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# LONGITUDINAL EVALUATION OF DEVELOPMENTAL PROTEIN MALNUTRITION RESEMBLING MARASMIC-KWASHIORKOR CONDITION IN WISTAR RATS

#### Badanthdka et al. Characterization of protein malnutrition rat model

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### ABSTRACT

**Objectives:** Protein malnutrition (PMN) is a significant public health concern that aggravates pathological states. Impact of early malnutrition on metabolism needs extensive evaluation. Current models employ short-term diet-restriction, and are neither ethically right nor clinically relevant. This study outlines the development of a PMN rat model to evaluate the effects of low protein diet (LPD) on physiological, hematological, biochemical, and histological changes affected by malnourishment from post-weaning to 40<sup>th</sup> week. **Materials and methods:** The PMN model was developed in Wistar rats (post weaning) by assigning randomized animals to patented LPD (10% protein) and the control group to normal diet (18% protein). **Results:** LPD-induced PMN showed stunted growth, altered biochemical and hematological markers, and significantly affected hepatic histology. Long-term study was conducted to analyze the pattern of developmental PMN and its stabilization over time.

**Conclusion:** The developed PMN rat model imitates the clinical condition and is confirmed as a stable, reproducible, and reliable model for short- and long-term studies. Its clinical relevance opens the avenue for research in treatment, drug development, molecular interactions, and disease model development. **Keywords:** Animal model, Biochemical parameters, Low protein diet, Marasmic- kwashiorkor, Protein malnutrition, Rats

# INTRODUCTION

Malnutrition is a condition caused by an imbalance in the intake of nutrients in terms of quantity, quality or both during any point of life.<sup>1,2</sup>A report by the Food and Agriculture Organization (FAO) stated that 728 million people around the globe were malnourished in 2020.<sup>3</sup>World hunger statistics 2021, reports a drastic increase of approximately 161 million malnourished people, between 2019 and 2020. This crisis can be attributed to climate change and COVID-19 consequences.<sup>4</sup>Malnutrition at critical growth period results in short or long-term metabolic impairments.<sup>5</sup>The metabolic activity of an individual is controlled by the nervous system, which generally develops in the early stage of life. Early under-nutrition processes information to the nervous system for the permanent self-programming to save energy in the form of fat and to reduce growth, this anatomical and physiological adaptation is to secure survival in possible adverse conditions.<sup>1,5</sup>

Malnutrition is not a disease, it is considered one of the prime concerns leading to disease burden in developing countries. PMN can be categorized into three forms based on the clinical manifestations: kwashiorkor, marasmus, and an intermediate stage named marasmic-kwashiorkor. Clinical features depend on severity, duration, stage of life and degree of nutritional deficiency.<sup>6</sup>Kwashiorkor is typically defined as edematous

malnutrition, with clinical characteristics such as skin lesions, hair loss, hypoalbuminemia<sup>7</sup> and hepatic abnormalities (hepatomegaly and fatty infiltrations). Whereas, marasmus is non-edematous malnutrition characterized by significant weight loss, lack of subcutaneous fat, muscular atrophy, and a poor weight-forheight ratio.<sup>8</sup>Marasmic-kwashiorkor is a clinical manifestation characterized by a combination of the clinical features of two types of malnutrition. Body composition, gastrointestinal tract, liver, kidney, tissue protein, body fluids, plasma, and hormones are targets of protein-energy malnutrition mediated physiological and functional changes.9

Experimental animal models serve as important sources of information to understand the effects and consequences of various diseases and drug action.<sup>10</sup>Laboratory animals are extensively used to assess the effects of variables in various degrees of malnutrition and individual pathologies related to malnutrition. The highly controlled evaluation of each nutritional parameter individually gives more consistent results in animal models over humans. Nutritional insults like protein malnutrition during the early and developmental phases of life induce weak hallmarks of metabolic malfunctions and can impair lifelong metabolism patterns.<sup>11</sup>Protein deficiency in Wistar rats produces changes in body weight, body and organ growth, and developed hepatic steatosis.<sup>12</sup> Malnutrition negatively affects biochemical parameters like total protein (TP) and albumin (ALB)<sup>13,14</sup>, phosphorous<sup>15</sup> and triglyceride (TG).<sup>16</sup>Liver function markers such as alkaline phosphatase (ALP), aspartate aminotransferase (AST), and Alanine aminotransferase (ALT) increase from malnutrition.<sup>17,18</sup> The global prevalence of PMN demands a better understanding of the underlying pathophysiological mechanism. Effects of under-nutrition in humans are not restricted to early development, but also produce a lasting effect. However, studies on the consequences of long-term malnutrition and its metabolic risks are scanty. It is necessary to have a stable, long-term, and clinically relevant malnutrition model in order to carry out this kind of research. Malnutrition models using different animals employ starvation/ diet restriction, which are generally short-term models developed for context-specific experiments. We present here the development of a long-term PMN model in female Wistar rats, which could mimic the potential impact of nutrition in preclinical studies, offering a scenario parallel to clinically malnourished populations. This tool would possibly help preclinical evaluation of novel therapeutic interventions.

#### MATERIALS AND METHODS

#### Chemicals and instruments

Semi-Auto Analyzer model: Star 21 Plus from Rapid Diagnostic Group of Companies, India, automated hematology analyzer: Nihon kohden, India, Biochemical reagent kits from Aspen Laboratories Pvt. Ltd., Formaldehyde (LobaChemie Pvt. Ltd., #01460). Normal diet (Amruth feeds, Pune, Maharashtra), Corn oil (Grainotch Industries Ltd., Cornlite), Sucrose (Shree Renuka Sugars Limited, Madhur sugars), Wheat bran (Liberty brand from local market), Vitamin mix (SIDDON BIOTECH, LBCE150205), Mineral mix (SIDDON BIOTECH, LBCE150203), Maize starch (SB-IMPEX, CHE150196).

#### Experimental animals

Healthy female Wistar rats were selected at weaning and housed in polypropylene cages using rice husk bedding at NUCARE (Nitte University Centre for Animal Research and Experimentation). Standard laboratory conditions were maintained (12-hour light/dark cycle; temperature  $22\pm2^{\circ}$ C; relative humidity  $60\pm5\%$ ) with free access to food and water. All experimental procedures were conducted as per the Institutional Animal Ethics Committee (NGSM Institute of Pharmaceutical Sciences) guidelines under approval no: NGSMIPS/Dec-2020/2022.

# Experimental design

Animals chosen above were divided into two groups: Normal diet (ND) and Low protein diet (LPD).<sup>19,20</sup>Details of the diet composition are mentioned in Table 1. ND group received normal diet (18% protein), and the LPD group received low protein diet (MB diet, 10% protein) ad libitum. Bodyweight and length (nose-to-anus length) were assessed weekly as biometrical parameters, and rats were classified into malnutrition categories according to Gomez classification of malnutrition.<sup>21,3</sup>

The body mass index (BMI) of rats were calculated using the standard formula:

# $\frac{Weight (g)}{Nose to anus length^2 (cm^2)}$ BMI =

Blood and tissue sampling

Blood was sampled on the first day of every alternative week by puncturing the retro-orbital plexus under isoflurane anesthesia. The collected blood samples were allowed to clot at room temperature, centrifuged at 3000 rpm for 5 minutes to separate serum. At the end of the experiment, rats were euthanized using isoflurane anesthesia. Liver tissue was excised, washed, weighed, and the tissue was fixed in 10% formalin for histopathological investigations.

Haematological analysis

Blood samples collected in EDTA coated tubes were analyzed immediately after collection using automated hematology analyzer.

# Serum biochemical analysis

Biochemical parameters were quantified using standard commercial kits as per the manufacturer's instructions. Stored serum samples were thawed and analysed of ALB, TP, TG, phosphorous, AST, ALT, and ALP. *Statistical analysis* 

The statistical analysis was done using the population of the mean and standard error of mean (n = 6). Test of significance or statistical analysis was by students T-test. P  $\leq 0.05$  was considered as statistically significant. Graph pad prism (version 8.4.3) (GraphPAD, San Diego, CA, USA) software was used for statistical analysis. **RESULTS** 

#### Biometric parameters

Bodyweight and BMI of LPD group was significantly decreased when compared to normal age-matched rats. Body weight rose in ND group during the first 6 weeks, after which it stabilized. The gradual rate of weight gain in LPD may be attributed to lower dietary protein (Figure 1A & 1B). The body weight and BMI in the LPD group confirm the development of a stable malnourished rat model compared to age-matched controls. LPD group animals were categorized into various degrees of malnutrition based on the principles of Gomez classification with respect to the body weight of ND group rats. By the end of week 1, LPD shows grade II malnutrition (moderate malnutrition), progressing to grade III malnutrition (i.e., severe malnutrition) from week 2 to 12. Subsequently, there was a slow improvement to moderate malnutrition from week 13 to 27. Later, it shifted to mild malnutrition or grade I malnutrition (Figure 1C), may be by survival adaptations.<sup>22</sup> *Haematological parameter* 

Haemoglobin (Hb), Haematocrit (HCT), Platelets (PLT), Platelet Crit (PCT) count in blood decreased in LPD rats compared to ND group (Table 2). Though Red Blood Cells (RBC) count was normal in LPD group, RBC indices like Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), and Mean Corpuscular Haemoglobin Concentration (MCHC) were significantly low. On the other hand, White Blood Cells (WBC) count increased compared to normal.

#### Biochemical parameters

TP and ALB being clinically relevant markers of PMN, both fell significantly in LPD owing to prolonged low protein intake. Average serum ALB ranged between 1.9 and 2.4 g/dL from week 4 to week 22, and TP ranged between 5.1 to 6.4 g/dL from week 10 to week 24 in LPD (Figure 2A & 2B), which corresponds to clinical data and previous reports.<sup>13,14</sup> ALT and AST levels increased in the LPD group (Figure 2C & 2D) because PMN alters liver function The significant increase in AST and ALT positively correlates with degree of malnutrition; grade II, III showed highest level of injury. Serum ALP also rose substantially in LPD group. Phosphorous levels were significantly higher in ND group initially but stabilized later. (Figure 2E & 2F). PMN remarkably depressed plasma TG levels (Figure 2G), possibly from lipid accumulation in the liver, as shown in histopathology results (Figure 4). The present study also demonstrates that the relative liver weight is higher in LPD than normal (Figure 2H). Fat accumulation and hydropic changes could have increased liver weight in LPD.

# Histopathology

Histopathological examination of liver at weeks - 10, 18, 20 & 26 capture the histological changes in the liver during development of PMN in rats. Liver sections of LPD group shows ballooning degeneration due to hydropic changes. Both Kupffer cells and dilated sinusoids around the central vein are evident. Nuclear displacement towards the periphery from fat deposition in hepatocytes with vacuoles. These remarkable changes in liver histology confirmed the development of a stable malnourished rat model. We have used only week 10 slides for comparison.

## Model validation

The model was validated by examining the biometric parameters, where malnourished rats underwent a refed process with ND (18% protein). Our results showed that LPD was able to induce PMN in rats, resulting in body weight loss, which could be reversed by refeeding. The BMI and body weight (Figure 3) of these animals increased significantly to match those of the ND group. During the 15 weeks period of diet rehabilitation, we observed that the refed rats presented catch-up growth of (60.4%) and recovered from grade II (65%) to grade I (89.36%) category of malnutrition according to Gomez classification. This finding supports the importance of protein content in the diet and demonstrates that treating protein deficiency malnutrition necessitates increasing dietary protein intake.

# DISCUSSION

As stated in the hypothesis, we developed a stable, reproducible, and clinically relevant PMN rat model, which could be used for short- and long-term studies. The morphology showed stunted growth and skeletal structure in the LPD group. Skeletal muscles are the main protein reservoirs in the body and are sensitive to protein deficiency. Therefore, depletion of differentiating muscle fibers weakens skeletal muscles<sup>23</sup> leading fall in weight gain. Opaque fur coating, voracious feeding, and a stooped posture were also noted.

The hematology markers such as MCV, MCH, MCHC, HGB, HCT, PLT, and PCT in the PMN group were significantly reduced. Despite a normal RBC count, the altered RBC indices (MCV, MCH, and MCHC) indicate reduced hemoglobin in the RBC, resulting in compromised cell size, resembles iron deficiency-induced hypochromic microcytic anemia. Red Cell Distribution Width (RDW), which increases in iron deficiency anemia<sup>24</sup> is possibly because total iron binding capacity is decreased in PMN.<sup>25</sup> Moderate anemia is prevalent in Kwashorkor<sup>26</sup> and marasmic conditions.<sup>27</sup> WBC count is high in the LPD group, indicating susceptibility to infection. Reduction in platelet count may be attributed to compromised bone marrow activity.<sup>28</sup> Depletion in amino acid precursors possibly led to a significant decrease in ALB levels in the LPD group.<sup>29</sup>ALB is synthesized solely in the liver on polysomes bound to the endoplasmic reticulum. After synthesis, albumin is transported from the rough endoplasmic reticulum to the golgi bodies and released directly into the systemic circulation. In PMN, loss of hepatic RNA, and disaggregation of polysomes together upsets ALB synthesis.<sup>30</sup>Clinically, hypoalbuminemia is more severe in children with kwashiorkor than with marasmus.<sup>31</sup>Serum ALB level of less than 2.3 g/dL is considered undernourished. Lower ALB levels in LPD correlate with earlier studies and are clinically relevant.<sup>14</sup>

Both AST and ALT are elevated in PMN possibly because of hepatic tissue damage. Malnutrition affect the hepatocytes and releases these enzymes into the bloodstream in clinical conditions.<sup>32</sup> Our PMN model mirrors with this clinical data and thereby validates the model.<sup>33</sup> Impairment in bone development and liver function raises ALP. Elevated ALP in PMN<sup>34</sup> has already been reported. Early rise (initial weeks) in ALP might be the result of compromised bone development rather than liver dysfunction.<sup>35</sup>This observation is strengthened by low phosphorous levels in early development. PMN depletes phosphorous levels, impairing bone development, and defects in bone maturation. Compensatory osteoblast activity is increased as a positive feedback mechanism to overcome this impairment, thereby elevating ALP.<sup>36</sup> From the later weeks, liver dysfunction accompanies elevated ALP levels, which correlates to AST and ALT data.

PMN decreases fatty acid oxidation, leading to increased lipogenesis and TG storage in the liver.<sup>7,2</sup>Hepatocytes recognize the amino acid profile, which is controlled by dietary protein intake that alters hepatocyte TG secretion<sup>37</sup>, resulting in decreased serum Further, a reduction in TG secretion can also be attributed to a reduction in the rate of very low-density lipoprotein (VLDL) synthesis, resulting in hypertriglyceridemia during protein deprivation<sup>38</sup>Triglycerides in the serum are frequently low in kwashiorkor, but they are normal or increased in marasmus condition.<sup>10</sup>

Severe but reversible changes in the liver are characterized by hepatocyte ballooning, due to the hydropic changes in the tissue. Edema enlarges the cell, characterized by irregular cytoplasmic accumulation of water and fat droplets in vacuoles. Initially, the vacuoles are small and surround the nucleus. Subsequently, vacuoles become more prominent and displace the nucleus to the periphery, forming a signet ring structure. The fat deposited in the vacuole is predominantly TG. The accumulation of TG in the liver leads to fatty changes that decrease hepatic TG secretion.<sup>39</sup>

In the present study LPD shows the presence of hepatomegaly, which, along with fatty liver constitutes an essential clinical feature of kwashiorkor. Likewise, children with marasmus also present with hepatic steatosis and hepatomegaly, demonstrating clinical relevance.<sup>40</sup> The fatty liver in kwashiorkor is more intense than in marasmus. In marasmus, liver increases the synthesis of plasma lipoproteins in response to excess of fatty acids, but in kwashiorkor, the liver, unable to dispose of fatty acids, end up accumulating lipid in the liver.<sup>10</sup> The clinical features of kwashiorkor include hepatomegaly, fatty liver, hair loss, stooped posture, hypoalbuminemia, low serum phosphate, TP and TG levels. However, there was no edema in PMN rats, a key indicator of kwashiorkor. Interestingly, PMN rats also exhibited marasmic features such as significant weight loss, low BMI, lack of subcutaneous fat, muscular atrophy, fat deposition in the liver, and a poor weight-forheight ratio. Hence, our model clinically represents the marasmic-kwashiorkor condition.

#### STUDY LIMITATIONS

The study focuses on biochemical, hematological, and histological changes but does not thoroughly investigate other molecular interactions involved in malnutrition.

#### CONCLUSION

The PMN rat model using 10% protein diet mimics clinical manifestations of marasmic-kwashiorkor condition. This model is inexpensive to develop, easy to maintain, repeatable, predictable, ethical, and clinically relevant. The model can be used for potential research in drug kinetics, disease model development, drug interaction studies, and drug discovery research. Being a long-term model, LPD induced malnutrition rat model will be appropriate for study on maternal and intragenerational malnutrition also. This PMN model may be appropriately and adaptively modeled to suit different experimental situations to evaluate multiple clinical/ biological attributes.

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# Ethics

Ethics Committee Approval: All the animal experiments were conducted as per the guidelines of the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) with approval no. NGSMIPS/Dec-2020/2022.

# **Authorship Contributions**

Concept: M.B., V.A., Design: M.B., V.A., Data Collection or Processing: V.A., V.D., M.R.J., Analysis or Interpretation: V.A., Literature Search: V.A., V.D. M.R.J., Writing: V.A., V.D., M.R.J., Reviewing and Editing: M.B., V.D., M.R.J., A.C., M.K., V.S.

Conflict of Interest: No conflict of interest was declared by the authors.

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Figure 1.Graphical representation of (A) rat body weight (g) from weaning to 40 weeks of ND and LPD group

(B) BMI of ND & LPD group (C) Percentage of change in body weight of LPD with respect to ND group, and (D) photographic representation of ND (a) and LPD (b) at week 10 (1) and week 34 (2) of the study (n = 6). Significant differences indicated by \*,  $P \le 0.05$ ; \*\*,  $P \le 0.01$ ; \*\*\*,  $P \le 0.001$ .



**Figure 2.** Serum biochemical parameters in ND and LPD (n =6): (A) ALB, (B) TP, (C) AST, (D) ALT, (E) ALP, (F) Phosphorous, and (G) TG & (H) relative liver weight at week 10 (n = 6). Significant differences indicated by \*,  $P \le 0.05$ ; \*\*,  $P \le 0.01$ ; \*\*\*,  $P \le 0.001$ .ALT, Alanine Aminotransferase; ALB, Albumin; ALP, Alkaline phosphatase; AST, Aspartate aminotransferase; TP, Total protein ;TG, Triglyceride.



**Figure 3.**Graphical representations of (A) body weight (B) BMI of ND, LPD & Refed groups during rehabilitation period. Significant differences indicated by \*,  $P \le 0.05$ ; \*\*,  $P \le 0.01$ ; \*\*\*,  $P \le 0.001$ .



**Figure 4.** Photomicrographs of H & E sections of liver (A) ND group showing normal cellular architecture with hepatocytes radiating from central vein and sinusoidal space at week 10 of study (20X) (B) Week 10 LPD group showing mild hydropic changes in the hepatocytes (20X) at week 10 (C) LPD group showing ballooning degeneration with irregular cytoplasm (40X) at week 18 (D) LPD group showing dilation of sinusoidal space (black arrows) with marked hydropic change (40X) at week 20 (E) LPD group showing central vein with

marked hydropic change and presence of kupffer cells (black circles) (40X) and (F) macro-vesicular fat droplets (black discontinued circle) occupying cytoplasm and displacing nucleus to periphery (40X) at week 26.

| Table 1. Composition of low protein diet (10% protein) for the development of malnourished rat model. |                     |      |                   |        |  |  |
|---|---------------------|------|-------------------|--------|--|--|
| Sl. no.   | Ingredients (ND)    | g/kg | Ingredients (LPD) | g/kg   |  |  |
| 1   | Wheat flour         | 56.2 | Normal diet       | 44.440 |  |  |
| 2   | DCP (rock base)     | 1.8  | Corn oil          | 2.415  |  |  |
| 3   | Calcite powder      | 1.0  | Sucrose           | 6.038  |  |  |
| 4   | LAF mix             | 1.0  | Wheat bran        | 3.019  |  |  |
| 5   | Linseed             | 5.0  | Vitamin mix       | 0.603  |  |  |
| 6   | Maize gluten        | 5.0  | Mineral mix       | 2.113  |  |  |
| 7   | Roasted gram flour  | 25.0 | Maize starch      | 41.360 |  |  |
| 8   | Skimmed milk powder | 5.0  |                   |        |  |  |

**Table 2** Hematological parameters of ND and LPD animal groups at  $10^{th}$  week (n=6)

| Table 2. Hematological parame | week (II-0)          |                        |
|-------------------------------|----------------------|------------------------|
| PARAMETER (unit)              | ND GROUP             | LPD GROUP              |
| WBC (10 <sup>3</sup> /uL)     | $10.533 \pm 0.974$   | $11.800 \pm 1.237$     |
| RBC $(10^{6}/ \text{ uL})$    | $8.363 \pm 0.252$    | $8.407 {\pm}\ 0.694$   |
| HGB (g/dL)                    | $14.750 \pm 0.531$   | $12.867 \pm 0.978$     |
| HCT (%)                       | $43.517 \pm 1.562$   | $39.817 \pm 3.016$     |
| MCV (fL)                      | $52.033 \pm 1.341$   | $47.450 \pm 0.439 **$  |
| MCH (pg)                      | $17.667 \pm 0.465$   | $15.366 \pm 0.182 ***$ |
| MCHC (g/dL)                   | $33.867 \pm 0.217$   | $32.367 \pm 0.236 ***$ |
| PLT $(10^{3}/uL)$             | $740.334 \pm 60.593$ | $681.5.167{\pm}60.702$ |
| RDWCV (%)                     | $12.300 \pm 0.339$   | $13.267 \pm 0.154$     |
| RDWSD (fL)                    | $25.616 \pm 0.849$   | $25.183 \pm 0.318$     |
| PCT (%)                       | $0.416 \pm 0.046$    | $0.371 \pm 0.320$      |
| MPV (fL)                      | $5.533 \pm 0.031$    | $5.483 \pm 0.071$      |
| PDW (%)                       | $15.400 \pm 0.068$   | $15.030 \pm 0.121$     |

All values are Mean  $\pm$  SEM. Bars represent the standard error. p< 0.05\*, p < 0.01\*\* and s p < 0.001\*\* when compared to the normal diet group.

HCT, Haematocrit; Hb, Haemoglobin; MCH, Mean Corpuscular Haemoglobin; MCHC Mean Corpuscular Haemoglobin Concentration; MCV, Mean Corpuscular Volume; MPV, Mean Platelet Volume; PCT, Platelet Crit; PDW, Platelet Distribution Width; PLT, Platelet Count; RBC, Red Blood Cells; RDWCV, Red Blood Cell Distribution Width; RDWSD, Standard Deviation Red Blood Cell Distribution Width; WBC, White Blood Cells.