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ANALYSIS OF TUMOR NECROSIS FACTOR G238A AND G308A GENE POLYMORPHISMS IN TUNISIAN PATIENTS WITH PRESSURE ULCER

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ABSTRACT: Pressure ulcer (PU) is a complex and multifactor disease in which the cellular and molecular mechanisms contributing to risk of a delay in healing. An imbalance between pro-oxidant and antioxidant systems has been suggested to be implicated in the physiopathology of PU. Polymorphisms in TNF-α gene are emerging as key determinants of many diseases. The TNF-α (-238 and -308) G/A single-nucleotide polymorphisms (SNPs) are the most extensively studied. This study aimed to assess the value of serum pro-oxidant and antioxidant levels and to determine the role of a TNF-α gene SNPs in the pathogenesis of PU. 100 Tunisian subjects suffering of PU and 213 controls were admitted. Oxidant status was evaluated by the measure of homocysteine and thiobarbituric reactive oxygen substances. Antioxidant status was evaluated by the measure of total antioxidant status, serum catalase activity and trace elements. G308A and G238A variants of TNF-α gene were screened by AS-PCR and RFLP-PCR. Our results suggest that the unbalance between pro-oxidants and antioxidants seems to be more aggravated in patients suffering from PU in comparison with normal volunteers. Thus, for the first time in Africa, we sought to investigate whether polymorphisms in TNF-α gene were associated with the development of PU pathology in Tunisia. The TNF-α G308A may contribute to susceptibility to PU disease but our results do not support an association between PU and TNF-α G238A polymorphism. Our study drives the attention to implication of oxidative stress in PU. TNF-α G308 A polymorphism might be genetic risk factor for PU.

INTRODUCTION: In 2007, the National Pressure Ulcer Advisory Panel (NPUAP) redefined the definition of a pressure ulcer (PU) as a "location injury to the skin and/or underlying tissue usually over a bony prominence, as a result of pressure, or pressure in combination with shear. A number of contributing or confounding factors are also associated with PU; the significance of these factors is yet to be elucidated" [1].

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PU can be dangerous and painful for a resident, in part because broken skin can allow many complications into the body. PU present a major health challenge worldwide than a major cause of morbidity, mortality and healthcare burden globally [2-3]. Studies suggest that PU is characterized by dysregulation of biological pathways that can result from infections, environmental factors and genetic mutations [4]. Some of the key regulators of wound healing include oxidative stress, pro-inflammatory cytokines, growth factors, proteases and protease inhibitors [5].

In the recent years, an increasing number of scientists studied oxidative stress (OS) that plays a major role in the pathogenesis of many systemic diseases [6-7]. OS is defined as a disturbance in the

balance between the production of reactive oxygen species (free radicals) and antioxidant defenses. Examples of the possible consequences of free radical damage are provided with special emphasis on lipid peroxidation, proteins and DNA [8]. The sources of the increased OS derive from an increased burden of inhaled oxidants, and from the increased amounts of reactive oxygen species (ROS) generated by immune and structural cells of the airways and several inflammatory [9]. Reactive oxygen and nitrogen molecules are also involved in inflammatory process that occur in PU [10]. The question of whether OS is increased in PU is discussed.

Tumor necrosis factor-alpha (TNF-α) is a cytokine with pleomorphic actions which plays a critical role in a wide range of pathological processes, including infection, inflammatory disease, ischaemia and immune responses, including those observed in PU [11]. However, the TNF-α gene mutations are seen in many diseases especially inflammatory diseases [12-13]. The TNF-α gene is located within the major histocompatibility complex (MHC) region in chromosome 6p21.1–6p21.3 and this gene consists of four exons which is a highly polymorphic region. Some single nucleotide polymorphisms (SNPs) have been recognized in its promoter, namely the substitution of guanine (G) by adenine (A) at residues 238 (rs361525) and 308 (rs1800629), located in a regulative motif of promoters of the MHC class II genes have been shown to alter the expression of TNF-α gene [13-14].

They are the most important in human disease susceptibility as these might influence the transcription of TNF-α gene. It has been found that TNF enhancer polymorphism influences the serum level of TNF-α in different human disease and thus affects the susceptibility to diseases [12]. However, to date, the association between genetic polymorphisms of the TNF-α gene and PU in the Tunisian population has not been well examined.

In this study, we aimed to evaluate pro and antioxidant stress capacity in serum samples from patients with PU in comparison with witnesses in our laboratory conditions. Furthermore, we have analyzed the TNF-α gene promoter -308G/A and -238G/A polymorphisms in Tunisian patients with PU to evaluate the contribution of these SNPs in genetic susceptibility to PU.

MATERIALS AND METHODS:

Subjects:

This case-control study involved 100 adult Tunisian patients (74 males and 26 females, mean age 55.5±20 years) suffering from PU that met the following inclusion criteria: presenting with at least of a wound and confirmed diagnosis of PU, age≥18 years old, bedridden, not feeds only and without trophic and mental disorders. Exclusion criteria: paediatric study populations, age >90 years old, allergy to wound products, malignant origin. Who followed in many departments (emergency, orthopedic, physical medicine) of three University Regional hospitals of Tunisia (Farhat Hached and Sahloul Sousse, Fattouma Bourguiba Monastir). The control group consisted of 213 healthy volunteers clinically free of PU and tissue necrosis and their mean of age was 51.5±17 years (125 males and 88 females). All patients and controls gave informed consent to participate in this study which was approved by the National Medical and research Ethics Committee. The methods carried out in the study are in accordance with the approved relevant guidelines and regulations.

Laboratory Analysis:

Biochemical parameters:

We obtained venous blood samples from 313 subjects, 100 PU patients and 213 healthy controls matched for age, sex and ethnicity. Fasting venous blood samples (10-15 ml) were collected in three tubes were made for each patient. Plasma levels glucose, a renal profile (urea, creatinine, uric acid**)** levels and serum total cholesterol (CH), triglycerides, high density lipoprotein cholesterol (HDL-C) were measured with colorimetric essay using an automated system (Cx5 and Cx9 Pro-Bechman Coulter-Fuller-Ton CA). Low density lipoprotein cholesterol (LDL-C) was determined by Friedewald formula. Serum albumin (Alb) and prealbumin (Pre-alb) were measured, as markers of nutrition, using the dry chemistry method (BN prospec, siemens). Serum copper was indicated spectrophotometrically with RANDOX kit (Cat. No. CU 2340; Randox Labs Ltd., Crumlin, UK) at 580 nm according to instructions introduced by manufacturer. Serum zinc was measured with

RANDOX kit (Cat. No. ZN 2341; Randox Labs Ltd, Crumlin, UK) at 560 nm. C-reactive protein (CRP) (COBAS INTEGRA 400 Roche, immunoturbidimetric methods) and α-1-acid glycoprotein (AAG) (BN prospec, Siemens, chemistry method) as markers of inflammation.

Determination of oxidative stress parameters:

Serum total homocysteine concentrations were measured by using an AxSYM (ABBOTT) homocysteine assay. Serum total antioxidant status (TAS) was measured with RANDOX kit (Cat. No. NZ 2332; Randox Labs Ltd., Crumlin, UK) by colorimetric method at 600 nm according to the method of Miller et al [15]. Lipid peroxidation level was estimated by measurement of thiobarbituric acid reactive substances (TBARS) in serum according to the fluorimetric method of Yagi [16]. For the quantitative in vitro of non esterified fatty acids (NEFA) in serum was determined by colorimetric method at 550 nm (Cat. No. FA 115; Randox Labs Ltd., Crumlin, UK). Serum catalase activity (Cat) was determined according to the spectrophotometric method of Goth [17] and principe based on the decomposition of H_2O_2. Briefly, this assay is based on the ability of a hydrogen peroxide/ammonium molybdate complex formation measured at 405 nm.

DNA isolation and genotyping of TNF- α (G308A and G238A):

Genomic DNA was extracted from whole blood using the salting out method. The genotypic analysis of the TNF-α G308A polymorphism was performed using Allele-specific PCR (AS-PCR) amplification, for each sample PCR was done in two separate reactions by specific primers [18].

Briefly, the PCR procedures were performed using a 18 µl reaction volume containing 3.6 µl of 5X buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.4), 0.72 µl of 25 mM $MgCl_2$, 0.36 µl of 2-50 uM of each allele specific primers, 0.36 µl of 10 uM common primer, 0.2 µl of 10 uM mix *internal* control primers, 0.36 µl of 20 mM dNTP, 0.09 U of Taq DNA Polymerase (Promega) and 1 µl of 100 ng of template DNA. The samples were incubated at 95°C for 10 min, followed by 30 cycles of 1 min denaturation at 95°C, 50 seconds annealing at 59°C, 50 seconds extension at 72°C, and a final extension at 72°C for 10 min. The amplicons were separated by electrophoresis in 2% agarose gel stained with ethidium bromide and were visualized and photographed using the Micro DOC gel documentation system (**Fig.1**).

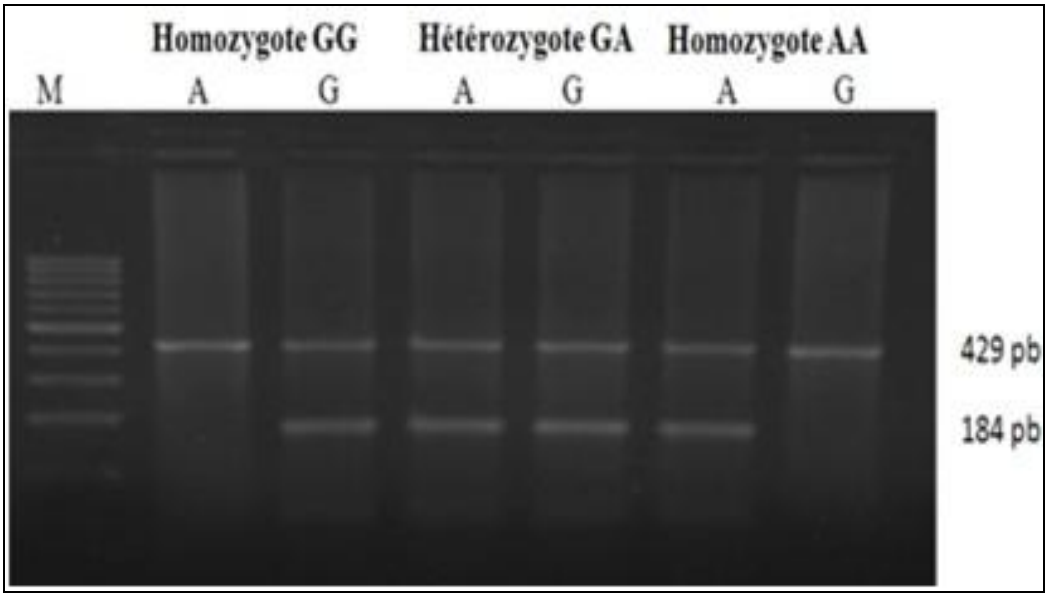

FIG. 1: TNF-α G308A (rs1800629) GENOTYPING BY ALLELE SPECIFIC PCR

TNF-α G238A promoter polymorphisms was determined by the polymerase chain reaction and restriction fragment length polymorphism (RFLP-PCR) method, as described previously and subsequent restriction enzyme analysis with BamHI [19] (**Fig.2**).

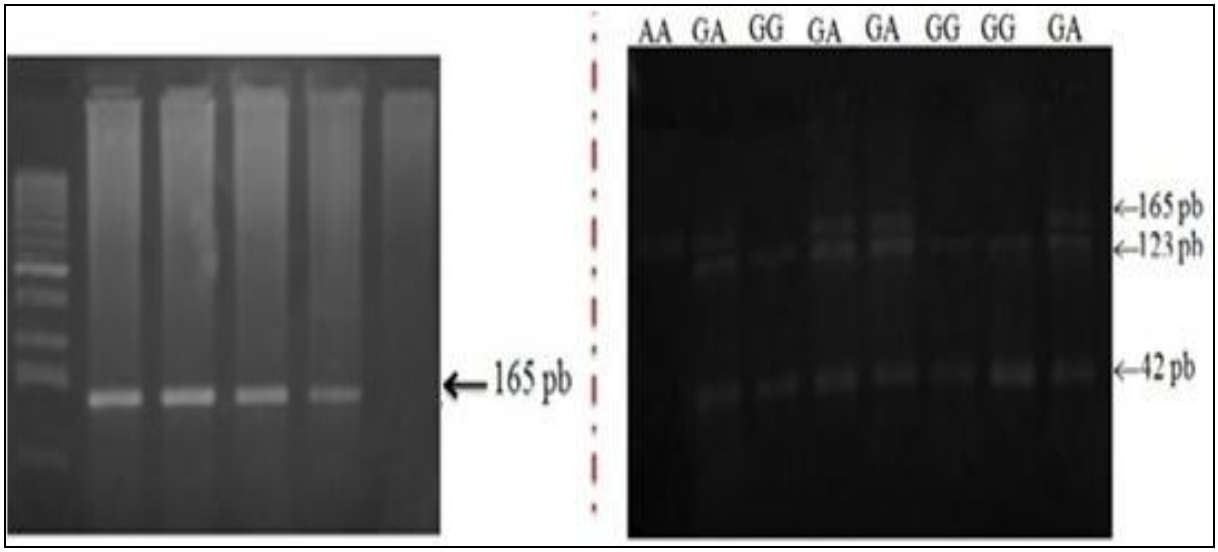

FIG. 2: TNF-α G238A (rs361525) GENOTYPING BY POLYMERASE CHAIN REACTION AND RESTRICTION FRAGMENT LENGTH POLYMORPHISM

Data analysis:

Statistical analysis for our data was performed using SPSS (SPSS for Windows, Release 18.0.0, 2009 - SPSS Inc –Chicago- IL, USA) and Epi info 6.0 (CDC, Atlanta, Georgia, USA) softwares. Continuous clinical data was compared by unpaired Student's t-test and presented as mean ± standard deviation (SD). The $\chi 2$ test was used to compare discrete variables and to compare genotype distributions. The Hardy-Weinberg equilibrium was confirmed using the $\chi 2$ test. A p value of <0.05 was considered to be statistically significant.

RESULTS:

The clinical characteristics:

Table 1 illustrate the variations data of glucose, renal, lipid and nutrition markers. In our study, glucose and renal markers concentrations were significantly elevated in patients compared to controls unlike uric acid and protide concentration was significantly higher among controls compared to patients. Lipid markers (CH, TG, HDL-C and LDL-C) showed significant difference between patients and controls. Contrary nutrition markers (Alb and Pre-alb) were statistically elevated in controls compared to patients.

TABLE 1: DEMOGRAPHIC DATA BY DIAGNOSTIC GROUPS

Variables	Patients (n=100)	Controls (n=213)	P
Women /Men	26/74	88//125	0.001
Age (years)	55.5±20	51.5±17	0.016
BMI (kg/m^2)	20.92±3.29	24.09±3.36	0.000
Glucose (mmol/l)	7.04±3.44	6.03±2.12	0.004
Urea (mmol/l)	6.95±6.13	4.68±1.79	0.000
Creatinine (µmol/l)	117.86±162.97	74.98±81.84	0.007
Uric acid (µmol/l)	202.68±143.68	214.01±75.8	0.41
Protide (g/l)	64.33±10.86	74.37±6.17	0.000
Cholesterol (mmol/l)	3.25±1.2	4.67±1.03	0.000
Triglyceride (mmol/l)	1.54±0.72	1.33±0.81	0.048
HDL- CH (mmol/l)	0.76±0.46	2.01±1.29	0.000
LDL- CH (mmol/l)	1.38±1.15	2.23±1.04	0.000
Albumin (g/l)	27.91±8.15	40.79±8.43	0.000
Prealbumin (g/l)	0.13±0.11	0.28±0.15	0.000

Oxidative status and inflammatory parameters

Oxidative status and inflammatory parameters of all subjects are listed in **Table 2**. In our study, a statistically significant elevation of the first-line inflammatory parameters was found among patients compared with controls. Contrary, anti and oxidant parameters were statistically elevated in controls compared to patients.

TABLE 2: OXIDANT AND INFLAMMATORY MARKERS FEATURES OF PATIENTS WITH PU AND CONTROLS

Variables	Patients (n=100)	Controls (n=213)	P
CRP (mg/l)	98.69±76.93	6.31±6.25	0.000
AAG (g/l)	1.85±0.62	1±0.5	0.000
Homocysteine (µmol/l)	27.22±12.50	10.46±3.64	0.003
TBARS (µmol/l)	0.47±0.31	1.11±1	0.000
NEFA (mmol/l)	0.637 ±0.305	1.042 ± 0.672	0.000
Cat (KU/l)	13.38± 7.15	38.11 ±7.5	0.000
TAS (mmol/l)	1.48±0.72	1.76±0.21	0.000
Copper (µmol/l)	12.97 ±1.88	16.24 ± 5.58	0.000
Zinc (µmol/l)	10.8 ±7.35	15.01±6.02	0.000

TNF- α (G308A and G238A) and risk of PU:

In this study, evaluation of Hardy-Weinberg equilibrium showed that the genotype frequencies of the TNF-α (G308A and G238A) polymorphisms were in Hardy-Weinberg equilibrium in the PU group and healthy blood donors. The genotypic and allelic frequencies of -308G/A and -238G/A were calculated and summarized in **Table 3**.

TABLE 3: GENOTYPE AND ALLELE DISTRIBUTIONS OF TNF-α POLYMORPHISMS IN CASE AND CONTROL GROUPS THE CONTROLS

Alleles /Genotypes *TNF-α-308* G → A				
	Patients (n=100)	Controls (n=213)	P-value	**OR (95 % CI)**
G	139 (69.5%)	330 (77.46%)	Ref	--
A	61 (30.5%)	96 (22.54%)	0.032	0.66 [0.45-0.96]
GG	48 (48%)	134 (62.91%)	Ref	--
GA	43 (43%)	62 (29.1%)	0.0105	0.51 [0.31-0.86]
AA	9 (9%)	17 (7.98%)	0.378	0.67 [0.28-1.61]
GA+AA	52 (52%)	79 (37.08%)	0.012	0.55 [0.33-0.88]
Alleles /Genotypes *TNF-α-238* G → A				
	Patients (n=100)	Controls (n=213)	P-value	**OR (95 % CI)**
G	142 (71%)	322 (75.59%)	Ref	--
A	58 (29%)	104 (24.41%)	0.221	0.79 [0.54-1.15]
GG	53 (53%)	131 (61.5%)	Ref	--
GA	36 (36%)	60 (28.16%)	0.138	0.67 [0.4-1.13]
AA	11 (11%)	22 (10.32%)	0.59	0.80 [0.36-1.78]
GA+AA	47 (47%)	82 (38.49 %)	0.154	0.70 [0.43-1.14]

The distribution of the GA and AA genotypes of the G308A polymorphism in the TNF- α gene was significantly different between the PU and control groups (p=0.012) with decreased frequency of GG genotype was observed in PU patients compared to controls (48% vs 62.91%). The corresponding TNF-α 308 alleles (G and A) also showed variations between patients and controls and both differences were significant at a P of 0.032. For the TNF-α G238A SNP, the distribution of the genotypes and allele neither demonstrated a significant difference between patients and controls.

DISCUSSION: PU is a major public health problem globally [20]. In Tunisian population, PU remains a serious health problem. Varied risk factors among patients confirm the multifactorial origin of the PU in which need to be more defined [21]. However, no study has been carried out on the role of the OS parameters and inflammatory parameters in PU. In this context, our study aims to evaluate the antioxidant capacity and to investigate the association of TNF-α promoter polymorphisms −308G /A and −238G/A with PU in the Tunisian population.

In the present study, underweight was a significant risk factor, as was hypoalbuminaemia which is a marker of poor nutrition. According to our observation, Berlowitz et al. and Allman et al. considered that the decreased body weight and low serum albumin are associated with the presence of PU [22-23]. Although the measure of trace elements (copper and zinc) in blood showed a significant increase in these parameters among patients

compared to controls. This could be consistent with several studies particularly [21-24]. Glucose and triglyceride concentrations were significantly elevated in patients compared to controls unlike lipid markers (CH, HDL-CH and LDL-CH) was significantly higher among controls compared to patients. The observation that elevated glucose and triglyceride levels can explain by occur in patients, suffering from PU, hospitalized with cardiovascular disease and age [25]. Long-term denutrition containing monounsaturated fatty acids have been shown to reduce platelet aggregation and decrease plasma LDL-cholesterol levels [26]. Undernutrition is demonstrated for the patients suffering from PU in our study.

Clinical, epidemiological and experimental studies provide evidence implicating the role of free radicals on the etiology of many diseases [27]. In our data, investigation about OS parameters shows a significantly decreased serum TBARS level, as a lipid peroxidation marker, in patients with PU compared to healthy subjects. Discording to our observation, Cordeiro et al. have considered that the increased serum TBARS is a consequence to excessive ROS generation at 12 PU subjects [27]. Therefore, decreased concentrations of NEFA in the PU group than in the healthy group consolidate their effects and these are consistent with previous results. Cat activity was measured based on the ability of the enzyme to break down H_2O_2.

In our study, we observed a significantly lower catalase activity ($P<0.001$) in patients compared to controls. Although, the quantize of TAS can evaluate the total capacity of all antioxidants found in serum to neutralize the oxidative action of free radicals in the human body, this status was significantly decreased in patients compared to controls which bring us back to promote the role of other antioxidant [10-28]. Furthermore, uric acid, as a sacrificial antioxidant, predicts development of obesity, hypertension, and cardiovascular disease, conditions associated with OS [10].

We also demonstrated that the plasma concentration of uric acid, a possible marker of radical generation in vivo, was markedly lowered in the patients suffering of PU. As observed in the present study significantly higher serum homocysteine, CRP (Inflammatory parameter) and AAG (Endogenous inflammatory marker) levels in patients with PU compared to witnesses. This lead to an excessive inflammatory reaction a result of the infection. Similarly, hyper-inflammation a related to nutritional or genetic factor led to increased risk of PU severity [27- 29].

While inflammation is an important and necessary part of the wound healing process, in wounds, such as PU, there is a persistent inflammatory stimulus that leads to chronicity. Also, genes encoding for cytokines have been reported as candidates for PU predisposition. TNF-α is a proinflammatory cytokine which exerts multiple biological effects and is thought to play a critical role in disease [30-31]. Various studies have demonstrated commonly varied SNPs in the TNF-α promoter region may have an impact on TNF-α transcriptional expression [32].

In that study, we assessed the association of two common polymorphisms of TNF-α promoter region at positions -238G/A and -308G/A with PU susceptibility in the Tunisian population. To our knowledge, no study in the world has investigated the association between the TNF-α promoter polymorphism and the risk of development of PU [33-34-35]. Interestingly, the distribution of -308G/A genotype and allele frequencies were significantly different between PU patients and controls ($p=0.032$) with the A allele conferring a lower risk for PU (odds ratio (OR) (GA and AA) =0.55; 95% CI= [0.33-0.88]). As we focused on studying the -238G/A polymorphism, we did not find any positive association between this SNP and PU disease.

CONCLUSION: Our retrospective study has shown important discrepancies for pro-oxidant and antioxidant status in patients suffering from PU in comparison with normal volunteers. Moreover, our data show that the TNF-α-308G>A polymorphism is a risk factor associated with PU susceptibility, although no significant association was observed between PU and TNF-α G238A gene polymorphism in Tunisian population. The study has some strengths but also limitations. These findings could be related to genetic heterogeneity or population stratification within each ethnicity

and/or samples size of studied populations. Further research to investigate the relationship between PU and other polymorphisms and we will study the molecular biology of how skin cells.

CONFLICTS OF INTEREST: The authors declare that they have no conflict of interest.

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