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CORRELATION BETWEEN VITAMIN B12 DEFICIENCY ANEMIA AND ARGININE METHYLATION IN ERYTHROCYTE BAND 3

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Abstract

Vitamin B12 deficiency is marked by megaloblastic anemia and abnormal red blood cell morphology. This study attempts to correlate B12 levels with RBC membrane proteins (especially Band 3) and arginine methylation. B12-deficient, iron-deficient and normal blood samples were analysed using SDS-PAGE and *in silico* using Alphafold3. SDS-PAGE revealed differences in band intensity and size, indicating altered membrane protein profiles in B12-deficient samples. AlphaFold3 modelling of known Band 3 mutations involving arginine substitutions revealed structural changes, especially in the N-terminal domain responsible for interaction with other membrane proteins. These preliminary observations suggest a possible correlation between B12 deficiency, arginine methylation, and changes in RBC membrane structure.

Keywords: Vitamin B12, arginine methylation, Band 3, red cell membrane

1. INTRODUCTION

Megaloblastic anemia, characterized by enlarged and dysfunctional RBCs, has been attributed to the role of vitamin B12 in DNA synthesis during erythropoiesis **[1]**. However, studies suggest that B12 levels may also influence post-translational modifications such as methylation, potentially affecting protein-protein interactions and erythrocyte membrane integrity **[2]**.

Vitamin B12 is essential for the synthesis of S-adenosylmethionine (SAM), the universal methyl donor **[3]**. Methylation of arginine residues in erythrocyte membrane proteins such as Band 3 could disrupt protein- protein interactions and cytoskeletal anchoring **[4]**. This is seen in natural variants of Band 3 where arginine mutations show spherocytosis and ovalocytosis **[5]**. This suggests that B12 deficiency may impair (arginine) methylation, destabilizing Band 3 leading to membrane fragility.

2. MATERIALS AND METHODS

Blood sample collection

Blood samples were obtained from Ragav's Diagnostic & Research Centre Pvt. Ltd., Jayanagar, Bengaluru. The samples, originally marked for disposal, had been tested for vitamin B12 and haemoglobin levels The samples were grouped into those with a) B12 deficiency b) adequate B12 levels c) low haemoglobin d) normal B12 and haemoglobin.

RBC Morphology Assessment

Red cell morphology was examined microscopically using a hemocytometer under 40× magnification. Observations focused on identifying morphological variations such as anisocytosis, poikilocytosis, and other abnormalities indicative of underlying haematological conditions.

Preparation of RBC Membrane fraction and SDS-PAGE Analysis

Whole blood samples were collected in 1.5 mL Eppendorf tubes and centrifuged to separate the serum, which was stored for subsequent analyses. 200 µL of red blood cells (RBCs) was mixed with 800 µL of sterile saline, mixed thoroughly and centrifuged at 4500 rpm for 8 minutes. The supernatant was discarded, and the pellet was washed three times with saline to remove residual plasma components. The RBC pellet was resuspended in sterile distilled water and incubated at 4 °C overnight to induce hemolysis. The hemolysed samples were then centrifuged at 10,00rpm for 10 minutes at 4 °C. The pellet was washed three times with buffer to isolate the RBC membrane fraction. For SDS-PAGE analysis, 10 µL of the membrane preparation was mixed with 2 µL of gel loading buffer (Genei SDS-PAGE kit) and heated at 95–100 °C for 5 minutes and electrophoresed on a 10% SDS-PAGE at 100volts **[6]**. Gels were stained in Ezee- Blue (Genei Labs) and destained in water.

3D Structural Analysis of Band 3 Protein

The amino acid sequence of the erythrocyte membrane protein Band 3 (UniProtKB accession number: P02730) and arginine mutations involved in hereditary spherocytosis (Table 1) were retrieved from the UniProt Knowledgebase. Wild type or mutant sequence was copied into the search box in the Alphafold3 server **[7]** and submitted for structural prediction.

Table 1. Naturally occurring variants of Band 3 whose structures were generated using Alphafold 3 (sourced from www.uniprot.org).

VAR_013792	490	R>C
VAR_058039	490	R>H
VAR_000802	518	R>C
VAR_013806	760	R>Q
VAR_013807	760	R>W
VAR_013810	808	R>C
VAR_013811	808	R>H
VAR_013816	870	R>W

Results and Discussion

Blood Sample Selection for the Study

In this study, blood samples with pre-determined levels of hemoglobin (Hb) and serum vitamin B12 (B12) were selected to facilitate a focused biochemical analysis. The classification of B12 status was based on clinically accepted reference ranges compiled from multiple diagnostic and regulatory sources, as summarized in Table 2**.**

Table 2. Clinically accepted reference ranges for serum vitamin B12 levels used in this study.

Definition	Apollo Hospitals	Dr. Lal PathLabs	USFDA	Europe
Normal Range	200-900 pg/mL	190-950 pg/mL	200-900 pg/mL	NA
Borderline/ Optimal/ Sufficient	400-600 pg/mL	200-300 pg/mL	300-400 pg/mL	200-500 pg/mL
Deficiency	< 200 pg/mL	< 200 pg/mL	< 200-250 pg/mL	< 200pg/mL
Reference	https://www.apollohospitals.com/diagnostics-investigations/	https://www.lalpathlabs.com/blog/	Strohle *et al.*, (2019)	https://www.clancymedicalgroup.com/

Samples with serum B12 concentrations below 200 pg/mL were categorized as deficient, while those within the range of 200–400 pg/mL were considered sufficient. To minimize confounding variables and maintain analytical consistency, demographic factors such as age, gender, and the presence of co-morbidities were deliberately excluded from the selection criteria.

Vitamin B12 status and RBC morphology

Red blood cell (RBC) morphology serves as a sensitive biomarker for hematological health and can reflect underlying micronutrient imbalances, particularly vitamin B12 deficiency. In this study, RBC morphology was examined across samples with varying serum B12 concentrations to assess structural changes associated with deficiency and sufficiency (Figure 1).

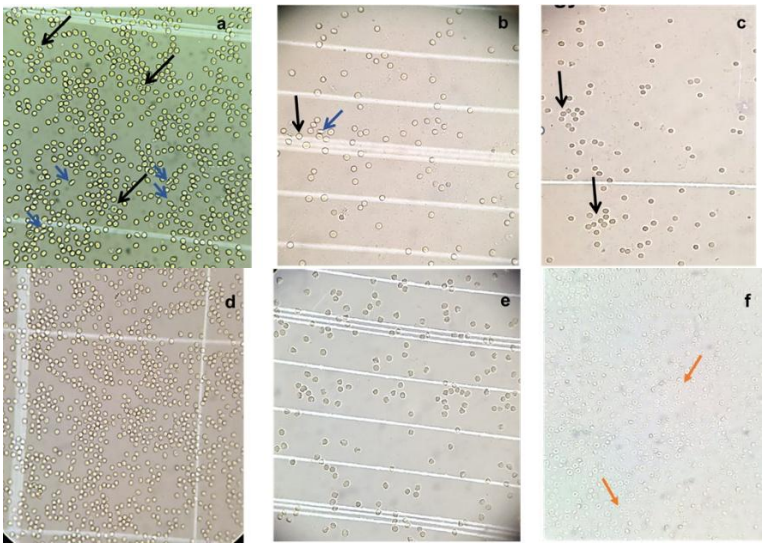

Figure 1. RBC morphology in samples with varying B12 and Hb levels. (a) B12: 130.20 pg/mL- burr cells and ovalocytes **(b)** B12: 178.20 pg/mL – mild poikilocytosis **(c)** B12: 183.20 pg/mL – fewer abnormal cells **(d)** B12: 365.70 pg/mL – normal biconcave morphology **(e)** B12: 385.50 pg/mL – ruptured cells, likely due to handling **(f)** Hb: 10.8 g/dL – biconcave cells.

Samples with serum B12 levels below 200 pg/mL (130.20, 178.20, and 183.20 pg/mL) consistently exhibited poikilocytosis, characterized by the presence of ovalocytes, burr cells, and acanthocytes. These abnormal shapes suggest impaired erythropoiesis and membrane instability, hallmark features of megaloblastic anemia. The presence of acanthocytes and ovalocytes may also reflect disrupted lipid metabolism and cytoskeletal remodeling processes indirectly influenced by methylation dynamics.

In contrast, samples with B12 levels above 200 pg/mL (365.70 and 385.50 pg/mL) showed predominantly normal biconcave RBCs. One sample (385.50 pg/mL) exhibited ruptured cells, likely due to mechanical damage during slide

preparation rather than intrinsic pathology. These observations reinforce the protective role of adequate B12 in maintaining erythrocyte integrity and morphology. The comparative analysis is summarized in Tables 3 and 4.

Table 3. Microscopic analysis of RBC morphology across B12-deficient and B12-sufficient samples.

Sample type	Parameter value	Presence of poikilocytes	Microscopic examination
B12 deficient	130.20	Yes	Ovalocytes, Acanthocytes
B12 deficient	178.20	Yes	Ovalocytes, Macrocytes
B12 deficient	183.20	Yes	Burr cells
B12 sufficient	365.70	No	Normal cells
B12 sufficient	385.50	No	Ruptured cells

Table 4. RBC morphology in an iron-deficient sample with low hemoglobin and normal sample.

Sample type	Hb value	Presence of poikilocytes	Microscopic examination
Iron deficient	10.8	Yes	Ovalocytes, biconcave normal RBCs
Normal	16	No	Biconcave cells

In a comparative analysis of RBC morphology in iron-deficient and normal samples, the iron-deficient sample (Hb = 10.8 g/dL) showed biconcave cells with mild poikilocytosis but lacked the pronounced morphological abnormalities seen in B12-deficient samples. This distinction highlights the unique structural impact of B12 deficiency on RBCs, as different from iron-deficiency anemia.

The observed morphological differences between B12-deficient and B12-sufficient samples suggest that vitamin B12 plays a role in maintaining normal RBC structure. Deficient samples showed clear signs of poikilocytosis, while sufficient samples largely retained the typical biconcave shape. These findings support the idea that low B12 levels may contribute to structural abnormalities in RBCs, potentially linked to impaired erythropoiesis.

RBC membrane protein profile from B12 deficient and sufficient samples

SDS-PAGE was employed to examine the membrane protein profiles of red blood cells (RBCs) under varying physiological conditions, specifically focusing on vitamin B12 and hemoglobin (Hb) levels as shown in Figure 2**.** The molecular weight marker used was bovine serum albumin (BSA), with a reference band at 66 kDa, serving as a calibration point.

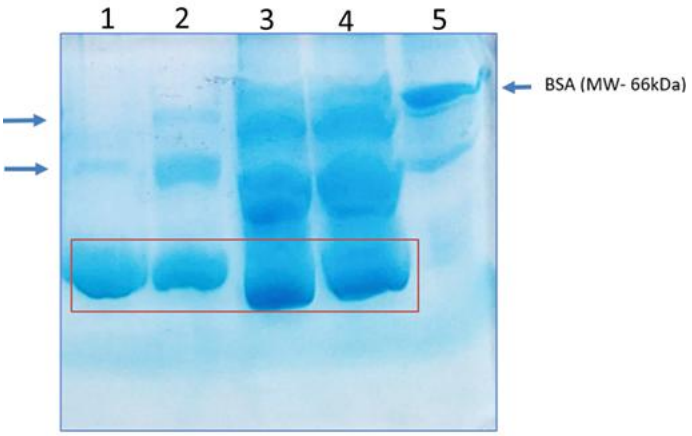

Figure 2. SDS-PAGE analysis of RBC membrane proteins from B12 deficient and sufficient samples. Lane M contains the molecular weight marker (BSA, 66 kDa). Lane 1 represents a B12 deficient sample (179.60 pg/mL), Lane 2 a B12 sufficient sample (365.70 pg/mL), Lane 3 a hemoglobin-deficient sample (10.8 g/dL), and Lane 4 a normal control. The bands boxed in red correspond to hemoglobin.

Prominent bands were observed in the high molecular weight region (~80–280 kDa), which likely correspond to key RBC membrane proteins such as spectrin (α: 280 kDa, β: 246.2 kDa), ankyrin (206 kDa), Band 3 (~100.8 kDa), and protein 4.1R (80.6 kDa), based on molecular weight references **[8]**. In Lane 1 (B12 deficient), several high molecular weight bands showed reduced intensity compared to Lane 2 (B12 sufficient), suggesting altered membrane protein expression. While the identity of individual bands was not confirmed in this study, the observed pattern is consistent with previous findings by Ballas (1978) **[9]**, which reported diminished expression of high molecular weight membrane proteins in B12-deficient RBCs.

Arginine Methylation, Band 3 Mutations, and RBC Membrane Structure

Vitamin B12 is essential for methylation reactions, including those modifying arginine residues in red blood cell (RBC) membrane proteins. Band 3, encoded by the *SLC4A1* gene, is a major structural and functional component of the RBC membrane. Methylation of arginine residues in Band 3 contributes to membrane stability, protein-protein interactions, and overall cell morphology. This has been suggested by experiments where arginine modification by phenylglyoxal renders the protein inactive **[10]**.

Natural variants in *SLC4A1* often involve substitutions at arginine sites, which may disrupt methylation and alter Band 3 conformation. These mutations are implicated in hereditary spherocytosis and Asian ovalocytosis conditions marked by abnormal RBC shapes and membrane fragility.

To investigate the structural consequences of these mutations, AlphaFold3 was used to model the full-length Band 3 protein and perform in silico mutagenesis. The available structures 1HYN and 4YZF corresponding to the N and C terminal domains lack the connecting loops and do not represent the full-length conformation (Figure 3).

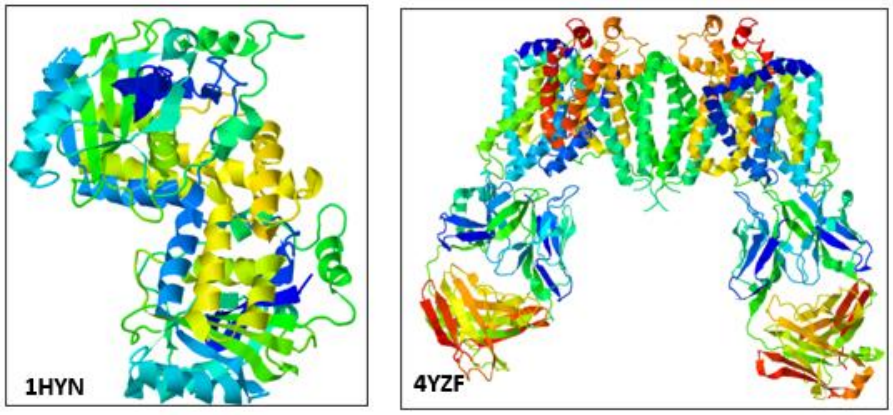

Figure 3. Crystal Structures of Band 3 Domains. (From www. rcsb.org). Left: N-terminal cytosolic domain (PDB: 1HYN). Right: C-terminal transmembrane domain (PDB: 4YZF).

These domain-level structures provide foundational insights but are insufficient for understanding inter-domain interactions or mutation-induced conformational shifts. Hence, AlphaFold3 was employed to generate a complete model (Figure 4).

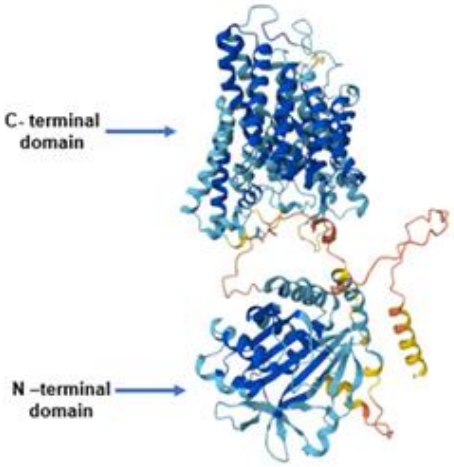

Figure 4. Full-Length Band 3 Predicted by AlphaFold3.

The AlphaFold3 model showed both N-terminal and C-terminal domains, connected by loops and helices (Figure 4). This structure was considered as the baseline for the structural analysis of the natural variants of Band 3. Specific arginine substitutions were then studied for changes in domain orientation, helix-loop conformation, and potential interaction surfaces. Substitution of R490 with cysteine or histidine is shown in Figure 5.

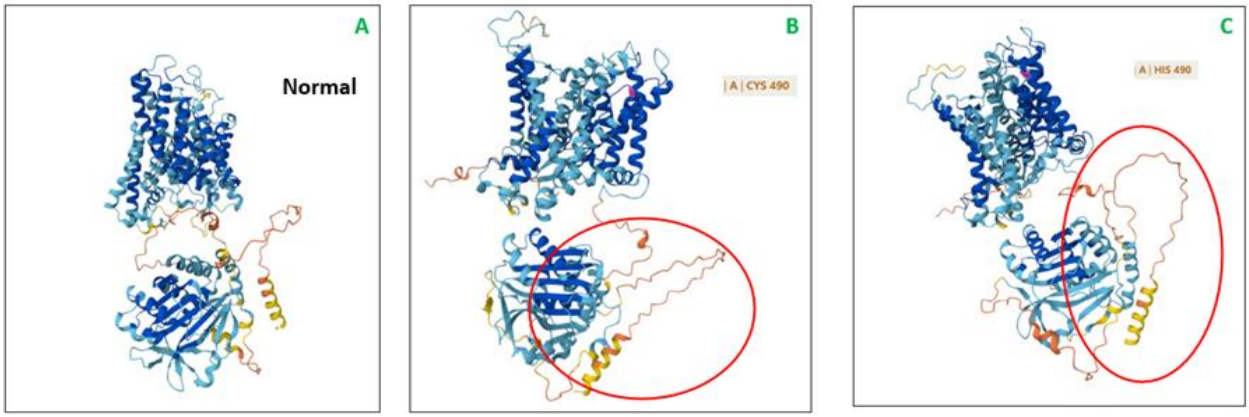

Figure 5. Mutagenesis of Arginine 490. (A) Wild-type structure. (B) R490C mutant. (C) R490H mutant. Conformational changes are circled in red.

The substitution of arginine with cysteine (Figure 5B) or histidine (Figure (5C) leads to significant distortion of the N-terminal helix-loop and misalignment of the C-terminal domain. These changes may impair protein-protein interactions and destabilize the cell membrane.

Replacing arginine 760 with glutamine or a bulky, hydrophobic amino acid like tryptophan also caused significant structural alterations (Figure 6).

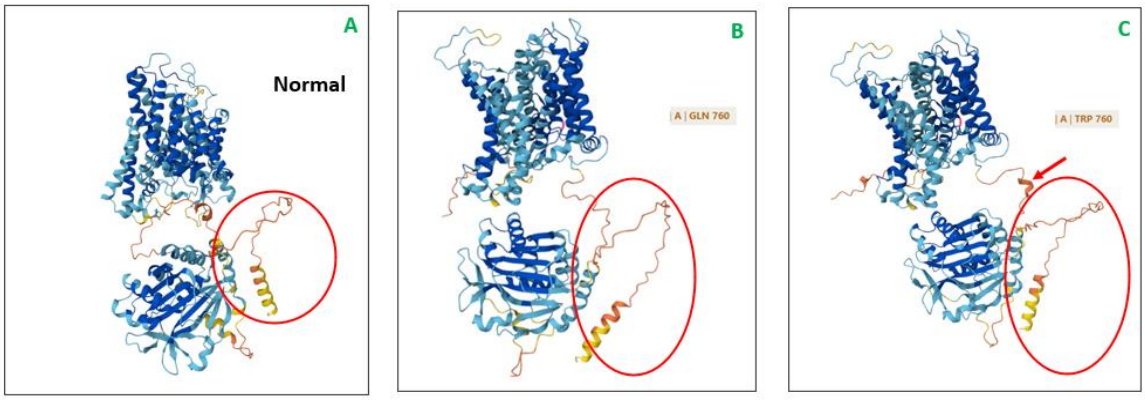

Figure 6. Mutagenesis of Arginine 760 (A) Wild-type structure. (B) R760Q mutant. (C) R760W mutant. Confirmational changes are circled in red.

Both the R760Q (Figure 6B). as well as R760W (Figure 6C) mutations disrupt the orientation between the N- and C-terminal domains The N-terminal loop shows altered curvature, suggesting compromised interaction with cytoskeletal elements or other membrane proteins.

When arginine 808 is replaced by a cysteine or histidine, drastic changes are seen in the N-terminal loop (Figure 7).

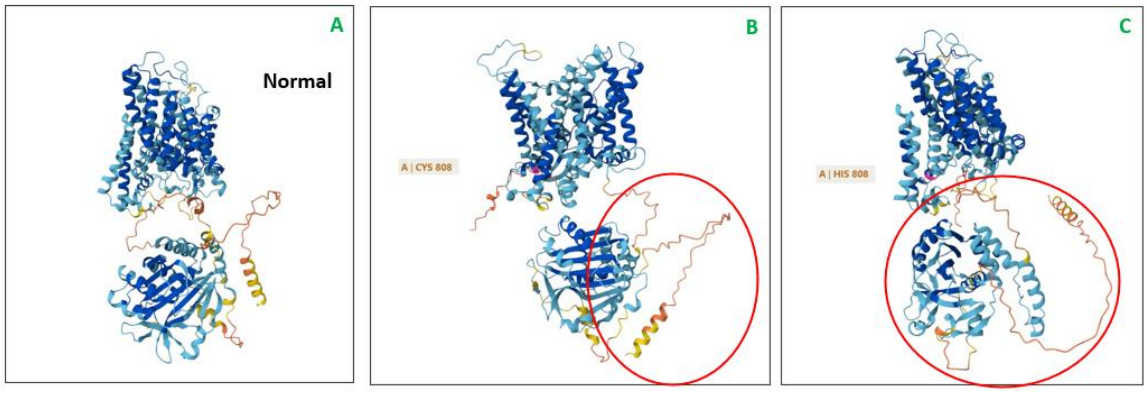

Figure 7. Mutagenesis of Arginine 808. (A) Wild-type structure. (B) R808C mutant. (C) R808H mutant. Structural changes are circled.

The R808C induces loop distortion (Figure 7B) while R808H mutation causes pronounced opening of the N-terminal domain (Figure 7C). These conformational shifts may affect membrane rigidity and anion exchange efficiency.

All these results suggests that the replacement of a positively charged arginine with a neutral residue (such as cysteine in R490C) or a highly hydrophobic residue (such as R760W and R870W) distorts the protein structure and disrupts its function.

Finally, substitution of arginines 518 and 870 with cysteine and tryptophan, respectively, also revealed significant structural changes (Figure 8).

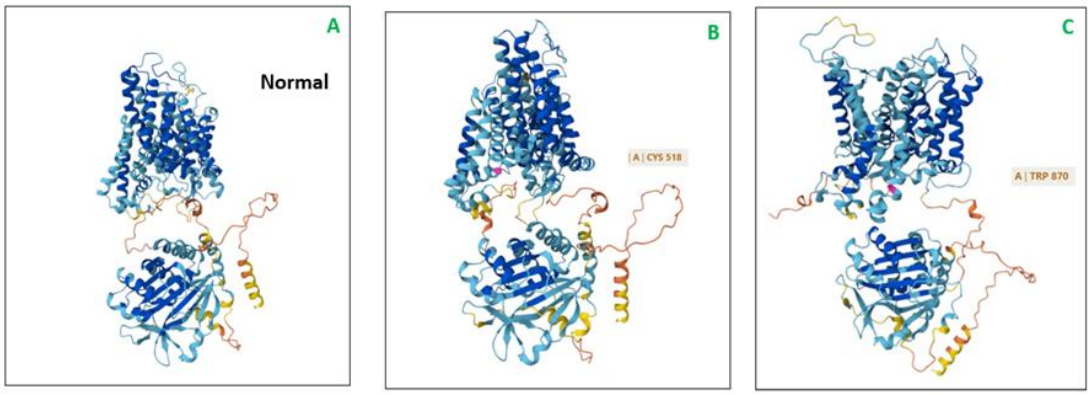

Figure 8.

Mutagenesis of Arg518 and Arg870. (A) Wild-type structure. (B) R518C mutant. (C) R870W mutant. Structural changes are circled.

R518C shows minimal domain disruption but alters the N-terminal loop (Figure 8B). R870W opens the C-terminal domain and modifies the N-terminal helix-loop, potentially affecting anion exchange activity and altering membrane curvature (Figure 8C).

The erythrocyte Band 3 protein has a total of 45 arginine residues (Figure 9).

MEELQDDYEDMMEENLEQEEYEDPDIPESQMEEPAAHDTEATATDYHTTSHPGTHKVYVE 60

LQELVMDEKN QELRWMEAAR WVQLEENLGE NGAWGRPHLS HLTFWSLLEL RRVFTKGTVL 120

LDLQETSLAG VANQLLDRFI FEDQIRPQDR EELLRALLLK HSHAGELEAL GGVKPAVLTR 180

SGDPSQPLLP QHSSLETQLF CEQGDGGTEG HSPSGILEKI PPDSEATLVL VGRADFLEQP 240

VLGFVRLQEA AELEAVELPV PIRFLFVLLG PEAPHIDYTQ LGRAAATLMS ERVFRIDAYM 300

AQSRGELLHS LEGFLDCSLV LPPTDAPSEQ ALLSLVPVQR ELLRRRYQSS PAKPDSSFYK 360

GLDLNGGPDD PLQQTGQLFG GLVRDIRRRY PYYLSDITDA FSPQVLAAVI FIYFAALSPA 420

ITFGGLLGEK TRNQMGVSEL LISTAVQGIL FALLGAQPLL VVGFSGPLLV FEEAFFSFCE 480

TNGLEYIVGR VWIGFWLILL VVLVVAFEGS FLVRFISRYT QEIFSFLISL IFIYETFSKL 540

IKIFQDHPLQ KTYNYNVLMV PKPQGPLPNT ALLSLVLMAG TFFFAMMLRK FKNSSYFPGK 600

LRRVIGDFGV PISILIMVLV DFFIQDTYTQ KLSVPDGFKV SNSSARGWVI HPLGLRSEFP 660

IWMMFASALP ALLVFILIFL ESQITTLIVS KPERKMVKGS GFHLDLLLVV GMGGVAALFG 720

MPWLSATTVR SVTHANALTV MGKASTPGAA AQIQEVKEQR ISGLLVAVLV GLSILMEPIL 780

SRIPLAVLFG IFLYMGVTSL SGIQLFDRIL LLFKPPKYHP DVPYVKRVKT WRMHLFTGIQ 840

IICLAVLWVV KSTPASLALP FVLILTVPLR RVLLPLIFRN VELQCLDADD AKATFDEEEG 900

RDEYDEVAMPV

Figure 9. Primary sequence of human red cell Band 3 (www.uniprot.org). Residues forming the N-terminal domain are highlighted in yellow. All arginine residues are in red except R730 and R901 which are required for catalytic activity. Naturally occurring variants are highlighted in teal blue.

As seen in Figure 9, the first 20 arginine residues are in the N-terminal domain (highlighted in yellow) while the rest fall in the C-terminal domain. Of the 25 arginine reidues in the C-terminal domain, Arg 730 and Arg901 have been shown to be crucial for anion exchange activity **[11]**. All the naturally occurring variants except for R490, are located on the cytosolic face of the protein (see Figure 10). This explains why the variants have a change in anion exchange activity.

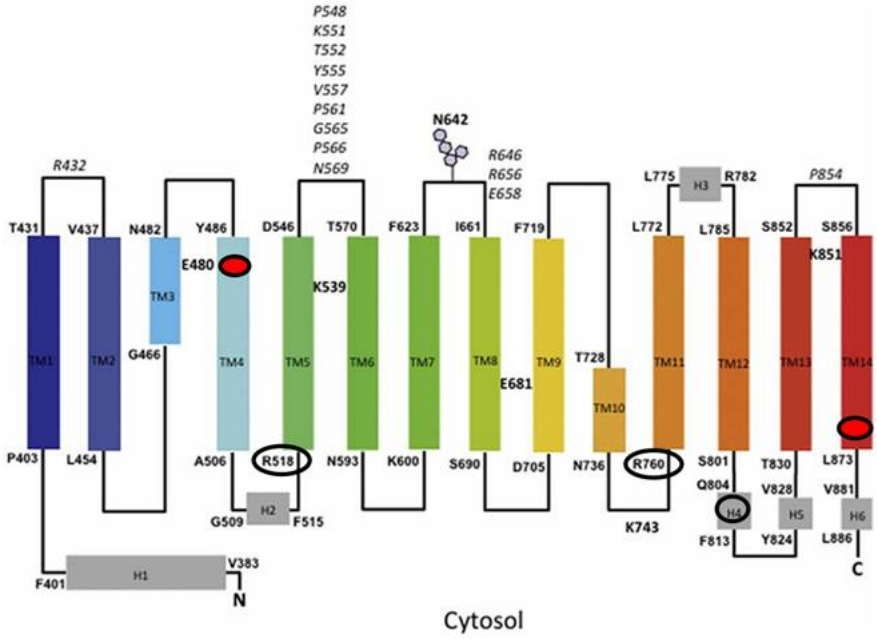

Figure 10. Linear representation of human red cell Band 3. The bands represent transmembrane helices and are linked by loops (lines). The positions of the naturally occurring variants is shown by black circles.

Our results with the *in silico* mutagenesis show that the N-terminal domain is sufficiently altered, which is why red cell morphology is affected in these variants, resulting in spherocytosis.
The question remains as to why red cell morphology is altered under conditions of vitamin B12. It is known that the N-terminal domain of Band 3 interacts via ankyrin and protein 4.1 with the spectrin- actin cytoskeleton. In the N-terminal domain, the RRRY motifs at positions 344-337 and 497-500 are critical for this interaction between Band 3 and ankyrin and protein 4.1 **[12]**. Both electrostatic and hydrophobic interactions have been implicated in stabilising these protein-protein interactions. It is, therefore, attractive to speculate that methylation of arginine(s) in the RRRY motif increases its hydrophobicity, leading to the normal biconcave shape of the red blood cell. Under B12 deficient conditions, it is possible that insufficient arginine methylation disrupts protein-protein interaction, leading to distortion in the cytoskeletal scaffolding and the formation of poikilocytes.
In conclusion, we find that *in silico* analysis makes it possible to correlate B12 dependent arginine methylation, especially that of Band 3, with erythrocyte morphology. The absence of high molecular weight bands in B12 deficient samples needs to be investigated with a larger sample size.

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4. STATEMENTS & DECLARATIONS

AI Statement: The authors declare that they have not used generative artificial intelligence, specifically ChatGPT, in the writing of this manuscript and/or in the creation of images, graphics, tables, or their corresponding captions.

Authorship Contribution: Anjana Francis Thevarmadom: Carrying out the data collection, data curation, and writing the original manuscript. Vinita Balasubramanya: Reviewing the draft and supervision.

Ethical Standards: All the ethical research standards were followed while writing this conceptual paper.

Conflict of Interest: The authors state that they do not have any conflict of interest.

Informed Consent / Ethical Compliance: As this is a conceptual paper, no consent is required.

Human or animal involvement in the article: None

Data Availability: All data included in this research article will be provided on request.

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