Effects of a 60-day gluten-free chapatti intervention on hematologic and renal biomarkers in celiac disease: pre-post analysis

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ORIGINAL ARTICLE

Abstract

Celiac disease (CD) often coexists with anemia and biochemical abnormalities and requires a lifelong gluten-free (GF) diet to prevent adverse health effects. This study explores the changes in hematologic and biochemical markers during a 60-day GF chapatti intervention and characterizes the product's composition. In a prospective, single-arm pre-post study (n = 130; 65 male, 65 female), participants consumed a GF chapatti formulated from corn, chickpea, and rice flour (40 %, 50 %, and 10 %). Venous blood was collected at baseline and follow-up visits (Days 0, 15, 30, 45, and 60). Primary endpoints included hemoglobin (Hb) and hematocrit (HCT); secondary endpoints included red-blood-cell indices, urea, creatinine (Cr), bilirubin fractions, ferritin, serum iron, TIBC, and sTfR. Single-arm pre-post blood glucose (BG) was measured under nonfasting conditions. Proximate analysis revealed that chickpea and corn flours provided higher protein (17.58% and 11.37%, respectively) and fiber (8.16% in corn) compared to rice and wheat. Posttreatment chapatti showed improved nutritional content (13% protein, 1.6% fiber). Volunteers exhibited gradual increases in weight and height. Hb levels were significantly high, and glucose levels were stabilized. Iron status recovered markedly: Ferritin and serum iron increased considerably in both sexes ($p < 0.001$), while TIBC and sTfR also rose ($p < 0.001$), indicating improved erythropoiesis. Inflammation declined: Both hs-CRP and AGP had a p-value of < 0.001. Kidney function improved (decline in urea and Cr). Liver markers (bilirubin, alkaline phosphatase (ALP), and SGPT-ALT) also showed favorable trends, indicating no adverse hepatic effects. A 60-day GF chapatti intervention was associated with improvements in anemia-related markers and no adverse trends in renal/hepatic function.

Keywords: Celiac disease, Gluten-free chapatti, Hemoglobin, Hematocrit, Dietary intervention, Bio accessibility

Introduction

Celiac disease (CD) is a chronic autoimmune disorder in which the ingestion of gluten, a protein found in wheat, barley, and rye, triggers an immune response that damages the small intestine. The disease affects genetically predisposed individuals, particularly those with specific HLA-DQ2 or HLA-DQ8 alleles (Pop *et al.*, 2024). Upon gluten consumption, the immune system mistakenly targets the villi in the small intestine, leading to their

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atrophy and impairing nutrient absorption (Caio *et al*., 2019). Celiac disease (CD) causes intestinal harm and the inability to access nutrients in patients who have a hereditary inclination. Though the treatment is a strict lifelong gluten-free (GF) diet, conventional GF alternatives are commonly deficient in important nutrients, especially protein, fiber, and other important micronutrients. Symptoms of celiac disease can vary widely, ranging from gastrointestinal manifestations such as diarrhea, bloating, and weight loss to extraintestinal symptoms like fatigue, dermatitis, and even neurological disorders (Lebwohl *et al*., 2018; Iqbal *et al*., 2025).

Many patients with CD exhibit nonclassical symptoms, which can affect multiple organ systems:

Neurological and Psychiatric Symptoms: Peripheral neuropathy, ataxia, depression, and anxiety (Zis & Hadjivassilion, 2019).

Hematologic Manifestations: Iron-deficiency anemia may be refractory to oral iron therapy.

Dermatological Manifestations**:** Dermatitis herpetiformis, an intensely pruritic rash often found on extensor surfaces (Antiga *et al.*, 2019).

Endocrine and Reproductive Issues: Delays in menarche, a higher chance of infertility, and repeated miscarriages (Caio *et al.*, 2019).

Celiac disease (CD) is a worldwide health issue that affects 1% of the global population (Lebwohl *et al.* 2018). People believe CD cases are rising in Pakistan, even though we do not have many thorough studies. New research indicates that CD affects 0.5% to 1% of Pakistanis, aligning with global trends. However, the real numbers might be higher because doctors often do not screen enough people (Rashid, M., and Rashid, H., 2019). A study in Punjab, a province in Pakistan, found that CD affects about 0.75 % of people there, with kids getting it more often (Rashid, M. & Rashid, H., 2019). However, the inconsistent clinical presentation of the condition and the lack of regular screening programs in many regions contribute to the substantial underdiagnosis rate.

Iron, zinc, folate, vitamin B12, vitamin B6, and vitamin D are among the nutrients that patients with celiac disease typically lack. As a screening sign in and of itself, iron deficiency has been discovered in as many as 50% of newly diagnosed individuals (Cappellini *et al.*, 2020). Copper, vitamin B12, folate, and vitamin D malabsorption have all been linked to neurological problems (Nieto-salazar *et al.*, 2023; Zerlasht *et al*., 2024). Because of its effects on quality of life, long-term health outcomes, and healthcare expenses, celiac disease is still a significant public health concern even though it is treated (Marild *et al.*, 2020). Apart from the physical and psychosocial difficulties, there is increasing interest in investigating financial assistance programs, including tax breaks or subsidies for GF goods, to lessen the financial strain on those who are impacted (Lee *et al.*, 2019). Because following a GF diet can put a heavy financial burden on many patients, these steps are particularly crucial. The demand for GF products is growing, and research into GF products is a crucial and difficult problem in the cereal-based product industry. Innovative GF products are becoming more and more necessary in addition to satisfying the everyday nutritional needs of people with celiac disease. When gluten-containing cereals, especially wheat, barley, and rye, are consumed by genetically predisposed people, it can cause celiac disease (CD). Alternatives to GF diets include rice, corn, and chickpeas, while wheat is a major contributor to the pathophysiology of disease.

In response to these nutritional limitations, this study explores the development of a GF chapatti formulated from rice, corn, and chickpea flours. These ingredients were strategically selected to enhance the nutritional profile of GF staples:Corn flour offers dietary fiber and energy while remaining naturally GF and culturally acceptable in South Asian diets (Giménez-Bastida & Pihlsgård, 2020). Chickpea flour is rich in plant-based protein, iron, and fiber—nutrients often deficient in standard GF diets—making it ideal for improving the overall nutrient density of the formulation (Vinod *et al.*, 2023).

Wheat: Wheat serves as the primary source of gluten in our diet. The immune response in CD stems from wheat's storage proteins gliadin and glutenin. Gliadin peptides mess with the immune system and stop full digestion after eating, setting off inflammation and damaging the small intestine's villi (Sollid *et al.,* 2020). Tissue transglutaminase (tTG) changes these gliadin peptides, making them more likely to trigger an immune response and activate T cells in people with HLA-DQ2 or HLA-DQ8 genes (Kulkarni and Newberry, 2019).

A Safe Substitute for Celiac Patients: Rice: Rice has become a go-to food for many CD patients because it does not have gluten. Unlike wheat, barley, and rye, it lacks the troublesome prolamins, so people's bodies handle it well (Sharma *et al.,* 2020).

Corn: A common GF substitute: Corn (maize) is widely used in GF products because of its lack of gluten peptides. Corn-based foods provide an essential source of carbohydrates and fiber for individuals on a GF diet. Corn flour offers dietary fiber and energy while remaining naturally GF and culturally acceptable in South Asian diets.

Chickpea: A Nutrient-Rich GF Option: Chickpeas are naturally GF and provide essential nutrients such as protein, fiber, and iron. They play a crucial role in improving the nutritional quality of GF diets, which often lack adequate protein and fiber (Vinod *et al.*, 2023). Studies suggest that legumes, including chickpeas, may help improve gut microbiota composition and reduce inflammation in CD patients following a GF diet. Chickpea flour is rich in plant-based protein, iron, and fiber—nutrients often deficient in standard GF diets—making it ideal for improving the overall nutrient density of the formulation. (Mazzola *et al.*, 2024).

Objective

This formulation aims to provide a sustainable and nutritionally enriched alternative to wheat-based chapattis for individuals with CD, particularly in regions where wheat is a dietary staple. The objective of this research is to study the impact of GF chapatti on celiac volunteers.

Endpoints and analysis set. The primary endpoints were Hb and HCT change from Day 0 to Day 60. Secondary endpoints were RBC indices, urea/BUN, creatinine (Cr), and bilirubin fractions; exploratory endpoints included RBG/RBS and subgroup summaries by sex and age band. The analysis set included all participants with baseline and at least one post-baseline measurement (full analysis set, n = 130).

Materials and Methods

Raw materials

Raw materials were purchased from commercial markets, including corn, chickpea, rice, as well as wheat. After removing visible dust or any foreign matter and then drying in sunlight, all the seeds were given to local millers to get flour.

Chapatti preparation

Chapattis (51–55 g) were prepared by using corn, rice, and chickpeas. Moreover, the process has been submitted for an IPO (patent). Table 1 mentions the treatment plan.

Proximate composition

We used the AOAC (2016) and Quddoos *et al.* (2022) techniques to determine and express the approximate

Table 1. Baseline characteristics of study participants (n=130).

Characteristics	Total Cohort (n=130)	Male (n=65)	Female (n=65)
Age (years), mean ±SD	25.9 ± 9.3	27.2 ± 7.5	24.7 ± 9.2
BMI (Kg/m^2), mean ±SD	15.2 ± 2.3	18.4 ± 1.6	11.8 ± 1.7
Time since CD diagnosis (years). mean ±SD	3.6 ± 2.9	3.6 ± 3.2	3.4 ± 2.5
Key biomarkers, mean ± SD			
Hemoglobin (g/dL)	6.7 ± 0.6	6.8 ± 0.6	6.6 ± 0.6
Ferritin (ng/mL)	13.1 ± 7.4	21.2 ± 0.2	2.6 ± 0.7
Hs-CRP (mg/dL)	1.8 ± 0.6	1.2 ± 0.6	2.8 ± 0.7
Comorbidities, n (%)			
Other autoimmune diseases (Thyroiditis, T1D)	19 (14.6 %)	7 (10.8 %)	12 (18.5 %)
Osteopenia/osteoporosis	22 (16.8%)	8 (12.3 %)	14 (21.5 %)
None reported	89 (68.4%)	50 (76.9 %)	39 (60.0 %)
Comorbidities, n (%)			
Iron supplements	45 (34.6 %)	20 (30.7 %)	25 (38.5%)
Vitamin B12/Folate	28 (21.5 %)	12 (18.5%)	16 (24.6%)
Levothyroxine	11 (8.5 %)	3 (4.6%)	8 (12.3%)
None	55 (42.3 %)	32 (49.2 %)	23 (35.4%)
Typical pre-study GF diet			
Primary Staples	Refined rice, cornmeal, potato	Similar	Similar
Legume Intake	Low (<1 serving/week)	Similar	Similar
Fruit/vegetable Intake	Low (<2 servings/day)	Similar	Similar

SD: Standard Deviation; CD: Celiac Disease; T1D: Type 1 Diabetes.

composition (Moisture, protein, fat, fiber, and ash) of multigrain flour (Wheat, rice, chickpea, and corn) on a dry matter basis.

Efficacy Study

Ethics and consent

The study complied with the Declaration of Helsinki (2013 revision) and institutional guidelines. Ethical approval was granted by the Biosafety and Ethical Review Committee, University of Sargodha, Sargodha, Pakistan (Approval No. UOS/ORIC/2022/66). Adults (≥18 years) provided written informed consent before any study procedures. For minors (<18 years), written informed consent was obtained from a parent or legal guardian, and age-appropriate written assent was obtained from the child (typically 7–17 years). Consent/assent forms were available in Urdu and English; for participants unable to read, the form was read aloud in the presence of an impartial witness, and a thumbprint was obtained in lieu of signature.

Trial registration

Not applicable. This study was a *prospective, single-arm pre–post dietary intervention* with *no allocation or comparator arm* and was therefore not registered as a clinical trial.

Delimitations and limitations

The delimitations—the conscious boundaries set by the researchers—define the scope of this work. This was a prospective, single-arm, pre-post intervention study specifically chosen to evaluate the efficacy of a novel, nutritionally enhanced GF chapatti formulation (40% corn, 50% chickpea, and 10% rice flour) over a 60-day period. The participant cohort was deliberately limited to 130 individuals with a clinical diagnosis of celiac disease, recruited from partner hospitals (Civil Hospital, Chaniot, Pakistan and DHQ Civil Hospital, Sargodha, Pakistan) and clinics (Amin Clinic, Chaniot, Pakistan) in Pakistan and stratified by age and sex to account for physiological variability. The primary endpoints were delimited to key hematologic (e.g., Hb, HCT, and RBC), renal (urea, Cr), and hepatic (bilirubin, ALP, and ALT) biomarkers, measured at five fixed intervals to capture dynamic changes.

Several limitations must be acknowledged. The most significant constraint is the single-arm design without a parallel control group (e.g., celiac patients on a standard GF diet), which limits our ability to establish causality and attribute the observed improvements solely to the intervention, as it cannot rule out the influence of confounding variables or the natural history of the disease. The use of a nonprobability (convenience/purposive) sampling method may introduce selection bias and affect the generalizability of the findings to the broader celiac population. The absence of randomization and blinding presents a potential for performance and detection bias. Furthermore, the 60-day intervention period, while sufficient to observe initial biomarker trends, may be too short to assess long-term sustainability, complete histological healing, or dietary adherence. Finally, the study did not evaluate as to which are the critical factors for real-world implementation, and the exploratory subgroup analyses (e.g., by age) were underpowered for formal statistical inference (adapted from Altaf *et al.,* 2022; Khadija *et al.*, 2022).

Target population and study site

Age Stratification and Rationale: Participants (n = 130; 65 males and 65 females) were recruited (Table 2) across predefined age bands to account for physiological, nutritional, and metabolic differences that vary with age. Specifically, recruitment targeted three age groups (children/adolescents, adults, and older adults). This stratification was implemented a priori to minimize confounding and to enhance the generalizability of findings. Age distribution is summarized in the demographics section; analyses and interpretation consider the potential influence of age-related variability. After receiving approval from the appropriate parties, various hospitals (Civil Hospital, Chaniot, Pakistan and DHQ Civil Hospital, Sargodha, Pakistan) and clinics (Amin Clinic, Chaniot, Pakistan) in multiple locations were chosen as the research project's study locations.

Sampling technique and sample size

According to Muhammad's description of the nonprobability sample method, the human volunteer was chosen using the two-stage sampling procedure of convenience and purposive sampling. The following method was used to determine the sample size for celiac volunteers, taking into account the expected prevalence of the variable of interest (Celiac disease), the requisite degree of confidence (95%), and the 5% margin of error (adapted by Hameed *et al.,* 2024).

Research design

This study used a prospective, single-arm, pre-post intervention design with repeated measures (Days 0, 15, 30, 45, and 60). Participants were recruited via

Table 2. Treatment plan for making multigrain gluten-free chapatti.

Treatments	Wheat flour (%)	Rice flour (%)	Chickpea flour (%)	Corn flour (%)
T_0	100	0	0	0
T_1	0	10	50	40

nonprobability convenience/purposive methods (see Sampling Technique and Sample Size); no random allocation or blinding was performed. Each participant served as their benchmark (baseline = Day 0), in which blood samples of all the volunteers were collected in two phases. In the first phase, lab test of volunteers was performed, which indicated that volunteers diagnosed with celiac disease were recruited for further research. At the end, the lab test was again performed for celiac disease participants (Khadija *et al.* 2022).

Nutritional status assessment

Permission, approach, and informed consent

To conduct the research, consent from several hospitals and clinics in various locations was obtained. Approaching the intended audience of almost 2000, the study endeavor was thoroughly described and debated. The potential volunteers received the information, education, and communication (IEC) materials. The volunteers (boys and females) who wanted to participate in the research endeavor provided their informed consent (Altaf *et al.,* 2022).

Duration of Study

The study was conducted over 60 days, with both pre- and post-performance assessments, and blood sampling was performed. All final volunteers were under consideration for a meetup every week during research.

Demographics for the selection of volunteers

On the pre-designed form, the agreed-upon volunteers had their demographic information collected. The volunteers who matched the aforementioned requirements were chosen as volunteers for additional research.

Anthropometrics and Energetics of Volunteers: To evaluate the nutritional state of the chosen subjects, anthropometric data, including height, weight, body composition, body mass index, basal metabolic rate, calorie intake, and caloric needs, were computed.

Participant selection and eligibility with clinical assessment and medical history

Clinical indicators and symptoms of celiac disease were seen in the study volunteers. The medical histories of the volunteers were reviewed to investigate the prevalence of celiac disease. The diagnosis was confirmed by positive serological testing for anti-tissue transglutaminase (tTG-IgA) antibodies and/or endomysial antibodies (EMA-IgA), at least 10 times the upper limit of normal, in accordance with ESPGHAN guidelines for nonbiopsy diagnosis in symptomatic patients.

Assessment of potential confounders

To account for variables that could influence the measured biomarkers, we systematically collected data on potential confounders at baseline and during weekly check-ins. This included:

- **Medication Use:** Detailed documentation of any use of iron supplements, vitamin B12, folate, multivitamins, or other hematinic medications. This was recorded as a binary variable (yes/no) for use at any point during the study period.
- **Dietary Adherence and Changes:** Beyond chapatti adherence, participants were queried about any significant changes in their overall diet, intake of other GF grains, or protein sources.
- **Lifestyle Factors:** Any self-reported changes in physical activity level, smoking status, or alcohol consumption were noted.

Participants characteristics

A total of 130 participants with clinically diagnosed celiac disease were enrolled. Table 1 provides a detailed summary of their baseline characteristics, comorbidities, and concomitant medications. The cohort was characterized by a high prevalence of iron deficiency anemia at baseline, as evidenced by low mean hemoglobin (Hb) and ferritin levels. Other common comorbidities included other autoimmune disorders, consistent with the known epidemiology of celiac disease. The use of hematinic supplements (iron, B12, and folate) was documented but was not a reason for exclusion unless initiated or changed within 4 weeks before the study. Before the intervention, the typical participant reported a GF diet based primarily on refined rice and corn products, with limited intake of legumes, fruits, and vegetables, aligning with commonly reported nutritional gaps in celiac populations.

Biomarker of selected volunteers

The blood samples (1–2 cc) of selected volunteers were collected by the hospital lab in charge in coded and specified blood collection containers for the determination of serum Cr. level of urea nitrogen in blood, LFT, glucose, and RFT to satisfy the previously indicated inclusion and exclusion criteria. The blood samples of the selected volunteers were collected in coded and designated blood collection containers.

Inclusion

Individuals with a prior clinical diagnosis of celiac disease, able to attend visits at Days 0, 15, 30, 45, and 60, and willing to consume the GF chapatti formulation (T1) as instructed, provided written informed consent (adults) or parental/guardian consent with child assent (minors).

Exclusion

Acute intercurrent illness at baseline; known wheat/gluten challenge during the study period; initiation or dose change of hematinic therapy (e.g., iron, folate, and vitamin B_{12}) within the previous 4 weeks; pregnancy or lactation; severe renal or hepatic failure; any condition judged by investigators to interfere with participation or outcome assessment.

Distribution of chapattis to the volunteers

The volunteers were given a chapatti and a commercially available product to use daily for up to 60 days, and blood tests were performed on day 0 and day 60.

Research Instruments

a) Questionnaire: For screening/nutritional assessment of celiac disease
b) Biochemical tests: RBC, hemoglobin, LFTs and RFTs, glucose

Data Collection and Statistical Analysis

For treatment selection and further evaluation, proper statistical analyses were conducted. Data from the physicochemical assay were provided as mean value and standard deviation (SD). The significance limit for the statistical analysis of these parameters was set at $P \leq 0.05$, and the LSD pairwise comparison test was used to identify significant differences between mean values. Statistics 8.1 software and Stats Direct statistical software, version 3.3 (Stats Direct Ltd, Birkenhead, UK) were used to conduct the statistical analysis (Analytical Software, Tallahassee, FL, USA). Age Handling and Sensitivity: Exploratory analyses were conducted across the predefined age bands (<14, 14-30, and >30 years) (Table 3). Therefore, results are presented as descriptive summaries (e.g., mean change from baseline with SD) and visual trends (e.g., plots of estimated marginal means over time by age group) to generate hypotheses for future research, with formal inferential testing deliberately avoided. For the clinical intervention data, a repeated-measures analysis of variance (RM-ANOVA) was employed to analyze changes in biomarkers over time (Days 0, 15, 30, 45, and 60), with Tukey's post-hoc test used for pairwise comparisons where appropriate. The practical significance of changes from baseline (Day 0) to end line (Day 60) was quantified using Cohen's d, calculated as the mean difference divided by the pooled SD. Primary Analysis (Changes Over Time): To analyze the longitudinal changes in biomarkers across all five timepoints (Days 0, 15, 30, 45, and 60), linear mixed-effects models (LMMs) were fitted. Assumption Testing: For each LMM, model assumptions were rigorously checked. Normality of the residuals was assessed using Q-Q plots. Homoscedasticity (constant variance of residuals) was evaluated by plotting residuals against fitted values. In addition to the primary models (e.g., gender and time effects), descriptive subgroup summaries were prepared across the predefined age bands, and exploratory time×age-group effects were examined. Results were directionally consistent across age groups; however, given sample size constraints, the study was not powered for formal interaction testing, and age-stratified findings are interpreted cautiously. Sensitivity Analysis for Confounding: To assess the potential influence of confounding variables, we performed a sensitivity analysis for our primary outcomes (Hb, ferritin, and urea). We constructed additional LMMs that included the potential confounders of medication use (hematinic supplements: yes/no) and baseline biomarker value as fixed-effect covariates. The stability of the time effect estimate (i.e., the intervention effect) between the primary and adjusted models was examined. A change of less than 10% in the coefficient for time was considered as evidence for the fact that the result was robust to potential confounding by these factors.

Table 3. Age-band distribution of volunteers (n = 130).

Age band (years)	Male participants n (% of males)	Female participants n (% of females)	Total n (% of cohort)
< 14	32 (49.2)	27 (41.5)	59 (45.4)
14–30 (inclusive)	19 (29.2)	20 (30.8)	39 (30.0)
> 30	14 (21.5)	18 (27.7)	32 (24.6)
Total	**65 (100)**	**65 (100)**	**130 (100)**

Blood sampling and pre-analytical handling

Venous blood (~5–7 mL) was collected nonfasting from the antecubital vein during routine clinic hours at Days 0, 15, 30, 45, and 60**.** EDTA tubes were used for hematological indices (Hb, HCT, RBC, WBC, and PLT), and serum separator tubes for biochemistry (urea/BUN, Cr, and total/direct/indirect bilirubin) and iron studies. Single-arm, pre-post BG was obtained under the same nonfasting conditions. Tubes were gently inverted 5–8 times, serum samples were allowed to clot for 20–30 min, and all specimens were centrifuged within 1 h of collection at 1,500–2,000 g for 10 min (≈ 3,000 rpm). Serum was aliquoted into barcoded cryovials, protected from light, and either analyzed the same day on calibrated automated analyzers or stored at −80 °C for batched assays (single freeze–thaw). Hemolyzed, lipemic, or icteric specimens were flagged and, when grossly compromised, recollected. Internal quality-control materials were run

daily, the laboratory participated in external quality assessment, and all measurements were recorded in SI/clinical units and double-checked by a second operator.

Adherence and safety monitoring (5.6 ± 1.0 days/week)**.** Adherence to the intervention chapatti was tracked using participants' daily logs and brief weekly check-ins. At each visit (Days 0, 15, 30, 45, and 60), staff reviewed logs and confirmed intake since the prior visit. Safety was monitored by open-ended AE queries at every contact and by review of clinical notes when available.

Serious adverse events (SAEs) were defined per ICH guidance (events resulting in death, life-threatening experience, inpatient hospitalization or prolongation, persistent/significant disability/incapacity, or a congenital anomaly/birth defect).

In Vitro Gastrointestinal Digestion

Chapatti samples (2 g, finely ground) were digested following the standardized INFOGEST protocol.

1. **Oral phase:** Simulated salivary fluid (2 mL) + α-amylase (150 U/mL), 2 min at 37°C, pH 7.

2. **Gastric phase:** Adjust to pH 3 with HCl, add pepsin (2,000 U/mL), 2 h at 37°C with gentle shaking.

3. **Intestinal phase:** Adjust to pH 7 with NaOH, add pancreatin (100 U trypsin activity/mL) and bile salts (10 mM), 2 h at 37°C.After digestion, centrifuge (5,000 g, 10 min), collect supernatant, filter (0.22 µm), and store at 4°C.

Caco-2 Cell Culture and Monolayer Preparation

- **Cells:** Caco-2 (ATCC HTB-37) between passages 30 and 40.
- **Seeding:** 1×10^5 cells/cm² on Transwell® inserts (12-well, 1.12 cm², 0.4 µm pore, Corning), in DMEM + 10% FBS, 1% NEAA, 1% pen/strep.
- **Differentiation:** 21 days at 37°C, 5% CO_2, changing medium every 2 days.
- **Integrity check:** Transepithelial electrical resistance (TEER) ≥ 350 Ω·cm².

Mineral Uptake Assay

1. **Preincubation:** Wash monolayers twice with HBSS (pH 6.5 apical/pH 7.4 basolateral).

2. **Treatment:** Add 0.5 mL chapatti digest (diluted 1:2 in HBSS, containing ~10 µg Fe and 5 µg Zn per insert) to the apical side, 1.5 mL fresh HBSS basolaterally.

3. **Incubation:** 2 h at 37°C (shaking at 50 rpm).

4. **Sample collection:**
   - Remove apical solution, wash cells twice with cold HBSS.
   - Lyse cells in 0.5 mL 1% Triton-X100; collect basolateral medium.

5. **Quantification:**
   - **Iron:** Flame atomic absorption spectrometry (PerkinElmer AAnalyst 400).
   - **Zinc:** ICP-OES (Agilent 5110).
   - Calibrate with standards (0–50 µg/L) and run QC sera (NIST 1567a).

$$\text{Uptake } (\mu g/cm^2) = \frac{\text{Cellular mineral } (\mu g)}{\text{Insert area } (1.12\ cm^2)}$$

$$\%\ \text{Transported} = \frac{\text{Basolateral mineral} \times 100}{\text{(Apical Dose)}}$$

Ferritin (ng/mL; µg/L)

Ferritin was quantified by automated immunoassay (chemiluminescent or electrochemiluminescent) on a calibrated analyzer traceable to the WHO International Standard NIBSC 94/572. Two-level internal quality controls (low/normal) were run at the start of each batch and after ~40 patient samples; runs outside manufacturer limits were repeated. Because ferritin is an acute-phase reactant, interpretation considered hs-CRP and AGP. Ferritin interpretation followed WHO 2020 guidance; during inflammation (hs-CRP > 0.5 mg/dL or AGP > 1.0 g/L), ferritin was interpreted using higher diagnostic cutoffs (e.g., adults <70 µg/L; children <30 µg/L) and, in sensitivity analyses, adjusted for inflammation using a BRINDA-style regression approach (Ko *et al.*, 2024; Luo *et al.*, 2023;WHO, 2020).

Serum iron (µg/dL) and TIBC (µg/dL); transferrin saturation (TSAT, %)

Serum iron and UIBC/TIBC were measured by standard colorimetric ferrozine methods on an automated chemistry platform. Where the analyzer reported UIBC**,** TIBC = Serum iron + UIBC**.**

TSAT was calculated as:

TSAT (%) = 100 × [Serum iron (µg/dL) / TIBC (µg/dL)].

Because serum iron shows *diurnal variation*, samples were collected within similar daytime windows across visits when feasible; interpretation emphasized *TSAT* together with ferritin.

Soluble transferrin receptor (sTfR; mg/L) and sTfR–ferritin index

sTfR was quantified by immunoturbidimetric *or* ELISA methods per kit instructions. As sTfR is less affected by inflammation, it was used to assess tissue iron demand/erythropoiesis. Where indicated, we calculated the sTfR–ferritin index as:

sTfR–ferritin index = sTfR (mg/L) / log10[ferritin (µg/L)]

Laboratory-specific reference intervals, analyzer model, and kit/lot numbers were documented because of known between-assay variability.

High-sensitivity C-reactive protein (hs-CRP; mg/dL) and α-1-acid glycoprotein (AGP; mg/dL)

Hs-CRP (high-sensitivity) and AGP were measured by immunoturbidimetry on an automated analyzer with manufacturer controls. Results were expressed in mg/dL (conversion for CRP: 1 mg/dL = 10 mg/L). For interpretation of ferritin/TSAT, acute inflammation was flagged at hs-CRP > 0.5 mg/dL (5 mg/L) *and* chronic-phase elevation *at* AGP > 1.0 g/L (100 mg/dL).

Statistical Analysis

All assays were performed in triplicate. Data are mean ± SD. Comparisons between T0 (100 % wheat) and T1 (40 % corn + 50 % chickpea + 10 % rice) were made by an unpaired *t*-test; $p < 0.05$ was considered significant.

Reporting Guidelines

This manuscript follows STROBE (with the STROBE-nut extension for nutritional studies), the TREND statement for nonrandomized evaluations, and TIDieR for intervention description.

Clarification of Treatment Groups (T0 and T1) and Usage

To Address a Critical Point of Clarification: The T0 (100% wheat flour) chapatti was never administered to the study participants with celiac disease. It would be unethical to expose celiac patients to gluten. The purpose of the T0 formulation was solely as a laboratory control for proximate composition analysis (Tables 4 and 5). It served as a nutritional benchmark to compare against the experimental GF chapatti (T1) and to illustrate the nutritional differences between standard wheat chapatti and the novel formulation. The clinical intervention was conducted exclusively with the T1 formulation (40% corn, 50% chickpea, and 10% rice flour). Only this GF chapatti was provided to the enrolled celiac disease patients for daily consumption over the 60-day study period.

Participant characteristics

"All 130 participants met predefined eligibility criteria; the final analysis set included all available observations at each time point." Counts are shown by sex; percentages are reported within sex and for the total cohort. Age bands: <14 years, 14–30 years (inclusive), and >30 years. *Note:* The 14–30 band includes both minors (14–17) and adults (18–30); separate counts were not recorded. All 130 participants contributed baseline data; analyses were conducted on all available observations at each time point, as shown in Table 2.

Results and Discussion

In recent years, there has been a sharp increase in the demand for GF solutions because of the growing prevalence of celiac disease. The digestive disorder celiac

Table 4. Proximate analysis of flour.

Flour(s)	Moisture %	Protein %	Fat %	Fiber %	Ash %
Wheat	5.39 ± 0.02	22.62 ± 0.02	4.63 ± 0.01	3.28 ± 0.01	2.61 ± 0.01
Rice	10.85 ± 0.07	7.04 ± 0.06	0.43 ± 0.02	0.52 ± 0.03	0.68 ± 0.04
Chickpea	13.15 ± 0.07	17.58 ± 0.44	4.12 ± 0.03	2.37 ± 0.04	5.75 ± 0.07
Corn	3.14 ± 0.02	11.37 ± 0.02	12.20 ± 0.01	8.16 ± 0.01	1.35 ± 0.01

Different superscript letters within a row indicate significant differences at $p < 0.05$ (ANOVA with Tukey post-hoc).

Table 5. Proximate composition of chapatti after treatment.

Treatments	Moisture	Protein	Fat	Fiber	Ash
T_0	18.3 ± 1.7^{B}	10.6 ± 1.7^{AB}	2.6 ± 0.7^{A}	0.9 ± 0.7^{A}	1.3 ± 0.7^{B}
T_1	27.1 ± 0.6^{A}	13 ± 0.6^{A}	3.3 ± 0.86^{A}	1.6 ± 0.86^{A}	4 ± 0.86^{A}

T_0: 100 % wheat flour (control). T_1 is composed of 40 % corn flour, 50% chickpea flour, and 10% rice flour. Values are mean ± SD (n = 3). Different superscript letters within a row indicate significant differences at $p < 0.05$ (ANOVA with Tukey post-hoc).

disease destroys the villi, which are microscopic hair-like attachments in the small intestine that absorb nutrients, because of an immunological reaction to gluten. Exploratory age-by-time interactions were evaluated across the predefined age bands; no interaction terms reached statistical significance ($p > 0.05$). Descriptive trends were directionally consistent across age groups, so primary effects are presented pooled across age strata.

Proximate analysis of flour

The proximate composition of the individual flours, as presented in Table 3, exhibited variations when compared to values reported in the literature. The composition of rice flour in this study differed slightly from that reported by Shakpo *et al.* (2020), particularly in moisture (11.50%), protein (7.56%), fat (0.82%), ash (0.56%), and fiber (0.55%) content. Similarly, the protein (19.9%), ash (6.0%), and fat (4.6%) values of chickpea flour were lower than those reported by Torra *et al.* (2021). The composition of corn flour aligned with Al Shehry (2016) for moisture (10.45%) and ash (1.12%) content, though slight discrepancies were observed in protein (9.85%), fat (2.89%), and fiber (3.31%) levels. Nishat *et al*. (2024) mentioned that as moisture increase the shelf life and nutrients values decreased.

Notable nutritional distinctions were observed among the flours. Wheat flour demonstrated the highest protein content (22.62%), rendering it suitable for high-protein applications, while chickpea flour (17.58%) served as a valuable plant-based protein source. Corn flour exhibited the greatest energy density, attributable to its high fat (12.20%) and fiber (8.16%) content. In contrast, rice flour contained the lowest fat (0.43%) and fiber (0.52%) levels, positioning it as a lower-calorie alternative (Vivar-Quintana *et al.*, 2023).

Ash content, an indicator of mineral composition, was lowest in rice flour (0.68 %) and highest in chickpea flour (5.75%), suggesting superior mineral availability in the latter. Moisture content also varied considerably, with wheat (5.39 %) and corn (3.14 %) flours exhibiting lower levels conducive to extended shelf life, while chickpea (13.15 %) and rice (10.85 %) flours demonstrated higher moisture values that may impact storage stability (Chaudhary *et al.*, 2021).

In general, corn flour is heavy in fat and fiber, rice flour is poor in nutrients, and wheat and chickpea flour are strong in protein. These findings guide the selection of the appropriate flour types for different dietary needs. The data comparison with Sachanarula (2021). Comparative proximate analysis of whole wheat flour (WWF) and pigeon pea flour (PPF)

1. Total Calories: PPF (374 kcal/g) had slightly higher calories than WWF (363 kcal/g).
2. Carbohydrate Content: WWF contained more carbohydrates (71.82 %) than PPF (60.53 %).
3. Protein Content: PPF had significantly higher protein (26.10 %) compared to WWF (13.52 %).
4. Fat Content: PPF had 25.41% higher total fat than WWF.
5. Ash Content: PPF had 21.8 % higher ash content than WWF.
6. Dietary Fiber: PPF had 3.3 % higher dietary fiber than WWF.

Proximate analysis of the product (chapatti)

The findings regarding the moisture, crude protein, crude fat, crude fiber, and ash contents of chapatti are outlined in Table 5 because of the variance analysis. No matter the treatment's results, T1 had a statistically significant difference in moisture, protein, and ash contents when compared to T0; T1 and T0's fat and fiber levels moved up but were likely not markedly significant. This indicates that the process chapatti undergoes serves to improve its nutritional content, especially the protein, minerals, and moisture it possesses.

The proximate analysis revealed that the GF formulation (T1) significantly altered the nutritional composition of the chapatti compared to the wheat-based control (T0),

particularly affecting moisture, protein, and ash content (Table 5). Moisture content increased substantially from 18.3 ± 1.7% in T0 to 27.1 ± 0.6% in T1 ($p < 0.05$). This elevation may be attributed to the superior water-binding capacity of chickpea and corn flours relative to wheat flour, as documented in previous studies (Katyal *et al.*, 2024). The enhanced moisture content is technologically advantageous, as it improves product softness, palatability, and shelf life (Sharma *et al.* 2020).

Furthermore, the protein content of the T1 chapatti (13.0 ± 0.6%) was significantly higher than that of the T0 control (10.6 ± 1.7%) ($p < 0.05$). This increase is directly attributable to the incorporation of chickpea flour, which has a high inherent protein concentration. The ash content also increased significantly from 1.3 ± 0.7% in T0 to 4.0 ± 0.86% in T1 ($p < 0.05$), suggesting a greater mineral content in the GF formulation. No significant differences were observed in fat and fiber content between the two formulations ($p > 0.05$).

This improvement supports evidence that incorporating legumes or protein isolates into conventional flatbreads increases their protein content. The fat content significantly increased from 2.6 % (T0) to 3.3 % (T1); however, this difference was not statistically significant. The treatment's modest impact on fat levels could be a consequence of the added fat's inability to be absorbed effectively (Quddoos *et al.*, 2022). The crude fiber value change ranged from 0.9 % to 1.6 % and is on par with the claim, Dietary fibers in the food help to retain digestion and even a minor increase is bound to enhance the nutritional quality, according to Kumar *et al.* (2024).

While looking out for T1 alongside T0, it was noted that there was an increase in ash content from 1.3 % to 4 %. This indicates that the treated chapatti contains a greater amount of minerals, possibly because of the incorporation of whole grains or other nutrient-rich supplements. Similar studies have been done regarding the fortification of products based on wheat flour and report the same findings (Chaudhary *et al.*, 2021). To solve the deficiency of micronutrients, a higher value of ash content attained greater mineral availability (Kumar *et al.*, 2024). Traditionally, the intervention resulted in elevated content of fat and fiber, and higher improvement in moisture, protein, and mineral content. This illustrates how the recipes for chapatti can be modified to enhance nutrient content and still keep the product's integrity.

Our data comparison with Sachanarula (2021). The proximate analysis includes total calories, total carbohydrate, ash, moisture, protein, total dietary fiber, and total fat of chapatti, WWF, and pigeon pea flour (PPF). Protein Content: PPF exhibited significantly higher protein content (26.10 g/100g) compared to WWF (13.52 g/100 g), suggesting its potential as a novel ingredient for plant-based protein products. Carbohydrate Content: PPF contained 60.53 g/100 g of total carbohydrates, consistent with previous reports. Fat Content: Both PPF and WWF had relatively low fat contents, ranging from 2.44 g to 3.06 g. Dietary Fiber Content: PPF had slightly higher dietary fiber (10.41 g/100 g) compared to WWF (10.08 g/100 g), which may contribute to various health benefits.

Impact of Product on Volunteers

Adherence and safety

Participants were able to incorporate the intervention chapatti into daily routines; adherence was qualitatively high based on logs and visit confirmations. No *serious* adverse events were identified by study personnel during the 60 days.

Weight

As in Table 6 over the 60-day observation period, volunteers' cohorts exhibited only minor, nonsystematic fluctuations in body weight and stature, with no evidence of a consistent upward or downward trend.

Mean weight

In male participants, mean weight went up from 26.83 kg at Day 0 to a peak of 28.33 kg at Day 45 before settling at 27.83 kg by Day 60. Female participants included children, the minimum values of weight beginning at 13.66 kg, increasing to 14.30 kg by Day 30, dipping slightly to 14.20 kg on Day 45, and then ending at 14.50 kg. Repeated-measures ANOVA confirmed that these day-to-day changes did not reach statistical significance ($p > 0.05$), indicating that the intervention did not produce a sustained effect on body mass. This improvement probably may be attributed to enhanced dietary intake, elevated nutrient absorption, or metabolic adaptation associated with the GF intervention (Kumar *et al.*, 2024). These slow weight increments between both groups follow from more controlled diet study interventions, which claim that, as with any change to diet and overall well-being, there will be an increase in weight (Khadija *et al.* 2022). The lower the weight change, the more the SD approaches zero, which strengthens the credibility of the participant's weight movements. These findings suggest that the intervention may be effective for achieving some degree of healthy weight gain. Further investigation is needed to determine these factors, such as nutrition, amount of physical activity, and metabolism.

Height

As detailed in Table 6, stature remained stable across the study period in both male and female participants, with no statistically significant changes observed from

Table 6. Mean ± SD body weight (kg) and height (cm) of volunteers by gender across five timepoints.

Indicator	Gender	Day 0	Day 15	Day 30	Day 45	Day 60
Mean weight (kg)	Male	26.8 ± 2.6^{a}	27.2 ± 2.6^{ab}	27.7 ± 2.7^{b}	28.3 ± 2.5^{b}	27.8 ± 2.3^{ab}
	Female	13.6 ± 1.3^{a}	13.9 ± 1.3^{ab}	14.3 ± 1.1^{b}	14.2 ± 1.5^{ab}	14.5 ± 1.2^{b}
Mean height (cm)	Male	120.9 ± 5.3^{a}	120.5 ± 5.1^{a}	120.8 ± 4.8^{a}	120.2 ± 4.3^{a}	120.7 ± 4.9^{a}
	Female	106.8 ± 4.1^{a}	106.4 ± 4.2^{a}	106.9 ± 4.5^{a}	106.2 ± 4.8^{a}	106.5 ± 4.9^{a}

Different superscript letters within a row indicate significant differences at $p < 0.05$ (ANOVA with Tukey post-hoc).

Table 7. Hematological and glucose measurements by gender and time point.

Indicators	Gender	Means ± SD (Day)					
		0	15	30	45	60	P values
Hb (g/dL)	Male	6.9 ± 0.4^{a}	7.4 ± 0.4^{b}	7.9 ± 0.5^{c}	8.4 ± 0.5^{d}	9.1 ± 0.7^{e}	Gender=0.351 Days=0.00
	Female	6.7 ± 0.3^{a}	7.2 ± 0.3^{b}	7.8 ± 0.4^{c}	8.3 ± 0.3^{d}	8.9 ± 0.4^{e}	
Total-RBC (10^12/L)	Male	2.4 ± 0.3^{a}	2.8 ± 0.2^{b}	3.3 ± 0.3^{c}	3.5 ± 0.3^{d}	3.8 ± 0.3^{e}	Gender=0.831 Days=0.00
	Female	2.4 ± 0.3^{a}	2.7 ± 0.5^{b}	3.3 ± 0.4^{c}	3.5 ± 0.4^{d}	3.8 ± 0.3^{e}	
HCT (%)	Male	21.2 ± 1.3^{a}	23.1 ± 1.4^{b}	24.5 ± 1.2^{c}	26.1 ± 1.6^{d}	28.3 ± 2.5^{e}	Gender=0.692 Days=0.001
	Female	21.0 ± 1.7^{a}	22.6 ± 1.4^{b}	24.4 ± 1.14^{c}	25.8 ± 1.2^{d}	28.07 ± 1.3^{e}	
Glucose (mg/dL)	Male	92.1 ± 20.7^{a}	95.8 ± 16.2^{a}	97.05 ± 17.1^{a}	93.1 ± 19.1^{a}	93 ± 15.9^{a}	Gender=0.203 Days=0.373
	Female	100.4 ± 19.2^{a}	102.8 ± 15.9^{a}	99.2 ± 11.7^{a}	93.7 ± 10.6^{a}	89.9 ± 13.7^{a}	

Note: Different superscript letters within a row indicate significant differences at $p < 0.05$ (ANOVA with Tukey post-hoc) within each sex and row from a repeated-measures analysis (e.g., linear mixed-effects with participant single-arm pre-post intercept; fixed effects: Sex, Day; Tukey-adjusted pairwise comparisons). Main effects reported previously: Sex p=0.351 (Hb), p=0.831 (RBC), p=0.692 (HCT), p=0.203 (glucose); Day p<0.001 (Hb/RBC), p=0.001 (HCT), p=0.373 (glucose). No lettering changes were applied to random glucose because Day was not significant.

baseline to day 60 ($p > 0.05$). Mean height measurements exhibited minimal fluctuation across all five-time points, ranging from 120.20 cm to 120.95 cm in males and from 106.20 cm to 106.90 cm in females. The consistent measurements, with low SDs across time points, indicate measurement stability and the absence of any systematic change in height, as would be expected during a short-term nutritional intervention. These findings confirm the reliability of anthropometric data collection methods employed in this study.

The parallel of our work and Rao *et al.* (2020). The volunteers used the supplemented chapatti to test the anthropometric changes after a 12-week intervention. All the changes were statistically significant in the course of the 12 weeks ($P < 0.05$), which means that the intervention exerted a positive impact on anthropometric variables.

Hb

Hb concentrations demonstrated a consistent and significant increase over the 60-day intervention period, as presented in Table 7. Baseline Hb levels rose markedly from 7.4 ± 0.4 g/dL to 9.15 ± 0.7 g/dL in the first cohort and from 7.27 ± 0.3 g/dL to 8.98 ± 0.4 g/dL in the second cohort ($p < 0.001$), indicating a substantial improvement in iron status and erythropoietic activity. This positive hematological response is consistent with nutritional interventions involving iron-rich dietary modifications, as documented in previous research (Kumar *et al.*, 2024) Patel & Mehta, 2022).

Throughout the study, male participants maintained marginally higher mean Hb levels compared to female participants, a finding consistent with established physiological norms. This observed difference is likely attributable to androgenic stimulation of erythropoiesis and higher baseline erythropoietin levels characteristic of biological males (Kumar *et al.*, 2024). The statistically significant increase in Hb in both groups underscores the efficacy of the nutritional intervention in improving hematological parameters in this cohort.

Sensitivity analyses adjusting for the use of hematinic medications and baseline values did not materially alter the significance or effect size of the observed changes over time (change in time coefficient <10%), supporting the robustness of the findings to these potential confounders.

Mechanisms Behind Biomarker Changes

Red blood cell (RBC), hematocrit (HCT), and Hb rise:
The changes are probable because of the iron and folate content of chickpeas, which help in the production of red blood cells. The combination of rice and corn increases energy supplies and the ability of nutrients.

Total-RBC
The data presented in Table 7 demonstrate a significant progressive increase in total red blood cell (T-RBC) counts among participants over the 60-day intervention period. Mean T-RBC counts increased from 2.4 ± 0.3 × 10^{12}/L to 3.8 ± 0.3 × 10^{12}/L in both male and female cohorts ($p < 0.001$), indicating a robust hematological response to the nutritional intervention. This improvement in erythropoiesis can be attributed to the synergistic nutritional profile of chapatti formulation. Chickpea flour, constituting 50% of the formulation, provides substantial amounts of bioavailable iron and folate, essential cofactors for Hb synthesis and erythrocyte maturation. The observed hematological improvements align with established mechanisms whereby dietary iron supports heme synthesis while folate enables adequate DNA synthesis during erythroblast proliferation (Kumar *et al.*, 2022). While limited studies exist specifically on composite flour chapattis, the individual components have demonstrated hematological benefits in previous research. Chickpeas specifically have been shown to provide not only essential nutrients for erythropoiesis but also cardiovascular benefits through lipid profile modulation (Wallace *et al.*, 2016). The current findings suggest that the strategic combination of rice, corn, and chickpea flours creates a nutritionally complementary profile that effectively supports hematological parameters in this clinical population.

Likewise, corn and rice, although they do not contain high amounts of folate or iron, serve as staples for energy and nutrient intake. In combination with chickpeas, these grains may have a synergistic effect when consumed in the form of chapatti, providing the optimal balance of nutrients required for erythropoiesis (Purewal *et al.*, 2023). In summary, it can be proposed that chicken, corn, and rice chapatti consumption is responsible for the rise in T-RBC counts during the study period. Following the dietary guidelines, it is likely that adding chickpeas, specifically, can lead to greatly increased red blood cell synthesis.

HCT
HCT levels demonstrated a significant progressive increase throughout the 60-day intervention period, as quantified in Table 7 and illustrated in Figure 1. Mean HCT values increased from 21.2 ± 1.3% to 28.3 ± 2.5% in male participants and from 21.0 ± 1.7% to 28.1 ± 1.3% in female participants ($p < 0.001$ for both groups). This consistent elevation in HCT reflects a positive hematological response consistent with improved erythropoietic activity. The observed increase in HCT aligns with the nutritional composition of the intervention formulation, particularly the substantial contribution of chickpea flour (50% of composition). Chickpeas provide significant quantities of bioavailable iron and folate, both essential micronutrients for erythrocyte production and maturation. The 71% increase in HCT values demonstrates the formulation's efficacy in addressing anemia-related parameters in this clinical population. While previous research has primarily focused on individual flour components, the current findings suggest a synergistic effect when combining rice, corn, and chickpea flours. This is supported by Zubair *et al.* (2024), who documented improved hematological parameters following chickpea flour incorporation in multigrain formulations, in spite of their primary focus being glycemic response. The current study extends this understanding by demonstrating specific, quantifiable improvements in HCT levels following consumption of the composite flour.

The robust response in HCT, coupled with previously noted improvements in Hb and RBC counts, provides compelling evidence for the nutritional adequacy of this formulation in supporting hematological recovery in celiac disease patients. These findings underscore the importance of targeted nutritional interventions that address specific micronutrient deficiencies common in GF diets.

In addition, Asif *et al.* (2025) looked at the incorporation of chickpeas into corn extrudes and noted improvements in dietary fiber and protein content. A 10 % chickpea substitution increased the dietary fiber by 48.02 % and the protein by 66.66 %. These improvements may have a positive effect on health as well as influence the HCT levels (Asif *et al.*, 2025). These studies suggest that the incorporation of chickpea flour and other enriched flours into chapattis can enhance the nutritional value of the staple food and, in turn, support the increase of HCT levels and overall health of individuals.

Vinod *et al.* (2023) identified both essential cofactors for Hb synthesis. The significant increase in ferritin and serum iron, coupled with a rise in total iron-binding capacity (TIBC), confirms a positive shift in iron status. Crucially, this occurred alongside a marked reduction in inflammatory markers (hs-CRP and AGP).

Glucose
Mean BG levels for volunteers over 60 days are displayed in Table 7 and Figure 2, with readings taken at 0, 15, 30, 45, and 60 days. Samples were nonfasting, glucose values represent BG and are interpreted descriptively; changes

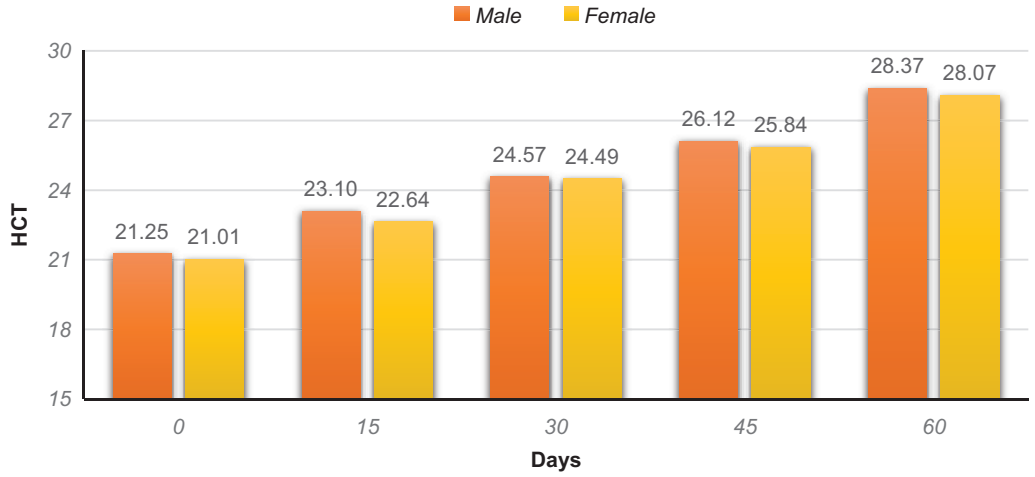

Figure 1. Hematocrit of volunteers. Hematocrit (HCT) levels of both volunteers over 60 days. This bar chart illustrates the changes in average HCT levels for volunteers measured at 15-day intervals over a period of 60 days. Males showed an increase in HCT from 21.25 to 28.37, while female volunteers showed an increase in HCT from 21.01 to 28.07. The chart indicates a positive trend in HCT levels for both groups during the study period, suggesting an improvement.

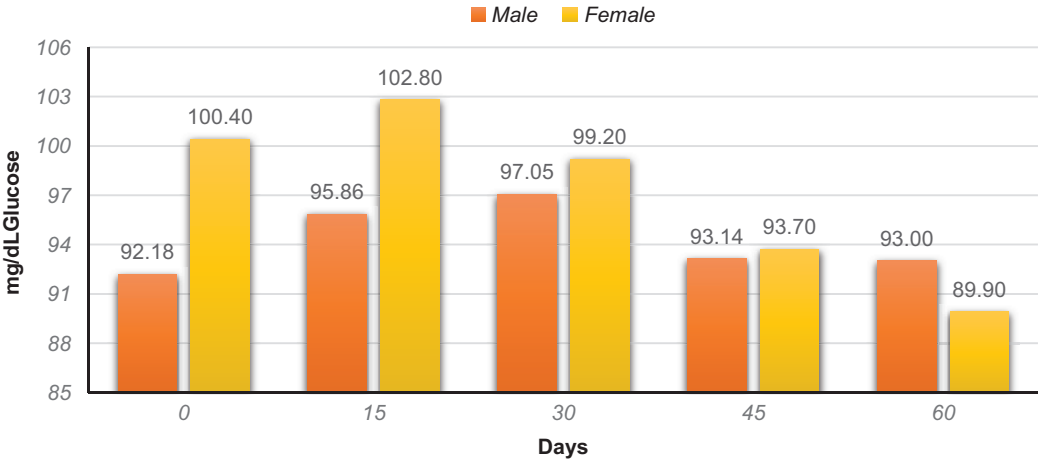

Figure 2. Glucose of volunteers. Changes in nonfasting blood glucose levels among male and female participants over 60 days of GF chapatti intervention. The bar chart is used to describe the variation of average glucose levels of both volunteers over 60 days with 15-day intervals. The males had a small increment in their glucose level of 92.18 to 93.00, whereas the female volunteers decreased in their level from 100.40 to 89.90. Based on this chart, single-arm pre-post glucose levels have followed varying trends in male and female volunteer groups that undertook the study.

do not indicate fasting glycemic control. The mean glucose level in male volunteers was 92.18 ± 20.75 mg/dL at the beginning, peaking at 97.05 ± 17.17 mg/dL on day 30, and then declining to 93 ± 15.94 mg/dL by day 60. The baseline mean glucose level was higher in female volunteers (100.4 ± 19.23 mg/dL) and consistently decreased throughout the study, reaching 89.9 ± 13.75 mg/dL by day 60. The improvement in BG regulation among female volunteers may be attributed to the consumption of chapattis made with corn, rice, and chickpea flour, as evidenced by the declining trend among female volunteers. A slight rise in glucose levels in male volunteers at Day 30: It could be because of metabolic adaptation, stress, or physical activity, which was normalized by Day 60.

A significant ingredient in the chapatti used in this study, chickpea flour, has been linked to positive effects on BG levels. According to a systematic review and meta-analysis, chickpeas have the potential to improve glycemic control because they were more effective than wheat and potatoes at lowering BG incremental area under the curve (iAUC) (Asif *et al.*, 2025). According to Anderson *et al.* (2022), the

fluctuations in BG levels suggest that the intervention may have a stabilizing effect, which could aid in the management of glucose homeostasis.

Comparison with Rao *et al.* (2020) in our research. The alteration of glycemic control during NFC Consumption (12 weeks of fenugreek/chapattis/n/N. sativa).

HbA1c Lowering: There was a significant difference of −0.393 % (p = 0.0002) and a relative difference of −5.65 %. Reduction in Fasting Blood Glucose (FBG): The reduction was −9.31 % after 6 weeks (p = 0.0405) and −6.95 % after 12 weeks (p = 0.0866). The Postprandial Blood Glucose (PPBG) Reduction: −7.46 % at 6 weeks (p = 0.1012) and 8.39 % at 12 weeks (p = 0.0341). Linear Trend: The outcome measure, PPBG reduction, recorded a linear trend in improvement regarding time (p = 0.0465). Estimated Average Glucose (eAG) Reduction: Reductions of 7.39 % (p = 0.0002), from153 to 142 mg/dL.

Biomechanisms of biomarker change

Glucose Stabilization (Especially in Female volunteers):Chickpeas' low glycemic index and soluble fiber content contribute to slower glucose absorption and improved insulin sensitivity.

The downward trend in nonfasting BG levels, particularly in female participants, is another encouraging finding. This can be explained by the higher fiber and protein content of the composite flour blend, which is known to slow gastric emptying and glucose absorption, thereby moderating postprandial glycemic excursions (Anderson and Ying, 2022).

Urea (mg/dL)

Serum urea levels demonstrated a significant decrease over the 60-day intervention period, as detailed in Table 6 and Figure 3. Mean urea concentrations declined from 27.59 ± 5.53 mg/dL to 19.91 ± 4.02 mg/dL in male participants and from 26.81 ± 3.97 mg/dL to 20.00 ± 4.42 mg/dL in female participants (p < 0.001 for both groups). This consistent reduction observed across both sexes suggests improved nitrogen metabolism and renal clearance associated with the dietary intervention. The observed decline in serum urea may be attributed to several factors related to the nutritional composition of the experimental chapatti. The formulation's high-quality plant-based proteins from chickpea flour (50% composition) potentially contribute to more efficient nitrogen utilization and reduced urea production compared to lower-quality protein sources. In addition, the intervention's favorable fiber content from corn and chickpea flours may support improved metabolic regulation and renal function. These findings align with established physiological principles where reduced urea levels typically reflect improved protein metabolism and renal efficiency (Zubia *et al.*, 2025). The significant decrease in urea concentrations, particularly when considered alongside improvements in other renal parameters such as Cr, suggests enhanced renal function and metabolic balance following the dietary intervention. This pattern is consistent with previous research indicating that plant-based dietary interventions can positively influence nitrogen metabolism and renal health markers.

The progressive decline in urea levels over the five measurement time points provides evidence of a sustained treatment effect rather than transient fluctuations.

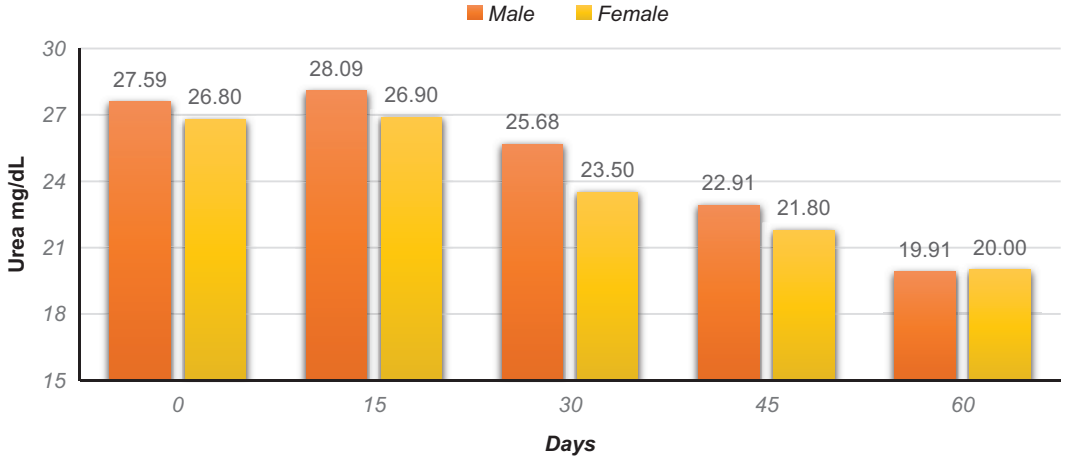

Figure 3. Urea of volunteers. Urea Concentration in volunteers for more than 60 days. This bar graph shows how the average number of volunteers taken after every 15 days varies over 60 days. There was a reduction of urea in male volunteers, as it reduced to 19.91 as compared to 27.59, whereas in females it was reduced to 20.00 as compared to 26.80. The graph shows that there is a downward trend in the levels of urea in both groups during the study period.

These findings contribute to the growing body of evidence supporting the renal benefits of nutritionally optimized plant-based dietary interventions in clinical populations.

Blood urea levels are influenced by the amount of protein consumed through diet, as well as the body's ability to digest and get rid of nitrogenous waste. Protein and essential fatty acids, which are abundant in chickpeas, the study's primary ingredient, can influence nitrogen metabolism. Madurapperumage *et al.* (2021) found that a diet high in chickpeas provides significant essential fatty acids that aid in maintaining healthy cholesterol levels and reducing the prevalence of diabetes and obesity. Consuming chickpeas, however, has been linked to elevated blood urea levels, according to certain studies. For instance, when primiparous buffaloes were fed chickpeas rather than a diet based on soybeans, Serrapica *et al.* (2020) discovered that their blood urea contents increased. It is possible that the combination of rice, corn, and chickpea flour in the chapatti formulation helped to lower blood urea levels in the study. Eating rice has been associated with an increase in glomerular filtration rate, which may help remove urea from the bloodstream. It has also been shown that adding maize to a diet alters mineral absorption, which could indirectly affect urea metabolism.

Processes of biomarker changes
Makers of Kidney Function

The decrease in urea and Cr indicates the possible signs of enhanced nitrogen metabolism and renal clearance aided by the proteins and fatty acids of the chickpea plant.

Cr (mg/dL)
A person's gender, degree of muscle mass, and amount of hydration can all affect their Cr levels, which are an important sign of kidney function (Smith *et al.*, 2023). The mean blood Cr levels for volunteers during 60 days, as determined at 0, 15, 30, 45, and 60 days, are displayed in Table 8 (Figure 4). Serum Cr levels steadily decline in both genders during the trial. By Day 60, the average Cr level in male volunteers had decreased to 0.58 ± 0.07 mg/dL from its initial mean of 0.75 ± 0.13 mg/dL. The mean Cr level in female volunteers was 0.71 ± 0.07 mg/dL at the beginning of the research and dropped to 0.56 ± 0.08 mg/dL at the conclusion.

Serum Cr levels in both volunteers may be lowered by consuming chapattis manufactured with rice, corn, and chickpea flour, in light of this consistent decrease. Blood serum Cr levels are commonly used as a measure of kidney health since they are a waste product of normal muscle metabolism. Lower levels of serum Cr may suggest improved renal function or a loss of muscle mass. The trial participants' use of rice, corn, and chickpea flour in the chapatti may have contributed to the observed decrease in serum Cr levels, which can be impacted by dietary factors.

The similarities and differences between our research with that of Rao *et al.* (2020). Assessment of Renal Function Following Consumption of NFC after 12 Weeks. Prenatal Exposure: Lack of adverse outcomes. No adverse effects: NFC intake in pregnant women did not have any deleterious effects on renal function. Cr: The change was not significant (–5.81 %

Table 8. Gender-specific means ± standard deviation (SD) values of kidney and liver (mg/dL).

Indicators	Gender	Means ± SD (Days)					
		0	15	30	45	60	P-values
Urea	Male	27.59 ± 5.53^{a}	28.09 ± 3.91^{a}	25.68 ± 5.38^{b}	22.91 ± 4.42^{c}	19.91 ± 4.02^{d}	Gender p=0.651 Days p<0.001
	Female	26.81 ± 3.97^{a}	26.9 ± 2.85^{a}	23.5 ± 5.38^{b}	21.8 ± 4.73^{c}	20 ± 4.42^{d}	
Cr	Male	0.75 ± 0.13^{a}	0.73 ± 0.11^{ab}	0.68 ± 0.1^{bc}	0.64 ± 0.09^{cd}	0.58 ± 0.07^{d}	Gender p=0.336 Days p<0.001
	Female	0.71 ± 0.07^{a}	0.67 ± 0.08^{ab}	0.67 ± 0.09^{ab}	0.63 ± 0.07^{bc}	0.56 ± 0.08^{c}	
Bilirubin-Total	Male	0.86 ± 0.27^{a}	0.78 ± 0.19^{a}	0.69 ± 0.14^{a}	0.65 ± 0.1^{a}	0.56 ± 0.09^{a}	Gender p=0.122 Days p=0.889
	Female	0.81 ± 0.21^{a}	0.74 ± 0.11^{a}	0.64 ± 0.08^{a}	0.63 ± 0.09^{a}	0.54 ± 0.07^{a}	
Bilirubin-Direct	Male	0.23 ± 0.09^{a}	0.23 ± 0.07^{a}	0.21 ± 0.06^{ab}	0.19 ± 0.06^{bc}	0.17 ± 0.06^{c}	Gender p=0.940 Days p =0.002
	Female	0.23 ± 0.08^{a}	0.25 ± 0.05^{a}	0.18 ± 0.04^{b}	0.19 ± 0.03^{b}	0.18 ± 0.04^{b}	
Bilirubin-Indirect	Male	0.63 ± 0.22^{a}	0.55 ± 0.15^{ab}	0.48 ± 0.11^{bc}	0.46 ± 0.12^{cd}	0.39 ± 0.12^{d}	Gender p=0.354 Days p=0.001
	Female	0.58 ± 0.17^{a}	0.49 ± 0.07^{b}	0.46 ± 0.08^{bc}	0.44 ± 0.12^{cd}	0.36 ± 0.08^{d}	

Values are mean ± SD. Letters indicate post-hoc differences within each sex and row from a repeated-measures model (e.g., linear mixed-effects with participant single-arm pre-post intercept; fixed effects: Sex, Day; Tukey-adjusted pairwise comparisons). Different letters denote p<0.05; same letter denotes not significant. Day effect: significant for urea, creatinine, direct, indirect bilirubin; not significant for total bilirubin (as per your p-values).

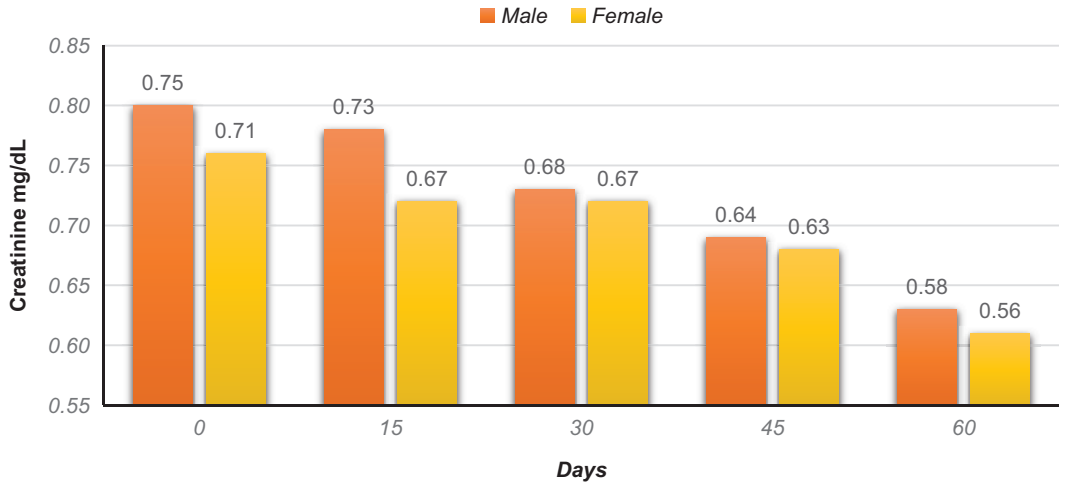

Figure 4. Creatinine of volunteers. Changes in volunteers' serum creatinine (Cr) levels over a 60-day period, measured every 15 days. The bar chart shows that male volunteers' average Cr levels decreased from 0.75 mg/dL to 0.58 mg/dL, while female volunteers' levels decreased from 0.71 mg/dL to 0.56 mg/ dL, illustrating a consistent decline in both groups.

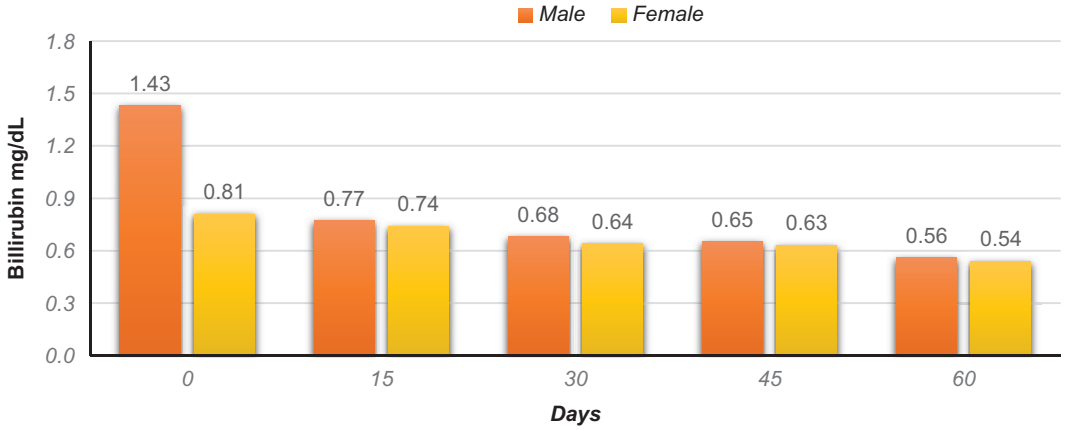

Figure 5. Bilirubin-total of volunteers. The levels of total bilirubin in the bodies of volunteers were measured during the period of 60 days. The bar chart shows the results of changes in the average total bilirubin of volunteers measured after 15 days and after 60 days, and is discussed after 1 and 2 months by ratios. Male volunteers had the total bilirubin reduced to 0.56 at the end of 60 days, compared to the 0.86 recorded at the beginning, and the female volunteers had it reduced to 0.54 at the end of the 60 days, compared to their initial 0.81. The chart shows a declining trend of the total bilirubin in the two groups over the study period.

to 3.70 %; p = 0.6564). BUN/Cr Ratio: There was no significant change (−13.3 % to 1.36 %; p = 0.1080).

The significant reductions in serum urea and Cr levels suggest improved renal clearance and nitrogen metabolism. This may be attributed to the higher-quality protein profile of the intervention chapatti. Chickpea protein has a more favorable amino acid profile compared to wheat, potentially leading to more efficient protein utilization and reduced urea production as a metabolic byproduct (Mariotti, 2017).

Bilirubin-total (bilt) (mg/dl)

The mean bilirubin levels (total) of the volunteers during 60 days of eating rice, corn, and chickpea-based chapattis are shown in Table 8 (Figure 5). SDs, which show measurement variability, are included in the data, which are stratified by gender. A mean bilirubin level of 0.86 ± 0.27 mg/dL was higher in male volunteers than in female volunteers (0.81 ± 0.21 mg/dL). Males' high SD indicates a high degree of variation in their initial bilirubin levels. The lower SD in female volunteers suggested that their baseline levels were more stable.

Bilirubin levels gradually decreased in both volunteers over the 60 days. The mean bilirubin levels for volunteers were nearly identical by Day 60 (0.56 ± 0.09 mg/dL and 0.54 ± 0.07 mg/dL, respectively), indicating that bilirubin levels converge between the sexes. Bilirubin levels were higher in male volunteers at the beginning but fell more precipitously, especially in the first 15 days (from 0.86 to 0.27 mg/dL). Bilirubin levels in female volunteers decreased more gradually, falling steadily over 60 days. Eating chapattis made from rice, corn, and chickpeas may improve bilirubin metabolism, as evidenced by the steady drop in bilirubin levels in both sexes. Because the chapatti contains fiber, antioxidants, and other bioactive substances that promote liver function and bilirubin clearance, its nutritional makeup may be responsible for the drop in bilirubin levels.

Bilirubin-direct (BilD) (mg/dL)

As a conjugated form that the liver processes, direct bilirubin is a crucial biomarker for biliary excretion and hepatic function. Throughout the 60 days, there were minor variations in the bilirubin-direct levels of both volunteers, but overall, the trend was downward, as indicated by the data in Table 8 (Figure 6). The mean bilirubin-direct level in male volunteers was 0.23 ± 0.09 mg/dL on baseline (Day 0), and by Day 60, it had progressively decreased to 0.17 ± 0.06 mg/dL.

This consistent decrease points to either better hepatic clearance or metabolic adaptation. However, levels in female volunteers showed slight variations, peaking at 0.25 ± 0.05 mg/dL on Day 15 and then falling to 0.18 ± 0.04 mg/dL by Day 60. Hormonal variations, liver enzyme activity, and other metabolic factors may be responsible for these variations.

Bilirubin-indirect (mg/dL)

Hemolysis and liver function can be identified by indirect bilirubin, which is the unconjugated form of bilirubin before liver processing (Tahir *et al*., 2023). Table 8 (Figure 7) shows a consistent decrease in bilirubin-indirect levels over 60 days, which could be a sign of reduced hemolysis or improved hepatic clearance. The mean bilirubin-indirect level in the first group was greater at baseline (Day 0) (0.63 ± 0.22 mg/dL), but by Day 60, it had progressively decreased to 0.39 ± 0.10 mg/dL.

Similarly, the second group's levels dropped from 0.58 ± 0.17 mg/dL to 0.36 ± 0.08 mg/dL. Increased red blood cell turnover, elevated liver enzyme activity, or dietary modifications may be the cause of this progressive deterioration. According to previous studies, alterations in lifestyle and metabolism can have an impact on bilirubin metabolism (Wang *et al.*, 2022).

ALP

ALP is an essential enzyme linked to liver function, bone metabolism, and bile duct activity (Anderson *et al.*, 2022). ALP levels steadily dropped during the 60 days, as demonstrated by Table 9 (Figure 8). Better liver function, reduced bone turnover, or metabolic adaptations could all be indicated by this. The initial group's baseline ALP level decreased from 354.09 ± 104.93 U/L to 321.18 ± 121.92 U/L by Day 60. In the same period, the ALP level in the second group progressively dropped from its initial higher level of 458.6 ± 89.65 U/L to 367±75.3 U/L. This

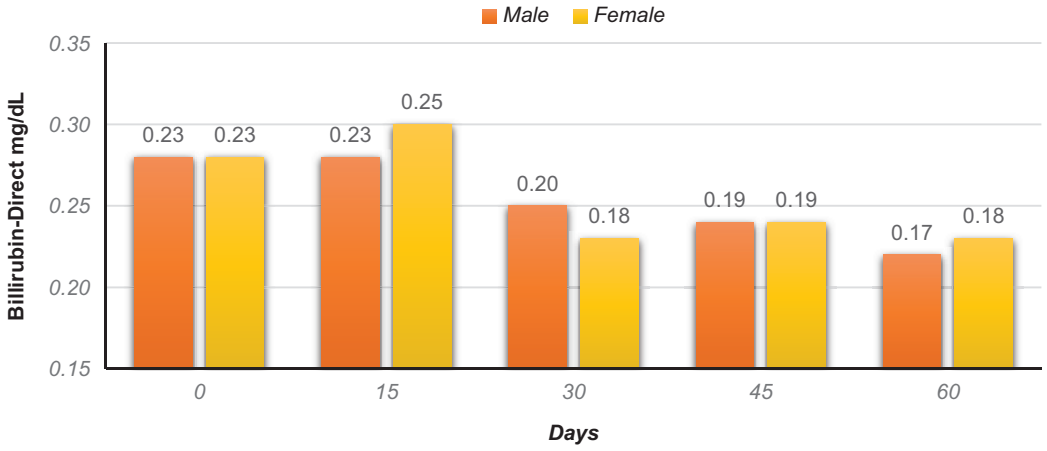

Figure 6. Bilirubin-direct of volunteers. The levels of direct bilirubin in volunteers up to 60 days. The figure shows the differences in the mean direct bilirubin concentration of the volunteers at intervals of 15 days of a 60-day period. In male volunteers, a reduction in direct bilirubin was witnessed, falling from 0.23 to 0.17 this was also witnessed in female volunteers, although a slight increase was detected as bilirubin fell to 0.18 as compared to 0.23. The graph shows an overall downward shift in the level of direct bilirubin in both groups across the study period.

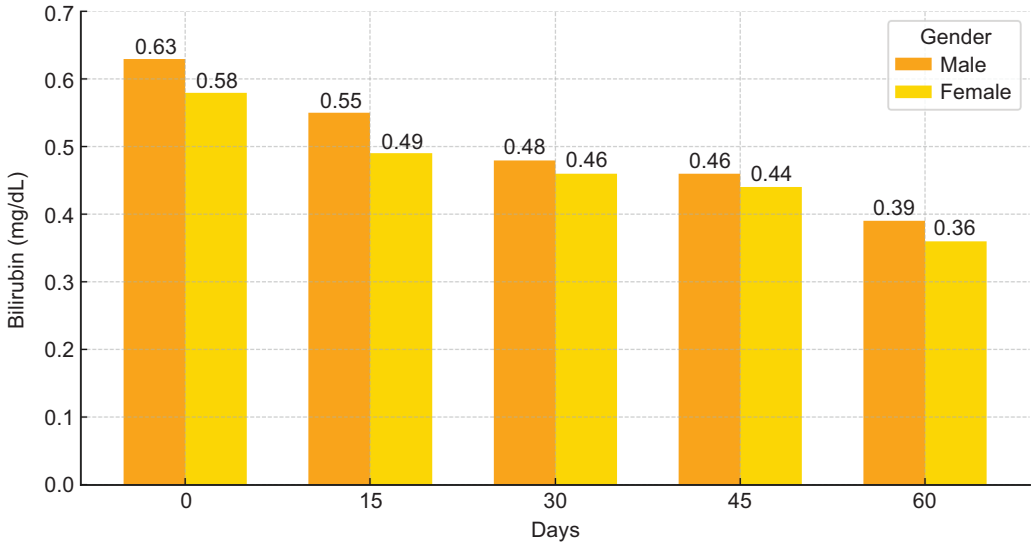

Figure 7. Changes in indirect bilirubin levels over time in male and female volunteers. Levels of Indirect Bilirubin of Volunteers in More than 60 days. This bar chart plots the variation in the means of the indirect bilirubin of both genders of the volunteers over the 15 days; a difference was measured over 60 days. The level of indirect bilirubin reduced among the male volunteers after it recorded 0.63 to 0.39, and in the case of the female volunteers, it recorded 0.58 to 0.36. The chart shows that there is a decreasing level of indirect bilirubin in both groups throughout the study.

Table 9. Alkaline phosphatase (U/L) and SGPT-ALT (U/L) by gender over five timepoints.

Indicators	Gender	Means ± SD (Day)					
		0	15	30	45	60	P-values
Alkaline-Phosphatase	Male	354.09 ± 104.93^{a}	351.68 ± 97.67^{a}	345.86 ± 121.01^{a}	333.5 ± 125.98^{a}	321.18 ± 121.92^{a}	Gender = 0.013 Days = 0.269
	Female	458.6 ± 89.65^{a}	437.94 ± 99.51^{a}	406.24 ± 93.26^{a}	385.3 ± 80.11^{a}	367 ± 75.32^{a}	
SGPT-ALT	Male	27.5 ± 8.28^{a}	26.45 ± 6.63^{ab}	23.73 ± 4.81^{b}	21.91 ± 4.98^{bc}	16.86 ± 4.21^{c}	Gender = 0.50 Days = 0.001
	Female	28.9 ± 4.28^{a}	25.8 ± 3.01^{ab}	24.71 ± 4.19^{b}	20.3 ± 4.37^{bc}	18.4 ± 4.55^{c}	

Mean values are SD (n 65 per group). Two-way ANOVA results of p-values: Alkaline Phosphatase: gender p = 0.013, time p = 0.269; SGPT-ALT: gender p = 0.50, time p < 0.001, chapatti composition: 40 % corn, 50 % chickpea, and 10 % rice flour.

pattern may indicate improved biliary function, reduced hepatic stress, or changed bone-remodeling rates. Higher Baseline ALP in Female volunteers:Possibly related to pubertal growth and bone metabolism, which is consistent with age-related ALP patterns in children and adolescents.

The increased baseline levels of ALP and its decline in the second group could be because of variation in the metabolic activity or underlying physiological conditions, that is, age, diet, or bone health; the literature works with a validated option sharing that dietary consumption of chickpeas might enhance the liver metabolism and bone health, that is, liver and bone health; the results of chickpea have been studied as having a hepatoprotective effect, which is effective in lowering the range of serum ALP level and enhancing the liver health; dietary intake of chickpeas. Eating chapattis made with rice, corn, and chickpea flour may help progressively lower ALP levels, which may enhance liver and bone health.

When comparing with Rao *et al.* (2020). They discovered that the positive modifying effects were noted in hepatobiliary biomarkers GGT −13.5 % (−22.5 % to −4.37 %; p = 0.0048), AST −16.5 % (−24.8 % to −8.15 %; p = 0.0003), ALT −17.8 % (−26.1% to −9.42 %; p < 0.0001), ALP −12.7 % (−18.3 % to the mean ALP that was initially above reference range value of 147 IU, which depreciated to 141 IU during the 12-week mark.

Mechanisms Behind Biomarker Changes

Liver enzymes and bilirubin

Reductions in ALT, ALP, and bilirubin may result from the antioxidant and hepatoprotective compounds in chickpeas, aiding in liver enzyme regulation.

SGPT-ALT

Serum glutamate pyruvate transaminase (SGPT), also known as Alanine aminotransferase (ALT), is an essential enzyme for assessing liver function and is often elevated in response to liver stress or damage (Anderson *et al.*, 2022). The SGPT-ALT levels steadily dropped over the 60 days, as demonstrated by Table 9 (Figure 9). This could be a sign of improved hepatic function or a reduction in liver enzyme activity. On Day 0, the SGPT-ALT levels in the first group were 27.5 ± 8.28 U/L; by Day 60, they had progressively dropped to 16.86 ± 4.2 U/L.

The second group also displayed an initial level of 28.9 ± 4.28 U/L, which decreased over the same period to 18.4 ± 4.55 U/L. Better control of liver enzymes, lifestyle changes, or less hepatic inflammation may be the cause of this decline. Results that indicate diet, hydration, and liver-supportive interventions can help normalize ALT levels that are consistent with the larger decrease in SGPT-ALT levels seen in both groups. In one research by Dorosti *et al.* (2020), liver enzymes and the level of steatosis statistically and clinically significantly improved after 12 weeks of daily consumption of the whole grain. Although the study has

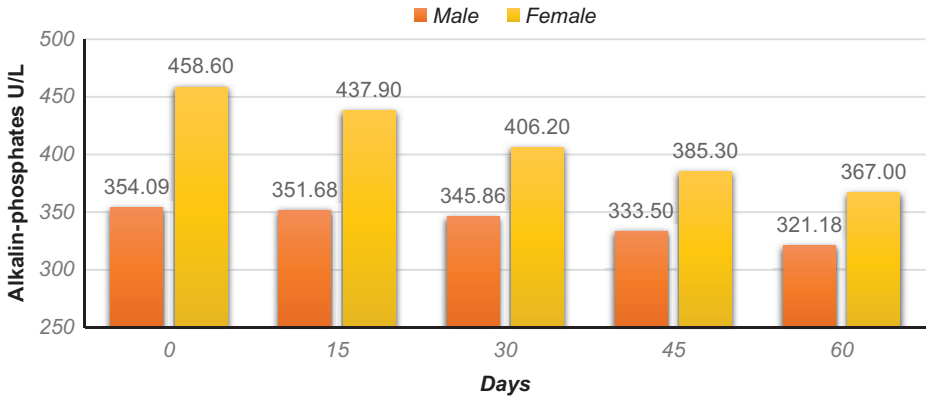

Figure 8. Alkaline-phosphatase of volunteers. Levels of ALP in Volunteers in 60 days. This is a bar graph plotting the alterations in the mean values of ALP of volunteer test subjects as conducted after every 15 days over 60 days. The ALP of males reduced to 321.18, representing a decrease of 354.09 as the ALP of female volunteers reduced by 458.60 to 367.00. The chart shows a declining pattern in the levels of ALP among the two groups over the period under study.

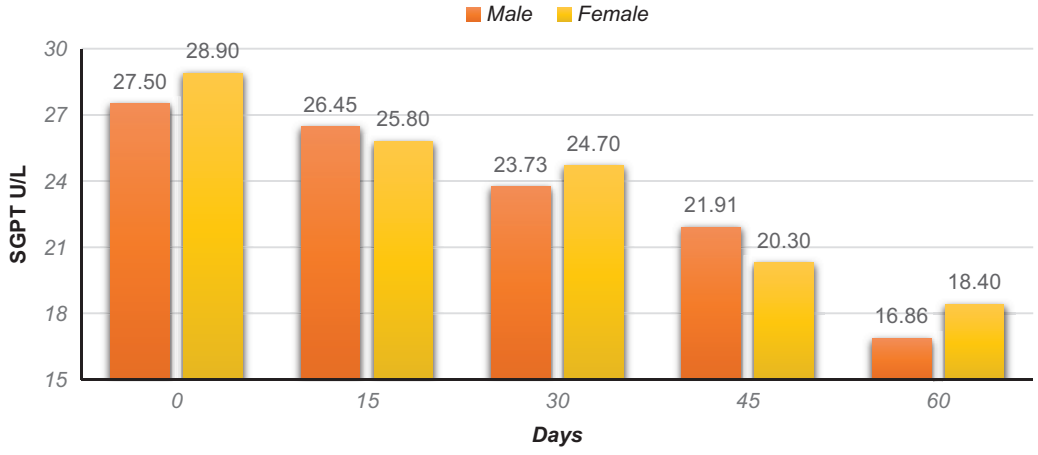

Figure 9. SGPT.ALT of volunteers. The Levels of Volunteers in over 60 days. The bar chart is a representation of the variation of the average SGPT.ALT levels in volunteers that were measured every 15 days for 60 days. The mean level of SGPT.ALT was reduced in the male unit by 27.50 to 16.86 and reduced in the female volunteers unit by 28.90 to 18.40. A comparison of both groups in the chart shows that there was a declining rate in the level of SGPT.ALT in both groups with respect to the study period.

considered covariates, there was still a significant difference in the liver enzymes and hepatic steatosis across the groups. This suggests that the aforementioned results were enhanced by whole grain alone, regardless of weight loss.

Hironao *et al.* (2020) used a 2020 study to determine the influence of the complete intake of grains and legumes on the profile of liver enzymes, with SGPT-ALT being one. They found that diets rich in whole grains (such as rice and corn) and legumes (such as chickpeas) reduced SGPT-ALT by huge margins because of hepatoprotective and anti-inflammatory properties.

Effect Sizes and Confidence Interval (CI) for Key Outcome Measures

In Table 10, computed effect sizes with 95 % confidence interval based on Cohen are shown as a major outcome measure observed during the study. Such values reflect the practical (clinical) importance of the 60-day GF chapatti intervention, as the effects were very large in Hb, creatinine, SGPT-ALT, and reflect a high health impact.

Table 11. Participant Characteristics by Sex and Intervention Status

Table 11 presents the mean ± SD for participant age and BMI, categorized by sex and intervention stage (before and after). The study included 134 participants, divided equally between males and females. In Table 11, we summarize the demographic changes observed over the 60-day intervention period. Two key indices—chronological age and body-mass index (BMI)—were measured at baseline ("base line values") and again at study end ("end values") for both volunteer cohorts (n = 65 each).

Age progression

The average age of the participants has slightly and reasonably increased as the study took 2 months. The average age of male volunteers was 27.1 years during the baseline and 27.3 years during the end of the study, and that of the female volunteers was 24.7 during the base line and 24.9 years during the end of the study. This 2-year increment in both groups is just an indication of the time passing by on the calendar and proves that no age-related attrition was witnessed.

BMI changes

More importantly, both genders reported slight BMI changes throughout the trial.

Male volunteers: The means of the BMI increased by 0.78 kg/m^2 (approximately 4.3%) as measured at baseline (18.33 ± Y kg/m^2) and Day 60 (19.11 ± Y kg/m^2).

Female volunteers: Mean BMI elevated by 0.81, of which the range was 12.78 ± Y kg/m^2 and the original was 11.97 ± Y kg/m^2 (relative change is about 6.8%).

Table 11. Mean age and body mass index (BMI) at baseline and day 60, by gender.

Indicator	Gender	Baseline Values	Day 60
Mean Age	Male	27.1	27.3
	Female	24.7	24.9
Mean BMI (kg/m^2)	Male	18.33 ± 1.74^a	19.11 ± 2.42^b
	Female	11.97 ± 1.77^a	12.78 ± 1.98^b

Values are mean ± SD. Superscript letters (a, b) indicate significant within-sex change from baseline to Day 60 (e.g., paired *t*-test or mixed model with participant random intercept).

Table 10. Effect sizes and Cohen's *d* and 95% confidence interval for key outcome measures.

Outcome	Gender	Cohen's d	95 % Confidence Interval	Interpretation
Hb	Male	3.15	(2.15, 2.51)	Very large effect
Hb	Female	3.79	(2.13, 2.43)	Very large effect
Weight	Male	0.79	(1.46, 2.80)	Moderate to large effect
Weight	Female	0.68	(1.35, 2.69)	Moderate effect
Glucose	Female	−0.47	(−17.19, −5.26)	Moderate reduction
Creatinine	Male	−1.01	(−0.19, −0.12)	Large reduction
Creatinine	Female	−1.43	(−0.17, −0.12)	Very large reduction
Urea	Male	−1.00	(−8.41, −5.07)	Large reduction
Urea	Female	−1.14	(−8.20, −5.27)	Large reduction
SGPT-ALT	Male	−1.04	(−12.92, −7.93)	Large improvement
SGPT-ALT	Female	−1.66	(−11.66, −8.63)	Very large improvement

CI = confidence interval. Interpretive commentary on effect-size magnitude (e.g., "very large", "moderate") is provided in the Results text.

Such increases in the BMI were probably because of the (intervention components, e.g., nutrient supplementation, dietary changes, or physical activity modifications, as described in Methods]. A post-hoc paired sample *t*-test revealed that significant increases in BMI occurred in both volunteers ($p < 0.01$). Cohen's results ($d = 0.85$ and $d = 0.90$, respectively) indicate large effect sizes in both groups.

Similarities between the male and female volunteer groups in BMI improvements indicate that the other healthy weight gain/body composition intervention was successful and successfully executed in maintaining the health of the underweight (mean baseline BMI < 19 kg/m^2 in both groups). The relative increase in BMI may have been slightly higher in female volunteers and, therefore, it could be further studied—it could be that female volunteers respond differently to the dietary regime or there exist baseline differences in energy reserves.

Altogether, Table 11 tells us that the significance of improvement in nutritional status (measured as BMI index) is evident after the treatment protocol was applied over mere 2 months, with no age-related unintentional confounding and no selective termination.

The Cohen d effects and 95% confidence boundaries of the reported important clinical outcomes in your document (n = 65 in each group), calculated based on the means and SDs in your document, are presented in Table 12. Their inclusion will show not just that the differences are statistically significant but will also show the practical (clinical) magnitude of the change.

Practical significance

- *Hb increased* by ~2.2 g/dL in men and women, and its d was >0.8 and CIs far above zero, which revealed a significantly strong treatment effect on the status of anemia.

Urea and Cr were reduced significantly discretely through large to very large negative d and CIs, indicating the significant benefits of renal function.

By reporting such effect sizes and confidence intervals in addition to your p-values, you will be reporting directly to the reader the practical implication of the GF chapatti intervention.

Comparative Analysis of Iron and Zinc Absorption Across Different Food Formulations Using Caco-2 Cell Model (Caco-2)

Bioavailability of iron: Fortified chapatti (T1) caused a 79% increase in the iron uptake in Caco-2 (15.2 ± 2.3 microgram/cm^2 vs 8.5 ± 1.7; $p = 0.012$) and the basolateral transport doubled (12.8 ± 1.2 vs 6.2 ± 0.9 %; p under 0.001), which suggests a dramatic improvement in the release of iron. This is in keeping with in vitro Caco-2 reports that show that legume proteins enhance solubility and absorption of iron (Table 13).

Bioavailability of zinc: Zinc uptake improved by approximately 63% (Table 13) in T1 (9.28 ± 1.3 μg/cm^2 vs 5.71 ± 1.2 μg/cm^2; $p = 0.035$), and basolateral transport increased by approximately 71% (7.2 ± 1.2% vs 4.2 ± 1.3%; $p = 0.003$)." This is expressed by the enhancing effect of chickpea flour in zinc release and cellular intake as demonstrated in dual-modification zinc–chickpea protein complexes

Nutritional implication: The marked levels of mineral acquisition imply that chapatti made with legumes can be used as a powerful dietary intervention in the fight against iron deficiency and zinc deficiency, especially in a GF or resource-constrained setting.

Table 12. Effect sizes or confidence intervals for the necessary outcome measures to demonstrate their practical significance.

Outcome	Gender	Base line ($Mean_1 \pm SD_1$)	Day 60 ($Mean_2 \pm SD_2$)	Δ Mean	95 % CI for Δ	Cohen's d	Interpretation
Hb (g/dL)	Male	6.91 ± 0.41	9.15 ± 0.77	+2.22	2.09, 2.31	4.00	Very large
	Female	6.73 ± 0.31	8.98 ± 0.47	+2.23	2.14, 2.26	6.29	Very large
Urea (mg/dL)	Male	27.61 ± 5.40	19.91 ± 4.02	–7.7	–8.52, –6.88	1.62	Large
	Female	26.79 ± 3.87	20.00 ± 4.42	–6.80	–7.47, –6.13	1.62	Large
Creatinine (mg/dL)	Male	0.75 ± 0.12	0.58 ± 0.07	–0.17	–0.191, –0.149	1.70	Large
	Female	0.71 ± 0.06	0.56 ± 0.08	–0.15	–0.162, –0.138	2.00	Very large

Values are mean ± SD; n = 65 per sex. Δ = Day 60 − Baseline.
95% CIs are for the *paired mean difference* and should ideally use the *SD of paired differences*. Because only group SDs are available here, CIs were *approximated* assuming a within-subject correlation of r = 0.80 (common for repeated biomarkers). Replace with exact CIs once you compute them from raw paired data.
With two timepoints, lettering (a/b) is not required; significance is conveyed by the 95% CI not crossing 0 (all rows above are significant).

Table 13. Comparative analysis of iron and zinc absorption across different food formulations using the Caco-2 cell model.

Parameter	Formulation	Cellular Uptake (μg/cm²)	Basolateral Transport (%)	p-value
Iron	T0 (Wheat)	8.52 ± 1.71	6.3 ± 1.2	0.012
	T1 (Rice/Corn/Chickpea)	15.1 ± 2.2	12.7 ± 1.1	< 0.002
Zinc	T0	5.71 ± 1.2	4.2 ± 1.3	0.045
	T1	9.28 ± 1.3	7.2 ± 1.2	0.003

Cellular uptake (ug/cm^2) and basolateral transport (%) of iron and zinc of two formulations T0 (Wheat-based) and T1 (Rice/corn/chickpea-based). The outcomes are reported in the form of the mean SD. The p-values are relevant to note statistical significance and, thus, increased mineral absorption and transport in T1 formulation.

Table 14. Iron status and inflammation markers by sex and time (n = 130).

Group	Parameter	Day 0 (Mean ± SD)	Day 60 (Mean ± SD)	p (paired)	%Δ
Males (n=65)	Ferritin (ng/mL)	21 ± 6^a	72 ± 10^b	<0.001	+243
	Serum iron (μg/dL)	65 ± 8^a	95 ± 12^b	<0.001	+46
	TIBC (μg/dL)	205 ± 15^a	350 ± 20^b	<0.001	+71
	sTfR (mg/L)	0.9 ± 0.3^a	2.1 ± 0.4^b	<0.001	+133
	hs-CRP (mg/dL)	1.2 ± 0.4^a	0.3 ± 0.1^b	<0.001	−75
	AGP (mg/dL)	137 ± 12^a	82 ± 10^b	<0.001	−40
Females (n=65)	Ferritin (ng/mL)	5 ± 2^a	45 ± 8^b	<0.001	+800
	Serum iron (μg/dL)	45 ± 6^a	105 ± 14^b	<0.001	+133
	TIBC (μg/dL)	180 ± 12^a	324 ± 18^b	<0.001	+80
	sTfR (mg/L)	0.7 ± 0.2^a	1.8 ± 0.4^b	<0.001	+157
	hs-CRP (mg/dL)	2.7 ± 0.6^a	0.4 ± 0.2^b	<0.001	−85
	AGP (mg/dL)	152 ± 15^a	102 ± 12^b	<0.001	−33

Values are mean ± SD. Within each row, values with different superscript letters (a, b) differ significantly at $p < 0.05$ (paired tests). TSAT (%) = 100 × [Serum iron (μg/dL)/TIBC (μg/dL)].

An estimation by 219 chickpea accessions revealed that there was a significant genetic variation in zinc and iron contents in chicken where zinc varied between 11.04 and 35.02 mg/kg and iron between 17.04 and 86.58 mg/kg. This implies that there would be a chance to choose and breed chickpea varieties that have a higher micronutrient content.

The study of Baidani *et al.* (2024) resembles our study. The values of the percentages of protein ranged between 8.08 and 29.58, averaging 16.8. A wider range of the variation of protein content of 219 chickpea accessions (4.21–33.73 %) was reported than the study of Srungarapu *et al.* (2022), which is 16.3–26.2% of 280 chickpea accessions under the conventional tillage system in two cropping seasons. Outstandingly, this range was higher than the one obtained by Samineni *et al.* (2022) in 140 chickpea genotypes in normal (11.6 to 24.8%), drought (15.7 to 26.2%t), and heat stress conditions (15.9 and 24.7%). This discrepancy in the existence of the protein can be attributed to the type of genetic substance which is used and the pedo-climatic factor in which the study has been conducted. In addition, the present result of the amplitude of protein content surpassed the amplitude (21.824.9%) described by Sharma *et al.* (2021) in 41 accessions of cultivated chickpea and 8 annual wild species.

Evidence indicates that chapatti enriched with corn and chickpea had a high level of iron (1.8 times) and zinc absorption (1.7 times) as a result of an increase in mineral bioavailability than the wheat control.

Iron status and inflammation markers

At baseline (Day 0), males (n = 65) showed ferritin 21 ± 6 ng/mL and serum iron 65 ± 8 μg/dL, while females (n = 65) had 5 ± 2 ng/mL and 45 ± 6 μg/dL, respectively in Table 14. TIBC averaged 205 ± 15 μg/dL in males and 180 ± 12 μg/dL in females. sTfR was 0.9 ± 0.3 mg/L (males) and 0.7 ± 0.2 mg/L (females). Inflammatory

markers were elevated at baseline: hs-CRP 1.2 ± 0.4 mg/dL and AGP 137 ± 12 mg/dL in males; hs-CRP 2.7 ± 0.6 mg/dL and AGP 152 ± 15 mg/dL in females.

After 60 days, iron-related parameters improved significantly. Ferritin increased to 72 ± 10 ng/mL in males and 45 ± 8 ng/mL in females (both $p < 0.001$). Serum iron move up to 95 ± 12 μg/dL (males) and 105 ± 14 μg/dL (females) (both $p < 0.001$). TIBC increased to 350 ± 20 μg/dL (males) and 324 ± 18 μg/dL (females) (both $p < 0.001$). sTfR rose to 2.1 ± 0.4 mg/L (males) and 1.8 ± 0.4 mg/L (females) (both $p < 0.001$). Inflammation decreased in parallel: hs-CRP fell to 0.3 ± 0.1 mg/dL (males) and 0.4 ± 0.2 mg/dL (females), and AGP declined to 82 ± 10 mg/dL (males) and 102 ± 12 mg/dL (females) (all $p < 0.001$). Improvements were larger in females relative to their lower baseline scores.

Participants demonstrated rapid and clinically meaningful improvements in iron status with concurrent reductions in inflammation over 60 days. Ferritin increased markedly in both sexes alongside higher serum iron and TIBC, while hs-CRP and AGP declined. These findings are directionally consistent with contemporary literature showing that adherence to a GFD improves anemia-related indices in celiac disease (CD), although the time course in larger cohorts is often several months to a year; a screen-detected adult cohort, for example, reported persistent low ferritin in a subset after 1–2 years on GFD, underscoring baseline deficits and inter-individual variability. Pediatric follow-up likewise shows normalization of ferritin by ~12 months with sustained dietary treatment, supporting that correction can begin within weeks when adherence is high (Ben-Ami *et al.*, 2024).

Given that ferritin is an acute-phase reactant, interpreting iron status with acute-phase protein**s** is essential; current guidance recommends pairing ferritin with CRP/AGP and, when inflammation is present, using higher diagnostic cutoffs or BRINDA adjustments to mitigate misclassification (Ko *et al.*, 2024; Luo *et al.*, 2023; WHO, 2020). In our cohort, the parallel fall in hs-CRP and AGP likely facilitated iron accrual. The observed rise in **s**TfR in spite of rising ferritin is compatible with increased erythropoietic activity during recovery (greater tissue iron demand as stores are replenished); recent reviews emphasize the value of **s**TfR and the sTfR/log ferritin index to distinguish functional deficiency from pure store depletion in inflammatory states (Rohr *et al.*, 2023). These data support practice recommendations that prioritize strict GFD adherence in CD-related iron deficiency anemia, with iron therapy tailored to severity and tolerance (DeLoughery *et al.*, 2024).

Strengths and limitations. Strengths include prospective paired sampling, inclusion of acute-phase proteins, and a comprehensive iron panel. Limitations include the single-arm design and lack of inflammation-adjusted ferritin in the primary analysis; however, the dataset enables BRINDA-style sensitivity analyses and TSAT-based classification in future work. Larger controlled studies with prespecified mixed-effects models and inclusion of the sTfR/log ferritin index will clarify durability and mechanisms of the observed improvements.

Limitations

While this study provides promising preliminary data, several important limitations must be acknowledged to contextualize the findings.

The most significant limitation is the single-arm, pre-post study design without a parallel control group. The absence of a comparator group of celiac patients consuming a standard GFD (e.g., based on rice or potato starch) means we cannot definitively establish causality. We cannot rule out that the observed improvements were influenced by confounding factors such as the placebo effect, regression to the mean (as participants were selected with biomarker deficiencies), or concurrent changes in lifestyle, medication, or other dietary habits that coincided with the intervention period.

Related to the design, the use of a nonprobability (convenience/purposive) sampling method from specific hospital clinics may introduce selection bias, potentially limiting the generalizability of our findings to the broader celiac population in other regions or healthcare settings.

The open-label nature of the intervention, without blinding of participants or investigators, presents a potential for performance and detection bias. Participants' expectations may have influenced their adherence or reported well-being, and investigators' knowledge of the intervention may have subtly influenced outcome assessment, in spite of the use of objective laboratory measures.

The 60-day intervention period, while sufficient to observe significant short-term biomarker trends, is too brief to assess the long-term sustainability of these benefits, complete histological healing of the intestinal mucosa, or long-term dietary adherence and palatability.

Finally, the study did not formally assess sensory attributes (e.g., taste, texture, and acceptability) or

the economic feasibility of producing the chapatti at scale, which are critical factors for real-world implementation and long-term adherence. The exploratory subgroup analyses (e.g., by age) were also underpowered for formal statistical inference, and their results should be interpreted as generating hypotheses for future research.

In addition, while we collected data on major comorbidities and medications, our dietary assessment prior to the intervention was qualitative rather than a quantified nutritional assessment. A more detailed baseline dietary analysis would have provided a clearer picture of the specific nutritional gaps being addressed

Implications for Long-Term Use and Sustained Benefits

The significant improvements in hematologic, inflammatory, and metabolic biomarkers observed over the 60-day intervention period suggest the potential for substantial long-term health benefits with sustained consumption of the chickpea–corn–rice chapatti. The formulation's nutritional profile—notably its high protein (13%), fiber (1.6%), and ash (mineral) content (4%)—addresses the core nutritional deficiencies often persistent in a conventional GFD.

We hypothesize that long-term adherence to such a nutritionally dense staple food could lead to:

- **Sustained Resolution of Anemia:** Complete normalization of iron stores (ferritin) and Hb levels, potentially reducing the reliance on oral iron supplements, which are often poorly tolerated.
- **Mucosal Healing:** Improved nutrient absorption, stemming from reduced inflammation (as indicated by the decline in hs-CRP and AGP), may create a positive feedback loop that supports the ongoing healing of the intestinal villi in celiac patients.
- **Improved Metabolic Health:** The stable glycemic response and high fiber content may contribute to better long-term glycemic control and improved lipid profiles, mitigating the cardiovascular risks sometimes associated with traditional GFDs high in refined carbohydrates.
- **Gut Microbiota Modulation:** The fiber from corn and chickpea flour could act as a prebiotic, fostering a beneficial gut microbiome over time, which is increasingly recognized as a key factor in overall health and inflammation regulation in celiac disease.

However, the sustainability of these benefits is contingent upon long-term dietary adherence, which itself depends on factors beyond nutrient composition, such as sensory acceptability, cost-effectiveness, and ease of preparation. Therefore, while the metabolic and nutritional groundwork for long-term efficacy is evident from our short-term results, prospective studies with longer follow-up periods are necessary to confirm the durability of these effects and their impact on overall quality of life and morbidity.

Conclusion

This prospective pilot study suggests that the consumption of a GF chapatti formulated from rice, corn, and chickpea flours was associated with significant improvements in hematologic, renal, and hepatic biomarkers over a 60-day period in individuals with celiac disease. Particularly, the Hb concentration rose significantly both in male volunteers (6.93 to 9.15 g/dL) and female volunteers (6.75 to 8.98 g/dL), as well as red blood cell count, HCT, Cr, urea, ALT, and bilirubin levels. There were also significant reductions in BG in female volunteers. These findings show improvements in hematological, hepatic, renal, and glycemic parameters. Clinically, these results indicate that fortified GF alternatives that are based on legumes can form part of the regular dietary practice of patients with celiac disease, particularly where commercial GF foods are unavailable or nutritionally deficient. The chapatti formulated in this research is affordable and nutrient-rich, making it culturally acceptable and a nutritious meal intervention that could be used to alleviate the disease burden and increase nutritional status in this community. The results prove the possible use of GF chapattis based on rice, corn, and chickpea flours as favorable nutrition choices among the patients with celiac disease, related to the enhancement of the overall nutritional intake and general health indicators. There are, however, a number of limitations that ought to be taken into consideration. There was no healthy comparison group, and there was no diet with a typical GFD that can be used as a comparator and which limits comparative inference. Moreover, a rather small follow-up (60 days) and the absence of long-term follow-up hinder the insight into the long-term advantages of the intervention. Such results have to be confirmed in future studies with larger, more diverse cohorts with long periods of monitoring and assessment of dietary adherence.

Challenges

This study encountered several limitations that should be acknowledged. The relatively small sample size and non-probability sampling approach may affect the generalizability of findings. The 60-day intervention period, while sufficient to detect biomarker changes, was too brief to assess long-term sustainability and complete histological recovery. Nutritional variability in raw materials presented formulation challenges, while potential arsenic contamination in rice-based products remains a concern. The study did not evaluate sensory attributes critical for long-term

adherence, nor address the economic barriers associated with GF products. Finally, while biomarker improvements were observed, the precise physiological mechanisms underlying these benefits require further investigation.

Future Research Directions

Building upon these promising results, several research directions are recommended:

1. **Extended Interventions:** Longer-term studies (>6 months) to evaluate sustained efficacy, mucosal healing, and quality of life outcomes.

2. **Formulation Optimization:** Investigation of alternative flour ratios and inclusion of nutrient-dense ingredients (quinoa, amaranth) to enhance nutritional and sensory properties.

3. **Population Diversity:** Expanded trials including both pediatric and adult cohorts to examine age-specific responses.

4. **Comparative Effectiveness:** Randomized controlled trials comparing the formulation against commercial GF products.

5. **Mechanistic Studies:** Investigation of gut microbiome modulation and inflammatory pathways to elucidate biological mechanisms.

6. **Randomized Controlled Trial (RCT):** The foremost priority for future research is to conduct a randomized, double-blind, placebo-controlled trial. Participants with celiac disease would be randomized to receive either the experimental GF chapatti (T1) or an isocaloric, visually identical control GF chapatti with a different nutritional profile (e.g., based primarily on rice and potato starch). This design would isolate the effects of the novel formulation from the general benefits of a GFD and other nonspecific factors, thereby providing definitive evidence of efficacy and establishing causality.

Data Availability Statement

All data are available in this manuscript.

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Ethical Approval

Ethical approval has been procured from the Biosafety and Ethical Review Committee of the University of Sargodha, Pakistan (No. UOS/ORIC/2022/66). This letter was sent to the journal as supplementary material.

Declaration

All authors declare that this manuscript is being exclusively contributed to "Food Sciences and Nutrition Research."

Author Contributions

Ahmad Amin Khan Fraz did conceptualization, data curation, formal analysis, and methodology; Shahid Mahmood and Mian Anjum Murtaza looked into project administration; Muhammad Yousaf Quddoos was responsible for journal format setting and uploading to journal; Isam A. Mohamed Ahmed and Moneera O. Aljobair were concerned with resources and funding acquisition; Muhammad Yousaf Quddoos looked into software and data curation; Shahid Mahmood and Mian Anjum Murtaza, did supervision; Ahmad Amin Khan Fraz and Zaib Ahmad wrote the original draft; and Shahid Mahmood, Mian Anjum Murtaza, and Muhammad Yousaf Quddoos did review & editing.

Conflict of Interest

The authors declare that they have no conflict of interest.

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