Available online at: www.basra-science-journal.org

ISSN –1817 –2695

An Association between *GSTM1* and *GSTT1* Gene Polymorphism with some physiological parameters among Haemodialysis Patients in AL-Qadisiya Province / Iraq

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Received 23-9-2013 , Accepted 12-5-2014

Abstract

Several studies have shown that high oxidative stress levels are associated with haemodialysis (HD) . Glutathione S- transferase (*GSTM1* and *GSTT11*) are members of the GST family of proteins , which protects cellular DNA against oxidative damage . This study tested the association of *GSTM1* and *GSTT1* gene polymorphism with some physiological parameters among haemodialysis patients.

GSTT1 and *GSTM1* genotypes and the estimations of some biomarkers were determined in 60 HD patients and 60 age- and gender – matched controls .There was a significant difference between males and females ($P<0.05$, χ^2 test) patient group. The biomarkers results of this study (the mean haemoglobin (Hb) concentration, packed cell volume (PCV) and plasma glutathione (GSH) level) were significantly lower ($P<0.05$, χ^2 test) in HD patients in comparison with healthy subjects, while levels of Urea, Creatinine, malondialdehyde (MDA) and GST were significantly higher ($P<0.05$) in HD patients compared with control. We observed that *GSTM1* (-) null genotype was present in 50% of the HD patients, While *GSTT1* (-) null genotype was present in 33.33% of the HD patients. There was a significant association of null alleles of the *GSTM1* (-) ($P= 0.031$, OR= 3.1, 95% CI= 25.0-27.14) and *GSTT1* (-) ($P = 0.015$, OR= 1.75, 95% CI= 34.82-46.82) with HD patients. The combinations of *GSTM1* (-) and *GSTT1* (-) null genotype were (33.33%) in the HD patients, the difference was significant ($P= 0.032$ OR= 2.58 , 95% CI= 32.11- 41.31) . It was concluded that there is an association between *GSTT1* and *GSTM1* gene polymorphisms and oxidative stress leading to functional deficiency in in haemodialysis patients .

Key words: Haemodialysis , *GSTT1*, *GSTM1* , polymorphisms, oxidative stress.

1-Introduction

Chronic renal failure (CRF) is a pathophysiologic process with multiple etiologies, resulting in the inexorable attrition of nephron number and function and frequently leading to end stage renal disease. This condition results from disease like diabetes mellitus, Arterial Hypertensions, Glomerulonephritis and Polycystic Kidney disease [1].

The primary event leading to renal failure is a free radical mediated injury to the endothelial cells in the outer medulla [2]. The patient involved a collection of symptom and clinical signs due to the accumulation of urea and other nitrogen metabolites in the body [1].Therefore, the patients are permanently dependent upon renal replacement therapy dialysis or transplantation in order to subsist[3, 1] .

Dialysis patients are subjected to an oxidative stress resulting from the dialysis sessions [4,5]. The uraemic stats and the bio-incompatibility of hemodialysis are associated with an increased oxidative stress in hemodialysis patients presumably caused by both an increased generation of oxygen free radical / reactive oxygen species and decreased levels of different antioxidants[6,7] . Oxygen free radicals and reactive – oxygen species are involved in the pathogenesis of many clinical disorders by damaging lipids, proteins and DNA or by altering cellular signal transduction [8] .

The glutathione S- transferase (GSTs) represents an important super family of phase II xenobiotic metabolizing enzymes that catalyse the conjugation of reduced glutathione and those potentially hazardous reactive electrophilic species to detoxify them and facilitate their excretion[9,10] .

The GST isoenzymes expressed in human tissues comprise alpha, Mu, Theta, Kappa, Sigma, Zeta and Omega gene families. Because many *GST* genes are polymorphic, there has been considerable interest in determent whether particular allelic variants are associated with altered risk of a variety of pathologies including cancers and cardiovascular and respiratory diseases [11] .

Human GSTs are divided into cytosolic, microsomial and mitochondrial families [12] . Because of the polymorphism of gene deletion , our study focused on only two genes, *GSTM1* and *GSTT1* from two classes of the cytosolic family GST Mu (*GSTM1* ; Chromosome 1p13.3) and GST Theta (*GSTT1*; Chromosomes 22p11.2), respectively. These polymorphisms depend on the gene deletion, finally resulting in a lack of protein , this lead to functional deficiency in the enzyme activity of circulating red cells [13,14] . The aim of the present study was to investigate the association of the genes *GSTT1* and *GSTM1* polymorphisms and some physiologic parameters caused by oxidative stress in HD patients .

2- Materials and methods

2.1 Collection of samples:

This study involved sixty chronic renal failure patients (end- stage renal disease) (46 male, 14 female) who were treated by haemodialysis at the Department of Nephrology in AL- Qadisiya hospital / Iraq . The mean age of the patients was (42.18±1.79) year. All patients were dialyzed three times a week and each session was at least four hours. The control group was composed of 60 healthy volunteers (40 male, 20 female) with a mean age of (43.11±1.22) year. Controls had no medical problems.

The blood samples were collected from the haemodilysis patients immediately after haemodialysis . The dialysis sessions are directly from antivirus fistula. The blood sample was divided into two tubes (with and without anticoagulant- EDAT) .

Blood was collected by EDTA divided into two parts , the first stored at -20 C^{o} till used for DNA extraction and the second part

was taken from each subject for the measurement of hematological parameters including determination of haemoglobin (Hb) concentration, packed cell volume (PCV) as described by [15] .

The blood in non-EDTA tubes was centrifuged at 2000 RPM for 20 minutes, The clear supernatants serum was frozen at -20˚C till the time of biochemical estimations including the levels of Creatinine and Urea, these were measured using an automatic analyser (Reflotron) (Germany). Estimations including the levels of serum MDA and GSH levels were determined respectively according to the method of [16] .

Estimations the levels of the activity of serum GST was done according to the method of [17] .

2.2 Isolation of genomic DNA:

Genomic DNA was isolated by DNA extraction kit (Genedia DNA Mini Kit , UKAS) and stored directly at- 4˚ C till used.

2.3 Polymerase Chain Reaction (PCR):

The *GSTM1* and *GSTT1* genotype was analysed by multiplex PCR according to the protocol of [18] .Genomic DNA was amplified by using six sets of primers table (1) .

Table-1- Oligonucleotide primer sequences used for PCR amplification of *GSTM1* and *GSTT1* genes .

Primers		Primer Sequences	Length	Tm	T_A
GSTM1	*F	5¯-GAA CTC CCT GAA AAG CTA AAG C-¯3	22	64ºC	59 ° C
	*R	5¯-GTT GGG CTC AAA TAT ACG GTC G-¯3	22	64ºC	59 ° C
GSTT1	F	5¯-TTC CTT ACT GGT CCT CAC ATC TC-¯3	23	64ºC	59 ° C
	R	5¯-TCA CCG GAT CAT GGC CAG CA-¯3	20	64ºC	59 ° C
Albumin	F	5¯-GCC CTC TGC TAA CAA GTC CTA C-¯3	22	64ºC	59 ° C
	R	5¯-GCC CTA AAA AGA AAA TCG CCA ATC-¯3	22	64ºC	59 ° C

***Albumin* was used as an internal control.** **Tm: Melting temperature** **T_A: Annealing Temperature**

*** F : Forward**

*** R : Reverse**

The reaction mix and PCR condition are given in table (2, 3).

Table-2- The reaction mix (25µl) for *GSTM1* and *GSTT1* genes.

Chemicals	Volume
Master Mix	12.5 µ l
Primer Forward	1 µ l
Primer Reverse	1 µ l
DNA	5 µ l
D.W.	5.5 µ l
Total Volume	25 µ l

D.W distilled water.

Table-3- PCR conditions for *GSTM1* and *GSTT1* genes.

NO. OF STEPS	STEPS	TEMPERATURE	TIME	NO. OF CYCLE
1	Denaturation 1	95 ° C	3 min	1 Cycle
2	Denaturation 2	95 ° C	1 min	30 Cycles
3	Annealing	58 ° C	1 min	
4	Extension 1	72 ° C	1 min	
5	Final Extension	72 ° C	5 min	1 Cycle

The PCR product was then subjected to electrophoresis on a 2% agarose gel. The presence of bands of 480 bps and 215 bps was indicative of the *GSTT1* and *GSTM1* genotypes; whereas the absence indicated the null genotypes for that gene. *Albumin*

indicated by a 350 bp product was used as an internal control.

2.4 Statistical Analysis :

The data were analysed by using students t- test taking (P<0.05) as the lowest limit of significance of difference .

X^2 tests were used to examine eth differences of allele and genotype frequencies between patients and controls. Fisher's exact test was used. ORS and the 95 % CI were calculated and P< 0.05 considered signification (SPSS software version 14).

4- Results and Discussion

The mean age of the control group was (43.112±1.220) years whereas HD patients was (42.183±1.790) years. There was no significant difference in the age of the control and HD patients. The results illustrated gender differences, among the control group, the numbers were 40 (66.66%) and 20 (33.33%) respectively , whereas for the patients the number of male 46 (76.66%) and female 14 (23.33%). There was a significant difference between males and females (P<0.05, χ^2 test) . The mean duration of HD patients was (10.466 ± 0.330) month. Table (4).

Haemoglobin concentration and PCV were significantly lower (P<0.05) in HD patients compared to those of healthy subjects as shown in table (5). Our results are in agreement with the study of [19] that showed lower Hb and PCV levels in HD patients. The decrease of PCV value in haemolyzed plasma HD patients indicates an increased destruction of erythrocytes.

Oxidative stress plays an important role in many diseases including renal, anaemia [20,21]. In uraemic patients , however, increased oxidative stress in RBC may result from multifarious factors such as uraemic toxin and haemodialysis [22] .

The membrane of the erythrocytes is rich in polyunsaturated fatty acid which are susceptible to lipid peroxidation which result in the loss of membrane fluidity and cellular lysis [23] . Lipid peroxidation of RBC membrane destroys the integrity and the activity of Na + - K+ ATPase [24] , and this leads to increased RBC osmotic fragility and haemolysis, due to increased RBC membrane permeability and the retention of sodium ion inside the cell.

[25] showed Chloramines contamination in the dialysis water supply may lead to massive haemolysis due to oxidation of the erythrocyte membrane .

Lipid peroxide possess an enzymatic antioxidant defence mechanism to cope effectively with oxygen free radical to block the lipid peroxidation . In chronic uraemia, a defective glutathione redox cycle in erythrocyte may play an important role in oxidative stress of red cells [26,27] . [5] found significant decrease in serum SOD activity in CRF patients undergoing haemodialysis . The formation of haemoglobin depends on the patient's nutritional status and drives for synthesis. Amino acid and iron are the main ingredients for haemoglobin synthesis and erythropoietin provides the drive for RBC formation [28] .

Table 4: Distribution of Demographic variables of the patients and controls.

Characteristics	Control (n = 60)	Patient HD (n =60)
Age (Years)	43.11± 1.22	* 42.18±1.79
Gender Male Female	40 (66.66 %) 20 (33.33 %)	* 46(76.66) % *14 (23.33)%
Duration of HD (Month)	_____	10.466±0.330

*** Significant difference between patients and control (P< 0.05).**

Table (5) Shows the values of serum creatinine and urea in HD patients' groups compared with the control health values, the levels of creatinine and urea were significantly increased (P<0.05) in the HD group than control health.

The results are in agreement with previous research which indicated that the HD leads to induce severe physiological and biochemical disturbances in CRF patients [29] . The data in the present study showed a significant increase in urea and creatine in chronic kidney disease on haemodialysis.

Table (5) Clearly shows that plasma GSH was significantly decreased (P<0.05) in HD patients when compared to the control. There was a significant increase (P<0.05) in serum MDA of HD patients compared to healthy controls.

Dialysis patients are subjected to an oxidative stress resulting from the dialysis sessions [5] . Oxidative stress occurs when there is an excessive free radical production and / or low antioxidant defence [30,6] .

It has been reported , in CRF patient , disturbances in enzymatic mechanisms of free radical detoxification lead to alteration in the antioxidant system [31] , and ROS attack on cell membranes also results in the formation of lipid peroxidation products such as MDA [32] .

6 and 33 have reported that free radicals formation increased lipid peroxidation in CRF patients and associated with dialysis caused an impairment of antioxidant defense and overproduction of oxidative stress markers [34,32]. We have found an increased MDA level in HD patients indicating that they are in a state of continuous oxidative stress [35,36] .

The reduced GSH is one of the most important scavengers of free radical in the red blood cell membrane [37] . In the absence of an efficient GSH generating system , GSH cannot be maintained in the reduced state when subjected to oxidative stress [32] . It has been shown that serum antioxidant activity is significantly decreased in HD patients , and that it is improved by HD treatment [38] .

The result of this study indicated a significant increased (P<0.05) in serum GST activity levels between control and HD patients. Glutathione S- tarnsferase (GST) comprises a multigene family their proteins involved in the metabolism of many disease causing electronic substrates and it protects the cells against oxidative stress [39] . As GST is involved in the detoxification of electrophiles and hydroperoxide [40] , It is generally expected that its plasma levels should have increased in CRF patients due to enhanced oxidative stress .

Oxidative stress could be included in the factors responsible for an over expression of GSH- dependents enzymes in dialysis patients. In fact, Lipophilic substance released during the oxidative damage to polyunsaturated lipids, such as short- chain aldehydes and alkenals, have been demonstrated to be substrates for GST [41] , and to accumulated in the plasma and RBCs of dialysis patients [27,40] .

This higher level of serum GST activity in HD patients may be due to increased synthesis of this enzyme under oxidative stress to protect the body from toxic compounds [42] . [43] found GST over expression has been document in the RBCs of patients with chronic renal failure , and the possibility that this cell type could function as a circulatory detoxification system has been proposed [44] . [45] found that the mean GST activity in uraemic patients was highly overexpressed with respect to control.

Different GST subclass are localized to specific parts of the body [46] α- GST is localized to specific parts of the renal tubule (proximal) and is readily released into the urine during injury, therefore, it is considered to be an excellent biomarker for proteinuria [47] . There was no significant change in serum GST levels in CRF patients, [48] also found no significant difference in the serum levels of GST in CRF patients when compared to healthy control.

Table-5- Results of hematological and biochemical tests of HD patients and control.

Parameters	Control (n = 60)	Patients (n = 60)
Hb (g / dl)	12.325 ± 0.161	* 9.991 ± 0.254
PCV (%)	38.889 ± 0.381	* 31.600 ± 0.660
Creatine (mg /dl)	0.804 ± 0.022	* 4.073 ± 0.136
Urea (mg /dl)	64.199±33.100	* 75.062±1.004
MDA (nmol /ml)	3.212 ± 0.107	* 81.126 ± 3.865
GSH (µmol /dl)	1.751 ± 0.823	* 0.397 ± 0.026
GST (nmol /min/mg)	2.939 ± 0.312	* 4.153 ± 2.590

*** Significant difference between patients and control (*P*< 0.05).**

Genotypic analysis by a multiplex PCR was used to analyse simultaneously the presence or absence of *GSTM1* and *GSTT1* . The *Albumin* gene was used as an internal control. The internal control amplified *Albumin* fragment was 350bp in length , whereas the presence of the *GSTM1* and *GSTT1* genes were identified by 215 and 480 bp fragments, respectively. (Fig 1).

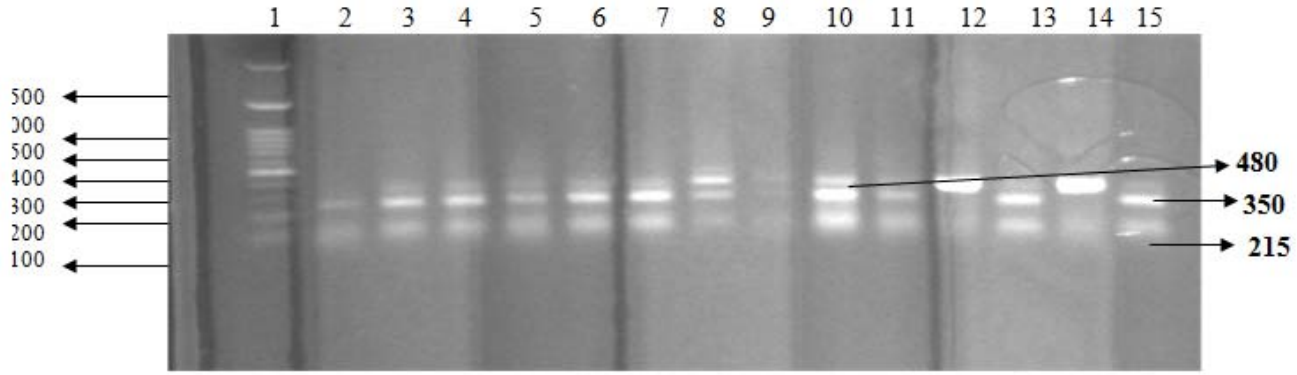

Fig 1: Electrophoresis of PCR products on agarose gel 2% .
Line 1= DNA marker.
Line 2,3,4,5,6,7,11,13,15 *GSTT1* null genotype.
Line 8,10 Normal genotype
Line 12,14 *GSTM1* null genotype.
Line 9 There was no band due to failure in DNA extraction or in the PCR process.

The frequencies of *GSTM1*- null genotypes were (50%) in the HD patients and (25%) in the control, the difference being statistically significant (P= 0.031, OR= 3.1, 95% CI= 25.0-27.14). The frequency of the *GSTT1*- null genotype was (33.33%) in the HD patients and (20%) in control. The difference was significant (P= 0.015 , OR= 2 , 95% CI= 34.82-46.82).

Table (6) showed the combinations of *GSTM1*- and *GSTT1*- null genotype was (33.33%) in the HD patients and (20%) in the control, the differences were significant (P=0. 032 , OR=2. 25 , 95% CI=32. 11-41.31).

Table-6- Distribution of polymorphism of *GSTM1* and *GSTT1* genes among patients and controls.

Genotypes	Control (n= 60)	Patients (n= 60)	OR	P- CI 95%	P-Value
GSTM1 (+)	45 (% 75)	29 (% 48.33)	1.0		
GSTM1 (-)	15 (% 25)	30 (% 50)	3.1	25.0-27.14	0.031
GSTT1 (+)	48 (% 80)	40 (% 66.66)	1.0		
GSTT1 (-)	12 (% 20)	20 (% 33.33)	2	34.82-46.82	0.015
GSTM1, GSTT1 (+)	45 (% 75)	29 (% 48.33)	1.0		
GSTM1, GSTT1 (-)	12 (% 20)	20 (% 33.33)	2.58	3232.11-41.31	0.032

The Hb and PCV were significantly lower in these with the absence of *GSTM1*- and *GSTT1*- genes compared to the presence of *GSTM1*+ and *GSTT1*+ . Levels of Creatin, Urea, MDA and GST were significantly higher in subjects with the genotypes of *GSTM1*- and *GSTT1*- than those with the genotype of *GSTM1*+ and *GSTT1*+ , While levels of GSH were significantly lower in those with the genotypes of *GSTM1*- and *GSTT1*- than those with the genotypes of *GSTM1*+ and *GSTT1*+ that were shown in table (7).

Table-7- Association between hemodialysis and combination of *GSTM1* and *GSTT1* genotype among the studied population.

Genotypes Parameters	*GSTM1* (+) (N = 29)	*GSTM1* (-) (N = 31)	P-Value	*GSTT1* (+) (N =40)	*GSTT1* (-) (N =20)	P-Value
Hb (g / dl)	0.283 ±10.937	0.341 ± 9.193	0.032	0.273 ± 10.540	0.436 ±9.030	0.043
PCV (%)	0.552 ±39.235	0.509 ±38.756	0.012	0.280 ±38.017	0.215 ±36.127	0.031
Creatine (mg /dl)	0.182 ±0.774	0.191 ±4.354	0.033	0.172 ±0.993	0.223 ±4.239	0.401
Urea (mg /dl)	1.470 ±64.967	1.396 ±75.151	0.021	0.603 ±62.251	0.383 ±71.037	0.041
MDA (nmol /ml)	5.528 ±8.327	5.490 ±81.873	0.844	4.463 ±7.962	7.547 ±83.454	0.011
GSH (µmol /dl)	0.105 ±0.434	0.022 ±0.354	0.071	0.759 ±1.033	0.073 ±0.312	0.008
GST (nmol /min/mg)	0.390 ± 1.053	0.211 ± 3.101	0.022	1.401 ± 2.34	1.620 ± 3.133	0.020

Dialysis patients are subjected to an oxidative stress resulting from the dialysis sessions [5].There is considerable evidence that HD patients are in a continuous state of oxidative stress, perhaps provoked by bioincompatibility of the dialysis membrane, Which may induce the formation of ROS [36] . HD patients may be exposed to potentially toxic substances in the dialysis water supply , and adverse reactions have

been reported in patients exposed dialysis water containing various contaminants [25] .

The present study was conducted to investigate the relationship between the genetic polymorphism of glutathione S-transferase *GSTM1* , *GSTT1* gene and HD which is involved in the metabolism of ROS and detoxifying xenobiotics. The contribution of the GST supergene family to oxidative stress resistance is well established [49] and therefore the absence of one or more of GST enzymes would result in increased ROS. *GSTM1* and *GSTT1* demonstrate activity towards phospholipids hydroperoxide [50] . It has been reported that an individual difference in metabolic activation and detoxification of xenobiotics partly depends on the genetic polymorphisms associated with *GSTT1* , *GSTM1* enzymes .

Studies reported the relationship between the combination of the GST genotypes and the risk of various diseases such as chronic renal failure [51,52] . There are inactive forms of the enzymes (null genotypes of *GSTM1, GSTT1* that reduce detoxification of oxidative metabolism, which may lead to the progression of the end stage renal disease (ESRD) . Therefore, it appears that the association ESRD risk with null alleles of *GSTM1,GSTT1* vary greatly in different populations [53,54] .

Previous studies demonstrated that GSH level in the blood are markers for evaluating oxidative stress HD patients [55,56] , and GST plays a vital role in phase II of biotransformation of many substances. Overexpression of GST has been documented into erythrocytes of patients with chronic renal failure [57] . GST protein catalysed the conjugation of electrophiles to GSH, charges in protein level due to variation at the genetic level may modulate the oxidative damage or inflammation caused by environmental exposure. Therefore, individual susceptibility to toxic agents may vary with GST polymorphisms [*58*,59].

GSTM1 and *GSTT1* genes are polymorphic in human and the null genotypes lead to the absence of enzyme function, contributing to inter individual differences in response to xenobiotics [60] . Adeletion polymorphism resulting in a total lack of enzyme activity in the homozygous null genotype, affects both *GSTM1*and *GSTTT1* .

In this study , we found that among HD patients , the *GSTM1* and *GSTT1* genotype were associated with a lower antioxidant capacity in terms of blood GSH, GST compared with *GSTM1+, GSTT1+* genotype. Glutathione is a major intracellular antioxidant, and a decrease in the whole blood level provides insight into a defective cellular redox state. We found that the lack of GST enzyme activity contributes to the augmented oxidative stress and that this subsequently leads to a further decrease in the levels of GSH among in HD patients.

There are few studies on the relation between GST genotypes and haemodialysis.[57] have shown the gene frequency of *GSTM1* and *GSTT1* polymorphism were evaluated, he observed that *GSTM1* null genotype was present in (46.74%) of the ESRD patients while *GSTT1* null genotype was present in (58.7%) of the ESRD subjects.[31] has shown that hemodialysis with *GSTM1* null genotype are more vulnerable to oxidative stress and damage , the frequency of the *GSTM1* genotype was (63.1%) in HD patients , while (60.2%) in healthy subjects.

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العلاقة بين التباين الوراثي للجينات *GSTM1* و *GSTT1* مع بعض المعايير الفسيولوجية لمرضى الغسيل الكلوي في محافظة القادسية – العراق

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الخلاصة

أوضحت العديد من الدراسات أن هناك علاقة بين الإجهاد التأكسدي العالي و الغسل الكلوي. إن الكلوتاثيون أس – ترانسفيريز تمثل مجموعة من البروتينات التي تحافظ على DNA ضد التلف الناتج من الأكسدة. تناولت هذه الدراسة دراسة العلاقة بين التباين الوراثي لجينات *GSTM1* و *GSTT1* مع بعض المعايير الفسيولوجية لمرضى الغسل الكلوي.

تم تحديد الأشكال الوراثية لجينات *GSTM1* و *GSTT1* و تقدير بعض المؤشرات الحيوية بين 60 مريض مع 60 آخرون أصحاء متماثلون في الجنس و العمر. ظهر فرق معنوي بين الإناث و الذكور (إختبار χ^2 ,P>0.05) لمجموعة المرضى. كانت نتائج المؤشرات الحيوية، نسبة الهيموغلوبين و حجم الخلايا المضغوط و تركيز الكلوتاثيون أقل في مجموعة المرضى بمقارنة بالسيطرة. أما مستوى اليوريا و الكرياتينين و MDA و GST أعلى (إختبار χ^2 ,P>0.05) في المرضى مقارنة بمجموعة السيطرة. لاحظنا أن التركيب الوراثي *GSTM1*(−) موجود في 50% من المرضى أما التركيب الوراثي *GSTT1*(−) موجود في 33.3% من المرضى. وجد أن هناك علاقة معنوية بين التراكيب الـوراثية *GSTM1*(−) – 25.00 =95% CI ,3.1 =OR ,0.031 =P) 27.14 و *GSTT1*(−) (46.82 – 34.82 =95% CI ,1.75 =OR ,0.015 =P) بين مرضى الغسل الكلوي. أما إرتباط التركيبين الوراثيين مع بعض *GSTM1*(−) و *GSTT1* (−) فكانت نسبته 33.33% في مرضى الفشل الكلوي و كان الإختلاف معنوياً (41.31 −32.11 =95% CI ,2.58 =OR ,0.032 =P) .

نستنتج من ذالك أن هناك علاقه بين التباين الوراثي لجينات GSTT1 و GSTM1 والاجهاد التأكسدي الذي يؤدي الى قصور في عمل الكلى لدى مرضى الغسيل الكلوي .

كلمات مفتاحية: الديلزة الدموية و جينات *GSTT1* و *GSTM1* و تعدد الأشكال و الأجهاد التأكسدي.