGR, Intrauterine Growth Restriction; F<sub>1</sub>, First filial generation; F<sub>2</sub>, Second filial generation; F<sub>0</sub>, Parent; PD, Protein Diet; PDD, Protein Deficient Diet; GD, Gestation Day; PND, Postnatal Day.

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and adulthood environment (Chan et al., 2015). Protein undernutrition can cause stunted growth, anemia, physical weakness, and edema (Abey et al., 2019). The ability to reproduce is central to the life history of all sexually reproducing organisms. The primordial follicle pool is built up at the early stage of development, and is therefore vulnerable to exposures at this stage of development. Therefore, reproductive maturation and capabilities depend on early-life events, (Chan et al., 2015). Previous reports from clinical and experimental studies have indicated that early-life problem is associated with a decline in ovarian follicular reserve, changes in ovulation rates, and altered age at onset of puberty. However, mechanisms underlying the regulation of the relationship between the early-life developmental environment and postnatal reproductive function are not clear (Hanson et al., 2015).

Nutrition has pivotal effects on reproduction as it can modulate reproductive activity at multiple levels (Silvestris et al., 2019). The success of reproduction in all animals depends on the function of the hypothalamus-pituitary-gonad axis. The interaction between nutrition and reproduction has been established to have important implications for the reproductive performance of ewes and goats (Nogueira et al., 2017). Also, Nutritional status of individuals influences virtually all aspects of female reproductive performance starting at the fertilization to their oocytes and embryo quality (Silvestris et al., 2019). More than 10% of the world’s population are affected by infertility (Rouchou, 2013). In developing countries, there are severe social, psychological and economic consequences for infertile men and women, especially in the low-resource settings where it may be associated with a significant risk of further impoverishing the health reserves and sustenance in the community, threatening survival and worsening poverty (WHO, 2020; Gerrits et al., 2012). This present work was undertaken to investigate perinatal dietary protein deficiency effects on the reproductive health of two (2) different generations (F<sub>1</sub> and F<sub>2</sub> – generations) in the rat model. As such, the pubertal attainment, oestrus cycle, fertility response, hormone profile and morphology of the ovary were used to validate this, the established consequence can inform some therapeutic interventions in cases of reproductive health issues resulting from persistent perinatal

dietary deficiency.

## 2. Materials and methods

### Ethical statement

The research was carried out following the guidelines of the Act 2004 health research standards for care and use of laboratory animal models. The College of Medicine University of Lagos Health Research Ethics Committee (local HREC; REC 11), approved the protocol to carry out the research (CMUL/HREC/11/18/462).

### 2.1. Animal grouping/maintenance

Averagely 6–8 weeks old virgin female Wistar rats (n = 50) were obtained from the animal house of the College of Medicine University of Lagos, Lagos Nigeria. Rats were grouped into four (4) according to the protein ration in the diets; All rats were maintained in clean capacious plastic cages (n = 10 per cage) under standard laboratory conditions (25 °C ± 2 °C at a 12-h light/dark cycle) according to the standard ethics guidelines of health research Act 2004. Feeding was done *ad libitum*, following the groupings; 21%PD: 21% protein ration diet (an upgrade/physiological protein requirement), Control: standard rat chow containing 16–18% protein (the recommended requirement for growth and maintenance), 10%PD: 10% protein ration diets (Mild protein deficiency), 5%PD: 5% protein ration diet (Severe protein deficiency), fed perinatally at F<sub>1</sub> and F<sub>2</sub> (see Fig. 1) so establish a mechanistic association between persistent perinatal malnutrition and intergenerational consequence.

### 2.2. Diet formulation

Non-purified isocaloric diet was formulated. The diet was formulated using non-purified constituents and standard formula, scored to requirements following adaptation from New Non-purified Diet (NTP-

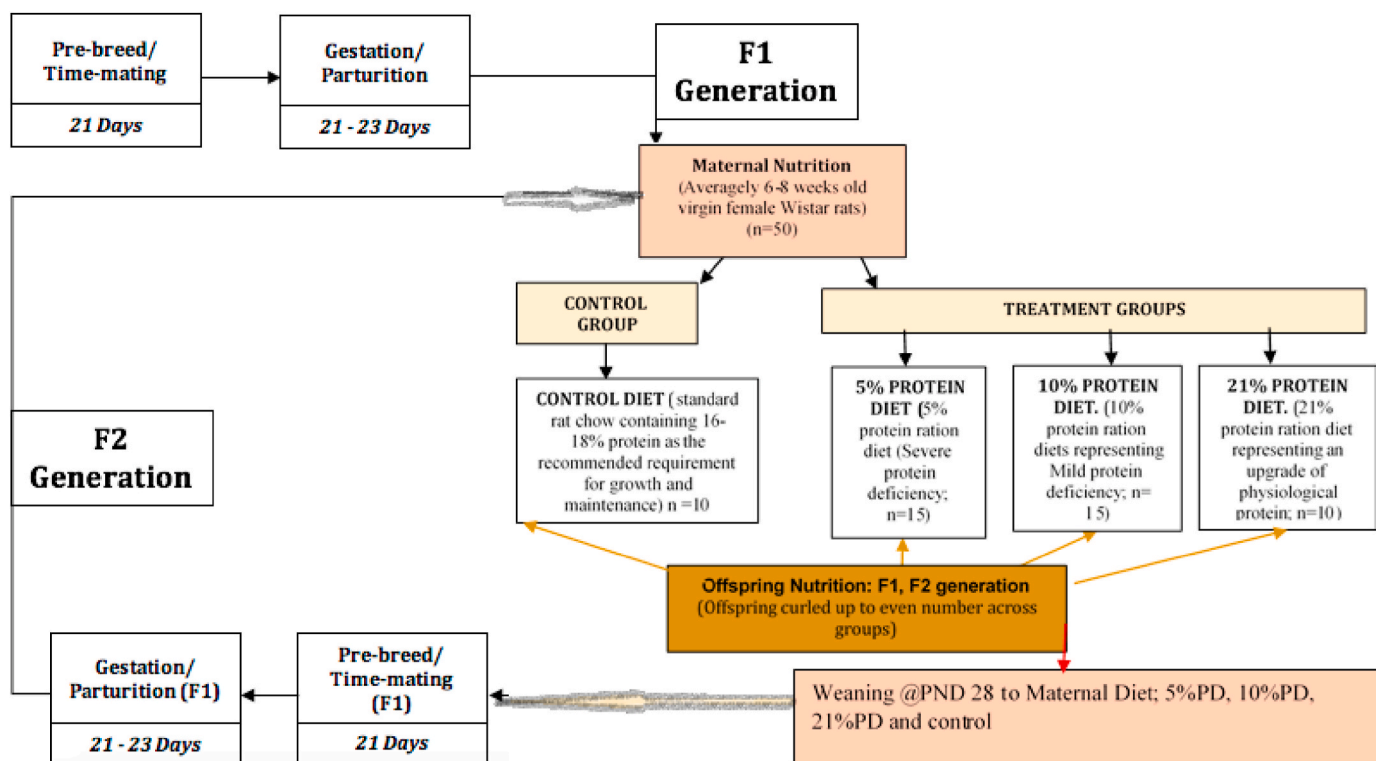


Fig. 1. Schematic diagram of feeding pattern.

2000) for Rodents by Rao (1997). The various protein ration diet for rat was formulated by scoring the proximate protein content to percentage in each dietary component and confirming the overall proximate analysis. Contents include: Maize, Fish meal, Soybean meal, cassava, wheat offal, Bone meal, Oyster shell, premix, methionine, lysine, Common salt in varying percentage towards the attainment of the experimental diet protein percentage for each grouping, in the modified method of Silas et al. (2014). Feed were fed to animals in pelletized form. Formulated diets were subjected to total proximate analysis as presented in the table below (Table 1).

### 2.3. Time-mating

Rats were maintained on group diet for about 6 weeks before mating. Three (3) weeks pre-breed, vaginal cytology was monitored to establish cycle pattern. At proestrus/oestrus phase, rats in each group were time-mated with certified reproductive male (in the ratio 2 female to 1 male). The presence of sperm plugs and/spermatozoa in the vagina smear was used to confirm day 0 of conception. the confirmed pregnant dams were separated into metabolic/nursing cages to produce F<sub>1</sub> generation, this process was repeated for F<sub>1</sub> generation to produce F<sub>2</sub> generation while the different diet group feeding continues. Pregnant rats were weighed every other day.

### 2.4. Assessment of pregnancy/pregnancy outcome

#### 2.4.1. Determination of fertility index

Fertility response and fertility index calculations were done following slightly modified procedures. Fertility Index (FI) = (Number of models mated- Number of model pregnant) \*100 (Watcho et al., 2009).

#### 2.4.2. Day 6 and 19 hormonal assay

The pregnancy hormone was monitored. Maternal blood at 6th and 19th day of gestation between 9th and 10th hour of the day, was collected through retro-orbital sinus using hematocrit capillary tubes cold centrifuged at 3500 rpm, and prepared for ELISA assay. Serum estradiol and progesterone levels were measured by ELISA following manufacturer's instructions on kit. This was also repeated for the F<sub>1</sub> generation.

#### 2.4.3. Implantation study

At gestation day 4 (GD4), four (4) dams in each group were injected at the tail, with Evans blue dye (1 mg/ml) and allowed to circulate for about 15 min, before sacrificing through cervical dislocation and the implantation sites (identified as distinct blue bands along the uterine horn) were observed, counted and recorded (Oludare and Iranloye, 2016). After culling, every other animal in each group were allowed to

**Table 1**

Proximate composition of the experimental diets.

SAMPLE ID	PPD	LPD	MPD	CONTROL
Moisture (%)	6.97 ± 0.22	7.43 ± 0.02	8.21 ± 0.04	8.51 ± 0.44
Crude Protein (%)	21.61 ± 0.14	5.76 ± 0.12	10.66 ± 0.02	15.98 ± 0.05
Crude Fat (%)	9.59 ± 0.70	13.76 ± 0.07	11.97 ± 0.12	10.16 ± 0.03
Crude Fiber (%)	1.56 ± 0.04	1.46 ± 0.01	1.60 ± 0.21	11.58 ± 0.73
Total Ash %	7.99 ± 1.58	6.49 ± 0.02	7.86 ± 0.92	7.31 ± 0.09
Carbohydrate (NFE) %	52.28 ± 1.13	65.10 ± 2.8	59.93 ± 1.11	46.46 ± 0.05
Energy Value (MJ/Kg)	14.92 ± 2.11	15.89 ± 1.71	16.74 ± 1.12	14.38 ± 2.51

**KEY:** PPD: Physiologic Protein Diet (21% PD), LPD: Low Protein Diet (5% PD), MPD: Mini Protein Diet (10% PD), NFE: Nitrogen Free Extract (Carbohydrate CHO).

go into delivery.

#### 2.4.4. Parturition/fetal outcome

Pregnant Dams underwent vaginal delivery naturally at parturition and pups' weight were also taken at birth within 24 h of birth. They were weighed on a sensitive electronic scale. Fetal outcomes including; birth weight, fetal appearance and average fetal outcome and survival rate in each treatment group were recorded.

#### 2.4.5. Weaning

Weaning was done at postnatal day 28 (Week 4/5) and anogenital distance (AGD) was used to determine and separate sex ( $\leq$  or  $\geq$  2.5 mm as the reference value (Zambrano et al., 2006)). Pups from each diet group were weaned to their maternal diets.

### 2.5. Reproductive function

#### 2.5.1. Onset of puberty

Caliper was used to take the anogenital distance (AGD) for determination of sex; using  $\leq$  or  $\geq$  2.5 mm as the reference value (Zambrano et al., 2006). Male and female progeny were separated and the female offspring were maintained *ad libitum* on the formulated diet until puberty. The onset of puberty defined as the age of vaginal opening (Goldman Jerome et al., 2007), was assessed by visual inspection every other day. The vagina opening was established as the complete separation of the membrane sheath covering the vaginal orifice.

#### 2.5.2. Assessment of oestrus cyclicity

Oestrus cycle monitoring follows vaginal opening. vaginal smears were collected from each treatment group using the lavage technique, to index the stage of the oestrous. To establish the timing of oestrus, cyclicity was recorded based on the proportion of leucocytes, epithelial cells and cornified cells, in the smear. The cycle was counted on intervals between proestrus and the next proestrus, and the average duration is calculated in each treatment group. (Marcondes et al., 2002). The cycle regularity was evaluated on; cycle length, percentage time spent in each cycle phase. Longer cycle length, more than 50% time in one stage is considered an irregular cycle. Prolonged diestrus also indicated acyclic period or pseudopregnancy (Li et al., 2017).

### 2.6. Determination of sex hormone level at puberty

F<sub>1</sub>-generation serum estradiol and progesterone, Follicle Stimulating Hormone, Luteinizing Hormone, Testosterone and cortisol levels were measured in each diet group at puberty (specifically the Oestrus phase) by EIA kit (Monobind Inc. Lake forest USA), using hormone standards and the specific antibodies following manufacturer's instructions of kit.

### 2.7. Ovarian morphometric analysis

Both ovaries were removed for morphological study at the diestrus phase. Tissue samples were fixed in formalin (10%) solution for 14–16 h, at 4 °C, the samples were dehydrated with alcohol solutions with increasing volume; followed by paraffin embedding. The samples were cut with a microtome 5  $\mu$ m diameter and consecutive staining with hematoxylin and eosin, sections were placed on slides (Ahmadi et al., 2017). In the final stage, ovarian morphology was assessed; the tissue sections taken from the ovaries were observed under a light microscope with a magnification of 40 and the number of each of the follicular groups were counted, the thickness of theca layer and granulosa were then measured (Roshangar et al., 2014).

### 2.8. Statistical analysis

Data were analyzed using diets as factors. Results are presented as the mean  $\pm$  SEM. Statistical analysis was performed using GraphPad

Prism 7.0 for analysis of variance (ANOVA) followed by posthoc Turkey's test and/or Dunnette's test, at  $P \leq 0.05$ , statistical difference is considered significant.

### 3. Results

#### 3.1. Pregnancy/pregnancy outcomes

In response to perinatal dietary protein deficiency, the reproductive response in Fig. 2A shows that there were significantly distinct features in protein-deficient groups for the fertility index. Also, in Fig. 2B, the circulating level of progesterone and estradiol at gestation day 6 and 19 in the 5%PD and 10%PD (deficient groups) were not significantly different from the 21% Protein diet and control group at F<sub>1</sub> generation, but at F<sub>2</sub> -generation, day 6 and day 19 estradiol and progesterone level were significantly altered in 5%PD and 10%PD groups, this was also reflected in the E2/PROG hormone ratio (Fig. 2). According to results presented in Table 2, there was a significant reduction in the gestation weight of the protein deficient pregnant dam at F<sub>1</sub> and F<sub>2</sub> generation, while the length of gestation across generations and between the groups did not change, also, the fetal resorption rate (↑), pups' birthweight (↓), and average fetal outcome (↓) at F<sub>1</sub> & F<sub>2</sub> -generations (Table 2) were statistically different compared to control diet group.

#### 3.2. Reproductive function

Reproductive function, defined as the postnatal date of puberty onset, and the characteristic oestrus cycle of rat in different group is represented in Figs. 3 and 4 respectively. The onset of puberty measured as date of vaginal opening (Fig. 3) in control and 21%PD groups were within the normal range (average of PND 36), while the protein-

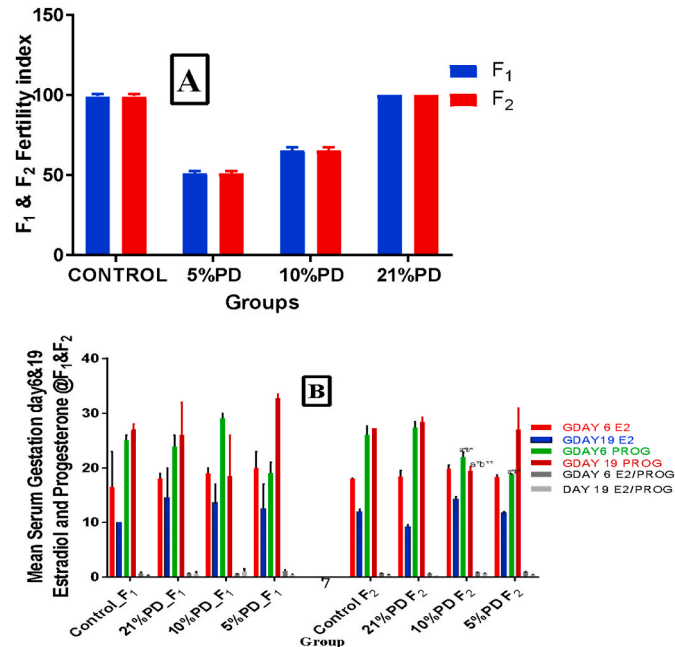


Fig. 2. Effect of perinatal protein deficiency on A: reproductive response in the different diet groups, at F<sub>1</sub> and F<sub>2</sub> generations. A: Fertility index, B: Mean Serum Estradiol and Progesterone levels on gestation days 6 and 19, Data are expressed as mean ± SEM, n = 6 in each group. Control (standard rat chow containing 16–18% Protein), 21%PD (Upgraded daily recommended intake), 5%PD (Protein deficient diet), 10%PD (Mild protein-deficient diet). E2-Estradiol, PROG- Progesterone, GD6 & GD19; Gestation Day 6 and 19. a; significantly different from 21% diet group, b; significantly different from the control diet group, c; significantly different from the 10% diet group (\* $P < 0.05$ , \*\* $P < 0.001$ , \*\*\* $P < 0.0001$ ).

deficient groups (10%PD and 5%PD) had a later date of pubertal onset (averagely PND 44 and 52 respectively), delayed onset of puberty persists across the generations (Fig. 3A and B). The pattern of the oestrus cycle (Fig. 4B) was disrupted in the nutritionally challenged groups. Within four (4) weeks of vaginal cytology, cycle irregularity was displayed for each of the protein deficient groups as the percentage of time spent in each oestrus phase (Fig. 4A and B), was of higher variance compared to control and 21%PD and the cycle length (Fig. 4C) was longer in the deficient group, across the generations. An irregular cycle is taken as more than 50% time spent in diestrus stage or cycle length above 7 days. The 10%PD group in F<sub>1</sub> had a persistent oestrus phase (i.e vaginal cornification), while the F<sub>1</sub> and F<sub>2</sub> 5%PD displayed prolonged diestrus compared to other groups (Fig. 4B).

#### 3.3. Sex hormone at puberty

Fig. 5 shows the pubertal sex hormone in all the groups, the protein deficient groups had significantly decreased levels of follicle-stimulating hormone (Fig. 5A), Progesterone (Fig. 5B), cortisol and testosterone (Fig. 5C), which are essential for the maturation of the ovarian structure. Some of the modulations in the sex hormone in the first generation persisted to the second generation. Although there was variation in the estradiol levels across the group, this was not significant. The Luteinizing hormone remained the same across the groups, in the first and second generations.

#### 3.4. Ovarian morphology

The quantification of the ovarian follicle and corpora lutea shows that at F<sub>1</sub>, all other follicle count was the same except the primary follicles which were significantly lowered and the cystic follicle which was higher significantly (Fig. 6A) in the protein-deficient groups. At F<sub>2</sub>; protein deficiency altered the number of primordial follicles (↑), cystic follicles (↑) and corpora lutea (↓) especially in the severely deficient group (5%PD), compared to control, significant at  $P < 0.05$  (Fig. 6B). The severely deficient group had significantly higher follicular diameter (Fig. 6C) and lowered theca (Fig. 6D) and granulosa thickness (Fig. 6E) across the generations.

### 4. Discussion

Perinatal exposure to a protein-deficient diet and general mother/child malnutrition during the critical period of development is known to lead to certain life-long psychopathological changes and increased susceptibility to dysfunction that underlies most origins of health defects and disease. This study is targeted at investigating the reproductive consequences of perinatal dietary protein deficiency in two (2) subsequent generations of rat models, in a bid to chart associated mechanisms. In the reproductive setup, the organ system, in an attempt to make up for the nutritional deficiency, may have undergone certain physiological and metabolic shifts which may directly or indirectly impact the functional units of the hypothalamic-pituitary-gonadal axis that controls an aspect of reproduction.

#### 4.1. Pregnancy hormone modulated by protein deficiency across generations

Models under nutritional inadequacy were observed to have grown at a very slow pace during gestation, thus corroborating a previous report by Carlin et al. (2019), The fetuses are exposed to a sub-optimum environment as a result of the competition that exists between the mother and the fetus for the available nutrient, and this may have resulted in the consequential imprints observed in later life. Maternal constraint alters the development of fetus within the uterine which was evident as IUGR conditioning in the severely deficient group (5%PD), and this corresponds with the low birthweight at both F<sub>1</sub> and F<sub>2</sub>

**Table 2**  
Pregnancy outcomes in F<sub>1</sub> and F<sub>2</sub> generations of the different diet groups, following protein deficiency.

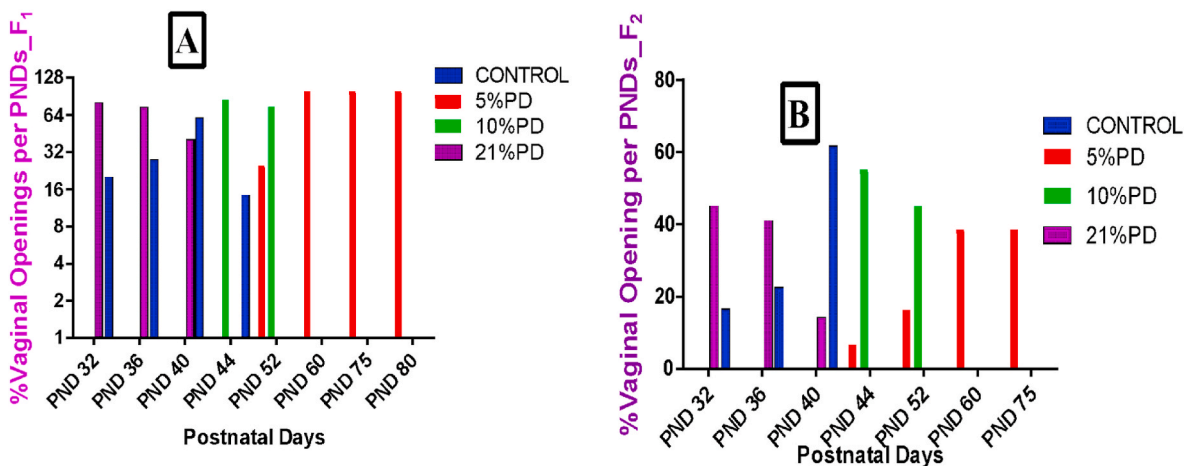
Parameter	Diet groups in each generation							
	F <sub>1</sub>				F <sub>2</sub>			
	Control	21%PD	10%PD	5%PD	Control	21%PD	10%PD	5%PD
Gestation Weight (g)	133.01 ± 5.9	134.35 ± 7.2	129.35 ± 6.06	99.44 ± 2.7 <sup>a**b*</sup>	145.64 ± 6.3	146.75 ± 6.4	117.24 ± 3.4	97.705 ± 3.5 <sup>a**b*c*</sup>
Gestation length (days)	23 ± 0.42	22 ± 0.68	22.1 ± 0.1	22.14 ± 0.56	21.62 ± 0.95	22.16 ± 0.6	22.04 ± 0.48	21.6 ± 0.11
Fetal resorption rate	0.11 ± 0.02	0.03 ± 0.03	0.27 ± 0.01 <sup>a*b**</sup>	0.33 ± 0.02 <sup>a*b**</sup>	0.007 ± 0.0	0.00 ± 0.00	0.32 ± 0.2 <sup>a*b*</sup>	0.32 ± 0.01 <sup>a*b*</sup>
Pups Birthweight	5.292 ± 0.1	5.476 ± 0.1	4.05 ± 0.2 <sup>a***b**</sup>	2.7 ± 0.2 <sup>a***b**c*</sup>	4.91 ± 0.1	5.06 ± 0.14	3.86 ± 0.2 <sup>a***b**</sup>	2.5 ± 0.2 <sup>a***b**c*</sup>
Fetal Outcome (Litter size)	8.330 ± 0.3	9.000 ± 0.6	5.67 ± 0.7 <sup>a*b**</sup>	5.33 ± 0.3 <sup>a*b**</sup>	7.000 ± 0.4	7.250 ± 0.5	3.67 ± 0.9 <sup>a*b*</sup>	4.000 ± 0.0 <sup>a*b*</sup>

Data are expressed as mean ± SEM, n = 6, Control (standard rat chow containing 16–18% Protein), 21%PD (Upgraded daily recommended intake), 5% (Protein deficient diet), 10% (Mild protein deficient diet).

a; significantly different from control group, b; Significantly different from the 21% Protein Diet group, c; Significantly different from the 10% Protein Diet group Asterisks indicate the level of significance (\*P < 0.05; \*\*P < 0.001), \*\*\*P < 0.0001.

Implantation Loss (Fetal resorption rate) = (Number of implantation- No of Fetuses alive)/Number of Implantation.

<sup>S</sup> IUGR Condition was set at: Pup Weight <10th percentile (i.e < 4 g).



**Fig. 3.** Effect of protein deficiency on the onset of puberty in each of the diet groups, across F<sub>1</sub> and F<sub>2</sub> generations. A: The Plot of percentage vaginal opening at different postnatal days (between PND 32–80), at F<sub>1</sub>. B: The Plot of percentage vaginal opening at different postnatal days (between PND 32–80), at F<sub>2</sub>. The onset of puberty taken as the postnatal date of complete removal of membrane sheath of the vaginal orifice. Data are expressed as mean ± SEM, Control (standard rat chow containing 16–18% Protein), 21%PD (upgraded daily recommended intake), 5%PD (Protein deficient diet), 10%PD (Mild protein-deficient diet). A statistically significant difference between individual group and 5% PD (\*P < 0.05) and the hypothetical average age of vagina opening (PND 36). PD: Protein Diet. PND: Postnatal Days.

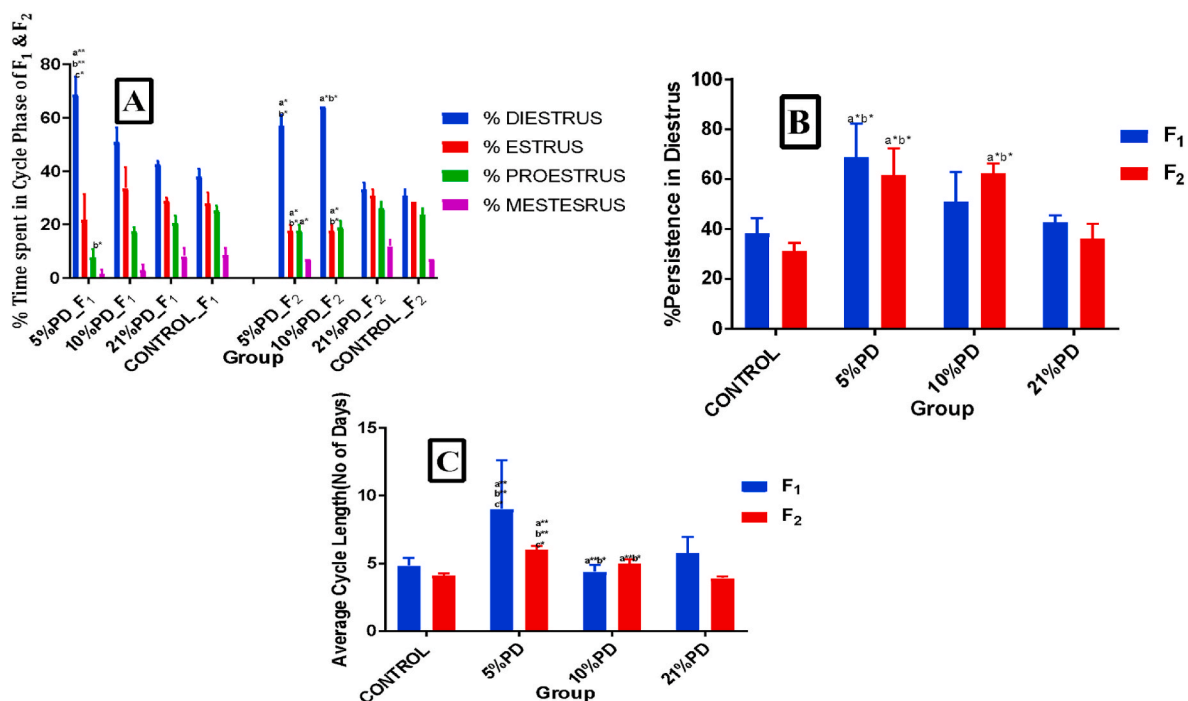
generations. The sub-optimal intrauterine condition that results from nutritional stress explains the low birthweight as earlier stated by Perez and Lehner (2019). The survival, growth and development depended critically on the nutritional status of the mother and the extent of protein deficiency, this result, therefore, emphasizes that maternal protein intake directly impart intrauterine growth and survival (Hanson and Gluckman, 2014) and could also be explained by fetal programming effects.

The fertility index is key to reproductive health, delayed conception and reduced fertility response are consequences of protein deficiency as reported in this study. Although dietary protein deficiency did not affect pregnancy hormone and gestation length, in the first generation, it is thought that there was a metabolic adjustment to cater for the pregnancy condition, and this must have accounted for the maintenance of the pregnancy till birth, for those dams that could carry a pregnancy to term, but in the second generation, the pregnancy hormone in the deficient group, was perturbed, this may explain the high fetal resorption rate, as well as the mortality rate, and the litter size in the deficient group. The high rate of fetal resorption and survival in the protein-deficient group has been hypothesized to share a common therefore

can be linked to the influence of dietary insufficiency on the maintenance of a viable pregnancy (Tsur et al., 2019).

#### 4.2. Protein deficiency delayed onset of puberty across generations

Delayed onset of puberty corresponds with lowered birth weight. We observed that vaginal opening (Onset of puberty) in the protein-deficient models did not occur within the normal age range earlier reported by Picut et al. (2015), The diversity and progression of puberty have been linked to lowered birthweight (Castellano and Tena-Sempere, 2016), this provides the basis for the fact that perinatal protein malnutrition is capable of programming the offspring’s sexual maturity (Reynolds and Vickers, 2019) since epigenetic factors play a role in sexual development (Bhandari et al., 2015). Burke et al. (2017), earlier stated that delayed onset of puberty may be inconsequential but the long-lasting differences associated with it may be detrimental, our result shows that the delayed puberty aligns with reduced fertility index in adults, therefore better-explaining fertility intricacies and pregnancy outcomes earlier reported in the F<sub>1</sub> generation. Monitoring of vaginal smear at adolescence at the onset of puberty in female rats provided a



**Fig. 4.** Effect of perinatal dietary protein deficiency on oestrus cyclicity across F<sub>1</sub> and F<sub>2</sub>-generations cycle phase in each of the diet groups. A: Percentage time spent in each cycle phase, B: Percentage persistence in diestrus phase, C: Average length of each cycle phase. Data are expressed as mean  $\pm$  SEM, n = 6, Control (standard rat chow containing 16–18% Protein), 21%PD (upgraded daily recommended intake), 5% (Protein deficient diet), 10% (Mild protein-deficient diet). A statistically significant difference between individual group and 5% PD (\*P < 0.05) and the hypothetical value of 7 day cycle length. A Persistent cycle length > 7 days reflects an irregular cycle. PD: Protein Deficient Diet. a < significantly different from 21% diet group > b < significantly different from the control c < significantly different from the 10% diet group > (\*P < 0.05, \*\*P < 0.001, \*\*\*P < 0.0001).

useful ancillary measure of the cycle pattern. The irregular pattern of the cycle following protein deficiency was characterized by increased cycle length, percentage time spent in the diestrus phase and prolonged oestrus (Li et al., 2017). Prolong diestrus (indicating acyclic period or pseudopregnancy (Egan et al., 2019) is a common pattern of disordered fertility (Li et al., 2017), this condition was prominent in 5%PD groups at F<sub>1</sub> and F<sub>2</sub> – generations. The 10%PD was labelled with persistent oestrus (vaginal cornification) in the F<sub>1</sub>- generation, this is also known as delayed ovulation or anovulation (Ann et al., 2019). Long term diestrus and oestrus phase, as well as altered cycle length, are common signatures of reproductive ageing (de Oliveira Ferreira et al., 2018; Li et al., 2017) and has also been implicated in prenatal androgenization of polycystic ovarian syndrome (Sullivan and Moenter, 2004). Perinatal protein deficiency according to earlier research findings (Abey et al., 2019) correlates with the altered dopaminergic and serotonergic systems. This result may also suggest why there is altered Intra and inter generation reproductive function since serotonin is established to stimulate ovarian maturation while hype in dopamine slows it down.

#### 4.3. Perinatal protein inadequacy disrupts sex hormone profile at puberty

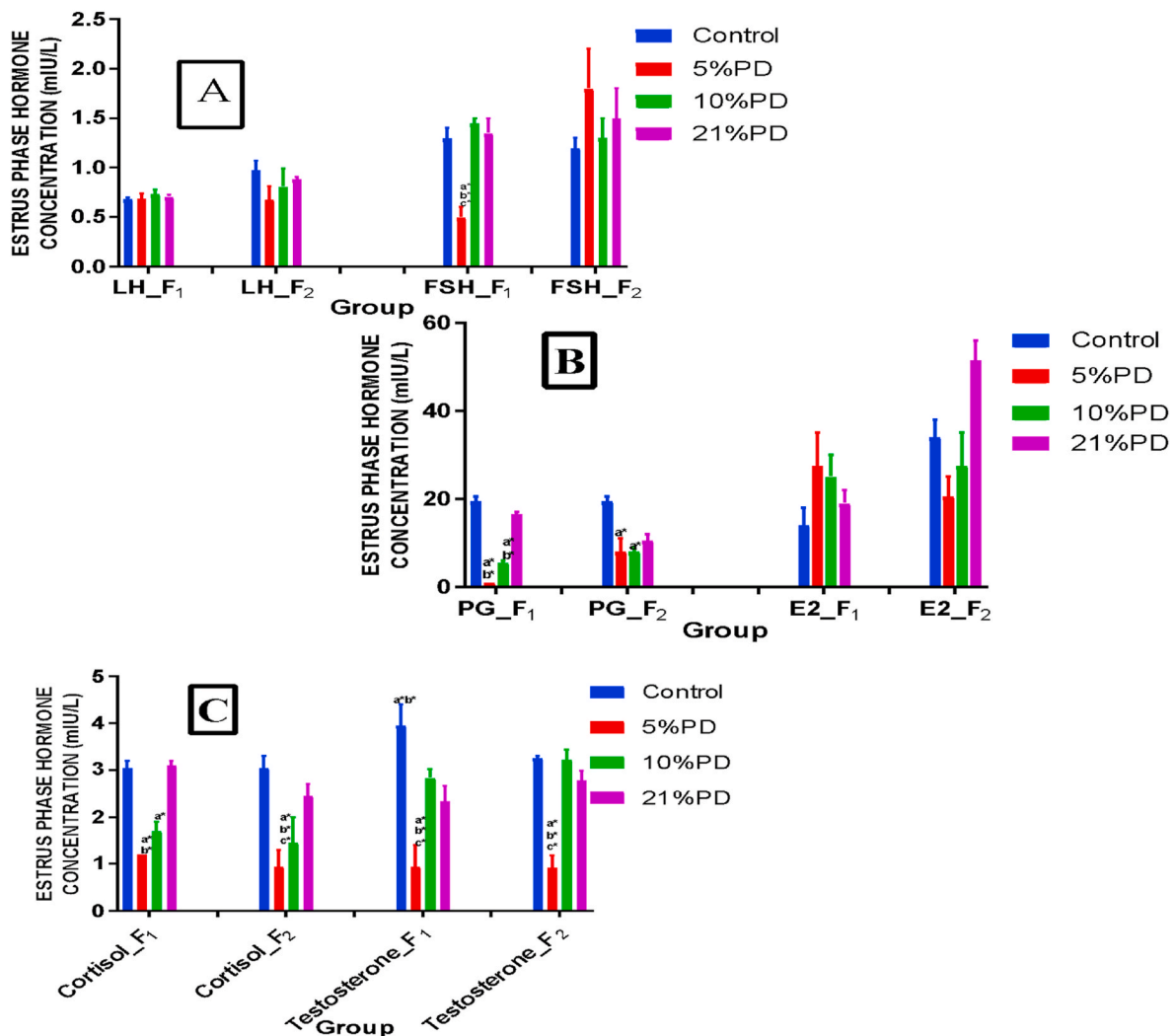
Puberty attainment, as well as ovulation, relies on the concerted and complex convolution of the hypothalamic-pituitary-gonadal axis. Any interference with luteinizing hormone (LH) and follicle-stimulating hormone (FSH) periodic surge at puberty will delay ovulation and puberty, resulting in increased cystic and antral follicles and decreased corpora lutea, as earlier reported by Picut et al. (2015). The pubertal sex hormone profiling at the oestrus phase in this study declined in the 10% PD and 5%PD FSH as well as the progesterone levels which are key to pubertal attainment. The endocrine dysfunction according to Parent et al. (2015), may underlie the cycle irregularity and delayed onset of puberty as seen in the deficient groups. Cortisol is expected to peak around ovulation and decrease rapidly fairly at the beginning of the

luteal phase (Cerdeira-Molina et al., 2013), in response to circadian rhythm, a high concentration of cortisol is usually available for biological actions preovulation, to reduce the inflammatory-like reactions that occur in connection with ovulation, while the rapid lowering during the luteal phase is important for implantations and maintenance of pregnancy (Fanson et al., 2015), rather in this study, cortisol level was lowered at this phase of oestrus, thereby underlining the anovulation and acyclic period displayed. Disruptions in this hormonal modulation may contribute to reproductive failures and ovarian dysfunction as observed in the cycling pattern of the protein deficiency groups.

#### 4.4. Perinatal protein deficiency altered ovarian morphology and function

The maintenance of reproductive status and overall health critically depends on the proper functioning of the ovary, and ovarian function is a subject of the normal development of ovarian follicles (Devine Patrick et al., 2012). In sexually matured rats, the ovarian cortex is characterized by the development of follicles at different stages. The subsequent transition of primordial follicles into primary follicles are critical process in normal ovulation physiology. Abnormalities in primordial transition can cause pathological conditions such as ovarian failure (Zhang et al., 2017; Phillip and Skinner, 2003). The ovarian morphometric analysis was presented with decreased primary follicles (at F<sub>1</sub>), an increased number of primordial follicles (at F<sub>2</sub>), decreased corpora lutea and increased cystic follicles (at both F<sub>1</sub> and F<sub>2</sub> - generation) in the protein-deficient groups as compared to control and 21%PD, which appeared normal showing the presence of follicles at all stages of development including corpora lutea. The persistent increase in the cystic follicles and decreased corpora lutea from one generation to another are signatures of the polycystic ovarian syndrome (PCOS) as reported by Osuka et al. (2018).

The altered ovarian architecture as well as the modulated mRNA expression levels of key ovarian genes (Abey et al., 2021), may directly



**Fig. 5.** Effect of protein deficiency on serum puberty sex hormone levels during oestrus phase of the F<sub>1</sub> and F<sub>2</sub> generation in different diet groups. A: Luteinizing Hormone (LH), and Follicle-stimulating Hormone (FSH), B: Progesterone and Estradiol, C: Cortisol and Testosterone. Data are expressed as mean ± SEM, Control (standard rat chow containing 16–18% Protein), 21%PD (upgraded daily recommended intake), 5% (Protein deficient diet), 10% (Mild protein-deficient diet). a < significantly different from 21% diet group and or control > b < significantly different from the 10% diet group > (\*P < 0.05, \*\*P < 0.001, \*\*\*P < 0.0001). LH: Luteinizing hormone, FSH; Follicle Stimulating Hormone, PG; Progesterone, E2; Estradiol.

impart its function and this may in part be responsible for some abnormalities in reproductive function and fertility response previously discussed. The Corpus luteum matures during the oestrus cycle and then regresses. A degenerating corpus luteal is characterized by an increased amount of fibrous tissue and yellow-brown lipofuscin pigment (Ahmadi et al., 2017), and Corpus luteal cyst is one of the functional cysts known to affect ovarian function in female domestic animals. Another underlying mechanism for the variation in ovarian morphology could be alteration in the follicular atresia process, this process regulates the size and number of follicles in the developing pool. An increase in follicular atresia can be observed following induced conditions of stress (which may be nutritional stress), where it continues to cause a decrease in corpora lutea and inflamed ovary (Chou and Chen, 2018).

**5. Conclusion**

Nutrition is an important factor affecting pubertal development. It can therefore be suggested from this study that intergenerational perinatal protein deficiency can consequently program reproductive developmental process, presenting a sub-optimal reproductive response and function in subsequent generations of adults, as a function of normal

development of the ovary, ovarian follicles, endocrine dysfunction, ovarian degeneration and morphological variation. These, therefore, underlie the reproductive responses and function in the perinatal dietary protein-deficient model from one generation to another.

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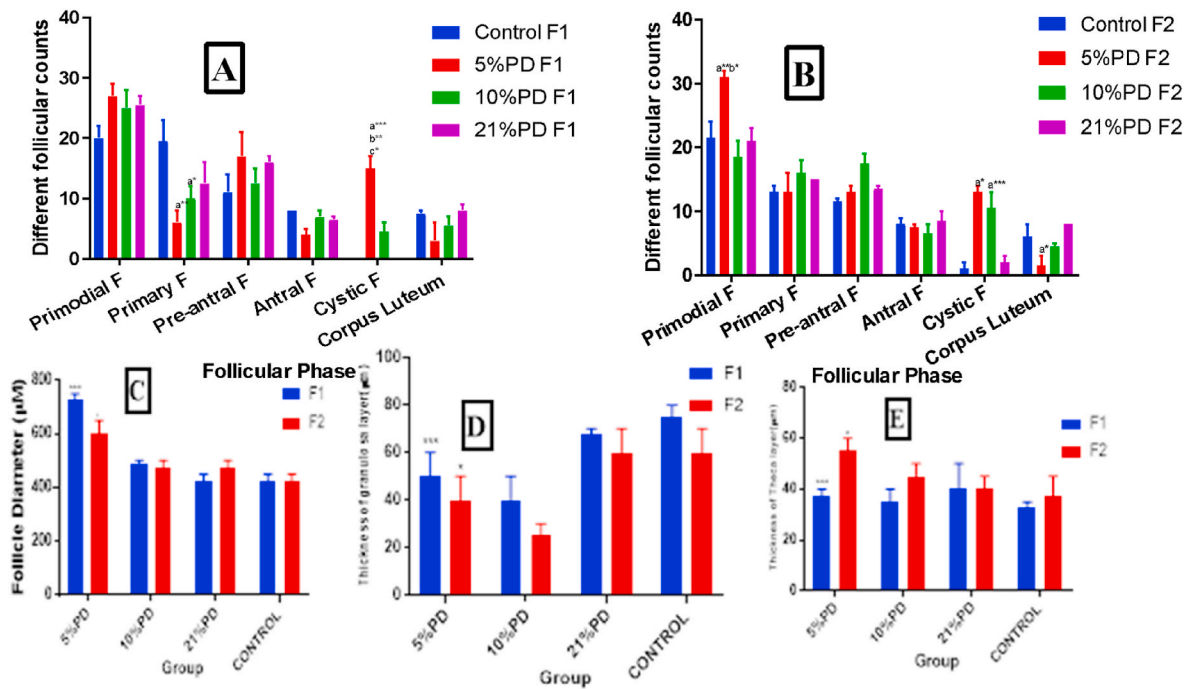
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**Author’s contribution**

NA, OE, and NI designed the study. OE and NI constructively supervised and review the benchwork, results and manuscript, while NA carried out the analyses, statistical analysis, the literature search, and wrote the first draft of the manuscript.

**CRedit authorship contribution statement**

**Nosarieme O. Abey:** Conceptualization, Methodology, Software, Data curation, Investigation, Writing – original draft, Writing – review & editing. **Osaretin A.T. Ebuehi:** Visualization, Investigation,



**Fig. 6.** Effect of protein deficiency on the morphometric analysis of ovarian follicles in various diet groups at F<sub>1</sub> and F<sub>2</sub> generations A: Follicular Phase count at F<sub>1</sub>, B: Follicular Phase count at F<sub>2</sub>, C: Follicle diameter at F<sub>1</sub> and F<sub>2</sub>, D: Granulosa layer thickness at F<sub>1</sub> and F<sub>2</sub>, E: Theca layer thickness at F<sub>1</sub> and F<sub>2</sub>. Data are expressed as mean ± SEM, n = 6, Control (standard rat chow containing 16–18% Protein), 21%PD (upgraded daily recommended intake), 5% (Protein deficient diet), 10% (Mild protein-deficient diet). a; significantly different from the 21% diet group, b; significantly different from the control diet group, c; significantly different from the 10% diet group, F<sub>1</sub> and F<sub>2</sub> are first and second generations respectively. (\*P < 0.05, \*\*P < 0.001, \*\*\*P < 0.0001).

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**Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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