بسم الله الرحمن الرحيم
Ischemia modified albumin in Beta TM: A new marker for an old problem

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Thalassemia is a hereditary anemia resulting from defects in hemoglobin production. A mutation of β-globin gene leads to a defective β chain production.

This defect leads to an imbalance in α/β globin synthesis as insufficient β-globin production results in the accumulation and precipitation of unpaired α-globin chains,

- Ineffective erythropoiesis
- Chronic hemolytic anaemia
- Iron overload
These changes lead to instability of the generated haemoglobin or to globin chain imbalance, which in turn impact the oxidative environment both intracellularly and extracellularly.

The resulting oxidative stress and the inability of the body to adequately overcome it are, to a large extent, responsible for the pathophysiology of the disease.
Patients with β-TM are under significant iron driven oxidative stress.

Many studies reported increased blood levels of the redox active fractions of non-transferrin bound iron (NTBI) and labile plasma iron (LPI) in patients with β-TM.

β-TM Patients experience decreased antioxidant capacity and increased products of peroxidative damage.
Secondary iron overload in transfusion-dependent (TD) β-thalassemia patients is commonly caused by increased dietary iron absorption and repeated blood transfusions.

This leads to the generation of labile iron at the inner and outer cell surfaces of the red blood cell.

Consequently, the excessive active iron catalyzes the production of a variety of reactive oxygen species (ROS), leading to cumulative cell damage and inflammatory changes.
Therefore, effective iron chelators are required to remove the toxic irons to prevent oxidative damage in the vital organs, particularly the heart and liver.

Chelators can act upon different iron pools, including:

- transferrin-bound iron,
- non-transferrin-bound iron
- labile plasma iron in plasma compartment,
- labile plasma iron in cytoplasm

To form iron-chelate (s), which will then be excreted in the urine and feces
Oxidative stress is defined as the interruption of balance between oxidants and reductants within the body due to the excess production of peroxides and free radicals. This imbalance will cause damage to cellular components and tissues which occurs as a result of increased levels of lipid peroxides and free-radical intermediates, as well as the decrease in total antioxidant capacity.
In thalassemia, there is excess production of reactive oxygen intermediates

superoxide anion ($O_2^-$), hydroxyl radical (OH·), singlet oxygen and hydrogen peroxide ($H_2O_2$) within the erythrocytes,

all these events lead to oxidative stress.

This oxidative stress and a possible consequential accelerated apoptosis

contribute to shortened life span of erythrocytes
1) Oxidative denaturation of Hb results in the production of ROS, free haeme and iron. Iron acts as a Fenton reagent for the generation of hydroxyl radical. Haeme promotes oxidation reactions and a proinflammatory effect by activating NF-κB. (2) Enzymatic generation of superoxide by NADPH oxidase. (3) ROS and ROS-induced increase of intra-cellular Ca$^{2+}$ activate caspase-3, which partially degrades band-3, affecting its interaction with the cytoskeleton. (4) Haemichromes mediate the oxidative crosslinking and phosphorylation of band-3 leading to band-3 clusterisation and dissociation from cytoskeletal proteins. This results in membrane blebbing and microparticle generation. (5) ROS promote oxidation of protein 4.1, actin and spectrin resulting in impaired interaction. (6) PS exposure results from ROS-induced disruption of normal membrane organisation.
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5) ROS promote oxidation of protein 4.1, actin and spectrin resulting in impaired interaction.

6) PS exposure results from ROS-induced disruption of normal membrane organisation.
1) Haptoglobin/Hb and haemopexin/haeme complexes are endocytosed by macrophages. Haeme is then degraded releasing biliverdin, CO and iron. Iron is then taken up by ferritin. (2) Antioxidant enzymes and molecules in RBCs. (3) Stress-response mechanisms in RBCs. FOXO3, FOXO4

Protein quality control pathways. Unpaired α-globins are selectively degraded via proteasomal degradation (UPS).
Oxidative stress and ineffective erythropoiesis

Ineffective erythropoiesis is well documented in β-thalassaemia and is attributed to the presence of excess unpaired α-globins.

Unpaired α-globins have the tendency to precipitate and autoxidise producing high amounts of ROS, free haeme and iron, these molecules can promote oxidation of membrane lipids and proteins resulting in band-3 clusterisation and PS exposure.

These are signs of apoptosis of RBCs, which are then removed by macrophages. Several studies have shown that there is an increased number of activated macrophages in the bone marrow of β-thalassaemia patients, indicative of enhanced apoptosis of erythroid precursors.

It have demonstrated the presence of α-globin precipitates in cells at the polychromatophilic erythroblast stage of mauration which is the stage of increased haemoglobinization and apoptosis in β-thalassaemia erythropoiesis.
1. Erythroid expansion.
2. Ineffective erythropoiesis.
3. Endocytosis of RBCs by macrophages through two different mechanisms: eryptosis and senescence.
4. Haemolysis. It leads to Hb release in the plasma, which subsequently autoxidises producing ROS, free haeme and iron.
5. ROS, free haeme and iron intercalate into plasma membranes producing oxidative damage to the endothelium and haematopoietic cells. ROS and haeme activate the production of pro-inflammatory cytokines (IL-1, IL-6, TNFα) and adhesion molecules on the endothelium. This increases the adhesion of RBCs, leucocytes and platelets to the endothelium. Activated leucocytes generate more ROS by their NADPH oxidase, creating a vicious cycle of oxidative stress and inflammation.
6. Reduction of NO bioavailability due to free Hb, ROS, and neutrophil activation.
7. Increased expression of plasma and endothelial enzymes gives rise to more ROS.
ROS implication in erythroid expansion and accelerated differentiation

In β-thalassaemia, ineffective erythropoiesis and haemolysis give rise to chronic anaemia, which results in tissue hypoxia and subsequent increase in EPO production.

This in turn leads to enhanced extramedullary and bone marrow erythropoiesis, which is characterised by increase erythroid proliferation and accelerated differentiation.

A recent study has implicated ROS in the increased proliferation and differentiation of β-thalassaemia/HbE erythroid precursors.

A model where an increase in aerobic respiration would result in increased levels of ATP. This could lead to an increase in Ca$^{2+}$ intracellular levels possibly through activation of Ca$^{2+}$ pumps. High Ca$^{2+}$ levels stimulate oxidative phosphorylation resulting in further increase of ATP.

ATP can directly or indirectly, through cAMP or Ca$^{2+}$, activate several effectors which are by-products of aerobic metabolism, are also able to activate these effectors, which in turn can increase erythroid proliferation and differentiation both directly or indirectly.

**Increased erythroid proliferation and differentiation lead to increased metabolic rates creating a feedback loop. Therefore even a small initial imbalance in ROS could rapidly result in a significant negative outcome.**
Iron overload in β-thalassaemia

Iron overload is well documented in β-thalassaemia. It is caused by the increased intra- and extra-cellular oxidative denaturation of Hb and subsequent iron release, the recurrent blood transfusions, and the enhanced absorption from the gastrointestinal (GI) tract due to the decreased hepcidin levels observed in β-thalassaemia.

Hepcidin is produced in the liver and binds to the iron exporter ferroprotein on the enterocytes, macrophages and liver cells, mediating its internalisation and subsequent degradation preventing iron export from these cells.
Hepcidin is regulated by:

- iron status,
- inflammation,
- hypoxia
- erythropoietic iron demand

Its levels are decreased in β-thalasseamia due to the ineffective erythropoiesis and subsequent enhanced erythropoietic activity, which increases iron demand.

Oxidative stress has been implicated in both ineffective erythropoiesis and erythroid expansion.
Recent findings support the role of an erythroid regulator on the reduction of hepcidin expression.

The erythroid regulator is possibly a factor that is secreted from proliferating or apoptotic precursors.

TWSG1 (twisted gastrulation 1), isolated from immature precursors in β-thalassaemia mice, and GDF15, found in the plasma of patients with β-thalassaemia or other diseases characterised by ineffective erythropoiesis, are two of the factors considered to play a role in down-regulation of hepsidin expression.
At high levels iron is an extremely toxic molecule. It can exit circulating RBCs and migrate from the plasma to haematopoietic and endothelial cells as well as various organs where it can promote oxidative reactions.

**Myocardial affection, liver cirrhosis, and endocrine** complications are among the long term consequences of iron overload. Excess iron absorbed from the GI travels to the liver through the portal vein and thus liver fibrosis occurs as iron promotes collagen synthesis.

Transfusional iron on the other hand first accumulates in the reticuloendothelial system and is then transferred to parenchymatous organs such as the heart and endocrine organs.

Interestingly, the transport of NTBI to organs is faster than that of transferrin bound iron leading to increased iron deposition and subsequent oxidative organ damage.

Moreover, the myocardium and endocrine tissues, unlike other tissues, have L-type voltage dependant calcium channels that transport NTBI and this possibly justifies the pattern of transfusional iron deposition in certain organs.
In β-thalassemic erythropoiesis ROS induces peroxiredoxin-2 (PRDX2) expression.

In the early stage of β- thalassemic erythropoiesis, ROS and heme levels are both increased and PRDX2 acts on both targets; immature cells,

when ROS levels are still high and heme levels are reduced, ROS might become the PRDX2 major target.

ROS promotes proteins activation results in phosphorylation of the α-subunit, an important regulatory translation initiating factor, which inhibits the α-, β-globin chain synthesis and activates the pathway towards redox genes such as heme-oxygenase-1, glutathione S-transferase and NAD(P)H quinone oxidoreductase

The upregulation of these genes in combination with the decrease in α-, β-globin chain synthesis might beneficially affect the ineffective erythropoiesis of β-thalassemia. The α chains (AHSP, α hemoglobin-stabilizing protein) is another cytoprotective system, which partially protects the erythroid precursors from the α chain excess. AHSP binds free α-globin chains, stabilizing their structure. AHSP prevents their precipitation and might be important in β-thalassemic erythropoiesis characterized by unbalance in globin chain synthesis (heme-regulated inhibitor of protein translation (HRI) that represses globin translation

Schematic model of novel cytoprotective mechanisms in response to oxidative stress in β-thalassemic (-βthal) erythroid precursors.
Lipids are susceptible targets of oxidation because of their molecular structure abundant with reactive double bonds. Due to the presence of double bonds, polyunsaturated fatty acid, is highly reactive towards free radicals mainly hydroxyl radical.

Two reaction mechanisms are proposed for lipid peroxidation as hydroxyl radical dependent and hydroxyl independent. Fe$^{2+}$ acts as a positive catalyst for the production of hydroxyl radical dependent lipid peroxidation. Polyunsaturated fatty acids (PUFAs) are mainly targeted by these radicals due to the presence of double bonds and produce polyunsaturated fatty acid-free radicals.

The resulting lipid hydroperoxides can affect membrane fluidity and membrane protein function.
Ischemia modified albumin:

- An altered type of serum albumin that forms under conditions of oxidative stress.
- The N-terminus residues of human serum albumin tend to bind with transition metals such as cobalt, copper and nickel.
- Alterations in this region of albumin hinder its binding capacity to such elements.
Overproduction of ROS resulting from conditions related to:

- Ischemia
- Hypoxia
- Acidosis
- Free radicles
- Free Iron

Play a major role in the formation of IMA
Ischemia modified albumin (IMA) is currently used as an early marker for myocardial ischemia and acute coronary syndrome.

Recently, it was found that it is an end product of oxidative stress.

Accordingly, it has been suggested that elevated levels of IMA may reflect a generalized rather than organ or tissue-specific state of oxidative stress.
Role of IMA in disease

Elevated in the following diseases:

- Ischemic heart disease.
- Type 2 diabetes.
- Hyperlipidemia.
- Chronic kidney disease.
- Mutiple sclerosis.

Thalassemia?
To measure the levels of IMA in β-thalassemia major patients as a marker of oxidative stress, and to assess its relation to lipid peroxidation as well as the effect on vascular complications, subclinical atherosclerosis and efficacy of iron chelation.
This study was carried out in Pediatric Hematology Clinic, Pediatric Hospital, Ain Shams University including:

- **Patients**: 45 Patients with β-thalassemia major
- **Controls**: 30 age- and sex-matched healthy subjects
Methods:

- **Detailed medical history:**
  - Age at onset and disease duration.
  - Family history of thalassemia or anemia.
  - Transfusion history.
  - Therapy.
  - History of splenectomy.
  - History of viral hepatitis.

- **Thorough clinical examination.**
Radiological examination:

**Echocardiography**
- EF, FS.
- Tricuspid regurgitant jet velocity.

**Carotid intima media thickness (CIMT):**
- Carotid scans were done for all patients and Mean values for RT and LT CIMT was calculated.
Laboratory investigations:

- CBC.
- Liver functions (total and direct bilirubin, ALT, AST and LDH).
- Serum ferritin.
- Measuring serum levels of IMA by enzyme linked immunosorbent assay (ELISA).
- Measuring serum levels of MDA by production of Thiobarbituric Acid Reactive Substances (TBARS).
Clinical data of the studied β-TM patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>β-TM patients (n=24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive family history of anemia, n(%)</td>
<td>27(60)</td>
</tr>
<tr>
<td>Disease duration</td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>12.9±3.2</td>
</tr>
<tr>
<td>Range</td>
<td>3-16</td>
</tr>
<tr>
<td>Transfusion index (ml/kg/ year)</td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>391.6±118.1</td>
</tr>
<tr>
<td>Range</td>
<td>120-480</td>
</tr>
<tr>
<td>Heart disease , n (%)</td>
<td>9 (20)</td>
</tr>
<tr>
<td>Pulmonary hypertension risk , n (%)</td>
<td>10 (22.2)</td>
</tr>
<tr>
<td>Viral hepatitis , n (%)</td>
<td>24 (53.3)</td>
</tr>
<tr>
<td>Splenectomized, n (%)</td>
<td>24 (53.3)</td>
</tr>
<tr>
<td>Compliance to chelation, n (%)</td>
<td></td>
</tr>
<tr>
<td>Good</td>
<td>18 (40)</td>
</tr>
<tr>
<td>poor</td>
<td>27(60)</td>
</tr>
</tbody>
</table>
Comparison between $\beta$-TM patients and control as regard both IMA and MDA levels

<table>
<thead>
<tr>
<th>Variable</th>
<th>Controls (n=30)</th>
<th>$\beta$- TM (n=45)</th>
<th>Test value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IMA (U/ml)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>5 (3-5) 1-8</td>
<td>18 (13-40) 10-130</td>
<td>-5.795</td>
<td>$&lt;0.001^*$</td>
</tr>
<tr>
<td><strong>MDA (nmol/ml)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>10 (7-15) 5-18</td>
<td>30 (20-45) 10-112</td>
<td>-5.334</td>
<td>$&lt;0.001^*$</td>
</tr>
</tbody>
</table>
IMA levels in patients with β-TM and controls

MDA levels in patients with β-TM and controls

P < 0.001
### IMA and MDA levels in relation to clinical characteristics of β-TM patients; PH risk: pulmonary hypertension

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients number</th>
<th>IMA (U/mL)</th>
<th>MDA (nmol/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
</tr>
<tr>
<td><strong>Puberty</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delayed</td>
<td>30 (66.7)</td>
<td>18.75 (15 – 80)</td>
<td>32.5 (20 – 60)</td>
</tr>
<tr>
<td>Appropriate</td>
<td>15 (33.3)</td>
<td>15 (12.5 – 17.5)</td>
<td>25 (20 – 35)</td>
</tr>
<tr>
<td><strong>Splenectomy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>24 (53.3)</td>
<td>17.5 (15 – 77.5)</td>
<td>30 (20 – 45)</td>
</tr>
<tr>
<td>Negative</td>
<td>21 (46.7)</td>
<td>15 (1.5 – 20)</td>
<td>30 (20 – 45)</td>
</tr>
<tr>
<td><strong>Heart disease</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>9 (20)</td>
<td>17.5 (15.0 – 77.5)</td>
<td>30 (20 – 50)</td>
</tr>
<tr>
<td>Negative</td>
<td>36 (80)</td>
<td>12.5 (12.5 – 17.5)</td>
<td>23 (20 – 45)</td>
</tr>
<tr>
<td><strong>PH risk</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>10 (22.2)</td>
<td>80 (17.5 – 100)</td>
<td>30 (20 – 65)</td>
</tr>
<tr>
<td>Negative</td>
<td>35 (77.8)</td>
<td>15 (12.5 – 20)</td>
<td>27.5 (20 – 45)</td>
</tr>
<tr>
<td><strong>Serum ferritin (µg/L)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥2500</td>
<td>24 (53.3)</td>
<td>60 (2 – 90)</td>
<td>40 (30 – 65)</td>
</tr>
<tr>
<td>&lt;2500</td>
<td>21 (64.7)</td>
<td>15 (12.5 – 17.5)</td>
<td>20 (16.5 – 35)</td>
</tr>
</tbody>
</table>
IMA levels in β-thalassemia major patients with heart disease and pulmonary hypertension risk
Correlation between IMA and the studied variables among β- TM patients
Correlation between IMA and MDA levels and serum ferritin among patients with β-TM
Correlation between IMA and echocardiography parameters among patients with β-TM

\[ r = 0.412; \ p = 0.014 \]

\[ r = 0.621; \ p = 0.008 \]
Correlation between IMA and MDA levels and mean CIMT among patients with β-TM

![Graph 1: IMA (U/mL) vs. Mean CIMT (mm)]

- $r = 0.607; p < 0.001$

![Graph 2: MDA (nmol/mL) vs. Mean CIMT (mm)]

- $r = 0.590; p < 0.001$
Multiple linear regression analysis for variables related to increased IMA levels in patients with β-TM

<table>
<thead>
<tr>
<th>Variable</th>
<th>Unstandardized coefficients</th>
<th>Standardized coefficients</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>Standard Error</td>
<td>Beta</td>
</tr>
<tr>
<td>(constant)</td>
<td>-178.488</td>
<td>560.018</td>
<td>0.804</td>
</tr>
<tr>
<td>Disease duration (year)</td>
<td>3.182</td>
<td>3.970</td>
<td>0.224</td>
</tr>
<tr>
<td>WBC (x 10⁹/L)</td>
<td>0.034</td>
<td>0.209</td>
<td>0.022</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>0.781</td>
<td>0.369</td>
<td>1.880</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>0.665</td>
<td>0.325</td>
<td>1.900</td>
</tr>
<tr>
<td>Serum ferritin (µg/l)</td>
<td>0.008</td>
<td>0.006</td>
<td>0.576</td>
</tr>
<tr>
<td>MDA (nmol/mL)</td>
<td>0.061</td>
<td>0.001</td>
<td>0.552</td>
</tr>
<tr>
<td>EF (%)</td>
<td>-0.018</td>
<td>0.07</td>
<td>-0.687</td>
</tr>
<tr>
<td>TRV (m/s)</td>
<td>0.099</td>
<td>0.011</td>
<td>0.464</td>
</tr>
<tr>
<td>Mean CIMT (mm)</td>
<td>0.012</td>
<td>0.008</td>
<td>0.518</td>
</tr>
</tbody>
</table>
IMA levels are significantly high in β-TM patients due to iron overload and oxidative stress mechanisms.

IMA is correlated with MDA and may be considered a promising marker of oxidative stress in those patients.
IMA is elevated in patients with cardiopulmonary complications and significantly correlated with echocardiographic parameters. Thus, it could be useful for screening of patients at risk of cardiac complications because this alteration occurs in early stage subclinical cardiac disease.

The positive correlation between CIMT and IMA suggests that it could be used as a marker of vascular dysfunction and subclinical atherosclerosis in β-TM patients. Our results highlight the role of oxidative stress in the pathophysiology of vascular complications in thalassmeia.
The oxidative stress plays a central role in the pathogenesis of anemia in β-thalassemia. The emerging picture for treatment of β-thalassemia is that abnormalities ranging from red cell membrane proteins structure and function and membrane ion transport pathways to novel cytoprotective systems in erythropoiesis might constitute new pharmacological targets for treating β-thalassemia.

Future studies should be designed to evaluate in vivo novel antioxidant strategies with multitarget effects on both mature β-thal red cells and erythropoiesis with the final goal to impact anemia of -β-thalassemia.
Thank You…