Original Article

Detoxification Genomics in Children with β-Thalassemia Major: Pilot Study of Glutathione S Transferase M1, Pi & Methyltetrahydrofolate Reductase Gene Polymorphisms Combinations in β-Thalassemia Major

Magd A. Kotb[1], Mona Hamdy[1], Khaled Eid[1], Mona Aziz[2], Mai Abd El Salam[1], Hend Abd El Baky [1]*, Nabil Lotfi[3], Niveen Salama[1]

[1] Department of Pediatrics, Faculty of Medicine, Cairo University, Egypt;
magdkotb@kasralainy.edu.eg, mona.hamdy@staff.cu.edu.eg, khaled.eid@netscape.net,
mai_abdelsalam84@yahoo.com, hend.elhossainy@cu.edu.eg, niveensab@yahoo.com
[2] Department of Clinical and Chemical Pathology, Faculty of Medicine, Cairo University, Egypt;
drmona16_16@hotmail.com.
[3] Faculty of Medicine, Cairo University, Egypt; nabil-lotfy@students.kasralainy.edu.eg.
* Correspondence: hend.elhossainy@cu.edu.eg.

Abstract:
Background: Children with β-thalassemia major differ regarding age at presentation, transfusion requirements and unpredictable timing, rate and severity of hemolytic crisis. The blood transfusions are associated with iron overload. Glutathione S transferase M1 (*GSTM1*) null mutation was reported to be associated with myocardial iron overload with low body iron.
Aim of the Work: To investigate if children with ß-thalassemia major have more than a detoxification enzyme defect.
Materials and Methods: *GSTM1*, glutathione S transferase Pi (*GSTPi)* and methyltetrahydrofolate reductase *(MTHFR)* polymorphism were studied among 97 children with β-thalassemia major in a cross-sectional study.
Results: The studied cohort comprised 24 (24.7%) girls and 73 (75.3%) boys. Mean hemoglobin was 5.9+/- 0.7gm%, serum iron was 145.69 +/- 58.6 mcg% and total iron binding capacity was 222.58 +/-50.66 mcg%. Of them, 68 (70.1%) demonstrated single or multiple polymorphisms (43 had *GSTM1*, 20 *GSTPi* and 32 with *MTHFR* polymorphisms respectively), while 29 (29.2%) did not demonstrate any polymorphism. There was no correlation between type, number of polymorphisms and clinical phenotype. Sample size and cross- sectional nature of our study did not allow genotype-phenotype correlation. Most of studied children express *GSTM1, GSTPi and MTHFR* gene polymorphism which was not consistent among them.
Conclusion: Children with ß-thalassemia major may have one or more than a detoxification/ regeneration potential enzyme gene *GSTM1, GSTPi and MTHFR* polymorphism. Every child with ß-thalassemia major has unique genetic detoxification and regeneration abilities. The detected detoxification defects might explain the lack of predictability of occurrence of hemolytic attacks and their severity. More studies are needed to highlight impact of detoxification and regeneration genomics in ß-thalassemia.

Level of Evidence of Study: III (*1*).
Keywords: Glutathione S transferase M1 (G*STM1*); glutathione S transferase Pi (*GSTPi*); methyltetrahydrofolate reductase **(***MTHFR*); polymorphism; β-thalassemia major.
Abbreviations: HBB: hemoglobin beta; *GSTM1*: Glutathione S transferase M1; *GSTPi*: glutathione S transferase Pi; *MTHFR*: methyltetrahydrofolate reductase.

Introduction

The gene hemoglobin beta (HBB) codes for the beta hemoglobin protein. It is located on chromosome 11 short arm 15.5. It is a multigene locus of ß-globin; yet, the expression of beta

globin is controlled by single locus control region (*2*). The HBB defects result in beta thalassemia that afflicts millions worldwide (*3, 4*).

It results in reduced or absent production of globin chains of hemoglobin with a clinical spectrum ranging from trait to severe hemoglobinopathy and subsequent development of lifelong chronic hemolytic anemia necessitating lifelong regular blood transfusions. Severe forms of ß-thalassemia on regular blood transfusions develop iron overload and are maintained on iron-chelation therapy (*5*).

It is the commonest single gene disorder in the world first noted in the Mediterranean population (*3, 6*). Yet children with ß-thalassemia have individual variations as regards age at presentation, transfusion requirements, number of hemolytic attacks, etc. The different phenotypes were reported to correlate with different ß-thalassemia genotype mutations (*4*). Complications such as iron overload vary as well (*7*). Detoxification is decreed by interaction of intoxicating material (*8*) and by host detoxification genomics (*9*). Host detoxification is decreed by the efficiency of cytochrome p 450, Glutathione S Transferase (GST) glucuronidation, detoxification super families and structural regeneration ability to resolve aftermath (*10–14*).

Detoxification abilities might contribute to pathogenesis and phenotype variation among thalassemia afflicted subjects. Hence, once a child with ß- thalassemia is exposed to a chemical that he cannot handle, the child would present by various clinical signs and symptoms related to the chemical, and it would be variable and not consistent with the ß- thalassemia genotype. In this work we aimed to investigate glutathione S transferase M1 (*GSTM1*), glutathione S transferase Pi (*GSTPi*) and methyltetrahydrofolate reductase (*MTHFR*) gene mutations in children with ß- thalassemia.

Subjects and Methods

Participants

This study was a single center cross sectional pilot study that included 97 consecutive children with documented ß-thalassemia major on regular blood transfusion and on iron chelation therapy. The study was approved by the Pediatric Department Committee for Post-Graduate Studies and Research, Faculty of Medicine, Cairo University, Egypt. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008.

Methods

GSTM1, GSTPi and MTHFR polymorphism were studied in 97 children with documented ß-thalassemia major. All blood samples were withdrawn on the day of blood transfusion, prior to the transfusion and during the cannulation process. The study was conducted at New Children Hospital, Cairo University, Egypt. Gene testing results were compared to the serum iron and TIBC of the studied children.

GSTM1 Polymorphism:

Whole peripheral blood was the source for DNA isolation using Qiagen. The polymorphic detection of GSTM1 gene was typed using the multiplex PCR (*10, 11*). The PCR primers used were as follows: P1: 5'CGCCATCTTGTGCTACATTGCCCG, P2: '5ATCTTCTCCTCTTCTGTCTC and P3: '5TTCTGGATTGTAGCAGATCA. P1 and P3 amplify a 230 bp product that is specific to GSTM1, whereas P1 and P2 anneal to GSTM1 and GSTM4 genes, yielding a 157 fragment that serves as an internal control. PCR was performed to 20ml containing 20 ng of genomic DNA, 0.5µmol/L of primer, 200 µmol of each dNTPs, 10mmol/L Tris Hcl (pH 8.3), 50 mmol/Kcl, 1.5 mmol/L Mg cl 2 and 0.5 U of amphitaq DNA polymerase (promega). After denaturation for 4 min at 94ºC, the PCR was performed for 35 cycles of 30 seconds at 94ºC, 1min at 58ºC and 1min at 72ºC. The last elongation step was 7 min. The presence of one or both GSTM1 allele identified by a 230 bp, or its complete deletion (null type) was analyzed by electrophoresis using 1.2% agarose gel. The absence of amplifiable GSTM1 (in the presence of the GST4 amplified control) indicated a null genotype.

GSTPi Polymorphism:

PCR assay adopted the method described by Harries and colleagues. The primers used were as follows: P105F: 5'ACC CCA GGG CTC TAT GGG AA-3', P105R: 5'- TGA GGG CAC AAG AAG CCCCT-3'. It detected single 176 base pair fragment (homozygous wild type), 91 and 85 base pair fragments (homozygous polymorphic) and 176, 91 and 85 base pair fragments (heterozygote) (*12*).

Genotype Analyses of the MTHFR 677:

DNA was extracted from the whole blood (5 ml sample) with a QIAamp DNA blood Mini Kit (Qiagen, Valencia, CA). MTHFR 677 genotyping was performed (*13, 14*). For the C→T polymorphism, extracted DNA was amplified with the forward primer 5′-TGA AGG AGA AGG TGT CTG CGG GA-3′ and reverse primer 5′- AGG ACG GTG CGG TGA GAG TG -3′. Polymerase chain reaction (PCR) thermal cycling conditions were 2-minute denaturation at 94ºC, then 40 cycles at 94ºC for 30 seconds, 62ºC for 30 seconds and finally 72ºC for 30 seconds. This was followed by 7-minute extension at 72ºC. Amplified 198-bp PCR products were digested with Hinf 1 (Fermentas) and were visualized under electrophoresis on 4% agarose gel with ethidium bromide. The C allele produced 198-bp band, and the T allele produced 175- and 23-bp fragments.

Statistical Analysis

All the statistical analyses in this study were conducted using Statistical Package for Social Sciences version 15 (SPSS, Chicago, Ill). Simple frequency, cross-tabulation, descriptive analysis, and tests of significance (t test for parametric data and Chi x^2 for non-parametric data) were used. Data are presented as mean +/- standard deviation (SD). To annul confounding effects of medicines, all children were compliant on iron chelation, and all were on regular blood transfusions. We relied upon historical Egyptian control group for comparison (*15–17*).

Results

This cross-sectional study included 97 children with known thalassemia major whose ages ranged between 3-15 years (mean=8.85 +/- 3.7years). The cohort comprised 24 (24.7%) girls and 73 (75.3%) boys. They were on iron chelation at least 6 months prior to enrollment. Patients who were not on iron chelation at least 6 months prior to the study were excluded from the study. They were on regular monthly transfusions. Their mean hemoglobin level was 5.9+/- 0.7 gm%, serum iron was 145.69 +/- 58.6 mcg% and total iron binding capacity was 222.58 +/-50.66 mcg%. There was no correlation between serum iron and any of the mutations. They all suffered repeated attacks of hemolytic crises. Of them 68 (70.1%) demonstrated single or multiple polymorphism(s) (43 had *GSTM1*, 20 *GSTPi* and 32 with *MTHFR* polymorphisms respectively), while 29 (29.2%) did not demonstrate any polymorphism to studied mutations. No correlation between type and number of polymorphisms and phenotype was detected. There was no difference between those with polymorphisms of *GSTM1* and *GSTPi*, as regards disease severity, response to chelation or blood transfusion requirements.
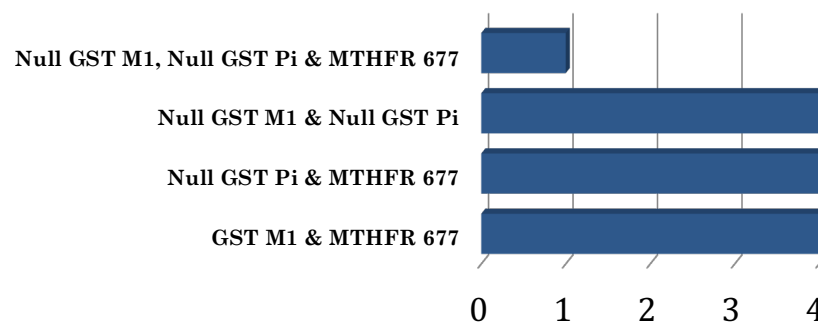

Figure (1): Combinations of homozygous multiple polymorphisms in studied cohort with β-Thalassemia Major.

Genotyping

Among the studied cohort, genotyping for *GSTM1*, *GSTPi*, *MTHFR 677* polymorphism was performed in 86, 49 and 46 children respectively. Only 19 underwent genotyping for the 3 mutations, 46 for 2 gene mutations, and 32 for a single gene mutation (Tables 1,2 and figure 1). GSTM1 polymorphism was detected in 43 (50%) compared to 40(54.5%) among historical controls (1-17) (p=0.800), *GSTPi* polymorphism was detected in 40.8% compared to 43.9% among historical controls (17) (p=0.11) and *GSTPi* polymorphism was detected in 69.6% compared to 30% among historical controls (18) (p=0.0001) (Figure 2).
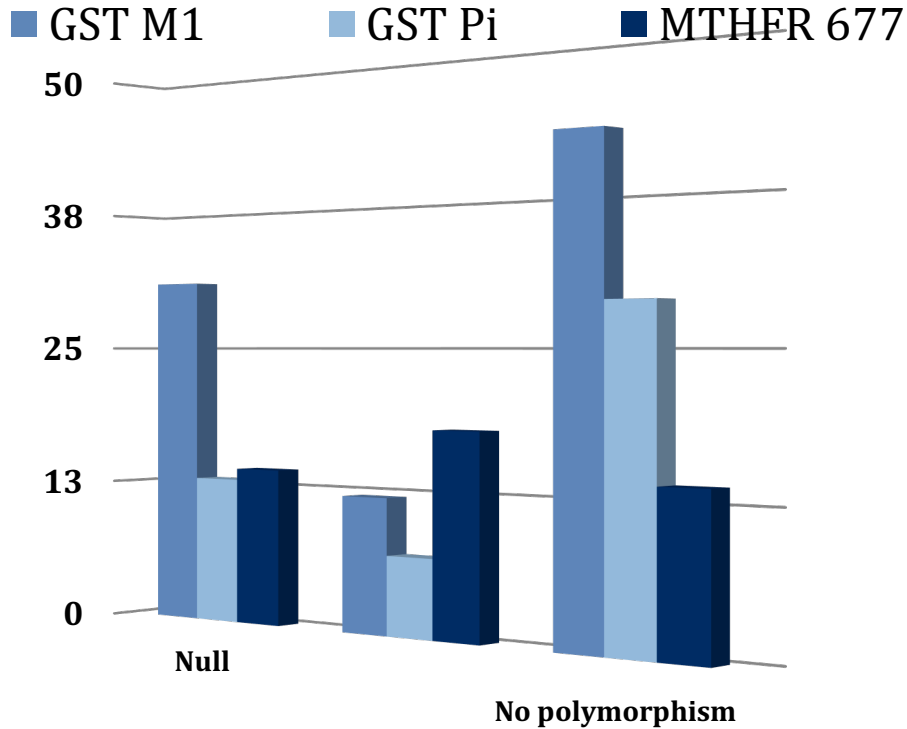
Figure (2): Glutathione S Transferase M1, Pi & Methyltetrahydrofolate Reductase Gene Polymorphisms Combinations in the studied cohort with β- Thalassemia Major.

Table (1): Types of gene polymorphisms detected among the studied cohort with β- Thalassemia Major.

		Gender		Total	*P* value
		Males	**Females**		
GSTM1 (86 children)	**Null**	26	5	31	
	Heterozygous	9	3	12	0.378
	No polymorphism	30	13	43	
GSTPi (49 children)	**Null**	8	5	13	
	Heterozygous	5	2	7	0.323
	No polymorphism	24	5	29	
MTHFR 677 (46 children)	**Null**	10	4	14	
	Heterozygous	15	3	18	0.462
	No polymorphism	10	4	14	

GST: glutathione S transferase, MTHFR 677: methyltetrahydrofolate reductase 677.

Table (2): Number of gene polymorphisms detected among the studied cohort of children with β- Thalassemia Major.

	Total		Gender		*P* value
	N	**%**	**Males**	**Females**	
Single mutation	44	45.5	34	10	0.430
Two mutations	23	30.9	19	4	0.261
Three mutations	1	1	0	1	0.284
Single heterozygous mutation	29	29.9	22	7	0.572
Two heterozygous mutations	5	5.2	5	0	0.233
Three heterozygous mutations	0	0	0	0	
Single null mutation	34	35.1	27	7	0.287
Two null mutations	12	12.3	9	3	0.529
Three null mutations	1	1	0	1	0.248
Heterozygous & null mutations	12	12.3	10	2	0.386

N= number.

Discussion

GST is a super family responsible for second tier hepatic detoxification especially of xenobiotics. Hence, GST superfamily members are responsible for detoxification of chemicals not naturally present or produced, or those chemicals produced in concentrations more than needed by human body (*18*). GST is distributed in cytosol, mitochondria and microsome. GST abnormalities are associated with many diseases, such as malignancies, neurodegenerative diseases e.g. parkinsonism, immune diseases e.g. diabetes and asthma. GST detoxifies a wide variety of compounds including aflatoxins, polycyclic aromatic hydrocarbons, drugs (*18*, *19*) and other environmental toxins. Genetic detoxification defect is seen as a host susceptibility factor that works only in presence of a specific type of toxin and its amount. Hence 50% of Egyptian children with diabetes have almost 50% deletions of *GSTM1*, which is comparable to the general population, yet an unforeseen factor seems to make these children develop the diabetes type 2 disease. In the same study, the children with type 2 diabetes had other detoxification defects as *GSTT1* and *GSTPi*. Hence development of a disease phenotype might prove to be the result of exposure to a specific chemical in an amount that cannot be handled by the dysfunction of detoxification of the host. This trilogy theory explains the individual peculiarities within a general phenotype spectrum (*20*, *21*). Our work provides more evidence of detoxification defects that confer host susceptibility, but are not the sole determinant factor of disease pathogenesis, clinical picture, march or outcome. It seems plausible that future research addresses specific effects of chemical exposure-host interaction as a cause of specific disease phenotype. Again the amount of toxin and duration of exposure is a well-known determinant of phenotype; those acutely exposed to bigger amount of cyanide will most probably die, while those who are exposed chronically in small amounts to cyanide will have different neurologic, cardiac, chest and hematologic symptoms and signs (*22*).

GSTM1 and *GSTPi* polymorphism were not a constant finding in our studied cohort of children with ß-thalassemia. There was no difference between those with polymorphisms of *GSTM1* and *GSTPi* as regards disease severity, response to chelation or blood transfusion requirements. As our study was a cross-sectional one, it remains to be seen if GST polymorphism affects each child natural history, long term response to treatment or susceptibility to complications. In any case, it was already reported that *GSTM1* null genotype was associated with increased myocardial iron as evident by MRI, but not unanimous increase in all studied cases,

suggesting that genetically determined loss of *GSTM1* function would increase susceptibility to cardiac iron deposition in some children with ß-thalassemia (*23*).

Serum iron represents the transferrin bound iron and not the tissue stored iron as ferritin. Is the detoxification enzyme deficiency or partial deficiency related to iron detoxification only? Many studies unravel that interaction of genetic host susceptibility and exposure to environmental specific toxin(s) dictate clinical phenotype (*8, 18, 21*). Interaction between time of exposure and ontogeny maybe another phenotype-determining factor; exposure during fetal, perinatal, or during infancy and childhood, etc. Ontogeny is the chronologic timing of biologic programmed events during a life time of a being (*24*). Hence, the detected detoxification deficiencies might be related to chemicals besides iron that subjects with ß- thalassemia major cannot handle (*25*), therefore the clinical picture and phenotype would be related to quantity and timing of exposure to this chemical if it ever happens. The detoxification defects noted in our study cannot be assumed to be the only defects, as genotyping of other members of detoxification GST superfamily and CYP450 would provide an answer to this assumption (*19*). In any case we can expect the march of disease, response to medications and chelation therapy to be very much individualized. Hence, the noted clinical heterogeneity of ß- thalassemia, with varied genotype/phenotype congruence (*26*).

MTHFR is a single carbon carrier essential for DNA synthesis, hence essential for regeneration of DNA in health and in disease (*27–29*). ß- thalassemia is a disease where children get hemolysis and intra-medullary ineffective erythropoiesis with unmatched regeneration of healthy red blood corpuscles series as dictated by the genotype. The contribution of *MTHFR* status to clinical phenotype of ß- thalassemia is not clear cut as well (*25, 30*), as it can occur in unique combination with other GST deficiencies and their respective compromised detoxification when challenged.

ß-Thalassemia genotype dictates ineffective intramedullary erythropoiesis, shortened red blood corpuscles life span and over-worked compensation, but do not account for the other phenotype variability. The implications of our work specifies that every child afflicted with ß-thalassemia has a unique detoxification weakness, or unique combination of weaknesses and unique regeneration potential. Hemolytic attacks, iron overload, other manifestations that cannot be explained by ß-thalassemia gene mutation among these children might be related to exposure to toxins that children with ß- thalassemia cannot detoxify (*31–34*). Since the combination of detoxification abilities seem to be inherent to all humans, then we assume that ß- thalassemia gene poses shortened life span of red blood corpuscles, and once that child with shortened red blood corpuscles gets exposed to a toxin (chemical) that causes minor hemolysis, the child cannot compensate for this hemolysis and would present by overt hemolytic crises. This scenario applies to exposure to heavy metals, vitamins, etc. (*35–38*).

Ferritin is an acute phase reactant that is affected by multiple factors other than mere detoxification. Upper level of serum iron however is almost always under very strict control, which seems to be under more stringent control that is not only governed by detoxification enzymes.

Our study has limitations. The study design was a pilot cross-sectional one that addressed the question of host detoxification and regeneration genomics in ß-thalassemia. And yes, the study proved that children with ß-thalassemia have individual host detoxification and regeneration ability/disability genomics, but not all cytochrome p450, GST glucuronidation detoxification superfamilies genomics were studied. The study did not address all factors affecting structural regeneration ability, apart from *MTHFR* of host as well. The study underscored that children with ß- thalassemia phenotype is a function of more than mutation of ß- thalassemia gene. The other short-comings of our study included the lack of assessment of specific toxins known to cause hemolysis in our studied group, and lack of long term follow up of the children studied to allow genotype/phenotype correlation in terms of clinical picture, disease progression, complications and outcome. Again the study did not aim or address phenotype genotype correlations. Another short-coming of the study was the lack of assessment of stored iron as ferritin among the studied cohort.

Conclusion

Children with ß-thalassemia may have one or more than a detoxification/ regeneration potential enzyme gene *GSTM1*, *GSTPi* and *MTHFR* polymorphism. Every child with ß-thalassemia has unique detoxification and regeneration abilities. Sample size and the cross-

sectional nature of our pilot study did not allow genotype-phenotype correlation definition. We assume that the unique genomics of each child might lead to phenotypical variation in ß-thalassemia symptoms and would be related to time of exposure or to type of chemical, and its quantity if it happens. Hence the unique clinical picture, the individuality of ß- thalassemia clinical picture and march among different children afflicted with the disease. Each child has its own genetically determined detoxification-inability combination and has unique DNA regeneration abilities. The children lifestyle differences with regards to nutrition and exposure to specific chemicals provide further innumerable causes of uniqueness and individualization of ß-thalassemia phenotype that need to be studied in future research.

Acknowledgment

Author Contributions: All authors searched medical literature, databases, conceptualized, conducted the case review and reviewed the final manuscript. All authors have read and agreed to the published version of the manuscript.

FUNDING

CONFLICT OF INTEREST

The authors declare no conflict of interest in connection with the reported study. Authors declare veracity of information.

References

1. S. Tenny, M. Varacallo, *Evidence Based Medicine.* (StatPearls Publishing; Treasure Island (FL), 2020; https://www.ncbi.nlm.nih.gov/books/NBK470182/).
2. C. Traivaree, C. Monsereenusorn, P. Rujkijyanont, W. Prasertsin, B. Boonyawat, Genotype-phenotype correlation among beta-thalassemia and beta-thalassemia/HbE disease in Thai children: predictable clinical spectrum using genotypic analysis. *J. Blood Med.* **9**, 35–41 (2018).
3. B. Modell, Global epidemiology of haemoglobin disorders and derived service indicators. *Bull. World Health Organ.* **2008**, 480–487 (2008).
4. S. J. Henderson, A. T. Timbs, J. McCarthy, A. E. Gallienne, M. Proven, M. J. Rugless, H. Lopez, J. Eglinton, D. Dziedzic, M. Beardsall, M. S. M. Khalil, J. M. Old, Ten Years of Routine **α** - and **β** -Globin Gene Sequencing in UK Hemoglobinopathy Referrals Reveals 60 Novel Mutations. *Hemoglobin*. **40**, 75–84 (2016).
5. A. T. Taher, A. Radwan, V. Viprakasit, When to consider transfusion therapy for patients with non-transfusion-dependent thalassaemia. *Vox Sang.* **108**, 1–10 (2015).
6. S. Mondal, S. Mandal, Prevalence of thalassemia and hemoglobinopathy in eastern India: A 10-year high-performance liquid chromatography study of 119,336 cases. *Asian J. Transfus. Sci.* **10**, 105 (2016).
7. A. T. Taher, A. N. Saliba, Iron overload in thalassemia: different organs at different rates. *Hematol. Am. Soc. Hematol. Educ. Program*. **2017**, 265–271 (2017).
8. M. E. Sears, Chelation: harnessing and enhancing heavy metal detoxification--a review. *ScientificWorldJournal*. **2013**, 219840 (2013).
9. R. W. Kalpravidh, T. Tangjaidee, S. Hatairaktham, R. Charoensakdi, N. Panichkul, N. Siritanaratkul, S. Fucharoen, Glutathione redox system in β -thalassemia/Hb E patients. *ScientificWorldJournal*. **2013**, 543973 (2013).
10. P. D. Josephy, Genetic variations in human glutathione transferase enzymes: significance for pharmacology and toxicology. *Hum. Genomics Proteomics HGP*. **2010**, 876940 (2010).
11. Kotb M.A., Kotb A, Glutathione S Transferase M1 Polymorphism in Extrahepatic Biliary Atresia. *Med J Cairo Univ*. **83**, 109–112 (2015).
12. L. W. Harries, M. J. Stubbins, D. Forman, G. C. Howard, C. R. Wolf, Identification of genetic polymorphisms at the glutathione S-transferase Pi locus and association with susceptibility to bladder, testicular and prostate cancer. *Carcinogenesis*. **18**, 641–644 (1997).

13. P. Frosst, H. J. Blom, R. Milos, P. Goyette, C. A. Sheppard, R. G. Matthews, G. J. Boers, M. den Heijer, L. A. Kluijtmans, L. P. van den Heuvel, A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat. Genet.* **10**, 111–113 (1995).

14. E. E. Habib, M. Aziz, M. Kotb, Genetic polymorphism of folate and methionine metabolizing enzymes and their susceptibility to malignant lymphoma. *J. Egypt. Natl. Cancer Inst.* **17**, 184–192 (2005).

15. I. Youssry, R. Hammoud, O. Ibrahim, M. Youssef, A. Beshlawy, Genetic polymorphisms of glutathione-S-transferase and microsomal epoxide hydrolase in egyptian acquired aplastic anemia patients. *J. Pediatr. Hematol. Oncol.* **33**, 89–92 (2011).

16. D. G. Aly, S. A. Salem, K. S. Amr, M. F. A. El-Hamid, A study of the association of glutathione S-transferase M1/T1 polymorphisms with susceptibility to vitiligo in Egyptian patients. *An. Bras. Dermatol.* **93**, 54–58 (2018).

17. R. M. Shawky, F. El-baz, T. M. Kamal, R. M. Elhossiny, M. A. Ahmed, G. H. El Nady, Study of genotype–phenotype correlation of methylene tetrahydrofolate reductase (MTHFR) gene polymorphisms in a sample of Egyptian autistic children. *Egypt. J. Med. Hum. Genet.* **15**, 335–341 (2014).

18. S. Dasari, M. S. Ganjayi, P. Yellanurkonda, S. Basha, B. Meriga, Role of glutathione S-transferases in detoxification of a polycyclic aromatic hydrocarbon, methylcholanthrene. *Chem. Biol. Interact.* **294**, 81–90 (2018).

19. M. Ansari, P. Huezo-Diaz, M. A. Rezgui, S. Marktel, M. Duval, H. Bittencourt, B. Cappelli, M. Krajinovic, Influence of glutathione S-transferase gene polymorphisms on busulfan pharmacokinetics and outcome of hematopoietic stem-cell transplantation in thalassemia pediatric patients. *Bone Marrow Transplant.* **51**, 377–383 (2016).

20. M. A. Zaki, T. F. Moghazy, M. M. K. El-Deeb, A. H. Mohamed, N. A. A. Mohamed, Glutathione S-transferase M1, T1 and P1 gene polymorphisms and the risk of developing type 2 diabetes mellitus in Egyptian diabetic patients with and without diabetic vascular complications. *Alex. J. Med.* **51**, 73–82 (2015).

21. M.A. Kotb, Kotb A, Extrahepatic Biliary Atresia is an Aflatoxin Induced Cholangiopathy in Infants with Null GSTM1 Geno-type with Disrupted P53 and GSTPi to Mothers Heterozygous for GSTM1 Polymorphism: Damage Control is Mediated through Neutrophil Elastase and CD14+ Activated Monocytes: Kotb Disease. *Med J Cairo Univ.* **83**, 137–145 (2015).

22. F. P. Simeonova, L. Fishbein, Hydrogen cyanide and cyanides : human health aspects. World Health Organization (2004), (available at https://apps.who.int/iris/handle/10665/42942).

23. S. Sclafani, G. Calvaruso, V. Agrigento, A. Maggio, V. Lo Nigro, E. D'Alcamo, Glutathione S transferase polymorphisms influence on iron overload in β-thalassemia patients. *Thalass. Rep.* **3**, 6 (2013).

24. R. H. Tuttle, Ontogeny and phylogeny. By Stephen Jay Gould. Harvard University Press, Cambridge, Massachusetts. 1977. xvi + 501 pp., figures, tables, notes, bibliography, glossary, index. $18.50 (cloth). *Am. J. Phys. Anthropol.* **49**, 287–288 (1978).

25. M. A. Abd-Elmawla, S. M. Rizk, I. Youssry, A. A. Shaheen, Impact of Genetic Polymorphism of methylenetetrahydrofolate reductase C677T on Development of Hyperhomocysteinemia and Related Oxidative Changes in Egyptian β-Thalassemia Major Patients. *PloS One.* **11**, e0155070 (2016).

26. I. Panigrahi, S. Agarwal, Genetic determinants of phenotype in beta-thalassemia. *Hematol. Amst. Neth.* **13**, 247–252 (2008).

27. K. Curtin, M. L. Slattery, C. M. Ulrich, J. Bigler, T. R. Levin, R. K. Wolff, H. Albertsen, J. D. Potter, W. S. Samowitz, Genetic polymorphisms in one-carbon metabolism: associations with CpG island methylator phenotype (CIMP) in colon cancer and the modifying effects of diet. *Carcinogenesis.* **28**, 1672–1679 (2007).

28. A. Fredriksen, K. Meyer, P. M. Ueland, S. E. Vollset, T. Grotmol, J. Schneede, Large-scale population-based metabolic phenotyping of thirteen genetic polymorphisms related to one-carbon metabolism. *Hum. Mutat.* **28**, 856–865 (2007).

29. W. Kim, H. D. Woo, J. Lee, I. J. Choi, Y. W. Kim, J. Sung, J. Kim, Dietary folate, one-carbon metabolism-related genes, and gastric cancer risk in Korea. *Mol. Nutr. Food Res.* **60**, 337–345 (2016).

30. N. Y. Mustafa, R. Marouf, S. Al-Humood, S. M. Al-Fadhli, O. Mojiminiyi, Hypercoagulable State and Methylenetetra- hydrofolate Reductase (MTHFR) C677T Mutation in Patients with Beta-Thalassemia Major in Kuwait. *Acta Haematol.* **123**, 37–42 (2010).

31. Y. Wang, S. Wang, Increased extracellular ATP: an omen of bacterial RTX toxin-induced hemolysis? *Toxins.* **6**, 2432–2434 (2014).

32. R. Y. Shimojo, W. T. Iwaoka, A rapid hemolysis assay for the detection of sodium channel-specific marine toxins. *Toxicology.* **154**, 1–7 (2000).

33. V. R. Osorio e Castro, E. R. Ashwood, S. G. Wood, L. P. Vernon, Hemolysis of erythrocytes and fluorescence polarization changes elicited by peptide toxins, aliphatic alcohols, related glycols and benzylidene derivatives. *Biochim. Biophys. Acta.* **1029**, 252–258 (1990).

34. M. Hashmi, S. Gräf, M. Braun, M. W. Anders, Enantioselective depletion of mitochondrial glutathione concentrations by (S)- and (R)-3-hydroxy-4-pentenoate. *Chem. Res. Toxicol.* **9**, 361–364 (1996).

35. I. H. Ibrahim, S. M. Sallam, H. Omar, M. Rizk, Oxidative hemolysis of erythrocytes induced by various vitamins. *Int. J. Biomed. Sci. IJBS*. **2**, 295–298 (2006).

36. R. Crupi, R. Morabito, A. Remigante, E. Gugliandolo, D. Britti, S. Cuzzocrea, A. Marino, Susceptibility of erythrocytes from different sources to xenobiotics-induced lysis. *Comp. Biochem. Physiol. Toxicol. Pharmacol. CBP*. **221**, 68–72 (2019).

37. E. V. Nevezhin, N. V. Vlasova, I. A. Pyatnitskiy, E. P. Lysenko, M. V. Malakhov, On the mechanism of erythrocyte hemolysis induced by photooxidized psoralen. *Biochem. Biokhimiia*. **80**, 763–768 (2015).

38. C. Pavan, M. Tomatis, M. Ghiazza, V. Rabolli, V. Bolis, D. Lison, B. Fubini, In search of the chemical basis of the hemolytic potential of silicas. *Chem. Res. Toxicol.* **26**, 1188–1198 (2013).